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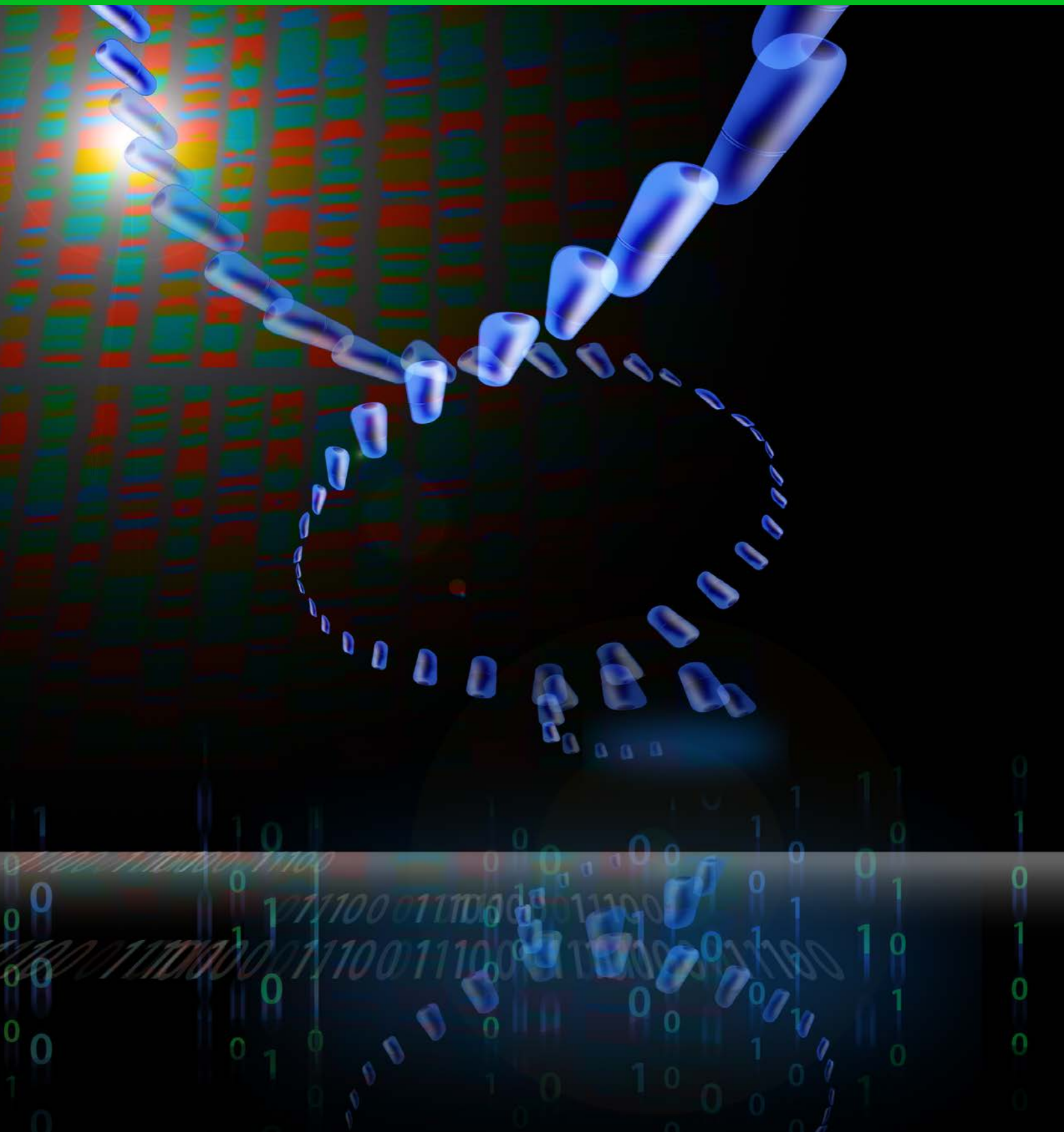

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'Translational Bioinformatics' is a collection of *PLOS Computational Biology* Education articles which reads as a "book" to be used as a reference or tutorial for a graduate level introductory course on the science of translational bioinformatics.

Translational bioinformatics is an emerging field that addresses the current challenges of integrating increasingly voluminous amounts of molecular and clinical data. Its aim is to provide a better understanding of the molecular basis of disease, which in turn will inform clinical practice and ultimately improve human health.

The concept of a translational bioinformatics introductory book was originally conceived in 2009 by Jake Chen and Maricel Kann. Each chapter was crafted by leading experts who provide a solid introduction to the topics covered, complete with training exercises and answers. The rapid evolution of this field is expected to lead to updates and new chapters that will be incorporated into this collection.

Collection editors: Maricel Kann, Guest Editor, and Fran Lewitter, *PLOS Computational Biology* Education Editor.

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Education

Introduction to Translational Bioinformatics Collection

Russ B. Altman*

Department of Genetics, Stanford University, Stanford, California, United States of America

This article is part of the "Translational Bioinformatics" collection for *PLOS Computational Biology*.

How should we define translational bioinformatics? I had to answer this question unambiguously in March 2008 when I was asked to deliver a review of "recent progress in translational bioinformatics" at the American Medical Informatics Association's Summit on Translational Bioinformatics. The lecture required me to define papers in the field, and then highlight exciting progress that occurred over the previous ~12 months. I have repeated this for the last few years, and the most difficult part of the exercise is limiting my review only to those papers that are within the field.

I have never worried much about definitions within informatics fields; they tend to overlap, merge and evolve. "Informatics" seems clear: the study of how to represent, store, search, retrieve and analyze information. The adjectives in front of "informatics" vary but also tend to make sense: medical informatics concerns medical information, bioinformatics concerns basic biological information, clinical informatics focuses on the clinical delivery part of medical informatics, biomedical informatics merges bioinformatics and medical informatics, imaging informatics focuses on…images, and so on. So what does this adjective "translational" denote?

Translational medical research has emerged as an important theme in the last decade. Starting with top-down leadership from the National Institutes of Health and its former Director, Dr. Elias Zerhouni, and moving through academic medical centers, research institutes and industrial research and development efforts, there has been interest in more effectively moving the discoveries and innovations in the laboratory to the bedside, leading to improved diagnosis, prognosis, and treatment. Translational research encompasses many activities including the creation of medical devices, molecular diagnostics, small molecule therapeutics, biological therapeutics, vaccines, and others. One of the main targets of translation, however, is revolutionary explosion of knowledge in molecular biology, genetics, and genomics. Some believe that the tremendous progress in discovery over the last 50+ years since elucidation of the double helix structure has not translated (there's that word!) into much practical health benefit. While the accuracy of this claim can be debated, there can be no debate that our ability to measure (1) DNA sequence (including entire genomes!), (2) RNA sequence and expression, (3) protein sequence, structure, expression and modification, and (4) small molecule metabolite structure, presence, and quantity has advanced rapidly and enables us to imagine fantastic new technologies in pursuit of human health.

There are many barriers to translating our molecular understanding into technologies that impact patients. These include understanding health market size and forces, the regulatory milieu, how to harden the technology for routine use, and how to navigate an increasingly complex intellectual property landscape. But before those activities can begin, we must overcome an even more fundamental barrier: connecting the stuff of molecular biology to the clinical world. Molecular and cellular biology studies genes, DNA, RNA messengers, microRNAs, proteins, signaling molecules and their cascades, metabolites, cellular communication processes and cellular organization. These data are freely available in valuable resources such as Genbank (http://www.ncbi.nlm.nih.gov/genbank/), Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), Protein Data Bank (http://www.wwpdb.org/), KEGG (http://www.genome.jp/kegg/), MetaCyc (http://metacyc.org/), Reactome (http://www.reactome.org), and many other resources. The clinical world studies diseases, signs, symptoms, drugs, patients, clinical laboratory measurements, and clinical images. The emergence of clinical and health information technologies has begun to make these clinical data available for research through biobanks, electronic medical records, FDA resources about drug labels and adverse events, and claims data. Therefore, a major challenge for translational medicine is to connect the molecular/cellular world with the clinical world. The published literature, available in PubMED (http://www.ncbi.nlm.nih.gov/pubmed), does this, as does the Unified Medical Language System (UMLS) that provides a *lingua franca* (http://www.nlm.nih.gov/research/umls/). However, it falls to translational bioinformatics to engineer the tools that link molecular/cellular entities and clinical entities. Thus, I define "translational bioinformatics" research as the development and application of informatics methods that connect molecular entities to clinical entities.

In this collection, Dr. Kann and colleagues have assembled a wonderful group of authors to introduce the key threads of translational bioinformatics to those new to the field. The collection first provides a conceptual overview of the key data and concepts in the field, and then introduces some of the key methods for informatics discovery and applications. Just by examining the table of contents on the collection page (http://www.ploscollections.org/translationalbioinformatics), it is clear that many exciting and emerging health topics are squarely within the scope of translational bioinformatics: cancer, pharmacogenomics, medical genetics, small molecule drugs, and diseases of protein malfunction. There is an unmistakable

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* E-mail: russ.altman@stanford.edu

flavor of personalized medicine here as well (genome association studies, mining genetic markers, personal genomic data analysis, data mining of electronic records): our molecular and clinical data resources are now allowing us to consider individual variations, and not simply population averages. I congratulate the editors and authors on creating an important collection of articles, and welcome the reader to an exciting field whose challenges and promise are unbounded.

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Education

Chapter 1: Biomedical Knowledge Integration

Philip R. O. Payne*

The Ohio State University, Department of Biomedical Informatics, Columbus, Ohio, United States of America

Abstract: The modern biomedical research and healthcare delivery domains have seen an unparalleled increase in the rate of innovation and novel technologies over the past several decades. Catalyzed by paradigm-shifting public and private programs focusing upon the formation and delivery of genomic and personalized medicine, the need for high-throughput and integrative approaches to the collection, management, and analysis of heterogeneous data sets has become imperative. This need is particularly pressing in the translational bioinformatics domain, where many fundamental research questions require the integration of large scale, multi-dimensional clinical phenotype and bio-molecular data sets. Modern biomedical informatics theory and practice has demonstrated the distinct benefits associated with the use of knowledge-based systems in such contexts. A knowledge-based system can be defined as an intelligent agent that employs a computationally tractable knowledge base or repository in order to reason upon data in a targeted domain and reproduce expert performance relative to such reasoning operations. The ultimate goal of the design and use of such agents is to increase the reproducibility, scalability, and accessibility of complex reasoning tasks. Examples of the application of knowledge-based systems in biomedicine span a broad spectrum, from the execution of clinical decision support, to epidemiologic surveillance of public data sets for the purposes of detecting emerging infectious diseases, to the discovery of novel hypotheses in large-scale research data sets. In this chapter, we will review the basic theoretical frameworks that define core knowledge types and reasoning operations with particular emphasis on the applicability of such conceptual models within the biomedical domain, and then go on to introduce a number of prototypical data integration requirements and patterns relevant to the conduct of translational bioinformatics that can be addressed via the design and use of knowledge-based systems.

This article is part of the "Translational Bioinformatics" collection for *PLOS Computational Biology*.

1. Introduction

The modern biomedical research domain has experienced a fundamental shift towards integrative and translational methodologies and frameworks over the past several years. A common thread throughout the translational sciences are needs related to the collection, management, integration, analysis and dissemination of large-scale, heterogeneous biomedical data sets. However, well-established and broadly adopted theoretical and practical frameworks intended to address such needs are still largely developmental [1–3]. Instead, the development and execution of multi-disciplinary, translational science programs is significantly limited by the propagation of "silos" of both data and knowledge, and a paucity of reproducible and rigorously validated methods that may be used to support the satisfaction of motivating and integrative translational bioinformatics use cases, such as those focusing on the identification of expression motifs spanning bio-molecules and clinical phenotypes.

In order to provide sufficient context and scope to our ensuing discussion, we will define translational science and research per the conventions provided by the National Institutes of Health (NIH) as follows:

> "***Translational research*** *includes two areas of translation. One is the process of applying discoveries generated during research in the laboratory, and in preclinical studies, to the development of trials and studies in humans. The second area of translation concerns research aimed at enhancing the adoption of best practices in the community. Cost-effectiveness of prevention and treatment strategies is also an important part of translational science."* [4]

Several recent publications have defined a ***translational research cycle***, which involves the translational of knowledge and evidence from "the bench" (e.g., laboratory-based discoveries) to "the bedside" (e.g., clinical or public health interventions informed by basic science and clinical research), and reciprocally from "the bedside" back to "the bench" (e.g., basic science studies informed by observations from the point-of-care) [5]. Within this translational cycle, Sung and colleagues [5] have defined two critical blockages that exist between basic science discovery and the design of prospective clinical studies, and subsequently between the knowledge generated during clinical studies and the provision of such evidence-based care in the clinical or public health settings. These are known as the T1 and T2 blocks, respectively. Much of the work conducted under the auspices of the NIH Roadmap initiative and more recently as part of the Clinical and Translational Science Award (CTSA) program is specifically focused on identifying approaches or policies that can mitigate these T1 and T2 blockages, and thus increase the speed and efficiency by which new biomedical knowledge can be realized in terms of improved health and patient outcomes.

The positive outcomes afforded by the close coupling of biomedical informatics

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* E-mail: philip.payne@osumc.edu

What to Learn in This Chapter

- Understand basic knowledge types and structures that can be applied to biomedical and translational science;
- Gain familiarity with the knowledge engineering cycle, tools and methods that may be used throughout that cycle, and the resulting classes of knowledge products generated via such processes;
- An understanding of the basic methods and techniques that can be used to employ knowledge products in order to integrate and reason upon heterogeneous and multi-dimensional data sets; and
- Become conversant in the open research questions/areas related to the ability to develop and apply knowledge collections in the translational bioinformatics domain.

with the translational sciences have been described frequently in the published literature [3,5–7]. Broadly, the critical areas to be addressed by such informatics approaches relative to translational research activities and programs can be classified as belonging to one or more of the following categories:

The management of multi-dimensional and heterogeneous data sets: The modern healthcare and life sciences ecosystem is becoming increasingly data centric as a result of the adoption and availability of high-throughput data sources, such as electronic health records (EHRs), research data management systems (e.g., CTMS, LIMS, Electronic Data Capture tools), and a wide variety of biomolecular scale instrumentation platforms. As a result of this evolution, the size and complexity of data sets that must be managed and analyzed are growing at an extremely rapid rate [1,2,6,8,9]. At the same time, the data management practices currently used in most research settings are both labor intensive and rely upon technologies that have not be designed to handle such multi-dimensional data [9–11]. As a result, there are significant demands from the translational science community for the creation and delivery of information management platforms capable of adapting to and supporting heterogeneous workflows and data sources [2,3,12,13]. This need is particularly important when such research endeavors focus on the identification of linkages between bio-molecular and phenotypic data in order to inform novel systems-level approaches to understanding disease states. Relative to the specific topic area of knowledge representation and utilization in the translational sciences, the ability to address the preceding requirements is largely predicated on the ability to ensure that semantics of such data are well understood [10,14,15]. This is a scenario often referred to as semantic interoperability, and requires the use of informatics-based approaches to map among various data representations, as well as the application of such mappings to support integrative data integration and analysis operations [10,15].

The application of knowledge-based systems and intelligent agents to enable high-throughput hypothesis generation and testing: Modern approaches to hypothesis discovery and testing primarily are based on the intuition of the individual investigator or his/her team to identify a question that is of interest relative to their specific scientific aims, and then carry out hypothesis testing operations to validate or refine that question relative to a targeted data set [6,16]. This approach is feasible when working with data sets comprised of hundreds of variables, but does not scale to projects involving data sets with magnitudes on the order of thousands or even millions of variables [10,14]. An emerging and increasingly viable solution to this challenge is the use of domain knowledge to generate hypotheses relative to the content of such data sets. This type of domain knowledge can be derived from many different sources, such as public databases, terminologies, ontologies, and published literature [14]. It is important to note, however, that methods and technologies that can allow researchers to access and extract domain knowledge from such sources, and apply resulting knowledge extracts to generate and test hypotheses are largely developmental at the current time [10,14].

The facilitation of data-analytic pipelines in in-silico research programs: The ability to execute *in-silico* research programs, wherein hypotheses are designed, tested, and validated in existing data sets using computational methods, is highly reliant on the use of data-analytic "pipelining" tools. Such pipelines are ideally able to support data extraction, integration, and analysis workflows spanning multiple sources, while capturing intermediate data analysis steps and products, and generating actionable output types [17,18]. Such pipelines provide a number of benefits, including: 1) they support the design and execution of data analysis plans that would not be tractable or feasible using manual methods; and 2) they provide for the capture meta-data describing the steps and intermediate products generated during such data analyses. In the case of the latter benefit, the ability to capture systematic meta-data is critical to ensuring that such *in-silico* research paradigms generate reproducible and high quality results [17,18]. There are a number of promising technology platforms capable of supporting such data-analytic "pipelining", such as the caGrid middleware [18]. It is of note, however, that widespread use of such pipeline tools is not robust, largely due to barriers to adoption related to data ownership/security and socio-technical factors [13,19].

The dissemination of data, information, and knowledge generated during the course of translational science research programs: It is widely held that the time period required to translate a basic science discovery into clinical research, and ultimately evidence-based practice or public health intervention can exceed 15 years [2,5,7,20]. A number of studies have identified the lack of effective tools for supporting the exchange of data, information, and knowledge between the basic sciences, clinical research, clinical practice, and public health practice as one of the major contributors to effective and timely translation of novel biological discoveries into health benefits [2]. A number of informatics-based approaches have been developed to overcome such translational impediments, such as web-based collaboration platforms, knowledge representation and delivery standards, public data registries and repositories [3,7,9,21]. Unfortunately, the systematic and regular use of such tools and methods is generally very poor in the translational sciences, again as was the prior case, due to a combination of governance and socio-technical barriers.

At a high level, all of the aforementioned challenges and opportunities correspond to an overarching set of problem statements, as follows:

- Translational bioinformatics is defined by the presence of complex, heterogeneous, multi-dimensional data sets;
- The scope of available biomedical knowledge collections that may be applied to assist in the integration

and analysis of such data is growing at a rapid pace;

- The ability to apply such knowledge collections to translational bioinformatics analyses requires an understanding of the sources of such knowledge, and methods of applying them to reasoning applications; and
- The application of knowledge collections to support integrative analyses in the translational science domain introduces multiple areas of complexity that must be understood in order to enable the optimal selection and use of such resources and methods, as well as the interpretation of results generated via such applications.

2. Key Definitions

In the remainder of this chapter, we will introduce a set of definitions, frameworks, and methods that serve to support the foundational knowledge integration requirements incumbent to the efficient and effective conduct of translational studies. In order to provide a common understanding of key terms and concepts that will be used in the ensuing discussion, we will define here a number of those entities, using the broad context of Knowledge Engineering (KE) as a basis for such assertions. The KE process (Figure 1) incorporates multiple steps:

1. Acquisition of knowledge (KA)
2. Representation of that knowledge (KR) in a computable form
3. Implementation or refinement of knowledge-based agents or applications using the knowledge collection generated in the preceding stages
4. Verification and validation of the output of those knowledge-based agents or applications against one or more reference standards.

In the context of the final phase of the KE cycle, comparative reference standards can include expert performance measures, requirements acquired before designing the knowledge-based system, or requirements that were realized upon implementation of the knowledge-based system. In this regard, verification is the process of ensuring that the knowledge-based system meets the initial requirements of the potential end-user community. In comparison, validation is the process of ensuring that the knowledge-based system meets the realized requirements of the end-user community once a knowledge-based system has been implemented [22]. Furthermore, within the overall KE process, KA can be defined as the sub-process involving the extraction of knowledge from existent sources (e.g., experts, literature, databases, etc.) for the purpose of representing that knowledge in a computable format [23–28].

The KE process is intended to target three potential types of knowledge, as defined below:

- **Conceptual knowledge** is defined in the education literature as a combination of atomic units of information *and* the meaningful relationships between those units. The education literature also describes two other types of knowledge, labeled as procedural and strategic;
- **Procedural knowledge** is a process-oriented understanding of a given problem domain [29–32];
- **Strategic knowledge** is knowledge that is used to operationalize conceptual knowledge into procedural knowledge [31].

The preceding definitions are derived from empirical research on learning and problem-solving in complex scientific and quantitative domains such as mathematics and engineering [30,32]. The cognitive science literature provides a similar differentiation of knowledge types, making the distinction between procedural and de-

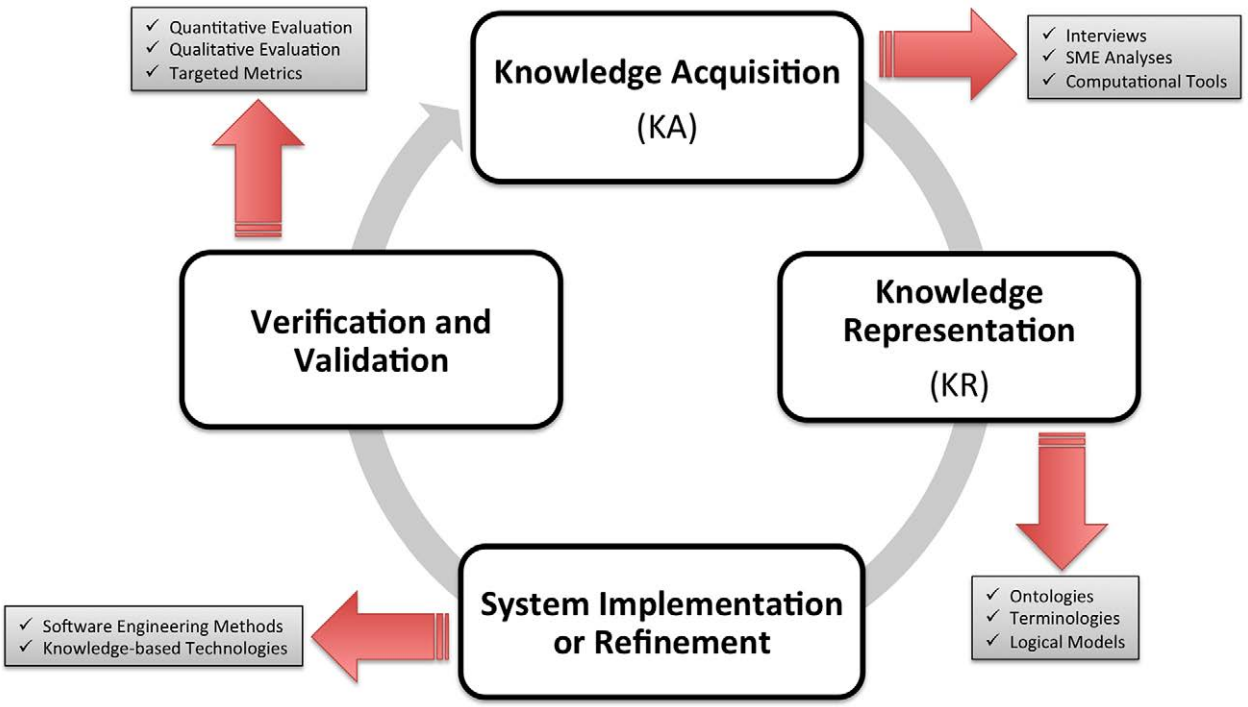

Figure 1. Key components of the KE process.
doi:10.1371/journal.pcbi.1002826.g001

clarative knowledge. Declarative knowledge is synonymous with conceptual knowledge as defined above [33].

Conceptual knowledge collections are perhaps the most commonly used knowledge types in biomedicine. Such knowledge and its representation span a spectrum that includes ontologies, controlled terminologies, semantic networks and database schemas. A reoccurring focus throughout discussions of conceptual knowledge collections in the biomedical informatics domain is the process of representing conceptual knowledge in a computable form. In contrast, the process of eliciting knowledge has received less attention and reports on rigorous and reproducible methods that may be used in this area are rare. It is also important to note that in the biomedical informatics domain conceptual knowledge collections rarely exist in isolation. Instead, they usually occur within structures that contain multiple types of knowledge. For example, a knowledge-base used in a modern clinical decision support system might include: (1) a knowledge collection containing potential findings, diagnoses, and the relationships between them (*conceptual knowledge*), (2) a knowledge collection containing guidelines or algorithms used to logically traverse the previous knowledge structure (*procedural knowledge*), and (3) a knowledge structure containing application logic used to apply or operationalize the preceding knowledge collections (*strategic knowledge*). Only when these three types of knowledge are combined, it is possible to realize a functional decision support system [34].

3. Underlying Theoretical Frameworks

The theories that support the ability to acquire, represent, and verify or validate conceptual knowledge come from multiple domains. In the following sub-section, several of those domains will be discussed, including:

- Computational science
- Psychology and cognitive science
- Semiotics
- Linguistics

3.1 Computational Foundations of Knowledge Engineering

A critical theory that supports the ability to acquire and represent knowledge in a computable format is the physical symbol hypothesis. First proposed by Newell and Simon in 1981 [35], and expanded upon by Compton and Jansen in 1989 [24], the physical symbol hypothesis postulates that knowledge consists of both symbols of reality, and relationships between those symbols. The hypothesis further argues that intelligence is defined by the ability to appropriately and logically manipulate both symbols and relationships. A critical component of this the theory is the definition of what constitutes a "physical symbol system", which Newell and Simon describe as:

> *"…a set of entities, called symbols, which are physical patterns that can occur as components of another type of entity called an expression (or symbol structure). Thus, a symbol structure is composed of a number of instances (or tokens) of symbols related in some physical way (such as one token being next to another). At any instant of time the system will contain a collection of these symbol structures."* [36]

This preceding definition is very similar to that of conceptual knowledge introduced earlier in this chapter, which leads to the observation that the computational representation of conceptual knowledge collections should be well supported by computational theory. However, as described earlier, there is not a large body of research on reproducible methods for eliciting such symbol systems. Consequently, the elicitation of the symbols and relationships that constitute a "physical symbol system", or conceptual knowledge collection, remains a significant challenge. This challenge, in turn, is an impediment to the widespread use of conceptual knowledge-based systems.

3.2 Psychological and Cognitive Basis for Knowledge Engineering

At the core of the currently accepted psychological basis for KE is expertise transfer, which is the theory that humans transfer their expertise to computational systems so that those systems are able to replicate expert human performance.

One theory that helps explain the process of expertise transfer is Kelly's Personal Construct Theory (PCT). This theory defines humans as "anticipatory systems", where individuals create templates, or constructs that allow them to recognize situations or patterns in the "information world" surrounding them. These templates are then used to anticipate the outcome of a potential action given knowledge of similar previous experiences [37]. Kelly views all people as "personal scientists" who make sense of the world around them through the use of a hypothetico-deductive reasoning system.

It has been argued within the KE literature that the constructs used by experts can be used as the basis for designing or populating conceptual knowledge collections [26]. The details of PCT help to explain how experts create and use such constructs. Specifically, Kelly's fundamental postulate is that *"a person's processes are psychologically channelized by the way in which he anticipated events."* This is complemented by the theory's first corollary, which is summarized by his statement that:

> *"Man looks at his world through transparent templates which he creates and then attempts to fit over the realities of which the world is composed… Constructs are used for predictions of things to come… The construct is a basis for making a distinction… not a class of objects, or an abstraction of a class, but a dichotomous reference axis."*

Building upon these basic concepts, Kelly goes on to state in his Dichotomy Corollary that *"a person's construction system is composed of a finite number of dichotomous constructs."* Finally, the parallel nature of personal constructs and conceptual knowledge is illustrated in Kelly's Organization Corollary, which states, *"each person characteristically evolves, for his convenience of anticipating events, a construction system embracing ordinal relationships between constructs"* [26,37].

Thus, in an effort to bring together these core pieces of PCT, it can be argued that personal constructs are essentially templates applied to the creation of knowledge classification schemas used in reasoning. If such constructs are elicited from experts, atomic units of information can be defined, and the Organization Corollary can be applied to generate networks of ordinal relationships between those units. Collectively, these arguments serve to satisfy and reinforce the earlier definition of conceptual knowledge, and provide insight into the expert knowledge structures that can be targeted when eliciting conceptual knowledge.

There are also a number of cognitive science theories that have been applied to inform KE methods. Though usually very similar to the preceding psychological theories, cognitive science theories specifically describe KE within a broader context where humans are anticipatory systems who engage in frequent transfers of expertise. The cognitive science literature identifies expertise transfer pathways as an existent medium for the elicitation of knowledge from domain experts. This conceptual model of expertise transfer is

often illustrated using the Hawkins model for expert-client knowledge transfer [38].

It is also important to note that at a high level, cognitive science theories focus upon the differentiation among knowledge types. As described earlier, cognitive scientists make a primary differentiation between procedural knowledge and declarative knowledge [31]. While cognitive science theory does not necessarily link declarative and procedural knowledge, an implicit relationship is provided by defining procedural knowledge as consisting of three orders, or levels. For each level, the complexity of declarative knowledge involved in problem solving increases commensurately with the complexity of procedural knowledge being used [28,31,39].

A key difference between the theories provided by the cognitive science and psychology domains is that the cognitive science literature emphasizes the importance of placing KA studies within appropriate context in order to account for the distributed nature of human cognition [25,40–46]. In contrast, the psychology literature is less concerned with placing KE studies in context.

3.3 Semiotic Basis for Knowledge Engineering

Though more frequently associated with the domains of computer science, psychology and cognitive science, there are a few instances where semiotic theory has been cited as a theoretical basis for KE. Semiotics can be broadly defined as "*the study of signs, both individually and grouped in sign systems, and includes the study of how meaning is transmitted and understood*" [47]. As a discipline, much of its initial theoretical basis is derived from the domain of linguistics, and thus, has been traditionally focused on written language. However, the scope of contemporary semiotics literature has expanded to incorporate the analysis of meaning in visual presentation systems, knowledge representation models and multiple communication mediums. The basic premise of the semiotic theory of "meaning" is frequently presented in a schematic format using the Ogden-Richards semiotic triad, as shown in Figure 2 [48].

A core component of semiotic triad is the hypothesis that there exist three representational formats for knowledge, specifically:

- **Symbol**: representational artifact of a unit of knowledge (e.g., text or icons).
- **Referent**: actual unit of knowledge, which is largely a conceptual construct.
- **Thought or Reference**: unit of knowledge as actually understood by the individual or system utilizing or acting upon that knowledge.

In addition, three primary relationships are hypothesized to exist, linking the three preceding representational formats:

- **"Stands-for" imputed relation**: relationship between the symbolic representation of the knowledge and the actual unit of knowledge
- **"Refers-to" causal relation**: relationship between the actual unit of knowledge, and the unit of knowledge as understood by the individual or system utilizing or acting upon that knowledge
- **"Symbolizes" causal relation**: relationship between the unit of knowledge as understood by the individual or system utilizing or acting upon that knowledge, and the symbolic representation of the knowledge

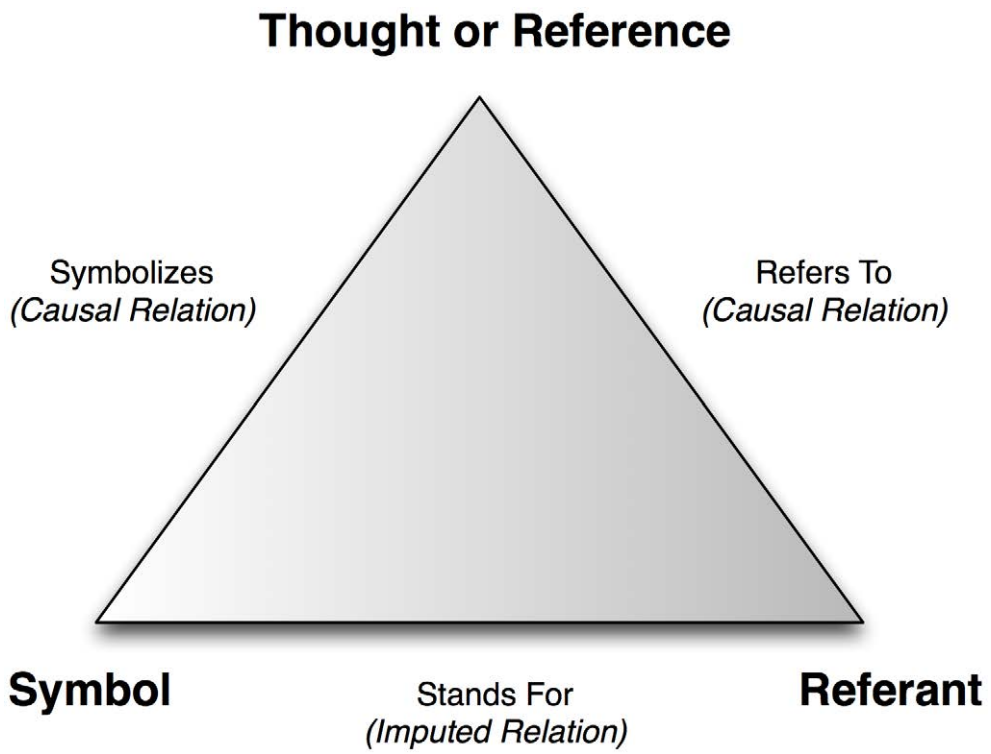

Figure 2. Ogden-Richards semiotic triad, illustrating the relationships between the three major semiotic-derived types of "meaning".
doi:10.1371/journal.pcbi.1002826.g002

- The strength of these relationships is usually evaluated using heuristic methods or criteria [48].

3.4 Linguistic Basis for Knowledge Engineering

The preceding theories have focused almost exclusively on knowledge that may be elicited from domain experts. In contrast, domain knowledge can also be extracted through the analysis of existing sources, such as collections of narrative text or databases. Sub-language analysis is a commonly described approach to the elicitation of conceptual knowledge from collections of text (e.g., narrative notes, published literature, etc.). The theoretical basis for sub-language analysis, known as sub-language theory was first described by Zellig Harris in his work concerning the nature of language usage within highly specialized domains [49]. A key argument of his sub-language theory is that language usage in such highly specialized domains is characterized by regular and reproducible structural features and grammars [49,50]. At an application level, these features and grammars can be discovered through the application of manual or automated pattern recognition processes to large corpora of language for a specific domain. Once such patterns have been discovered, templates may be created that describe instances in which concepts and relationships between those concepts are defined. These templates can then be utilized to extract knowledge from sources of language, such as text [51]. The process of applying sub-language analysis to existing knowledge sources has been empirically validated in numerous areas, including the biomedical domain [50,51]. Within the biomedical domain, sub-language analysis techniques have been extended beyond conventional textual language to also include sub-languages that consist of graphical symbols [52].

4. Knowledge Acquisition Tools and Methods

While a comprehensive review of tools and methods that may be used to facilitate the knowledge acquisition (KA) is beyond the scope of this chapter, in the following section, we will briefly summarize example cases of such techniques in order to provide a general overview of this important area of informatics research, development, and applications.

As was introduced in the preceding section, KA can be defined as the sub-process involving the extraction of knowledge from existent sources (e.g., experts, literature, databases, etc.) for the purpose of representing that knowledge in a computable format [23–28]. This definition also includes the verification or validation of knowledge-based systems that use the resultant knowledge collections [27]. Beyond this basic definition of KA and its relationships to KE, there are two critical characteristics of contemporary approaches to KA that should be noted, as follows:

- By convention within the biomedical informatics domain, KA usually refers to the process of eliciting knowledge specifically for use in "knowledge-bases" (KBs) that are integral to expert systems or intelligent agents (e.g., clinical decision support systems). However, a review of the literature concerned with KA beyond this domain shows a broad variety of application areas for KA, such as the construction of shared database models, ontologies and human-computer interaction models [23,53–57].
- Verification and validation methods are often applied to knowledge-based systems only during the final stage of the KE process. However, such techniques are most effective when employed iteratively throughout the entire KE process. As such, they also become necessary components of the KA sub-process.

Given the particular emphasis of this chapter on the use of conceptual knowledge collections for the purpose of complex integrative analysis tasks, it is important to understand that the KA methods and tools available to support the generation of conceptual knowledge collections can be broadly divided into three complementary classes:

- **Knowledge unit elicitation**: techniques for the elicitation of atomic units of information or knowledge
- **Knowledge relationship elicitation**: techniques for the elicitation of relationships between atomic units of information or knowledge
- **Combined elicitation**: techniques that elicit both atomic units of information or knowledge, and the relationships that exist between them

There are a variety of commonly used methods that target one or more above these KA classes, as summarized below:

4.1 Informal and Structured Interviewing

Interviews conducted either individually or in groups can provide investigators with insights into the knowledge used by domain experts. Furthermore, they can be performed either informally (e.g., conversational exchange between the interviewer and subjects) or formally (e.g., structured using a pre-defined series of questions). The advantages of utilizing such interviewing techniques are that they require a minimal level of resources, can be performed in a relatively short time frame, and can yield a significant amount of qualitative knowledge. More detailed descriptions of interviewing techniques are provided in the methodological reviews provided by Boy [58], Morgan [59], and Wood [60].

4.2 Observational Studies

Ethnographic evaluations, or observational studies are usually conducted in context, with minimal researcher involvement in the workflow or situation under consideration. These observational methods generally focus on the evaluation of expert performance, and the implicit knowledge used by those experts. Examples of observational studies have been described in many domains, ranging from air traffic control systems to complex healthcare workflows [61,62]. One of the primary benefits of such observational methods is that they are designed to minimize potential biases (e.g., Hawthorne effect [63]), while simultaneously allowing for the collection of information in context. Additional detail concerning specific observational and ethnographic field study methods can be found in the reviews provided by John [62] and Rahat [64].

4.3 Categorical Sorting

There are a number of categorical, or card sorting techniques, including Q-sorts, hierarchical sorts, all-in-one sorts and repeated single criterion sorts [65]. All of these techniques involve one or more subjects sorting of a group of artifacts (e.g., text, pictures, physical objects, etc.) according to criteria either generated by the sorter or provided by the researcher. The objective of such methods is to determine the reproducibility and stability of the groups created by the sorters. In all of these cases, sorters may also be asked to assign names to the groups they create. Categorical sorting methods are ideally suited for the discovery of relationships between atomic units of information or knowledge. In contrast, such methods are

less effective for determining the atomic units of information or knowledge. However, when sorters are asked to provide names for their groups, this data may help to define domain-specific units of knowledge or information. Further details concerning the conduct and analysis of categorical sorting studies can be found in the review provided by Rugg and McGeorge [65].

4.4 Repertory Grid Analysis

Repertory grid analysis is a method based on the previously introduced Personal Construct Theory (PCT). Repertory grid analysis involves the construction of a non-symmetric matrix, where each row represents a construct that corresponds to a distinction of interest, and each column represents an element (e.g., unit of information or knowledge) under consideration. For each element in the grid, the expert completing the grid provides a numeric score using a prescribed scale (defined by a left and right pole) for each distinction, indicating the strength of relatedness between the given element-distinction pair. In many instances, the description of the distinction being used in each row of the matrix is stated differently in the left and right poles, providing a frame of reference for the prescribed scoring scale. Greater detail on the techniques used to conduct repertory grid studies can be found in the review provided by Gaines et al. [26].

4.5 Formal Concept Analysis

Formal concept analysis (FCA) has often been described for the purposes of developing and merging ontologies [66,67]. FCA focuses on the discovery of "natural clusters" of entities and entity-attribute pairings [66], where attributes are similar to the distinctions used in repertory grids. Much like categorical sorting, FCA is almost exclusively used for eliciting the relationships between units of information or knowledge. The conduct of FCA studies involves two phases: (1) elicitation of "formal contexts" from subjects, and (2) visualization and exploration of resulting "concept lattices". It is of interest to note that the "concept lattices" used in FCA are in many ways analogous to Sowa's Conceptual Graphs [68], which are comprised of both concepts and labeled relationships. The use of Conceptual Graphs has been described in the context of KR [68–70], as well as a number of biomedical KE instances [48,71–73].

Recent literature has described the use of FCA in multi-dimensional "formal contexts" (i.e., instances where relational structures between conceptual entities cannot be expressed as a single many-valued "formal context"). One approach to the utilization of multi-dimensional "formal contexts" is the agreement context model proposed by Cole and Becker [67], which uses logic-based decomposition to partition and aggregate *n*-ary relations. This algorithmic approach has been implemented in a freely available application named "Tupleware" [74]. Additionally, "formal contexts" may be defined from existing data sources, such as databases. These "formal contexts" are discovered using data mining techniques that incorporate FCA algorithms, such as the open-source TOSCANA or CHIANTI tools. Such algorithmic FCA methods are representative examples of a sub-domain known as Conceptual Knowledge Discovery and Data Analysis (CKDD) [75]. Additional details concerning FCA techniques can be found in the reviews provided by Cimiano et al. [66], Hereth et al. [75], and Priss [76].

4.6 Protocol and Discourse Analysis

The techniques of protocol and discourse analysis are very closely related. Both techniques are concerned with the elicitation of knowledge from individuals while they are engaged in problem-solving or reasoning tasks. Such analyses may be performed to determine the unit of information or knowledge, and relationships between those units of information or knowledge, used by individuals performing tasks in the domain under study. During protocol analysis studies, subjects are requested to "think out loud" (i.e., vocalize internal reasoning and thought processes) while performing a task. Their vocalizations and actions are recorded for later analysis. The recordings are then codified at varying levels of granularity to allow for thematic or statistical analysis [77,78]. Similarly, discourse analysis is a technique by which an individual's intended meaning within a body of text or some other form of narrative discourse (e.g., transcripts of a "think out loud" protocol analysis study) is ascertained by atomizing that text or narrative into discrete units of thought. These "thought units" are then subject to analyses of both the context in which they appear, and the quantification and description of the relationships between those units [79,80]. Specific methodological approaches to the conduct of protocol and discourse analysis studies can be found in the reviews provided by Alvarez [79] and Polson et al. [78].

4.7 Sub-Language Analysis

Sub-language analysis is a technique for discovering units of information or knowledge, and the relationships between them within existing knowledge sources, including published literature or corpora of narrative text. The process of sub-language analysis is based on the sub-language theory initially proposed by Zellig Harris [49]. The process by which concepts and relationships are discovered using sub-language analysis is a two-stage approach. In the first stage, large corpora of domain-specific text are analyzed either manually or using automated pattern recognition techniques, in an attempt to define a number of critical characteristics, which according to Friedman et al. [50] include:

- Semantic categorization of terms used within the sub-language
- Co-occurrence patterns or constraints, and paraphrastic patterns present within the sub-language
- Context-specific omissions of information within the sub-language
- Intermingling of sub-language and general language patterns
- Usage of terminologies and controlled vocabularies (i.e., limited, reoccurring vocabularies) within the sub-language

Once these characteristics have been defined, templates or sets of rules may be established. In the second phase, the templates or rules resulting from the prior step are applied to narrative text in order to discover units of information or knowledge, and the relationships between those units. This is usually enabled by a natural language processing engine or other similar intelligent agent [81–85].

4.8 Laddering

Laddering techniques involve the creation of tree structures that hierarchically organize domain-specific units of information or knowledge. Laddering is another example of a technique that can be used to determine both units of information or knowledge and the relationships between those units. In conventional laddering techniques, a researcher and subject collaboratively create and refine a tree structure that defines hierarchical relationships *and* units of information or knowledge [86]. Laddering has also been reported upon in the context of structuring relationships between domain-specific processes (e.g., procedural knowledge). Therefore, laddering may also be suited for discovering strategic knowledge in the

form of relationships between conceptual and procedural knowledge. Additional information concerning the conduct of laddering studies can be found in the review provided by Corbdridge et al. [86].

4.9 Group Techniques

Several group techniques for multi-subject KA studies have been reported, including brainstorming, nominal group studies, Delphi studies, consensus decision-making and computer-aided group sessions. All of these techniques focus on the elicitation of consensus-based knowledge. It has been argued that consensus-based knowledge is superior to the knowledge elicited from a single expert [27]. However, conducting multi-subject KA studies can be difficult due to the need to recruit appropriate experts who are willing to participate, or issues with scheduling mutually agreeable times and locations for such groups to meet. Furthermore, it is possible in multi-subject KA studies for a forceful or coercive minority of experts or a single expert to exert disproportionate influence on the contents of a knowledge collection [25,27,59,87]. Additional detail concerning group techniques can be found in reviews provided by Gaines [26], Liou [27], Morgan [59], Roth [88], and Wood [60].

5. Integrating Knowledge in the Translational Science Domain

Building upon the core concepts introduced in Section 1–4, in the remainder of this chapter we will synthesize the requirements, challenges, theories, and frameworks discussed in the preceding sections, in order to propose a set of methodological approaches to the data, information, and knowledge integration requirements incumbent to complex translational science projects. We believe that it is necessary to design and execute informatics efforts in such context in a manner that incorporates tasks and activities related to: 1) the identification of major categories of information to be collected, managed and disseminated during the course of a project; 2) the determination of the ultimate data and knowledge dissemination requirements of project-related stake-holders; and 3) the systematic modeling and semantic annotation of the data and knowledge resources that will be used to address items (1) and (2).

Based upon prior surveys of the state of biomedical informatics relative to the clinical and translational science domains [3,89], a framework that is informative to preceding design and execution pattern can be formulated. Central to this framework are five critical information or knowledge types involved in the conduct of translational science projects, as are briefly summarized in Table 1.

The preceding framework of information and knowledge types ultimately informs a conceptual model for knowledge integration in the translational sciences.

Table 1. Overview of information and knowledge types incumbent to the translational sciences.

Information or Knowledge Type	Description	Examples Sources or Types
Individual and/or Population Phenotype	This information type involves data elements and metadata that describe characteristics at the individual or population levels that relate to the physiologic and behavioral manifestation of healthy and disease states.	• Demographics • Clinical exam findings • Qualitative characteristics • Laboratory testing results
Individual and/or Population Bio-markers	This information type involves data elements and metadata that describe characteristics at the individual or population levels that relate to the bio-molecular manifestation of healthy and disease states.	• Genomic, proteomic and metabolomic expression profiles • Novel bio-molecular assays capable of measuring bio-molecular structure and function
Domain Knowledge	This knowledge type is comprised of community-accepted, or otherwise verified and validated [17] sources of biomedical knowledge relevant to a domain of interest. Collectively, these types of domain knowledge may be used to support multiple operations, including: 1) hypothesis development; 2) hypothesis testing; 3) comparative analyses; or 4) augmentation of experimental data sets with statistical or semantic annotations [15,17,125].	• Literature databases • Public or private databases containing experimental results or reference standards • Ontologies • Terminologies
Biological Models and Technologies	This knowledge type typically consists of: 1) empirically validated system or sub-system level models that serve to define the mechanisms by which bio-molecular and phenotypic processes and their markers/indicators interact as a network [6,20,124,126]; and 2) novel technologies that enable the analysis of integrative data sets in light of such models. By their nature these tools include algorithmic or embedded knowledge sources [124,126].	• Algorithms • Quantitative Models • Analytical "Pipelines" • Publications
Translational Biomedical Knowledge	Translational biomedical knowledge represents a sub-type of general biomedical knowledge that is concerned with a systems-level synthesis (i.e., incorporate quantitative, qualitative, and semantic annotations) of pathophysiologic or biophysical processes or functions of interest (e.g., pharmacokinetics, pharmacodynamics, bionutrition, etc.), and the markers or other indicators that can be used to instrument and evaluate such models.	• Publications • Guidelines • Integrative Data Sets • Conceptual Knowledge Collections

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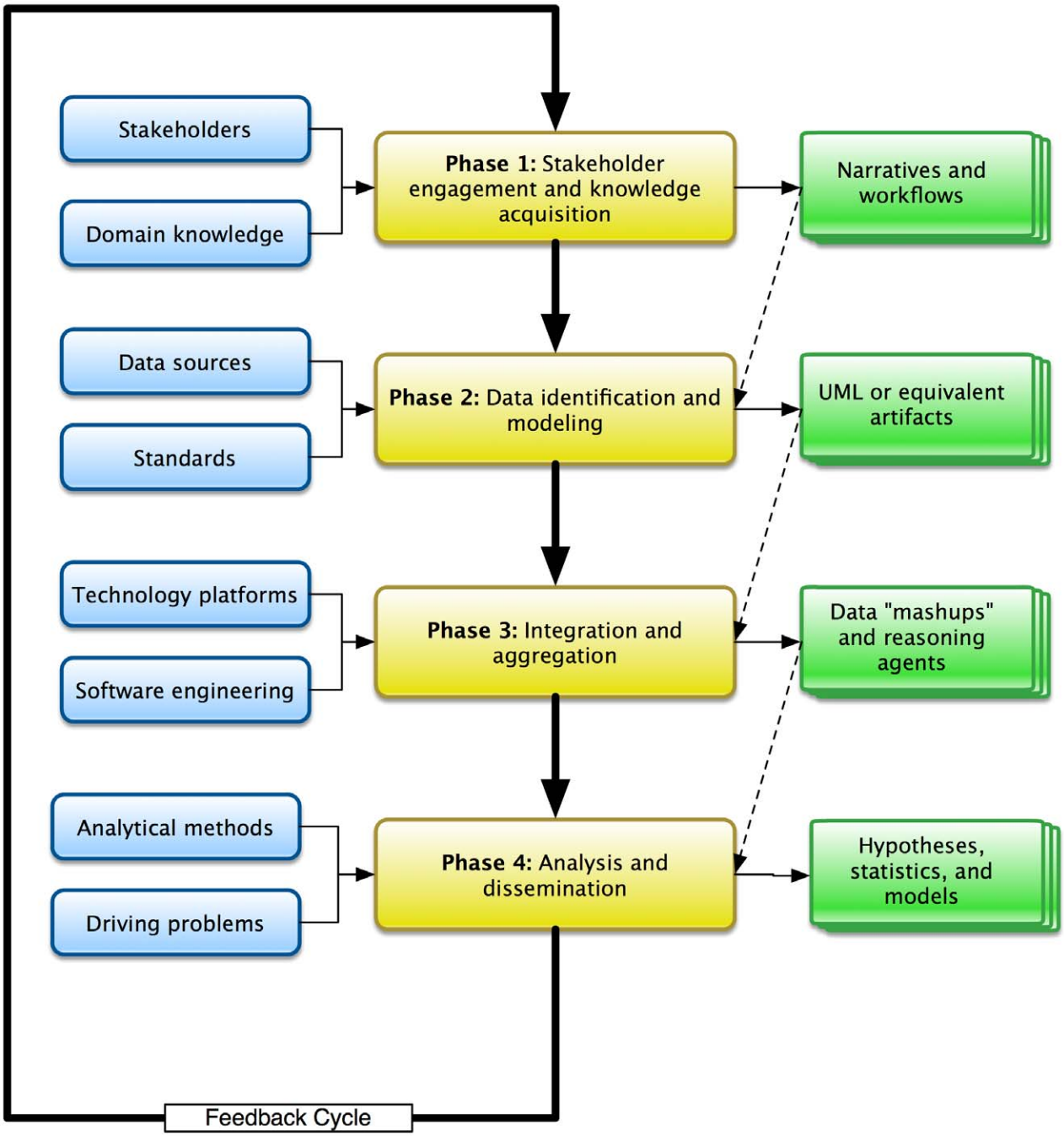

Figure 3. Practical model for the design and execution of translational informatics projects, illustrating major phases and exemplary input or output resources and data sets.
doi:10.1371/journal.pcbi.1002826.g003

The role of Biomedical Informatics and KE in this framework is to address the four major information management challenges enumerated earlier relative to the ability to generate ***Translational Biomedical Knowledge***, namely: 1) the collection and management of high throughput, multi-dimensional data; 2) the generation and testing of hypotheses relative to such integrative data sets; 3) the provision data analytic "pipelines"; and 4) the dissemination of knowledge collections resulting from research activities.

5.1 Design Pattern for Translational Science Knowledge Integration

Informed by the conceptual framework introduced in the preceding section and illustrated in Figure 3, we will now summarize the design and execution pattern used to address such knowledge integration requirements. This design pattern can be broadly divided into four major phases that collectively define a cyclical and iterative process (which we will refer to as a ***translational research cycle***,). For each phase of the pattern, practitioners must consider both the required inputs and anticipated outputs, and their interrelationships between and across phases.

Phase 1 - Stakeholder engagement and knowledge acquisition: During this initial phase, key stakeholders who will be involved in the collection, management, analysis, and dissemination of project-specific data and knowledge are identified and engaged in both formal and informal knowledge acquisition, with the ultimate goal of defining the essential workflows, processes, and data sources (including their semantics). Such knowledge acquisition usually requires the use of ethnographic, cognitive science, workflow modeling, and formal knowledge acquisition techniques [14]. The results of such activities can be formalized using a thematic narratives [90–92] and workflow or process artifacts [92–94]. In some instances, it may be necessary to engage domain-specific subject matter experts (SMEs) who are not involved in a given project in order to augment available SMEs, or to validate the findings generated during such activities [14,92].

Phase 2 - Data identification and modeling: Informed by the artifacts generated in Phase 1, in this phase, we focus upon the identification of specific, pertinent data sources relative to project aims, and the subsequent creation of models that encapsulate the physical and semantic representations of that data. Once pertinent data sources have been identified, we must then model their contents in an implementation-agnostic manner, an approach that is most frequently implemented using model-driven architecture techniques [95–99]. The results of such MDA processes are commonly recorded using the Unified Modeling Language (UML) [16,100–102]. During the modeling process, it is also necessary to identify and record semantic or domain-specific annotation of targeted data structures, using locally relevant conceptual knowledge collections (such as terminologies and ontologies), in order to enable deeper, semantic reasoning concerning such data and information [16,103,104].

Phase 3 - Integration and aggregation: A common approach to the integration of heterogeneous and multi-dimensional data is the use of technology-agnostic domain or data models (per Phases 1–2), incorporating semantic annotations, in order to execute data federation operations [105] or to transform that data and load it into an integrative repository, such as a data warehouse [106–108]. Once the mechanisms needed to integrate such disparate data sources are implemented, it is then possible to aggregate the data for the purposes of hypothesis discovery and testing – a process that is sometimes referred to as creating a data "mashup" [109–115]. Data "mashups" are often created using a variety of readily available reasoners, such as those associated with the semantic web [109–115], which directly employ both the data models and semantic annotations created in the prior phases of the Translational Informatics Cycle, and enable a knowledge-anchored approach to such operations.

Phase 4 - Analysis and dissemination: In this phase of the Translational Informatics Cycle, the integrated/aggregated data and knowledge created in the preceding phases is subject to analysis. In most if not all cases, these analyses make use of domain or task specific applications and algorithms, such as those implemented in a broad number of biological data analysis packages, statistical analysis applications, and data mining tools, and intelligent agents. These types of analytical tools are used to address questions pertaining to one or more of the following four basic query or data interrogation patterns: 1) to generate hypotheses concerning relationships or patterns that serve to link variables of interest in a data set [116]; 2) to evaluate the validity hypotheses and the strength of their related data motifs, often using empirically-validated statistical tests [117,118]; 3) to visualize complex data sets in order to facilitate human-based pattern recognition [119–121]; and 4) to infer and/or verify and validate quantitative models that formalize phenomena of interest identified via the preceding query patterns [122,123].

6. Open Research Questions and Future Direction

As can be ascertained from the preceding review of the theoretical and practice bases for the integration of data and knowledge in the translational science domain, such techniques and frameworks have significant potential to positively impact the speed, efficacy, and impact of such research programs, and to enable novel scientific paradigms that would not otherwise be tractable. However, there are a number of open and ongoing research and development questions being addressed by the biomedical informatics community relative to such approaches that should be noted:

Dimensionality and granularity: the majority of knowledge integration techniques being designed, evaluated, and applied relative to the context of the translational science domain target low-order levels of dimensionality (e.g., the integration of data and knowledge corresponding to a single type, per the definitions set forth in Table 1). However, many translational science problem spaces require reasoning across knowledge-types and data granularities (e.g., multidimensional data and knowledge collections). The ability to integrate and reason upon data in a knowledge-anchored manner that addresses such multi-dimensional context remains an open area of research. Many efforts to address this gap in knowledge and practice rely upon the creation of semantically typed "vertical" linkages spanning multiple integrative knowledge networks, as is illustrated in Figure 4.

Scalability: Similar to the challenge of dimensionality and granularity, the issue of scalability of knowledge integration methods also remains an open area of research and development. Specifically, a large number of available knowledge integration techniques rely upon semi-automated or human-mediated methods or activities, which significantly curtail the scalability of such approaches to large-scale problems. Much of the research targeting this gap in knowledge and practice has focused on the use of artificial intelligence and semantic-reasoning technologies to enable the extraction, disambiguation, and application of conceptual knowledge collections.

Reasoning and visualization: Once knowledge and data have been aggregated and made available for hypothesis discovery and testing, the ability to reason upon and visualize such "mashups" is highly desirable. Current efforts to provide reusable methods of doing so, such as the tools and technologies provided by the semantic web community, as well as visualization techniques being explored by the computer science and human-computer interaction communities, hold significant promise in addressing such needs, but are still largely developmental.

Applications of knowledge-based systems for *in-silico* science paradigms: As has been discussed throughout this collection, a fundamental challenge in Translational Bioinformatics is the ability to both ask and answer the full spectrum of questions possible given a large-scale and multi-dimensional data set. This challenge is particularly pressing at the confluence of high-throughput bio-molecular measurement methods and the translation of the findings generated by such approaches to clinical research or practice. Broadly speaking, overcoming this challenge requires a paradigm that can be described as *in-silico* science, in which informatics

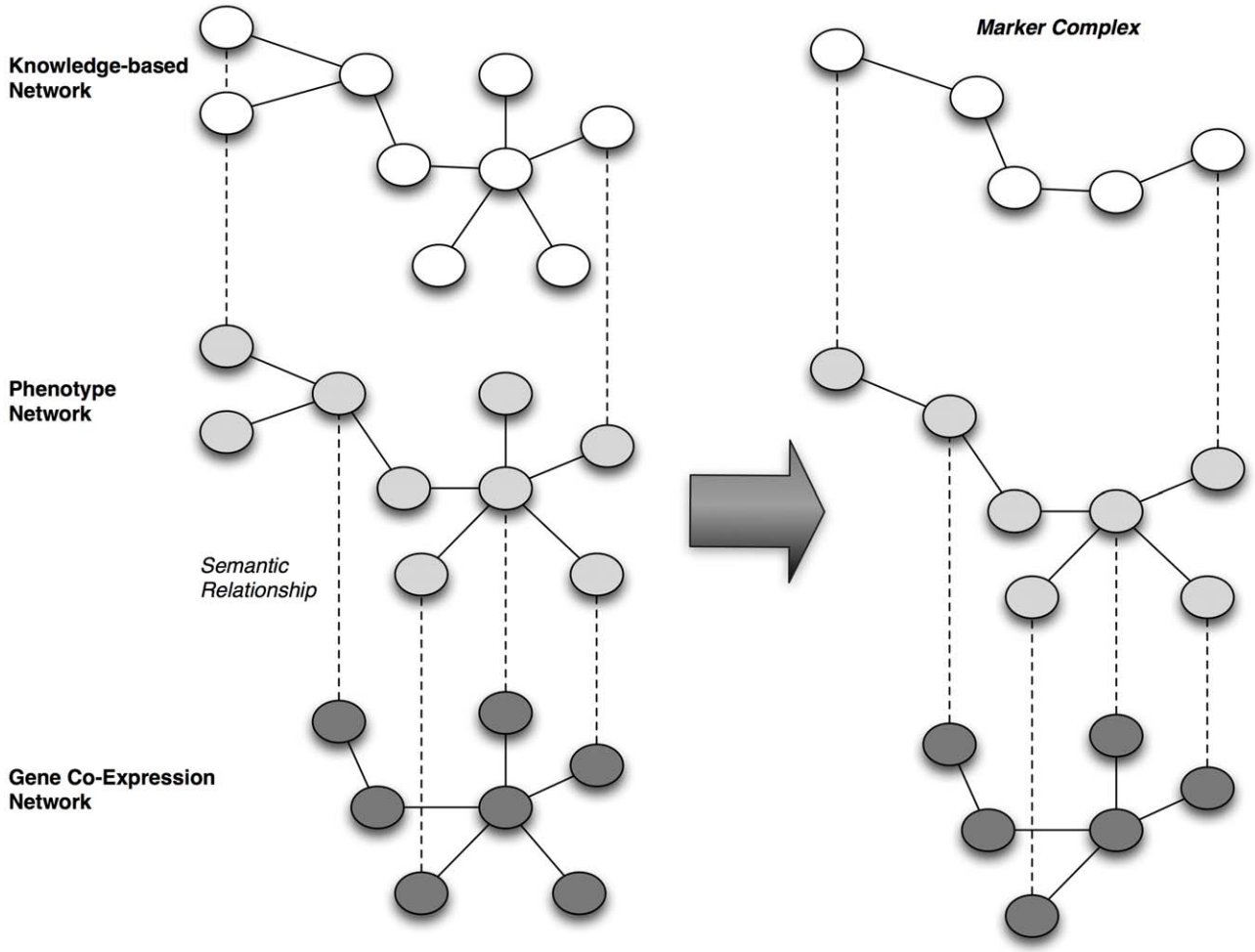

Figure 4. Conceptual model for the generation of multi-network complexes of markers spanning a spectrum of granularity from bio-molecules to clinical phenotypes.
doi:10.1371/journal.pcbi.1002826.g004

methods are applied to generate and test integrative hypotheses in a high-throughput manner. Such techniques require the development and use of novel KA and KR methods and structures, as well as the design and verification/validation of knowledge-based systems targeting the aforementioned intersection point. There are several exemplary instances of investigational tools and projects targeting this space, including RiboWeb, BioCyc, and a number of initiatives concerned with the modeling and analysis of complex biological systems [6,113,114,120,124]. In addition, there are a number of large-scale conceptual knowledge collections focusing on this particular area that can be explored as part of the repositories maintained and curated by the National Center for Biomedical Ontologies (NCBO). However, broadly accepted methodological approaches and knowledge collections related to this area generally remain developmental.

7. Summary

As was stated at the outset of this chapter, our goals were to review the basic theoretical frameworks that define core knowledge types and reasoning operations with particular emphasis on the applicability of such conceptual models within the biomedical domain, and to introduce a number of prototypical data integration requirements and patterns relevant to the conduct of translational bioinformatics that can be addressed via the design and use of knowledge-based systems. In doing so, we have provided:

- Definitions of the **basic knowledge types and structures** that can be applied to biomedical and translational research;
- An overview of the **knowledge engineering cycle**, and the products generated during that cycles;
- Summaries of basic **methods, techniques, and design patterns that can be used to employ knowledge products** in order to integrate and reason upon heterogeneous and multi-dimensional data sets; and
- An introduction to the **open research questions and areas related to the ability to apply knowledge collections** and knowledge-anchored reasoning processes across multiple networks or knowledge collections.

Given that the translational bioinformatics is defined by the presence of complex, heterogeneous, multi-dimensional data sets, and in light of the growing volume of biomedical knowledge collections, the ability to apply such knowledge collections to biomedical data sets requires an understanding of the sources of such knowledge, and methods of applying them to reasoning applications. Ultimately,

these approaches introduce both significant opportunities to advance the state of translational science, while simultaneously adding areas of complexity to the design of translational bioinformatics studies, including the methods needed to reason in an integrative manner across multiple networks or knowledge constructs. As such, these theories, methods, and frameworks offer significant benefits as well as a number of exciting and ongoing areas of biomedical informatics research and development.

8. Exercises

Instructions: Read the following motivating use case and then perform the tasks described in each question. The objective of this exercise is to demonstrate how available and open-access knowledge discovery and reasoning tools can be used to satisfy the information needs incumbent to biomedical knowledge integration needs commonly encountered in the clinical and translational research environment.

Use Case: *The ability to identify potentially actionable phenotype-to-biomarker relationships is of critical importance in the translational science domain. In the specific context of integrative cancer research, it is regularly the case that structural and functional relationships between genes, gene products, and clinical phenotypes are used to design and evaluate diagnostic and therapeutic approaches to targeted disease states. Large volumes of domain specific conceptual knowledge related to such hypothesis generation processes can be found in publically available literature corpora and ontologies.*

1) **Task One:** Select a specific cancer and perform a search for a collection of recent literature available with full free text via PubMed Central (the resulting corpora should contain 5 manuscripts published within the last three years, selected based upon their publication dates beginning with the most recent articles/manuscripts). Download the free text for each such article.
2) **Task Two:** For each full text article in the corpora established during Task One, semantically annotate genes, gene products, and clinical phenotype characteristics as found in the Abstract, Introduction, and Conclusion (or equivalent) sections using applicable Gene Ontology (GO) concepts, using the NCBO annotator found at: http://bioportal.bioontology.org/annotator)
3) **Task Three:** Identify the top 10 most frequently occurring Gene Ontology (GO) concepts found in your annotations, per Task Two. For each such concept, perform a search of PubMed Central for articles in which both the appropriate terms describing the cancer selected in Task One as well as these concepts co-occur. For the top 5 (most recent) articles retrieved via each search, retrieve the associate abstract for subsequent analysis
4) **Task Four:** Using the NCBO Ontology Recommender (http://bioportal.bioontology.org/recommender), analyze each of the abstracts retrieved in Task Three to determine the optimal ontology for annotating those abstracts, noting the top "recommended" ontology for each such textual resource.
5) **Task Five:** For each abstract identified in Step Three, again using the NCBO annotator (found at: http://bioportal.bioontology.org/annotator), annotate those abstracts using the recommended ontologies identified in Step Four (selecting only those ontologies that are also reflects in the NLM's UMLS). Then, for the top 2–3 phenotypic (e.g., clinically relevant) concepts identified via that annotation process, use the UMLS UTS (https://uts.nlm.nih.gov/) in order to identify potential phenotype-genotype pathways linking such phenotypic concepts and the genes or gene products identified in Task Two. Please note that performing this task will require exploring multiple relationship types reflected in the UMLS metathesaurus (documentation concerning how to do perform such a search can be found here: http://www.ncbi.nlm.nih.gov/books/NBK9684/).

Answers to the Exercises can be found in Text S1.

Supporting Information

Text S1 Answers to Exercises
(DOCX)

Further Reading

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Glossary

- **Data:** factual information (as measurements or statistics) used as a basis for reasoning, discussion, or calculation [127]
- **Information:** knowledge obtained from investigation, study, or instruction [127]
- **Knowledge:** the circumstance or condition of apprehending truth or fact through reasoning [127]
- **Knowledge engineering:** a branch of artificial intelligence that emphasizes the development and use of expert systems [128]
- **Knowledge acquisition:** the act of acquiring knowledge
- **Knowledge representation:** the symbolic formalization of knowledge
- **Conceptual knowledge**: knowledge that consists of atomic units of information and meaningful relationships that serve to interrelate those units.
- **Strategic knowledge:** knowledge used to infer procedural knowledge from conceptual knowledge.
- **Procedural knowledge:** knowledge that is concerned with a problem-oriented understanding of how to address a given task or activity.
- **Terminology:** the technical or special terms used in a business, art, science, or special subject [128]
- **Ontology:** a rigorous and exhaustive organization of some knowledge domain that is usually hierarchical and contains all the relevant entities and their relations [128]
- **Multi-dimensional data:** data spanning multiple levels or context of granularity or scope while maintaining one or more common linkages that span such levels
- **Motif:** a reproducible pattern
- **Mashup:** a combination of multiple, heterogeneous data or knowledge sources in order to create an aggregate collection of such elements or concepts.
- **Intelligent agent:** a software agent that employs a formal knowledge-base in order to replicate expert performance relative to problem solving in a targeted domain.
- **Clinical phenotype:** the observable physical and biochemical characteristics of an individuals that serve to define clinical status (e.g., health, disease)
- **Biomarker:** a bio-molecular trait that can be measure to assess risk, diagnosis, status, or progression of a pathophysiologic or disease state.

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Chapter 2: Data-Driven View of Disease Biology

Casey S. Greene, Olga G. Troyanskaya*

Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, New Jersey, United States of America

Abstract: Modern experimental strategies often generate genome-scale measurements of human tissues or cell lines in various physiological states. Investigators often use these datasets individually to help elucidate molecular mechanisms of human diseases. Here we discuss approaches that effectively weight and integrate hundreds of heterogeneous datasets to gene-gene networks that focus on a specific process or disease. Diverse and systematic genome-scale measurements provide such approaches both a great deal of power and a number of challenges. We discuss some such challenges as well as methods to address them. We also raise important considerations for the assessment and evaluation of such approaches. When carefully applied, these integrative data-driven methods can make novel high-quality predictions that can transform our understanding of the molecular-basis of human disease.

This article is part of the "Translational Bioinformatics" collection for *PLOS Computational Biology*.

1. Introduction

Researchers are using genome-scale experimental methods (i.e. approaches that assay hundreds or thousands of genes at a time) to probe the molecular mechanisms of normal biological processes and disease states across systems from cell culture to human tissue samples. Data of this scale can provide a great deal of information about the process or disease of interest, the tissue of origin, and the metabolic state of the organism, among other factors. To understand biological processes on a systems level one must combine data from measurements across different molecular levels (e.g. proteomic, metabolomic, and genomic measurements) while incorporating data from diverse experiments within each individual level. An effective integrative analysis will take advantage of these data to develop a systems level understanding of diseases or tissues.

Human genome-scale experimental data include microarrays [1,2,3], genome-wide association studies [4,5], and RNA interference screens [6,7] among many other experimental designs [8]. These experiments range from those targeted towards tissue specificity [9] to those targeted towards specific diseases such as cancer [10]. The NCBI Gene Expression Omnibus (GEO) [11], a database of microarrays alone, contains over 700 human datasets collected under diverse experimental conditions encompassing more than 8000 individual arrays. The human PeptideAtlas [12], a similar resource for proteomics experiments, currently contains almost 6.7 million MS/MS spectra representing almost 84,000 non-singleton peptides across 220 samples. In addition to these high throughput experiments, there are databases of biochemical pathways [13], gene function [14], pharmacogenomics [15], and protein-protein interactions [16,17,18].

Integrating heterogeneous genome-scale experiments and databases is a challenging task. Beyond the straightforward concern of experimental noise in each individual dataset, integrative approaches also face particular challenges inherent to the process of unifying heterogeneous data types. Specifically we are concerned with biological and computational sources of heterogeneity. Biological heterogeneity among experiments emerges from the measurement of many different processes or the unique probing of biological systems. The source of biological material (e.g. whether experiments measure cells in culture or biopsied tissues) can also lead to systematic biological heterogeneity. Computational heterogeneity (e.g. some datasets have discrete value measurements while others are continuous) comes from the diversity of experimental platforms used to assay biological processes. Integrative approaches that bring together diverse data types and experiments must address the challenge of effectively combining these data for inference.

There are many strategies for combining these diverse and heterogeneous data. These include ridge regression [19,20], Bayesian inference [21,22,23,24,25], expectation maximization [26], and support vector machines [27]. This chapter focuses on the strategy of Bayesian integration, which is capable of both predicting the probability of an interaction between gene pairs and providing information on the contribution of each experiment to that prediction. Bayesian integration allows for datasets to be combined based on the strength of evidence from individual datasets, which can be either learned from the data [28] or expert annotated [29]. Intuitively the Bayesian strategy works by evaluating the accuracy and coverage of each individual dataset and the relevance of each source of data to the disease or tissue of interest and using this information to weight each dataset's impact on resulting predictions. Here we discuss Bayesian methods that infer genome-scale functional relationship networks from high throughput experimental data by building on exiting gold standards. We discuss how these methods work, how to develop high quality gold standards, and how to evaluate networks of predicted functional relationships.

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* E-mail: ogt@genomics.princeton.edu

What to Learn in This Chapter

- What a functional relationship network represents.
- The fundamentals of Bayesian inference for genomic data integration.
- How to build a network of functional relationships between genes using examples of functionally related genes and diverse experimental data.
- How computational scientists study disease using data driven approaches, such as integrated networks of protein-protein functional relationships.
- Strategies to assess predictions from a functional relationship network

2. Combining Diverse Data Using Bayesian Inference

Bayesian inference is a powerful tool that can be used to make predictions based on experimental evidence. If we want to calculate the probability that a gene of unknown function is involved in a disease, we can begin by developing a list of genes known to be involved in the disease (positive examples) and a list of genes not involved in the disease (negative examples). These positive and negative examples are termed a "gold standard" in the field of machine learning. Figure 1 shows, under three conditions, how the measurements for positive genes and negative genes are distributed in datasets measuring three hypothetical conditions. From this, we can observe that genes having a higher (more to the right) score in Condition A and a lower (more to the left) score in Condition C appear to be involved in the disease.

Bayesian inference allows us to use these distributions to quantify the probability that a gene is involved in disease given these data. Table 1 shows experimental results from Condition A where the median has been used to divide the continuous values into discrete bins.

From this contingency table we can calculate the probability that a gene i is involved in disease, $P(D_i)$, given the experimental results for gene i, E_i. Mathematically this can be written as $P(\mathrm{D}_i|\mathrm{E}_i)$. Bayes' theorem states that

$$P(\mathrm{D}_i|\mathrm{E}_i)=\frac{P(\mathrm{E}_i|\mathrm{D}_i)P(\mathrm{D}_i)}{P(\mathrm{E}_i)}.$$

The probability that a gene is involved in disease ignoring any evidence, $P(\mathrm{D}_i)$, is known as the prior probability. We can conservatively estimate this as, for instance, the proportion of positive examples to the proportion of total genes. If the organism of interest has 20,000 genes, this would be

$$P(\mathrm{D}_i)=\frac{\text{Positive Examples}}{\text{Genes in Organism}}=\frac{200}{20{,}000}=0.01.$$

This is likely to be too conservative as it assumes that there are no unknown genes that are involved in the disease of interest. In practice, however, as evidence accumulates the impact of the prior probability on individual predictions is diminished.

With knowledge of the state of gene i in Condition A we can calculate $P(\mathrm{E}_i|D_i)$. In this example, assume that the measurement for gene i is above the median. This probability of observing the experimental result for gene i given that a gene is involved in disease can be calculated as

$$P(\mathrm{E}_i|D_i)=\frac{\text{Positive Examples Above Median}}{\text{Positive Examples}}=\frac{150}{200}=0.75.$$

The final component of this formula is the probability of observing the experimental result that was observed for gene i, $P(\mathrm{E}_i)$. This value is the proportion of genes from the standard measured above the median to the total number of genes in the standard,

$$P(\mathrm{E}_i)=\frac{\text{Above Median}}{\text{Total in Standard}}=\frac{211}{422}=0.5.$$

It is important to note that, if the prior is adjusted from the proportion observed in the data, $P(\mathrm{E}_i)$ must also be adjusted to present the probability of the evidence under the new prior. With these components we can calculate the probability of disease given the experimental evidence for gene i as

$$P(\mathrm{D}_i|\mathrm{E}_i)=\frac{P(\mathrm{E}_i|\mathrm{D}_i)P(\mathrm{D}_i)}{P(\mathrm{E}_i)}=\frac{0.75\times0.01}{0.5}=0.015.$$

This probability is still small in large part due to our conservative prior, but by assuming that experimental results from different datasets are independent, we can perform this same calculation for gene i in experimental condition B using this probability as the prior, and the calculation for condition C using the probability from condition B as the prior. This procedure exploits Bayes' theorem to bring together diverse evidence sources through the common framework of probabilities.

3. Defining a Functional Relationship Gold Standard

Going beyond gene lists to networks of genes requires a different type of gold standard. While the inference approach described in Section 2 can be used to implicate genes in a disease or process, the specific roles of those genes remain unclear. In the strategy from Section 2, positive and negative genes make up the

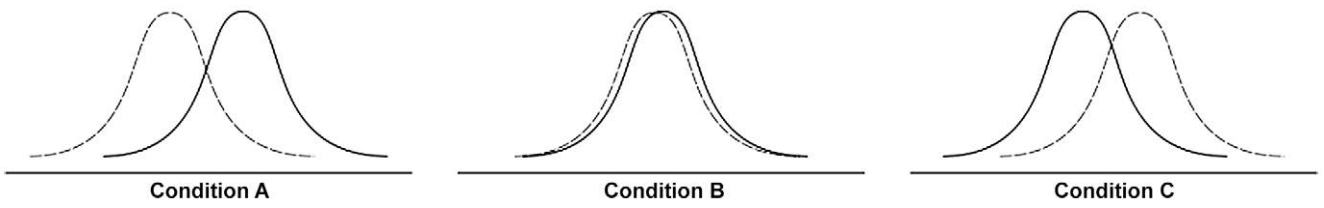

Figure 1. Potential distributions of experimental results obtained for datasets collected under three different conditions. The dotted line indicates the distribution of negative examples and the solid line indicates the distribution of positive examples. In condition A the positive examples more often occur to the right of the negative examples, in condition B both sets overlap, and in condition C the positive examples occur more often to the left of the negative examples.
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Table 1. A contingency table for the experimental results for Condition A.

	Below Median	Above Median	Total
Positive Examples	50	150	200
Negative Examples	161	61	222
Total	211	211	422

Genes are discretized into values above or below the median. The numbers of positive and negative examples come from the gold standard. These values can be used to predict the probability that a gene with unknown status is involved in the disease.
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gold standard. By building a gold standard of positive and negative relationships, it becomes possible to predict whether or not a pair of genes interacts.

As with all machine learning strategies, the gold standard determines what type of relationship can be discovered. Here we will describe the process of building a gold standard of functional relationships, but a different standard of only physical or only metabolic interactions could be used to develop a network with those types of connections. Here we define two genes as having a functional relationship if they work together to carry out a biological process (e.g. a KEGG pathway) that can be assayed by definitive experimental follow-up. This definition allows us to capture diverse types of relationships, while discovering relationships suitable for biological follow-up. The Gene Ontology's biological process ontology provides annotations of genes to process, but includes both very broad and very narrow processes. Two examples of broad terms would be "biological regulation" and "response to stimulus." Two examples of narrow terms would be "positive regulation of cell growth involved in cardiac muscle cell development" and "cell-matrix adhesion involved in tangential migration using cell-cell interactions." The broad terms are not specific enough to provide a meaningful gold standard, while the narrow terms have too few annotations to provide sufficient examples of known relationships.

To address this shortcoming, Myers et al. [30] used a panel of experts to select terms from the biological process ontology that were appropriate for confirmation or refutation through laboratory experiments such as "response to DNA damage stimulus" and "aldehyde metabolism." These terms can be downloaded and used to build a positive functional relationship standard. Gene pairs where both pairs share one of these terms can be considered to have a functional relationship. Gene pairs which do not share an annotation are of unknown status. For Bayesian inference we must also have a negative standard. One potential way to develop a negative standard would be to randomly select pairs of genes. This assumes that most pairs of genes do not interact.

It is possible to add additional high quality experimentally annotated relationships to these standards from other databases. Databases like KEGG [13], Reactome [31], and HPRD [32] have previously been used to identify additional functional relationships [33]. The positive and negative relationships from the standard determine the type of relationship that will be predicted by the Bayesian integration. Here we use functional relationships, but a gold standard built strictly from physical protein-protein interactions will infer only physical interactions relationships between genes.

4. Building a Network of Functionally Related Genes

Given a gold standard of gene-gene relationships, the probability that two genes of unknown status have a relationship can be calculated from diverse data using Bayesian inference. The process is similar to the integration process described for single-gene prediction, but there are differences. For each dataset, appropriate scores for each gene pair must be calculated. Furthermore, these scores should not require any manual intervention or adjustment that would make an analysis of hundreds or thousands of datasets time consuming. For datasets that are naturally made up of pair-wise scores such as yeast two-hybrid assays, this task is straightforward. For datasets made up of individual gene measurements, such as microarray experiments, a useful measure must be found.

One measure that can provide pair-wise scores across arrays is correlation. Correlation quantifies the amount that two genes vary together and can be a useful indicator of functional relationships. Comparing correlation across datasets in a regular manner is difficult however, because datasets may display more or less correlation based on both true biology (e.g. under some conditions more genes vary together) or experimental error (e.g. systematic biases due to hybridization conditions) and the variance of gene-wise correlations would vary based on these dataset dependent effects. Fisher's z-transform provides a means to convert these correlation coefficients (r) to z-scores by calculating z as

$$z = \frac{1}{2}\ln\frac{1+r}{1-r}.$$

These z-scores provide a familiar framework to work with correlation and allow correlation measures between genes to be compared across datasets. It is then possible to categorize genes pairs as negatively correlated, uncorrelated, or positively correlated based on whether their z-score is less than, approximately equal to, or greater than zero.

These pairs can then be used as evidence in an integration. In the single

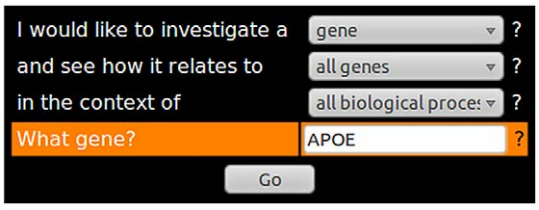

Figure 2. An example of querying HEFalMp for the role of APOE across all biological processes (http://hefalmp.princeton.edu/).
doi:10.1371/journal.pcbi.1002816.g002

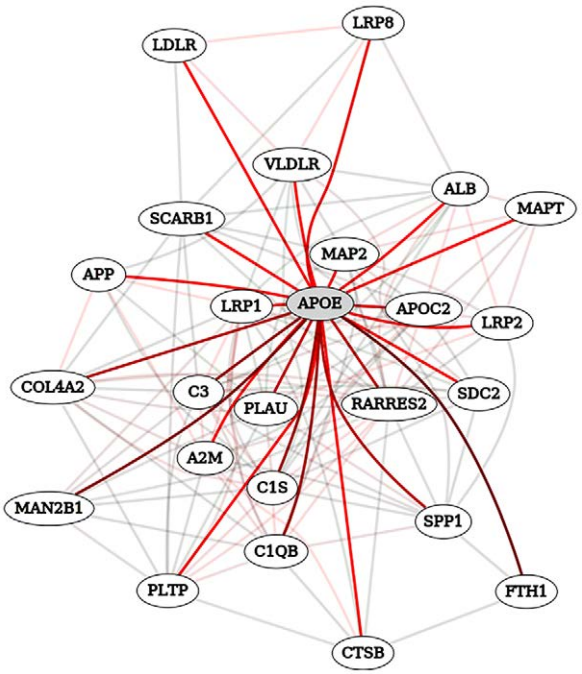

Figure 3. The result of querying HEFalMp for the role of APOE across all biological processes. Red links indicate that there is a high probability of a functional relationship between the two genes.
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gene situation, we were interested in $P(\mathrm{D}_i|\mathrm{E}_i)$, or the probability of gene i causing disease given its evidence. Here we are interested in the probability of a functional relationship between genes i and j, $P(\mathrm{FR}_{i,j})$, given some pair-wise evidence (e.g. correlation), $E_{i,j}$. As in the single gene situation, this can be calculated with

$$P(\mathrm{FR}_{i,j}|E_{i,j}) = \frac{P(E_{i,j}|\mathrm{FR}_{i,j})P(\mathrm{FR}_{i,j})}{P(E_{i,j})}.$$

Like before, a contingency table is used. The difference in this situation is that the table is based on pair-wise gene measures instead of measurements for individual genes. This process, when used to calculate pair-wise probabilities of functional relationships for all of the genes in the genome of interest, results in a functional relationship network for the organism of interest.

Huttenhower et al. [33] performed Bayesian integration and prediction using human gold standards and datasets. This tool allows users to query the network and also displays what datasets contribute to the relationships predicted from the integrated approach. As an example we can query HEFalMp to find out how the APOE protein relates to all genes across all biological processes as shown in Figure 2. The result is shown in Figure 3. The red links indicate that there is a high probability of a functional relationship between the two genes and green links indicate a low probability. Black links indicate a probability of approximately 0.5.

The probability of a functional relationship between any pair of genes is calculated as described previously. As such, this probability is dependent on evidence from each individual dataset. By clicking on a link, the contributions for each dataset towards that gene pair are provided as shown in Figure 4 for APOE and PLTP. This figure indicates the value of including high quality databases such as BioGRID as input data. While the microarray datasets are informative, in this case the three highest weighted datasets were non-microarray data sources.

These functional relationships can then be used to connect genes to diseases through guilt by association approaches. Guilt by association approaches work by finding genes or diseases that are highly connected to query genes. How exactly this is done depends on the underlying network, the size and type of the query sets, whether or not the task must be done in real time. An example approach would be to consider as positives only relationships with a probability from the inference stage of greater than 0.9. A Fisher's exact test p-value [34] can then be calculated using the counts of genes connected to the query, the number of genes connected to the query and annotated to the disease of interest, as well as the total number of genes in the network and the number of those genes annotated to the disease [34]. The approach used by the HEFalMp online tool is more complicated because the network-specific calculations must be done in real time for the web interface. Figure 5 shows diseases significantly associated with the APOE protein through the HEFalMp online tool, while the procedure used to generate the results for Figure 6 flips the analysis and shows genes significantly associated with Alzheimer disease based on their connectedness to genes annotated to this disease in OMIM [35].

5. Evaluating Functional Relationship Networks

After performing a Bayesian integration it is appropriate to assess the quality of the inference approach. One straightforward way to evaluate the network would be measure the concordance of the gold standard and predictions from the network. This is easily done by ordering gene pairs by their probabilities in the network from highest to lowest. For each gene pair in the gold standard, the true positive rate (TPR) to that point can be calculated as

$$\mathrm{TPR} = \frac{\text{Positive Pairs Thus Far}}{\text{Total Positives in Standard}}.$$

The false positive rate (FPR) can be calculated with the same values for negative pairs. These values can then be plotted with FPR on the horizontal axis and TPR on the vertical access. This provides one type of receiver-operator characteristic (ROC) curve which can be used to assess the quality of predictions from the network. The area under this curve (AUC) summarizes to a single number the quality of predictions.

Unfortunately this approach to evaluation uses the same evaluation standard as the gold standard used for learning and therefore it tests the ability of the inference approach to match the gold standard, and not its ability to make new predictions. One way to avoid this circularity is to hold a group of genes out of the gold standard during the integration process. Connections between these held out genes can then be used after the networks are generated to assess the quality of predictions from the network (in this case the concordance between the predictions and

Dataset ?	Score ?	Evidence ?
BioGRID, in vitro/in vivo assay	0.9761	Interaction
Transfac transcription factor binding site profile similarity	0.09784	Very low TFBS similarity (<-1.5 SD)
GSEA set C2 (chemical/genetic perturbations)	0.04942	Interaction
MA, Fibroblast response to adenoviral infection (Miller et al 2007)	0.02196	High correlation (1.5 to 2.5 SD)
MA, CD4+ lymphocyte polarization into Th1 and Th2 cells in the presence of TGFbeta: time course (HG-U95A) (GDS1290)	0.01588	Moderately high correlation (0.5 to 1.5 SD)
MA, Monozygotic twins (GDS1040)	0.01464	Moderately high correlation (0.5 to 1.5 SD)
MA, Testicular diffuse large B cell lymphoma (GDS1960)	0.01456	High correlation (1.5 to 2.5 SD)
MA, Testicular diffuse large B cell lymphoma (GDS1960)	0.01456	High correlation (1.5 to 2.5 SD)
MA, Tamoxifen effect on breast cancer cell line expressing estrogen receptor alpha and beta (GDS2367)	0.01381	Moderately high correlation (0.5 to 1.5 SD)
MA, B-cell chronic lymphocytic leukemia progression (GDS1388)	0.01147	High correlation (1.5 to 2.5 SD)
MA, Methyl-CpG-binding protein 2 binding disruption during neuronal maturation (GDS2125)	-0.02345	Moderately low correlation (-1.5 to -0.5 SD)
MA, Macrophage response to hypoxia (GDS2036)	-0.02386	Moderately low correlation (-1.5 to -0.5 SD)
MA, Obesity: adipocyte expression profile (HG-U95A) (GDS1493)	-0.02548	Moderately low correlation (-1.5 to -0.5 SD)
MA, Acute rotavirus infection: peripheral blood mononuclear cells (GDS2048)	-0.0261	Average correlation (-0.5 to 0.5 SD)
MA, Anemia induced by acute renal rejection: peripheral blood lymphocytes (GDS1700)	-0.02717	Average correlation (-0.5 to 0.5 SD)
MA, Vascular smooth muscle response to voltage-dependent and store-operated calcium channel activation (GDS1783)	-0.02821	Moderately low correlation (-1.5 to -0.5 SD)
MA, Melanoma, cutaneous malignant, classification (GDS2)	-0.03094	Moderately low correlation (-1.5 to -0.5 SD)
MA, Polyethylene glycol-conjugated G-CSF mobilized CD34+ cells (GDS2321)	-0.04976	Moderately low correlation (-1.5 to -0.5 SD)
MA, Bone and soft tissue sarcomas (GDS1268)	-0.0501	Low correlation (<-1.5 SD)

Figure 4. The highest and lowest contributing datasets for the pair of APOE and PLTP are shown (http://hefalmp.princeton.edu/gene/one_specific_gene/18543?argument=21697&context=0). These contributions are based on how well the bin containing the queried gene pair separated known positive functional relationships from known negative functional relationships.
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the known relationship status of the held out genes are used). While the holdout approach is effective for large gold standards, when gold standards are small this can result in too few known relationships for assessment of the network. This assessment problem can be alleviated at the cost of computation time by using a cross-validation approach. With cross-validation, the gene sets are divided up into groups. Like the hold-out approach, all but one group is used to train the network

Exploring APOE in relation to diseases in the context of all biological processes

Disease ?	Score ?	Between / Background ?
Alzheimer disease	0	0.5143 / 0.1774
Macular degeneration	0.004162	0.4223 / 0.1792
Nemaline myopathy	0.004933	0.3514 / 0.1707
Waardenburg syndrome	0.01264	0.3808 / 0.1879
Anemia	0.01881	0.2654 / 0.1653
Glioblastoma	0.02059	0.3168 / 0.173
Multiple sclerosis	0.02334	0.5377 / 0.1787
High density lipoprotein cholesterol level QTL	0.04972	0.4022 / 0.1832

Figure 5. The diseases that are significantly connected to APOE through the guilt by association strategy used in HEFalMp. Alzheimer disease and Macular degeneration are both annotated to the disease in OMIM as noted by the gold bars to the left of the disease (http://hefalmp.princeton.edu/gene/diseases?context=0&name=APOE). The other diseases are implicated by APOE's functional relationships to genes annotated to that disease in OMIM.
doi:10.1371/journal.pcbi.1002816.g005

Exploring Alzheimer disease
in relation to all genes in the context of all biological processes

Gene ?	Score ?	Description ?
APP	0	amyloid beta (A4) precursor protein (peptidase nexin-II, Alzheimer disease)
CTNND2	0	catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein)
GFAP	0	glial fibrillary acidic protein
CD34	0	CD34 molecule
APOE	0	apolipoprotein E
THY1	0	Thy-1 cell surface antigen
APBA1	5.96e-08	amyloid beta (A4) precursor protein-binding, family A, member 1 (X11)
KLK3	5.96e-08	kallikrein-related peptidase 3
FLT1	5.96e-08	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
C2	5.96e-08	complement component 2
COL1A2	1.192e-07	collagen, type I, alpha 2
MMP2	1.192e-07	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)

Figure 6. The genes that are most significantly connected to Alzheimer disease genes using the HEFalMp network and OMIM disease gene annotations (http://hefalmp.princeton.edu/disease/all_genes/55?context=0). The gold bars to the left of APP and APOE indicate that both genes were annotated Alzheimer disease according to OMIM.
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while the evaluation is performed on the left out group. In contrast to the hold-out approach, the process of training and evaluation is performed iteratively with each group of genes being evaluated, but like the hold-out approach, only the predictions generated on held out genes are used for evaluation.

When standards are incomplete, existing literature can also be used for evaluation. This can be incorporated in a number of ways. One way is to use a blind literature evaluation. Pairs predicted with high probability or genes highly connected to members of the standard can be selected for follow-up. These are combined with randomly selected genes to create a gene list for evaluation. Literature evidence for genes on this list can be assessed, and a comparison can be performed for genes selected from the network and genes selected randomly. If the proportion of literature based positives of genes or pairs selected from the network is substantially higher than those selected randomly, this provides evidence that the network recapitulates true biology.

Fundamentally the goal of this data driven functional genomics strategy is to create a network of predictions useful for designing biological experiments [36]. If these predictions lead to a higher success rate in molecular biology experiments, an integrative analysis can dramatically lower the cost per discovery. Hibbs et al. [37] used a data driven approach to direct experimental biology and found that computational predictions could be experimentally validated at a substantially higher rate than randomly selected genes. Furthermore, those genes that were found by computational methods were more likely to exhibit a subtle phenotype than the genes already known to be involved. This study provides evidence that computational predictions combined with experimental science can lower the cost of experimental discoveries while finding subtle phenotypes that high throughput experimental designs may miss.

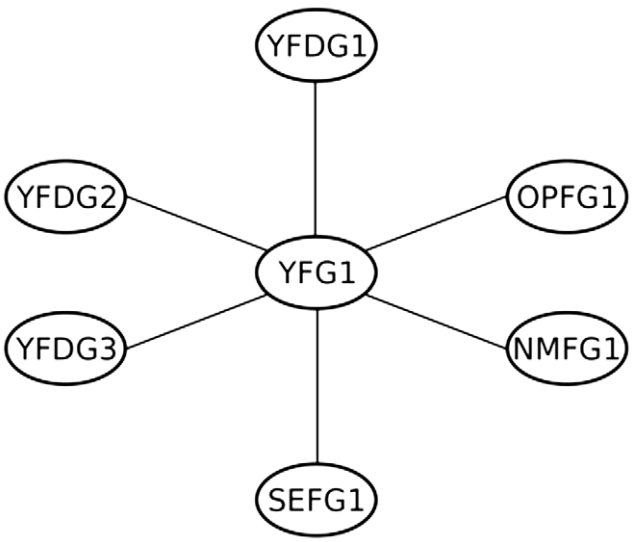

Figure 7. The functional relationship network discovered by a data driven integration for the YFG gene in YFO.
doi:10.1371/journal.pcbi.1002816.g007

6. Summary

Data driven functional genomics strategies combine methods from statistics and computer science to integrate diverse experimental data for the purpose of making novel biological predictions. By bringing diverse data together, these methods are capable of discovering patterns of biological relevance not well characterized in individual studies [38]. Furthermore, because these methods rely on existing data, they can be used to efficiently direct definitive low throughput experimental studies in a cost effective manner [37,39].

Integrative data driven approaches are often compared to publicly available databases of knowledge or experiments or to the statistical analysis of results from

Table 2. A contingency table for gene-pairs based on correlation in a gene expression dataset.

	Negatively Correlated	Uncorrelated	Positively Correlated
Known Positive Relationships	20	30	50
Known Negative Relationships	400	300	200

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Table 3. A contingency table for gene-pairs based on a database of physical interactions.

	Not Physically Interacting	Physically Interacting
Known Positive Relationships	10	90
Known Negative Relationships	900	100

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individual high throughput experiments, but they are distinct from both of these. Databases generated by literature curation are by their nature not well suited to the discovery of new knowledge and databases of experimental results require researchers to know *a priori* which datasets are relevant to the biological question of interest. Integrative data driven approaches combine high throughput experiments and databases of diverse types and in so doing can make predictions beyond those discovered using single data sources.

The flexibility of the data driven approach also gives rise to its greatest challenge. This strategy relies upon gold standards that are a representation of high quality current knowledge. When these standards are of high quality and appropriate to the biological question of interest, the resulting answers are likely to be useful. If the standards are of lower quality, the utility of the predictions will be lessened. In many cases the gold standard quality is the critical determinant of success for these algorithms. With careful use, these methods can generate predictions capable of efficiently directing experimental biology [37,40].

7. Exercises

1. All proteins connected to the protein Your Favorite Gene (YFG) in the functional relationship network of Your Favorite Organism (YFO) are shown in Figure 7. Three of them are known to be associated with Your Favorite Disease (YFD). These genes are YFDG1, YFDG2, and YFDG3. YFD has six genes annotated to it among the 100 genes present in YFO. Using a Fisher's exact test to evaluate guilt by association, is YFG significantly associated with YFD ($\alpha < 0.05$)?
2. Does the gene expression dataset described by the contingency table in Table 2 provide any information about whether or not the genes YFG and MFG are likely to have a functional relationship if they are uncorrelated in this dataset? What if they are negatively correlated?
3. Using the contingency tables from Tables 2 and 3 and the knowledge that 20% of gene-pairs in the organism of interest have a functional relationship, what is the probability that genes YFG and MFG have a functional relationship if they are positively correlated in the experiment that Table 2 is derived from and physically interacting in the database from which Table 3 is derived?
4. What is the major difference between databases and integrative data driven approaches?

Answers to the Exercises can be found in Text S1.

Supporting Information

Text S1 Answers to Exercises
(DOCX)

Further Reading

- Kanehisa M, Bork P (2003) Bioinformatics in the post-sequence era. Nat Genet 33 Suppl: 305–310.

Glossary

- Functional Relationship: The type of interaction that two genes have if they participate in the same biological process.
- Gold Standard: A set of genes or gene-pairs with a known status (positive or negative) in the tissue, process, disease, or phenotype of interest.
- Hypergeometric/Fisher's Exact Test: A test of independence appropriate for categorical count data when the number of items in each cell is small.

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Education

Chapter 3: Small Molecules and Disease

David S. Wishart[1,2,3]*

1 Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada, **2** Department of Computing Science, University of Alberta, Edmonton, Alberta, Canada, **3** National Research Council, National Institute for Nanotechnology (NINT), Edmonton, Alberta, Canada

Abstract: "Big" molecules such as proteins and genes still continue to capture the imagination of most biologists, biochemists and bioinformaticians. "Small" molecules, on the other hand, are the molecules that most biologists, biochemists and bioinformaticians prefer to ignore. However, it is becoming increasingly apparent that small molecules such as amino acids, lipids and sugars play a far more important role in all aspects of disease etiology and disease treatment than we realized. This particular chapter focuses on an emerging field of bioinformatics called "chemical bioinformatics" – a discipline that has evolved to help address the blended chemical and molecular biological needs of toxicogenomics, pharmacogenomics, metabolomics and systems biology. In the following pages we will cover several topics related to chemical bioinformatics. First, a brief overview of some of the most important or useful chemical bioinformatic resources will be given. Second, a more detailed overview will be given on those particular resources that allow researchers to connect small molecules to diseases. This section will focus on describing a number of recently developed databases or knowledgebases that explicitly relate small molecules – either as the treatment, symptom or cause – to disease. Finally a short discussion will be provided on newly emerging software tools that exploit these databases as a means to discover new biomarkers or even new treatments for disease.

This article is part of the "Translational Bioinformatics" collection for *PLOS Computational Biology*.

1. Introduction

For most of the past 100 years, the fields of toxicology, pharmacology and clinical biochemistry have focused on identifying the chemicals that cause (toxins), cure (drugs) or characterize (biomarkers) most human diseases. Historically, this kind of work has been reliant on the slow, careful and sometime tedious approaches of classical analytical chemistry and classical biochemistry. Nevertheless, it has led to important discoveries and enormous advances in our understanding of the actions of chemicals on genes, proteins and cells. With the recent emergence of high throughput "omics" technologies, our ability to detect, identify, and characterize small molecules along with their large molecule targets has been radically changed [1,2]. Now it is possible to perform as many sequencing experiments, mass spectrometry (MS) experiments or compound identifications in a single day as used to be done in a single year. As a result, traditional fields such as toxicology, pharmacology and biochemistry have been transformed into totally new fields called toxicogenomics, pharmacogenomics and metabolomics. This transformation has changed not only the fundamentals of these disciplines, but also the fundamentals of their data. Rather than trying to manage a few samples, a few sequences or a few compounds in a paper notebook or on an Excel spreadsheet, researchers are confronted with the task of handling hundreds of samples, thousands of compounds, thousands of spectra and thousands of genes or protein sequences. This has led to the development of novel computational tools and entirely new bioinformatic disciplines to facilitate the handling of this data. This particular chapter focuses on an emerging field of bioinformatics called "chemical bioinformatics" – a discipline that has evolved to help address the blended chemical and molecular biological needs of toxicogenomics, pharmacogenomics, metabolomics and systems biology.

Chemical bioinformatics combines the sequence-centric tools of bioinformatics with the chemo-centric tools of "cheminformatics". The term cheminformatics, which is an abbreviated form of "chemical informatics", was first coined by Frank Brown nearly 15 years ago [3]. Cheminformatics (as it is known in North America) or chemoinformatics (as it is known in Europe and the rest of the world) is actually a close cousin to bioinformatics. Just as bioinformatics is a field of information technology concerned with using computers to analyze molecular biological data, cheminformatics is a field of information technology that uses computers to facilitate the collection, storage, analysis and manipulation of large quantities of chemical data.

However, there are some distinct "cultural" differences between bioinformatics and cheminformatics. For instance, cheminformatics software is mostly designed for use by chemists, while bioinformatics software is designed for use by molecular biologists. Consequently there is often a terminology gap that makes it difficult for biologists to use cheminformatic software and chemists to use bioinformatics software. Likewise, most cheminformatic software is structure-based or picture-driven while most bioinformatic software is sequence-based or text-driven. As a result, different search and query interfaces have evolved that are quite specific to either cheminformatic or bioinformatic software.

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* E-mail: david.wishart@ualberta.ca

What to Learn in This Chapter

- The meaning of chemical bioinformatics
- Strengths and limitations of existing chemical bioinformatic databases
- Using databases to learn about the cause and treatment of diseases
- The Small Molecule Pathway Database (SMPDB)
- The Human Metabolome Database (HMDB)
- DrugBank
- The Toxin and Toxin-Target Database (T3DB)
- PolySearch and Metabolite Set Enrichment Analysis

Further compounding this culture gap is the fact that most cheminformatics software and chemical compound databases were developed without the expectation that this information would ever be biologically or medically relevant. Likewise, most bioinformatics software and bioinformatic databases were developed without the intention of using this data to facilitate small molecule biomarker identification or small molecule drug discovery. Consequently most biological sequence data is not linked in any meaningful way to drug or disease information – and vice versa. However, thanks to the emergence of new fields such as pharmacogenomics, toxicogenomics, systems biology and metabolomics, there is now a growing desire to bring bioinformatics and cheminformatics closer together. This has spawned the new field of chemical bioinformatics.

In this chapter we will cover several topics related to chemical bioinformatics. First, a brief overview of some of the most important chemical bioinformatic resources will be given. This will include a discussion of some of the major databases and classes of databases. Second, a more detailed overview will be given on those particular resources that allow researchers to connect small molecules to diseases. This section will focus on describing a number of recently developed databases or knowledgebases that explicitly relate small molecules – either as the treatment, symptom or cause – to disease. Finally a short discussion will be provided on newly emerging software tools that exploit these databases as a means to discover new biomarkers or even new treatments for disease.

2. Databases for Chemical Bioinformatics

Electronic databases lie at the heart of almost any subdiscipline of bioinformatics – and chemical bioinformatics is no exception. Indeed, without databases there is essentially no foundational knowledge to the discipline, and consequently, no compelling reason to write software. Programs such as BLAST [4] would be useless without GenBank [5], likewise, PSIPRED [6] couldn't exist without the Protein Databank [7] and Gene Set Enrichment Analysis – GSEA [8] would be impossible without the GEO and KEGG databases [9,10]. Given their importance, it is perhaps worthwhile to briefly review the different types of chemical-bioinformatic databases that are available and discuss some of their particular strengths and limitations.

Currently there are four major classes of chemical-bioinformatic databases. These include: 1) small molecule (or metabolic) pathway databases; 2) metabolite or metabolomic databases; 3) drug databases; and 4) toxin or toxic substance databases. In an ideal world each of these database classes could/should be useful for relating small molecules to human diseases or disease treatments. For instance, metabolic pathway databases would be expected to be most useful for understanding the "big-picture" relationship between small molecules and disease – either with regard to those small molecule compounds causing disease (i.e. toxins), indicating disease (i.e. biomarkers) or being used in the treatment of disease (i.e. drugs). On the other hand, metabolite or metabolomic databases would be expected to be most useful for associating small molecule biomarkers with specific diseases, such as inborn errors of metabolism or a variety of chronic or infectious diseases characterized by metabolite imbalances. Drug databases would obviously be most relevant for identifying small molecules with disease treatments, although they could also be used to identify small molecule drugs causing adverse drug reactions. Finally toxin or toxic compound databases would be expected to be most useful for identifying the compounds causing diseases or causing symptoms associated with certain poisoning or environmental exposure incidents. This could include acute poisonings or more long-term, environmentally influenced conditions such as cancer, allergies or birth defects.

However, as detailed below, not all of the available chemical-bioinformatic databases are particularly suited for these kinds of disease-associated queries. This likely reflects the relatively nascent stage of this field (it's less than five years old) and the fact that disease-related information is much more difficult to gather and codify than either chemical structure or gene sequence information. Certainly all of today's existing chemical-bioinformatic databases contain information about different classes of chemicals (metabolites, drugs or poisons) and most contain some limited information about the corresponding protein and/or genetic targets. However, only a very small number of these databases actually include information on the diseases or physiological effects that may be caused, cured or characterized by these chemicals.

2.1 Metabolic Pathway Databases

Among the four major classes of chemical-bioinformatic databases that are available, metabolic pathway databases are perhaps the best known and most widely used. They include a number of popular web-based resources such as the Kyoto Encyclopedia of Genes and Genomes – also known as KEGG [10], the "Cyc" databases [11,12], the Reactome database [13], WikiPathways [14], the Small Molecule Pathway Databases or SMPDB [15] and the Medical Biochemistry Page [http://themedicalbiochemistrypage.org/]. Several commercial pathway databases also exist such as TransPath (from BioBase Inc.), PathArt (from Jubilant Biosys Inc.), MetaBase (from GeneGo Inc.) and Ingenuity Pathways Analysis (Ingenuity Systems Inc.), many of which provide nicely illustrated metabolic pathway diagrams. Most of these pathway databases were designed to facilitate the exploration of metabolism and metabolites across many different species. This broad, multi-organism perspective has been critical to enhancing our basic understanding of metabolism and our appreciation of biological diversity. Metabolic pathway databases also serve as the backbone to facilitate many practical applications in biology including comparative genomics and targeted genome annotation. Table 1 lists the names, web addresses and general features for these and other useful pathway databases.

Those metabolic pathway databases that strive for very broad organism coverage, such as KEGG and Reactome, tend to use pathway diagrams that are very generic and highly schematic, while

those that are organism-specific (i.e. human-only), such as SMPDB and the Medical Biochemistry Page, tend to use diagrams that are very specific and much richer in detail, colour and content. Most pathway databases support interactive image mapping with hyperlinked information content that allows users to view chemical information (if a compound is clicked) or brief summaries of genes and/or proteins (if a protein is clicked). Almost all of the databases support some kind of limited text search and a few, such as Reactome, SMPDB and the "Cyc" databases, support the mapping of gene, protein and/or metabolite expression data onto pathway diagrams. As might be expected, the major focus of most of today's small molecule pathway databases is on basic metabolism. As a result, only one of these databases (SMPD) actually includes any pathways associated with drug action or disease.

2.2 Metabolomic Databases

The second major class of chemical-bioinformatic databases are metabolomic or metabolite databases. These databases tend to have a major focus on chemicals and chemical descriptors with a lesser (or even absent) focus on biological data. They are primarily used for metabolite identification – especially in metabolomic studies. Some databases are almost exclusively chemical in nature, containing primarily information on the chemical name(s), synonyms, InChI (International Chemical Identifier) identifier, structure, and molecular weight. These include Lipid Maps [16], a comprehensive database of biological lipids; ChEBI [17], a database of biologically interesting compounds; PubChem [18], a collection of most known organic chemicals with links to PubMed articles and more than 500,000 bioassays; ChemSpider [19], a chemical databases that is similar in size to PubChem; KNApSAcK [20], a database of plant phytochemicals and METLIN [21], a database of known and presumptive human metabolites. All of these databases support a variety of text search options and a few (such as PubChem, ChemSpider, LipidMaps and ChEBI) support structure and structure similarity searches. In addition to these biochemical databases, there are a number of smaller databases that contain spectral (NMR or MS) data of small molecule metabolites. These include the BioMagResBank or BMRB [22] which contains experimental NMR spectra of mammalian metabolites, MassBank [23] which contains MS spectra of a variety of metabolites, drugs and toxic compounds, MMCD [24] which contains experimental and predicted NMR spectra of *Arabadopsis* metabolites, and the Golm Metabolome database [25] which contains MS spectra of different plant metabolites. These spectral databases are frequently used to facilitate compound identification via spectral comparison. More recently, a much more comprehensive kind of metabolomic database has emerged which attempts to combine chemical data, spectral data, protein target data, biomarker data and disease data into a single resource. Perhaps the best example of this is the Human Metabolome Database (HMDB). The HMDB is a database containing comprehensive data on most of the known or measurable endogenous metabolites in humans [26]. Table 2 presents a summary of the names, web addresses and general features for the major metabolite/metabolomic databases.

2.3 Pharmaceutical Product Databases

The third major class of chemical bioinformatic databases are the drug or pharmaceutical product databases. In particular, two types of electronic drug databases have started to emerge over the past five years: 1) clinically oriented drug databases and 2) chemically oriented drug databases. Examples of some of the better-known clinically oriented drug databases include DailyMed [27] and RxList [28]. These resources typically offer very detailed clinical information (i.e. their formulation, metabolism and indications) about selected drugs derived from their FDA labels. As a result, these kinds of databases are targeted more towards pharmacists, physicians or consumers. Examples of chemically or genetically oriented drug databases include the TTD [29], PharmGKB [30] and SuperTarget [31]. TTD (which stands for Therapeutic Target Database) contains information on 5028 drugs (both approved and experimental) with 1894 identified targets and links to 560 different diseases or indications. PharmGKB (which stands for Pharmacogenomics Knowldege Base) has information on 1587 approved drugs (with descriptions and indications), including pharmacogenomic data on 287 drugs. SuperTarget contains information on more than 2500 target proteins, which are annotated with about 7300 literature-mined relations to 1500 different drugs. All three of these databases provide synoptic data (5–10 data fields per entry) about the nomenclature, structure and/or physical properties of small molecule drugs and, in the case of SuperTarget and TTD, their drug targets. Both TTD and SuperTarget support text, sequence and chemical structure searches, while PharmGKB provides mechanistic, pharmacodynamic and pharmacokinetic pathway information for 68 different drugs or drug classes. As a general rule, chemically oriented drug databases tend to appeal to medicinal chemists, biochemists and molecular biologists. In addition to these somewhat specialized databases, a much more comprehensive "hybrid" database, known as DrugBank [32] has recently been developed. Drugbank combines the clinical/disease information of the clinically oriented drug databases with the biochemical/chemical information of the chemically oriented drug databases. As a result, a typical DrugBank entry contains 80–100 different data fields, instead of 5–10 as seen with the other kinds of databases. Like TTD and SuperTarget, DrugBank supports very extensive text, sequence and chemical structure searches. It also provides detailed pathway information on the mechanism of action for >200 different drugs or drug classes. Table 3 provides a short summary of the names, descriptions and website addresses of the more popular drug or pharmaceutical product databases.

2.4 Toxic Substance Databases

The final class of chemical-bioinformatic databases we will discuss are the toxic substance databases. These include the Animal Toxin Database (ATDB), SuperToxic [33], ACToR [34], the Comparative Toxicogenomics Database [35] and T3DB [36]. Table 4 presents a summary of the names, web addresses and general features for these databases. The Animal Toxin Database (ATDB), with >3800 peptide toxins, provides data on the sequence of many peptide/protein toxins from venomous insects and animals as well as information on the channel targets to which these toxins bind. Both ACToR (which stands for the Aggregated Computational Toxicology Resource) and SuperToxic provide bioassy data and chemical structure information for a very large number of industrial or pharmaceutically interesting chemicals (>60,000 for SuperToxic, >500,000 for AcTOR). The Comparative Toxicogenomics Database (CTD), with >5000 chemicals, provides literature-derived information on chemical-gene interactions. This includes microarray information on genes that are up/down-regulated upon contact or exposure to these chemicals. T3DB (which stands for the Toxin, Toxin-Target Database) provides very extensive structural, physio-

Table 1. Alphabetical List of Popular Metabolic Pathway Databases.

Database Name	URL or Web Address	Comments
HumanCyc (Encylopedia of Human Metabolic Pathways)	http://humancyc.org/	-MetaCyc adopted to human metabolism -No disease or drug pathways
KEGG (Kyoto Encyclopedia of Genes and Genomes)	http://www.genome.jp/kegg/	-Best known and among the most complete metabolic pathway databases -Covers many organisms -A Few disease and drug pathways
The Medical Biochemistry Page	http://themedicalbiochemistrypage.org/	-Simple metabolic pathway diagrams with extensive explanations -A few drug and disease pathways
MetaCyc (Encyclopedia of Metabolic Pathways)	http://metacyc.org/	-Similar to KEGG in coverage, but different emphasis -Well referenced -No disease or drug pathways
Reactome (A Curated Knowledgebase of Pathways)	http://www.reactome.org/	-Pathway database with more advanced query features -Not as complete as KEGG or MetaCyc
Roche Applied Sciences Biochemical Pathways Chart	http://www.expasy.org/cgi-bin/search-biochem-index	-The old metabolism standard (on line) -Describes most human metabolism
Small Molecule Pathway Database (SMPDB)	http://www.smpdb.ca/	-Pathway database with disease, drug and metabolic pathways for humans -Extensive search, analysis and visualization tools
Wikipathways	http://www.wikipathways.org	-Community annotated pathway database for 19 model organisms -Contains 175 human pathways -Few drug or disease pathways

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logical, mechanistic, medical and biochemical information on about 3100 commonly encountered (i.e. household or environmental) toxins and poisons.

Each of these databases addresses the needs of certain communities such as animal physiologists (ATDB), toxicogenomics or toxicology specialists (CTD and T3DB), environmental or industrial regulators (ACToR) or medicinal chemists interested in toxicity prediction (SuperToxic). However, with the exception of T3DB, most of these online toxin or toxic compound databases are relatively lightly annotated, with fewer than a dozen data fields per compound and essentially no physiological, disease or disease symptom information.

Clearly not all of the chemical-bioinformatic databases we have described in this section are suitable for deriving information about small molecules and disease. Likewise, many of the databases mentioned above are not exactly suitable for translational bioinformatic questions or for applications relating to medicine, medical biochemistry or clinical research. However, there is at least one database in each of the four major chemical-bioinformatic database classes that does generally meet these criteria. In particular: 1) SMPDB is a pathway database that explicitly relates small molecules to disease and disease treatment; 2) HMDB is a metabolomic database that associates metabolites to disease biomarkers or disease diagnosis; 3) DrugBank is a drug database that links drugs and drug targets to symptoms, diseases and disease treatments and 4) T3DB is a toxic substance database that associates toxins and their biological targets with symptoms, conditions, diseases and disease treatments. A more detailed description of each of these databases is provided below.

3. SMPDB – A Pathway Database for Drugs and Disease

As noted earlier, SMPDB is a pathway database specifically designed to facilitate clinical ''omics'' studies, with a specific emphasis on clinical biochemistry and clinical pharmacology. Currently SMPD consists of more than 450 highly detailed, hand-drawn pathways describing small molecule metabolism or small molecule processes that are specific to humans. These pathways can be placed into four different categories: 1) metabolic pathways; 2) small molecule disease pathways; 3) small molecule drug pathways and 4) small molecule signaling pathways. An example of a typical SMPDB pathway (Phenylketonuria) is shown in Figure 1. As seen in this figure, all SMPDB pathways explicitly include the chemical structure of the major chemicals in each pathway. In addition, the cellular locations (membrane, cytoplasm, mitochondrion, nucleus, peroxisome, etc.) of all metabolites and the enzymes involved in their processing are explicitly illustrated. Likewise the quaternary structures (if known) and cofactors associated with each of the pathway proteins are also shown. If some of the metabolic processes occur primarily in one organ or in the intestinal microflora, this information is also illustrated. The inclusion of explicit chemical, cellular and physiological information is one of the more unique and useful features of SMPDB. SMPDB is also unique in its inclusion of significant numbers of metabolic disease pathways (>100) and drug pathways (>200) not found in any other pathway database. Likewise, unlike other pathway databases, SMPDB supports a number of unique database querying and viewing features. These include simplified database browsing, the generation of protein/metabolite lists for each pathway, text querying, chemical structure querying and sequence querying, as well as large-scale pathway mapping via protein, gene or chemical compound lists.

The SMPDB interface is largely modeled after the interface used for DrugBank [32], T3DB [36] and the HMDB [26], with a navigation panel for Browsing, Searching and Downloading the database.

Table 2. Alphabetical List of Metabolomic, Chemical or Spectral Databases.

Database Name	URL or Web Address	Comments
BioMagResBank (BMRB – Metabolimics)	http://www.bmrb.wisc.edu/metabolomics/	-Emphasis on NMR data, no biological or biochemical data -Specific to plants (Arabadopsis)
Chemicals Entities of Biological Interest (ChEBI)	http://www.ebi.ac.uk/chebi/	-Covers metabolites and drugs of biological interest -Focus on ontology and nomenclature not biology
ChemSpider	http://www.chemspider.com/	-Meta-database containing chemical data from 100+ other databases -20+ million compounds -Good search utilities
Golm Metabolome Database	http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html	-Emphasis on MS or GC-MS data only -No biological data -Few data fields -Specific to plants
Human Metabolome Database	http://www.hmdb.ca	-Largest and most completely annotated metabolomic database -Specific to humans only
KNApSAcK	http://kanaya.naist.jp/KNApSAcK/	-A phytochemical database containing data for 50,000 compounds
LipidMaps	http://www.lipidmaps.org/	-Contains 22,500 different lipids found in plants & animals -Nomenclature standard
METLIN Metabolite Database	http://metlin.scripps.edu/	-Human specific metabolite database -Name, structure, ID only
PubChem	http://pubchem.ncbi.nlm.nih.gov/	-Database containing 27 million unique chemicals with links to Bioassays and PubMed abstracts

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Below the navigation panel is a simple text query box that supports general text queries of the entire textual content of the database. Mousing over the Browse button allows users to choose between two browsing options, SMP-BROWSE and SMP-TOC. SMP-TOC is a scrollable hyperlinked table of contents that lists all pathways by name and category. SMP-BROWSE is a more comprehensive browsing tool that provides a tabular synopsis of SMPDB's content with thumbnail images of the pathway diagrams, textual descriptions of the pathways, as well as lists of the corresponding chemical components and enzyme/protein components. This browse view allows users to scroll through the database, select different pathway categories or re-sort its contents. Clicking on a given thumbnail image or the SMPDB pathway button brings up a full-screen image for the corresponding pathway. Once ''opened'' the pathway image may be expanded by clicking on the Zoom button located at the top and bottom of the image. An image legend link is also available beside the Zoom button.

At the top of each pathway image is a pathway synopsis contained in a yellow

Table 3. Alphabetical List of Pharmaceutical Compound or Drug Databases.

Database Name	URL or Web Address	Comments
DailyMed	http://dailymed.nlm.nih.gov/	-A drug database containing FDA label (package inserts) for most approved drugs
DrugBank	http://www.drugbank.ca/	-Comprehensive database of 1480 drugs with 1700 drug targets -Contains chemical, biological & clinical data -Extensive search utilities
PharmGKB	http://www.pharmgkb.org/	-Data on 1587 approved drugs including pharmacogenomic data on 287 drugs. -Provides mechanistic, pathway information for 68 different drugs
SuperTarget	http://bioinf-tomcat.charite.de/supertarget/	-Searchable database of drugs and drug targets -Includes 2500 target proteins, which are annotated with about 7300 literature-mined relations to 1500 different drugs.
TTD (Therapeutic Target Database)	http://xin.cz3.nus.edu.sg/group/ttd/ttd.asp	-Contains data on 1894 drug targets for 5126 drugs -Limited chemical data -No clinical or pharmacological data

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box while at the bottom of each image is a list of references. On the right of each pathway image is a grey-green Highlight/Analyzer tool with a list of the key metabolites/drugs and enzymes/proteins found in the pathway. Checking on selected items when in the SMP-Highlight mode will cause the corresponding metabolite or protein in the pathway image to be highlighted with a red box. Entering concentration or relative expression values (arbitrary units) beside compound or protein names, when in the SMP-Analyzer mode, will cause the corresponding metabolites or proteins to be highlighted with differing shades of green or red to illustrate increased or decreased concentrations. As with most pathway databases, all of the chemical structures and proteins/enzymes illustrated in SMPDB's diagrams are hyperlinked to other on-line databases or tables. Specifically, all metabolites, drugs or proteins shown in the SMP-BROWSE tables or in a pathway diagram are linked to HMDB, DrugBank or UniProt [37] respectively. Therefore, clicking on a chemical or protein image will open a new browser window with the corresponding DrugCard, MetaboCard or UniProt table being displayed.

The most powerful search option in SMPDB is SMP-MAP, which offers both multi-identifier searches as well as "Omic" (transcriptomic, proteomic or metabolomic) mapping. In contrast to SMP-BROWSE, which is used for data browsing and single entity highlighting, SMP-MAP can be used for multi-entity highlighting and mapping. In particular SMP-MAP allows users to enter lists of chemical names, gene names, protein names, UniProt IDs, GenBank IDs, Agilent IDs or Affymetrix IDs (with or without concentration data) and to have a table generated of pathways containing those components. The resulting table, like the SMP-BROWSE table, displays a thumbnail image of the matching pathways along with the list of matching components (metabolites, drugs, proteins, etc.). The table is ordered by the number of matches and a significance score (calculated via a hypergeometric function), with the pathway having the most matches being placed at the top. Clicking on the thumbnail image or the SMPDB pathway button brings up a full-screen image for the corresponding pathway with all the matching components (metabolites, drugs, proteins, etc.) highlighted in red. Concentration data can be displayed using a red-to-yellow gradient by entering concentration data in a text box located beside the map image.

SMPDB's Search menu also offers users a choice of searching the database by chemical structure (ChemQuery), text (TextQuery) or sequence (SeqSearch). The ChemQuery option allows users to draw (using MarvinSketch applet) or write (using a SMILES string) a chemical compound and to search SMPDB for drugs and metabolites similar or identical to the query compound. The TextQuery button supports a more sophisticated text search (partial word matches, data field selection, Boolean text searches, case sensitive, misspellings, etc.) of the text portion of SMPDB, including the accompanying pathway explanations and reference sections. The SeqSearch button allows users to conduct BLASTP (protein) sequence searches of the protein sequences contained in SMPDB. SeqSearch supports both single and multiple sequence BLAST queries.

To summarize, SMPDB allows users to interactively explore, through detailed pathway diagrams, the linkage between metabolites, genes or proteins and metabolic diseases. It also allows users to investigate the connection between drugs and their protein or gene targets through comprehensive illustrations of their mechanism of action. Because of its detailed depictions of both disease and drug pathways and its extensive use of visualization and query tools, SMPDB can potentially support a variety of translational bionformatic/cheminformatic questions. For example, through SMPDB it is possible for users to: 1) identify a metabolic disease or medical condition given a list of metabolites (via SMP-MAP); 2) use experimental gene expression data to identify which diseases, conditions or pathways are most affected by a given drug, dietary or chemical treatment (via SMP-MAP); 3) use metabolomic or metabolite expression data to help understand or rationalize specific metabolic diseases, conditions or biomarkers (through SMP-MAP); 4) determine the similarity of a newly found/synthesized compound to an existing drug (via the ChemQuery search); 5) determine the possible mechanism of action or protein targets for a newly found/synthesized compound (via the ChemQuery search); 6) ascertain whether a certain protein found in bacteria, fungi or viruses

Table 4. Alphabetical Listing of Toxic Compound Databases.

Database Name	URL or Web Address	Comments
ACToR (Aggregated Computation Toxicology Resource)	http://actor.epa.gov/actor/faces/ACToRHome.jsp	-Contains aggregated data on 2,500,000 environmental chemicals -Searchable by chemical name and structure -Data includes chemical structure, physico-chemical values, in vitro assay data and in vivo toxicology data.
ATDB (Animal Toxin Database)	http://protchem.hunnu.edu.cn/toxin/index.jsp	-Database with >3800 peptide toxins -Provides sequence data on peptide/protein toxins from venomous insects and animals
CTD (Comparative Toxicogenomic Database)	http://ctd.mdibl.org/	-Data on >5000 chemicals with literature-derived information on chemical-gene interactions
SuperToxic	http://bioinformatics.charite.de/supertoxic/	-Contains data on 60,000 toxic compounds and some target data -Provides chemical and toxicity information -Can predict the toxicity of query compounds
T3DB (Toxin, Toxin-Target Database)	http://www.t3db.org/	-Searchable database of 3100 common toxins and 1400 target proteins -Provides extensive structural, physiological, mechanistic, medical and biochemical information

doi:10.1371/journal.pcbi.1002805.t004

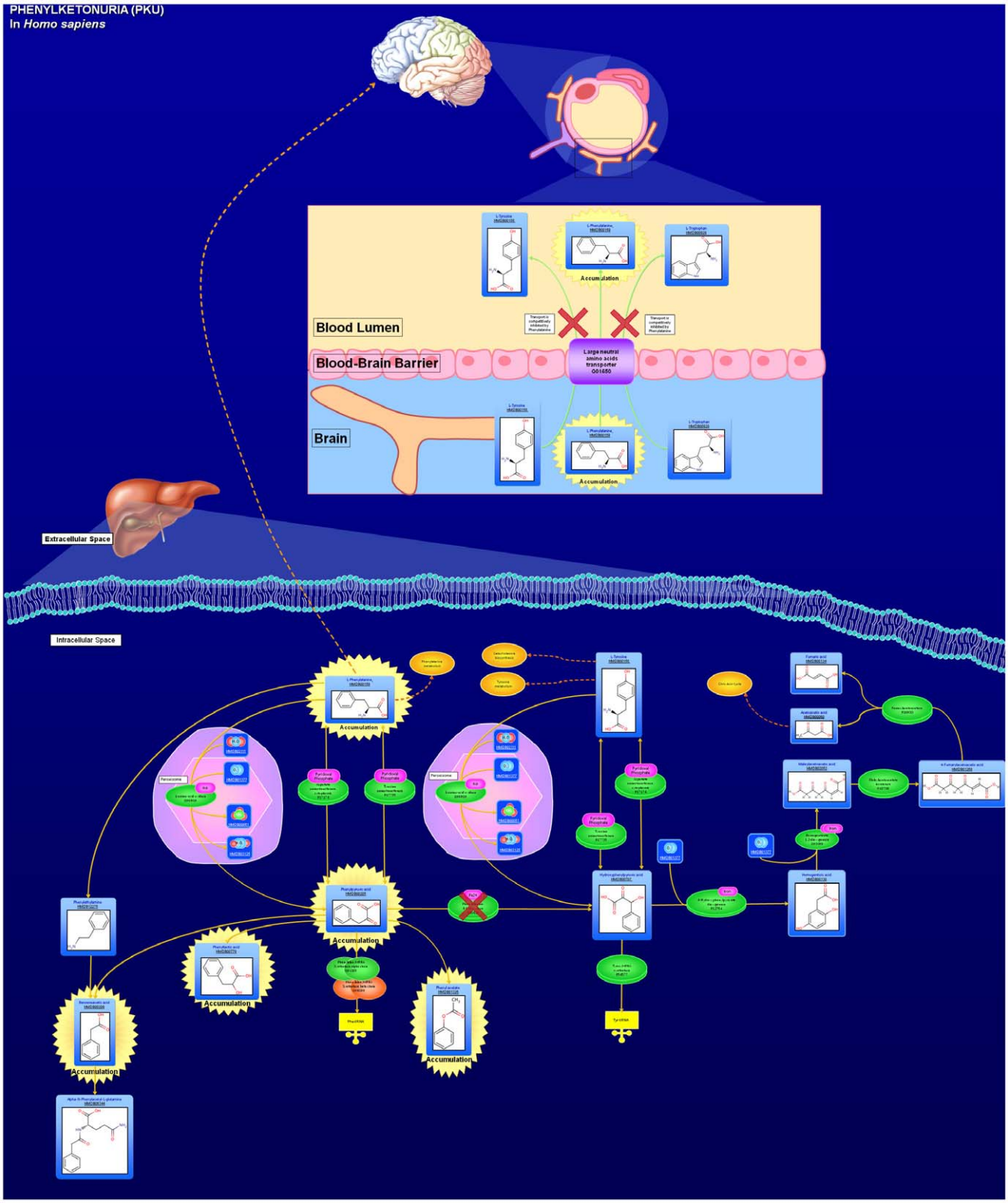

Figure 1. A pathway diagram for Phenylketonuria as taken from SMPDB (http://www.smpdb.ca).
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could be a drug target (via the SeqSearch query); 7) ascertain whether a newly identified human protein, such as an isoform or paralogue, may be a drug target or a disease indicator (through the SeqSearch query); or 8) use the pathway visualization and mapping tools to explain or teach others about metabolic diseases, basic metabolism or drug action.

4. HMDB – A Resource for Biomarker Discovery and Disease Diagnosis

The Human Metabolome Database (HMDB) is the by-product of the Human Metabolome Project – a 3-year (2005–2008), $7.5 million dollar project that was aimed at collating, identifying and annotating all the endogenous metabolites in the human body [38]. The HMDB is actually the largest and most comprehensive, organism-specific metabolomic database assembled to date. It contains spectroscopic, quantitative, analytic and molecular-scale information about human metabolites, their associated enzymes or transporters, their abundance and their disease-related properties. The HMDB currently contains more than 8000 human metabolite entries that are linked to more than 45,000 different synonyms. These metabolites are further connected to 3360 distinct enzymes, which in turn, are linked to nearly 100 metabolic pathways and more than 150 disease pathways. More than 1000 metabolites have disease-associated information, including both normal and abnormal metabolite concentration values. These diagnostic metabolites or metabolite signatures are linked to more than 500 different diseases (genetic and acquired). The HMDB also contains experimental metabolite concentration data for "normal" plasma, urine, CSF and/or other biofluids for more than 5000 compounds. More than 900 compounds are also linked to experimentally acquired "reference" ^{1}H and ^{13}C NMR and MS/MS spectra. The entire database, including text, sequence, structure and image data occupies nearly 30 Gigabytes of data – most of which can be freely downloaded.

The HMDB is a fully searchable database with many built-in tools for viewing, sorting and extracting metabolites, biofluid concentrations, enzymes, genes, NMR or MS spectra and disease information. As with any web-enabled database, the HMDB supports standard text queries (through the text search box located near the top of each page). It also offers extensive support for higher-level database search and selection functions through a navigation bar (located at the top of each page). The navigation bar has six pull-down menu tabs ("Home", "Browse", "Search", "About", "Download" and "Contact Us"). The "Browse" tab allows users to select from six browsing options including "HMDB Browse", "Disease Browse", "PathBrowse", "Biofluid Browse", "HML Browse" and "ClassBrowse". "HMDB Browse" allows users to search through the HMDB compound by compound through a series of hyperlinked, synoptic summary tables. These metabolite tables can be rapidly browsed, sorted or reformatted in a manner similar to the way PubMed abstracts may be viewed. Clicking on the MetaboCard button found in the leftmost column of any given HMDB summary table opens a webpage describing the compound of interest in much greater detail. Each MetaboCard entry contains more than 100 data fields with half of the information being devoted to chemical or physico-chemical data and the other half devoted to biological or biomedical data. These data fields include a comprehensive compound description, names and synonyms, structural information, physico-chemical data, reference NMR and MS spectra, biofluid concentrations (normal and abnormal), disease associations, pathway information, enzyme data, gene sequence data, protein sequence data, SNP and mutation data as well as extensive links to images, references and other public databases such as KEGG [10], BioCyc [12], PubChem [18], ChEBI [17], PubMed, PDB [7], SwissProt/UniProt [37], GenBank [5], and OMIM [39].

Outside of "HMDB Browse", there are five other browsing options that allow users to explore or navigate the database. "Disease Browse" allows users to view known metabolic disorders (as well as other diseases) and the metabolites that are typically associated with these conditions. It also allows users to enter lists of metabolites and to identify which diseases are characterized by perturbations to these metabolite levels. "PathBrowse" allows users to browse through the custom-drawn HMDB pathway images. Each pathway is named and each image is zoomable and extensively hyperlinked. Users may also search PathBrowse using lists of compounds (obtained from a metabolomic experiment) and view hyperlinked tables that display all of the pathways that are potentially affected. "Biofluid Browse" allows users to browse metabolite entries based on their concentrations and the biofluids in which they are found. Users may select entries by biofluid type and sort the table by compound name, HMDB ID, concentration, disease, age, or gender. "HML Browse" allows users to browse or search through the Human Metabolome Library (HML). The HML is a library of ~1000 reference metabolites stored in −80°C freezers at the Human Metabolome Project Centre in Edmonton, Canada. "ClassBrowse", is designed to allow users to view compounds according to their chemical class designation. Each displayed compound name is hyperlinked to an HMDB MetaboCard. Users may search for compounds (via a text box) or select to view certain compound classes using a pull-down menu located at the top of the ClassBrowse page.

In addition to the data browsing and sorting features already described, the HMDB also offers a chemical structure search utility, a local BLAST search [4] that supports both single and multiple sequence queries, a Boolean text search based on KinoSearch (http://www.rectangular.com/kinosearch/), a chemical structure search utility based on ChemAxon's MarvinView, a relational data extraction tool, an MS spectral matching tool and an NMR spectral search tool (for identifying compounds via MS or NMR data from other metabolomic studies). These can all be accessed via the database navigation bar located at the top of every HMDB page.

HMDB's simple text search supports text matching, text match rankings, misspellings (offering suggestions for incorrectly spelled words) and highlights text where the word is found. In addition to this simple text search, HMDB's TextQuery function uses the same KinoSearch engine, but also supports more sophisticated text querying functions (Boolean logic, multi-word matching and parenthetical groupings) as well as data-field-specific queries such as finding the query word only in the "Compound Source" field.

The HMDB's structure similarity search tool (ChemQuery) is the equivalent to BLAST for chemical structures. Users may sketch (through MarvinView's chemical sketching applet) or paste a SMILES string (40) of a query compound into the ChemQuery window. Submitting the query launches a structure similarity search tool that looks for common substructures from the query compound that match the HMDB's metabolite database. High scoring hits are presented in a tabular format with hyperlinks to the corresponding MetaboCards (which in turn links to the protein target). The ChemQuery tool allows users to quickly determine whether their compound of interest is a known metabolite or chemically related to a known metabolite. In addition to these structure similarity searches, the ChemQuery utility also supports compound searches on the basis of chemical formula and molecular weight ranges.

HMDB's BLAST search (SeqSearch) allows users to search through the HMDB via sequence similarity as opposed to chemical similarity. A given gene or

protein sequence may be searched against the HMDB's sequence database of metabolically important enzymes and transporters by pasting the FASTA formatted sequence (or sequences) into the SeqSearch query box and pressing the "submit" button. A significant hit reveals, through the associated MetaboCard hyperlink, the name(s) or chemical structure(s) of metabolites that may act on that query protein. With SeqSearch metabolite-protein interactions from model organisms (chimp, rat, mouse, dog, cat, etc.) may be mapped to these organisms via the human data in the HMDB.

The HMDB's data extraction utility (Data Extractor) employs a simple relational database system that allows users to select one or more data fields and to search for ranges, occurrences or partial occurrences of words or numbers. The Data Extractor uses clickable web forms so that users may intuitively construct SQL-like queries. The data extraction tool allows users to easily construct complex queries as "find all diseases where the concentration of homogentisic acid in urine is greater than 1 mM".

The NMR and MS search utilities allow users to upload spectra (for the MS search) or peak lists (for the NMR search) and to search for matching compounds from the HMDB's collection of MS and NMR spectra. In particular, the HMDB contains more than 2000 experimentally collected ^{1}H and ^{13}C NMR spectra for 900 pure compounds (most collected in water at pH 7.0). It also contains approximately 3800 predicted ^{1}H and ^{13}C NMR spectra for 1900 other compounds for which authentic samples could not be acquired. The HMDB's mass spectra library contains 2400 MS/MS (Triple-Quad) spectra collected at 3 different collision energies for more than 800 pure compounds. The HMDB's spectral search utilities allow both pure compounds and mixtures of compounds to be identified from their MS or NMR spectra via peak matching algorithms. Compounds may also be identified or searched for by entering their chemical formula or their mass (either their exact mass or a mass range). Figure 2 provides a screenshot montage illustrating the types of viewing and searching options available in HMDB.

To summarize the HMDB allows users to link endogenous metabolites (both their identity and their concentration) to a variety of disease conditions, including metabolic disorders, genetic diseases, chronic (age-related) disorders and a variety of infectious diseases. It also provides links between metabolites and their targets – both through descriptions of the compounds and their known biological roles and through the identification of known pathways or catalyzing enzymes. In addition, the HMDB also supports the direct identification of potential diagnostic biomarkers based on their mass, mass spectra or NMR spectra. Because of this linkage, the HMDB can potentially support a variety of translational bionformatic or cheminformatic queries. For example, through the HMDB it is possible for users to: 1) identify a novel biomarker for a given condition or disease given an NMR or GC/MS or MS/MS spectrum of the purified compound (via the MS/NMR search tools); 2) identify metabolites from a biofluid mixture that has been analyzed by NMR, GC/MS or MS/MS (via the MS/NMR search tools); 3) identify a disease or condition given a list of metabolites (via Disease Browse); 4) identify a pathway or process that has been altered/perturbed given a list of metabolites obtained from a metabolomic experiment (via PathBrowse); 5) determine normal and abnormal concentration ranges for metabolites in different biofluids (via Biofluid Browse); 6) obtain authentic standards of unique metabolites to confirm the diagnosis of a certain disease (via HML Browse); 7) determine the similarity of a newly found/synthesized compound to an existing metabolite (via the structure similarity search); 8) determine the possible mechanism of action or protein targets for a newly discovered/synthesized metabolite or metabolite analogue (via the structure similarity search); 9) diagnose or determine the cause of illnesses thought to be brought on by metabolite changes (through the text search); 10) extract detailed information on metabolites, metabolic diseases or metabolic pathways (via the data extractor); 11) extract information on common metabolite classes (via the data extractor or ClassBrowse); 12) ascertain whether a certain protein or protein homologue may also be involved in a metabolic process or pathway (via the sequence search).

5. DrugBank – A Resource for Drug Discovery and Disease Treatment

As previously noted, DrugBank [32] is essentially a hybrid clinically AND chemically oriented drug database that links sequence, structure and mechanistic data about drug molecules with sequence, structure and mechanistic data about their drug targets. DrugBank was one of the first electronic databases to provide the explicit linkage between drugs and drug targets and this particular feature made DrugBank particularly popular. Another important innovation in this database was the presentation of drug and drug target data in synoptic DrugCards (in anology to library cards or study flash-cards). This concept (which is now used in many other chemical-bioinformatic databases) helped make DrugBank particularly easy to view and navigate. Currently DrugBank contains detailed information on 1480 FDA-approved drugs corresponding to 28,447 brand names and synonyms. This collection includes 1281 synthetic small molecule drugs, 128 biotech (mostly peptide or protein) drugs and 71 nutraceutical drugs or supplements. DrugBank also contains information on the 1669 different targets (protein, lipid or DNA molecules) and metabolizing enzymes with which these drugs interact. Additionally the database maintains data on 187 illicit drugs (i.e. those legally banned or selectively banned in most developed nations) and 64 withdrawn drugs (those removed from the market due to safety concerns). Chemical, pharmaceutical and biological information about these classes of drugs is extremely important, not only in understanding their adverse reactions, but also in being able to predict whether a new drug entity may have unexpected chemical or functional similarities to a dangerous or highly addictive drug.

As with the HMDB, the DrugBank website contains many built-in tools and a variety of customized features for viewing, sorting, querying and extracting drug or drug target data. These include a number of higher-level database searching functions such as a local BLAST [4] sequence search (SeqSearch) that supports both single and multiple protein sequence queries (for drug target searching), a boolean text search (TextSearch) for sophisticated text searching and querying, a chemical structure search utility (ChemQuery) for structure matching and structure-based querying as well as a relational data extraction tool (Data Extractor) for performing complex queries.

The BLAST search (SeqSearch) is particularly useful for drug discovery applications as it can potentially allow users to quickly and simply identify drug leads from newly sequenced pathogens. Specifically, a new sequence, a group of sequences or even an entire proteome can be searched against DrugBank's database of known drug target sequences by pasting the FASTA formatted sequence (or sequences) into the SeqSearch query box and pressing the "submit" button. A

significant hit can reveal the name(s) or chemical structure(s) of potential drug leads that may act on that query protein (or proteome). The structure similarity search tool (ChemQuery) can be used in a similar manner to SeqSearch. For instance, users may sketch a chemical structure or paste a SMILES string [40] of a possible drug lead or a drug that appears to be causing an adverse reaction into the ChemQuery window. After submitting the query, the database launches a structure similarity search that looks for common substructures from the query compound that match DrugBank's database of known drug or drug-like compounds. High scoring hits are presented in a tabular format with hyperlinks to the corresponding DrugCards. The ChemQuery tool allows users to quickly determine whether their compound of interest acts on the desired protein target or whether the compound of interest may unexpectedly interact with unintended protein targets.

In addition to these search features, DrugBank also provides a number of general browsing tools for exploring the database as well as several specialized browsing tools such as PharmaBrowse and GenoBrowse for more specific tasks. For instance, PharmaBrowse is designed to address the needs of pharmacists, physicians and medicinal chemists who tend to think of drugs in clusters of indications or drug classes. This particular browsing tool provides navigation hyperlinks to more than 70 drug classes, which in turn list the FDA-approved drugs associated with the drugs. Each drug name is then linked to its respective DrugCard. GenoBrowse, on the other hand, is specifically designed to address the needs of geneticists or those specialists interested in specific Drug-SNP relationships. This browsing tool provides navigation hyperlinks to more than 60 different drugs, which in turn list the target genes, SNPs and the physiological effects associated with these drugs.

In addition to its general utility as a general drug encyclopedia, DrugBank also contains several tables, data fields or data types that are particularly useful for pharmacogenomic or pharmacogenetic studies. These include synoptic descriptions of a given drug's Pharmacology as well as its Mechanism of Action, Contraindications, Toxicity, Phase I Metabolizing Enzymes (name, protein sequence and SNPs), and associated Drug Targets (names, protein sequence, DNA sequence, chromosome location, locus number and SNPs). The information contained in DrugBank's Pharmacology, Mechanism of Action, Contraindications and Toxicity fields often includes details about any known adverse reactions. This may include descriptions of known phase I or phase II enzyme interactions, alternate metabolic routes or the existence of secondary drug targets. Secondary drug targets represent proteins (or other macromolecules) that are different than the primary target for which the drug was initially designed or targeted towards. Some drugs may have five or more targets, of which only one might be relevant to treating the disease. DrugBank uses a relatively liberal interpretation of drug targets in order to help identify these secondary drug targets. In particular, for DrugBank a drug target is defined as any macromolecule identified in the literature that binds, transports or transforms a drug. The binding or transformation of a drug by a secondary drug target or an "off-target" protein is one of the most common causes for unwanted side effects or adverse drug reactions (ADRs) [41]. By providing a fairly comprehensive listing of secondary drug targets (along with their SNP information and other genetic data), DrugBank is potentially able to provide additional insight into the underlying causes of a patient's response to a given drug.

DrugBank also provides detailed sequence and SNP data on known drug metabolizing enzymes and known drug targets. In particular DrugBank contains detailed summary tables about each of the SNPs for each of the drug targets or drug metabolizing enzymes that have been characterized by various SNP typing efforts, such as the SNP Consortium [42] and HapMap [43]. Currently DrugBank contains information on 26,292 coding (exon) SNPs and 73,328 non-coding (intron) SNPs derived from known drug targets. It also has data on 1188 coding SNPs and 8931 non-coding SNPs from known drug metabolizing enzymes. By clicking on the "Show SNPs" hyperlink listed beside either the metabolizing enzymes or the drug target SNP field, the SNP summary table can be viewed. These tables include: 1) the reference SNP ID (with a hyperlink to dbSNP); 2) the allele variants; 3) the validation status; 4) the chromosome location and reference base position; 5) the functional class (synonymous, non-synonymous, untranslated, intron, exon); 6) mRNA and protein accession links (if applicable); 7) the reading frame (if applicable); 8) the amino acid change (if existent); 9) the allele frequency as measured in African, European and Asian populations (if available) and 10) the sequence of the gene fragment with the SNP highlighted in a red box.

The purpose of these SNP tables is to allow one to go directly from a drug of interest to a list of potential SNPs that may contribute to the reaction or response seen in a given patient or in a given population. In particular, these SNP lists may serve as hypothesis generators that allow SNP or gene characterization studies to be somewhat more focused or targeted. By comparing the experimentally obtained SNP results to those listed in DrugBank for that drug (and its drug targets) it may be possible to ascertain which polymorphism for which drug target or drug metabolizing enzyme may be contributing to an unusual drug response. Obviously these database-derived SNP suggestions may require additional experimental validation to prove their causal association.

Drugbank also includes two tables that provide much more explicit information on the relationship between drug responses/reactions and gene variant or SNP data. The two tables, which are accessible from the GenoBrowse submenu located on DrugBank's Browse menu bar, are called SNP-FX (short for SNP-associated effects) and SNP-ADR (short for SNP-associated adverse drug reactions). SNP-FX contains data on the drug, the interacting protein(s), the "causal" SNPs or genetic variants for that gene/protein, the therapeutic response or effects caused by the SNP-drug interaction (improved or diminished response, changed dosing requirements, etc.) and the associated references describing these effects in more detail. SNP-ADR follows a similar format to SNP-FX but the clinical responses are restricted only to adverse drug reactions (ADR). SNP-FX contains literature-derived data on the therapeutic effects or therapeutic responses for more than 70 drug-polymorphism combinations, while SNP-ADR contains data on adverse reactions compiled from more than 50 drug-polymorphsim pairings. All of the data in these tables is hyperlinked to drug entries from DrugBank, protein data from SwissProt, SNP data from dbSNP and bibliographic data from PubMed. A screen shot of the SNP-ADR table is shown in Figure 3. As can be seen from the figure, these tables provide consolidated, detailed and easily accessed information that clearly identifies those SNPs that are known to affect a given drug's efficacy, toxicity or metabolism.

To summarize, DrugBank allows users to link drugs to a variety of disease conditions or health indications. It also provides links between drugs and their

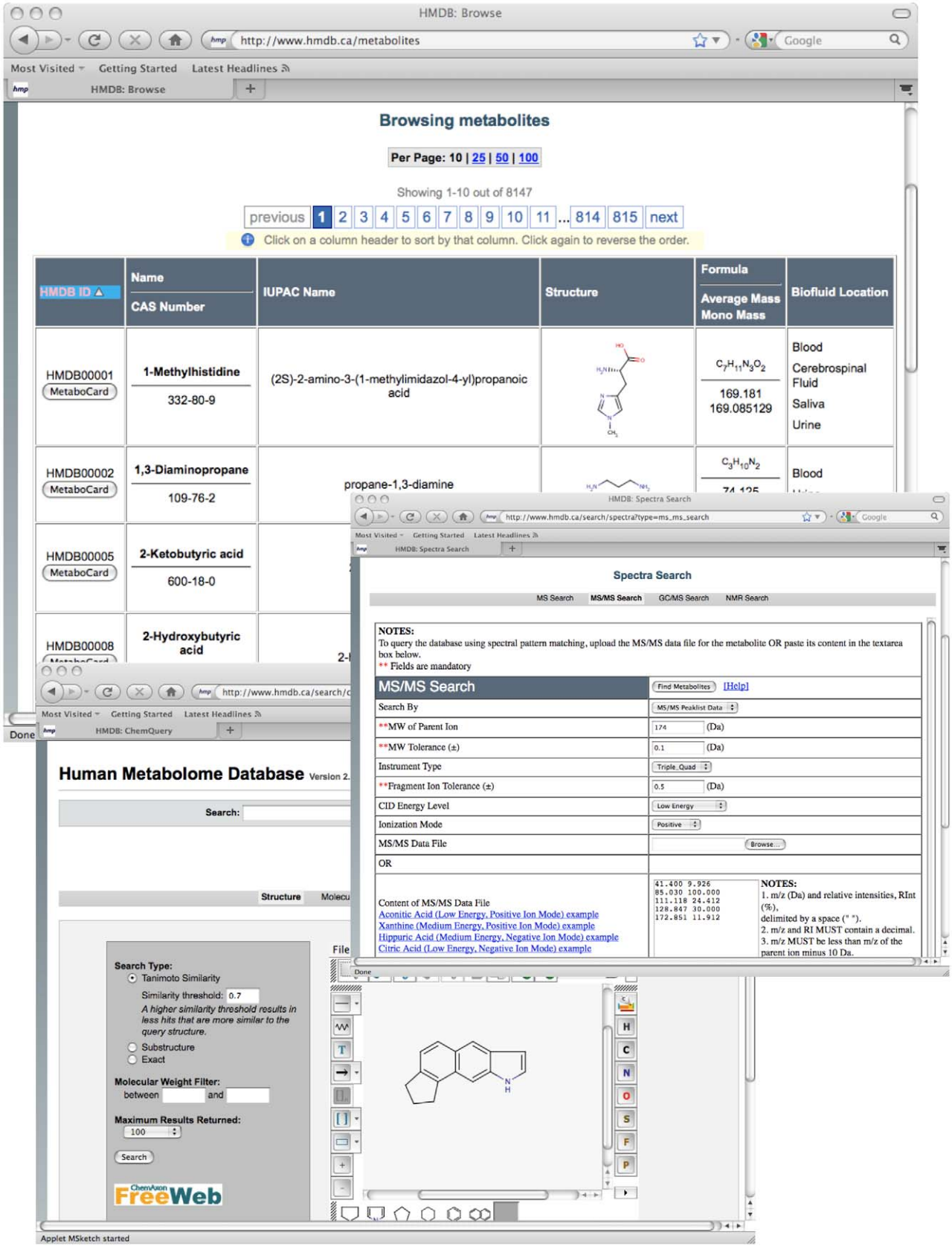

Figure 2. A screenshot montage illustrating the types of viewing and searching options available in HMDB (http://www.hmdb.ca).
doi:10.1371/journal.pcbi.1002805.g002

targets – both through descriptions of the mechanism of action and through the identification of known protein (or gene) targets. Because of this kind of extensive data linkage, DrugBank can potentially support a number of translational bionformatic or cheminformatic questions. For example, through DrugBank it is possible for users to: 1) determine the similarity of a newly found/synthesized compound to an existing drug (via the structure similarity search); 2) determine the possible mechanism of action or protein targets for a newly found/synthesized compound (via the structure similarity search); 3) diagnose or determine the cause of illnesses thought to be brought on by adverse drug reactions (through the text search or SNPADR/SNPFX); 4) treat or find references to the treatment of illnesses based on symptoms or disease diagnosis (via the text search); 5) extract information on common drug targets (via the data extractor or the sequence search); 6) extract information on common drug classes or structures (via the data extractor or the structure search); 7) ascertain whether a certain protein found in bacteria, fungi or viruses could be a drug target (via the sequence search); or 8) ascertain whether a newly identified human protein, such as an isoform or paralogue, may be a drug target (through the sequence search).

6. T3DB – A Resource linking Small Molecules to Disease & Toxicity

A toxic substance is a small molecule, peptide, or protein that is capable of causing injury, disease, genetic mutations, birth defects or death. Toxins, both natural and man-made, represent an important class of poisonous compounds that are ubiquitous in nature, in homes, and in the workplace. Common toxins include pollutants, pesticides, preservatives, drugs, venoms, food toxins, cosmetic toxins, dyes, and cleaning compounds. Because toxic compounds are essentially disease-causing agents, it has long been recognized that there is a need to associate toxic compound data with molecular toxicology and clinical symptomology. While this has been done in a variety of toxicology textbooks and medical reference manuals, it has only recently been done using electronic databases and the tools associated with bioinformatics and cheminformatics.

T3DB [36] is currently the only chemical-bioinformatic database that provides in-depth, molecular-scale information about toxins, their associated targets, their toxicology, their toxic effects and their potential treatments. T3DB currently contains over 3000 toxic substance entries corresponding to more than 34,000 different synonyms. These toxins are further connected to some 1450 protein targets through almost 35,500 toxin and toxin-target associations. These associations are supported by more than 5400 references. The entire database, including text, sequence, structure and image data, occupies nearly 16 Gigabytes of data – most of which can be freely downloaded.

As with HMDB and DrugBank, the T3DB is designed to be a fully searchable web resource with many built-in tools and features for viewing, sorting and extracting toxin and toxin-target annotation, including structures and gene and protein sequences. A screenshot montage illustrating the types of viewing and searching options available is shown in Figure 4. As with HMDB and DrugBank, the T3DB supports standard text queries through the text search box located on the home page. It also offers general database browsing using the "Browse" button located in the T3DB navigation bar. To facilitate browsing, the T3DB is divided into synoptic summary tables which, in turn, are linked to more detailed "ToxCards"- in analogy to the DrugCard concept found in DrugBank [32] or the MetaboCard in HMDB [26]. All of the T3DB's summary tables can be rapidly browsed, sorted or reformatted in a manner similar to the way PubMed abstracts may be viewed. Clicking on the ToxCard button, found in the leftmost column of any given T3DB summary table, opens a webpage describing the toxin of interest in much greater detail. Each ToxCard entry contains over 80 data fields, with ~50 data fields devoted to chemical and toxicological/medical data and ~30 data fields (each) devoted to describing the toxin target(s).

A ToxCard begins with various identifiers and descriptors (names, synonyms, compound description, structure image, related database links and ID numbers), followed by additional structure and physico-chemical property information. The remainder of data on the toxin is devoted to providing detailed toxicity and toxicological data, including route of delivery, mechanism of action, medical information, and toxicity measurements. All of a toxin's targets are also listed within the ToxCard. Each of these targets are described by some 30 data fields that include both chemical and biological (sequence, molecular weight, gene ontology terms, etc.) information, as well as details on their role in the mechanism of action of the toxin. In addition to providing comprehensive numeric, sequence and textual data, each ToxCard also contains hyperlinks to other databases, abstracts, digital images and interactive applets for viewing the molecular structures of each toxic substance.

A key feature that distinguishes the T3DB from other on-line toxin or toxicology resources is its extensive support for higher-level database search and selection functions. In addition to the data viewing and sorting features already mentioned, the T3DB also offers a local BLAST search that supports both single and multiple sequence queries, a boolean text search based on KinoSearch, a chemical structure search utility based on ChemAxon's MarvinView, and a relational data extraction tool similar to that found in DrugBank and the HMDB [26,32]. These can all be accessed via the database navigation bar located at the top of every T3DB page.

T3DB's simple text search box (located at the top of most T3DB pages) supports text matching, text match rankings, misspellings and highlights text where the word is found. In addition to this simple text search, T3DB's TextQuery function supports more sophisticated text querying functions including "and" and "or" queries, multi-word matching and parenthetical groupings as well as data-field-specific queries such as finding the query word only in the "Compound Source" field. Additional details and examples are provided on the T3DB's TextQuery page.

T3DB's sequence searching utility (SeqSearch) allows users to search through T3DB's collection of 1450 known (human) toxin targets. This service potentially allows users to identify both orthologous and paralogous targets for known toxins or toxin targets. It also facilitates the identification of potential toxin targets from other animal species. With SeqSearch, gene or protein sequences may be searched against the T3DB's sequence database of identified toxin-target sequences by pasting the FASTA formatted sequence (or sequences) into the SeqSearch query box and pressing the "submit" button.

T3DB's structure similarity search tool (ChemQuery) can be used in a similar manner as its SeqSearch tool. Users may sketch a chemical structure (through ChemAxon's freely available chemical sketching applet) or paste a SMILES string of a query compound into the ChemQuery window. Submitting the query launches a structure similarity search that looks for common substructures from the query

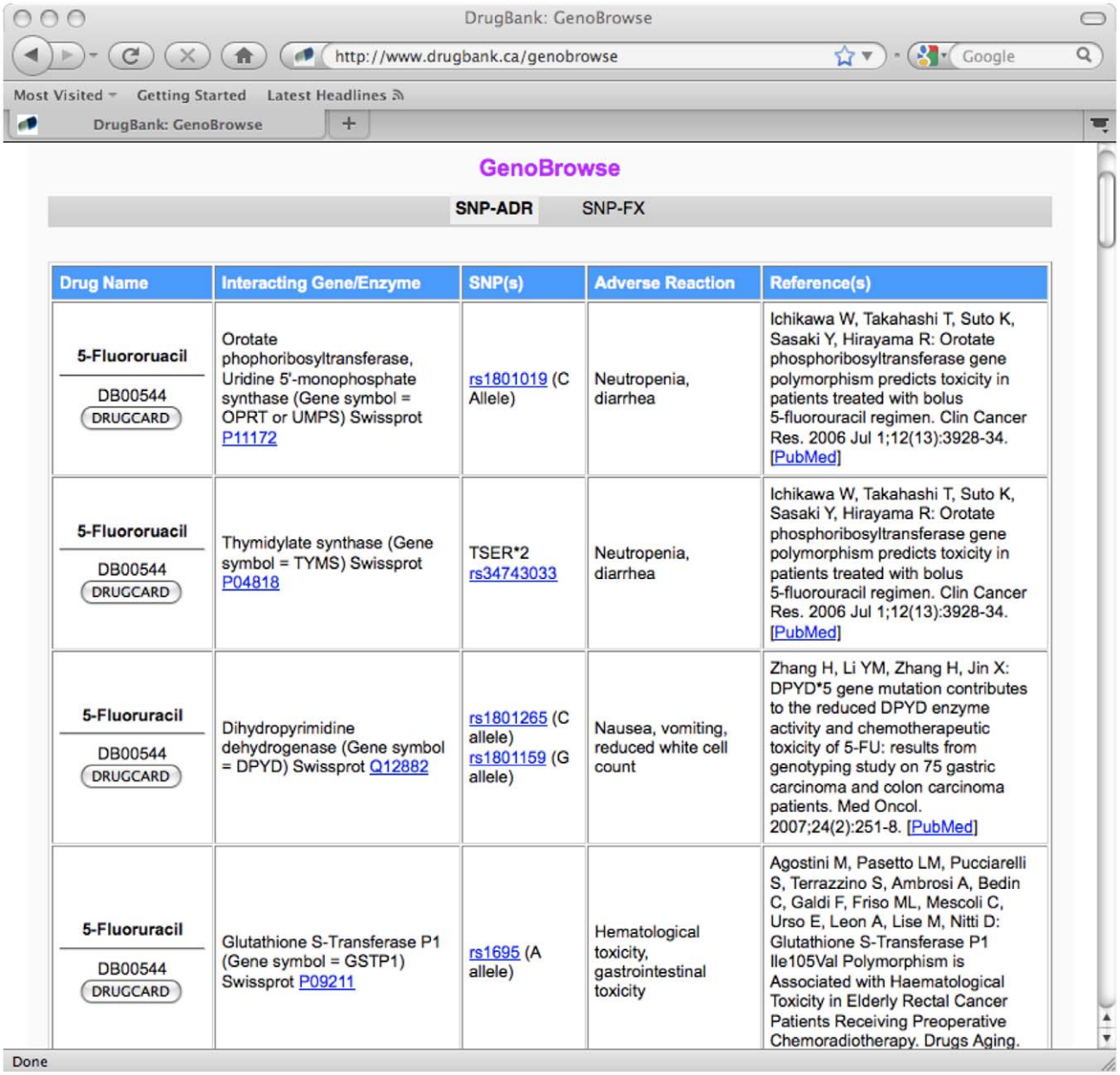

Figure 3. A screen shot of DrugBank's SNP-ADR table. This displays the information on the adverse drug reactions (ADRs) and associated SNP (single nucleotide polymorphisms) with certain drugs and drug targets (http://www.drugbank.ca).
doi:10.1371/journal.pcbi.1002805.g003

compound that matches the T3DB's database of known toxic compounds. Users can also select the type of search (exact or Tanimoto score) to be performed. High scoring hits are presented in a tabular format with hyperlinks to the corresponding ToxCards (which, in turn, links to the targets). The ChemQuery tool allows users to quickly determine whether their compound of interest is a known toxin or chemically related to a known toxin and which target(s) it may act upon. In addition to these structure similarity searches, the ChemQuery utility also supports compound searches on the basis of SMILES strings (under the SMILES tab) and molecular weight ranges (under the Molecular Weight tab).

The T3DB's data extraction utility (Data Extractor) employs a simple relational database system that allows users to select one or more data fields and to search for ranges, occurrences or partial occurrences of words, strings, or numbers. The data extractor uses clickable web forms so that users may intuitively construct SQL-like queries. Using a few mouse clicks, it is relatively simple to construct complex queries ("find all toxins that target acetylcholinesterase and are pesticides") or to build a series of highly customized tables. The output from these queries is provided in HTML format with hyperlinks to all associated ToxCards.

To summarize, T3DB allows users to link toxic substances to a variety of disease conditions, including acute toxicity, long-term toxicity, birth defects, cancer, other illnesses. It also provides links between toxic substances and their targets – both through descriptions of the mechanism of action and through the identification of

known protein (or gene) targets. Because of this kind of extensive data linkage, T3DB can potentially support a variety of bioinformatic or cheminformatic queries. For example, through T3DB it is possible for users to: 1) determine the similarity of a newly found/synthesized compound to an existing toxin (via the structure similarity search); 2) determine the possible mechanism of action or protein targets for a newly found/synthesized compound (via the structure similarity search); 3) diagnose or determine the cause of illnesses thought to be brought on by exposure to a given toxin (through the text search); 4) treat or find references to the treatment of illnesses brought on by exposure to a given toxin (via the text search); 5) extract information on common toxin targets (via the data extractor); 6) extract information on common toxin classes (via the data extractor); 7) ascertain whether a certain protein or protein homologue may also be a toxin target (via the sequence search); or 8) ascertain whether a newly identified peptide or protein may be a toxin (through the sequence search).

7. Software for Interpreting Small Molecule and Disease Data

With the recent emergence of chemical-bioinformatic databases having a solid translational (i.e. biomedical) functionality, the way has been cleared for the development of software tools that exploit these databases. This is a natural process in both bioinformatics and cheminformatics as databases always appear before any software applications are typically developed. Given that the field of chemical bioinformatics is still quite young and the number of databases with disease and small molecule information is still relatively small, it is not surprising to find that the number of software tools developed to exploit these databases is still quite small. Here we will briefly describe two recently developed software tools – PolySearch and MSEA – that exploit the data in SMPDB, HMDB and DrugBank to perform a number of useful applications.

7.1 Text Mining with PolySearch

PolySearch [44] is a freely available, web-based text-mining tool that allows users to search through large numbers of PubMed abstracts to make large-scale linkages or associations. Examples of large-scale associations are: "Find all genes associated with breast cancer" or "Find all diseases treatable by tamoxifen". In order to conduct the first query using PubMed, one would have to have a list of all known human genes and perform 25,000+ queries with each gene name and the words "breast cancer". To conduct the second query, it would be necessary to have a list of all known diseases (more than 5000 are known) and perform 5000+ queries with the word "tamoxifen" included in each query. Obviously this would take a person a very long time. However, using a computer to perform these repeated queries would be much less tedious and much faster. PolySearch is designed to rapidly perform these types of expansive queries by exploiting the PubMed application programming interface (API) and a special collection of dictionaries and thesauruses compiled from various bioinformatic and chemical-bioinformatic databases. In particular, the typical query supported by PolySearch is "Given X, find all Y's" where X or Y can be diseases, tissues, cell compartments, gene/protein names, SNPs, mutations, drugs and metabolites. The disease names and synonyms in PolySearch are derived from medical dictionaries and MeSH (medical subject headings), gene and protein names/synonyms are derived from UniProt, drug names/synonyms are derived from DrugBank while metabolites and metabolite synonyms are derived from the HMDB. Obviously, without these small molecule dictionaries or thesauruses, many of PolySearch's queries could not be performed.

PolySearch also exploits a variety of techniques in text mining and information retrieval to identify, highlight and rank informative abstracts, paragraphs or sentences. A central premise to PolySearch's search strategy is the assumption that the greater the frequency with which an X and Y association occurs within a collection of abstracts, the more significant the association is likely to be. For instance, if COX2 is mentioned in PubMed as being associated with colon cancer 510 times but thioredoxin is associated with colon cancer only once, then one is more likely to have more confidence in the COX2-colon cancer association. Frequency alone is not always the best way to rate a paper or a website for its relevancy. Therefore, in addition to counting the frequency of apparent associations, PolySearch employs a specially developed text-ranking scheme to score the most relevant sentences and abstracts that associate both the query and match terms with each other.

In summary, PolySearch is able to exploit the name and synonym sets from a number of small-molecule and disease databases (HMDB, DrugBank, MeSH, OMIM) thereby allowing users to perform a range of text mining queries on the PubMed abstract database. In particular, PolySearch allows users to find newly described or previously unknown (to the user, at least) associations between: 1) drugs and disease; 2) metabolites and disease; 3) genes/proteins and disease; 4) drugs and drug targets; 5) metabolites and metabolizing enzymes; 6) SNPs and disease and 7) mutations and disease. In addition, through its other query fields or query options, PolySearch is able to perform a large number (>50) of other text queries that may be relevant to a variety of applications in translational bioinformatics.

7.2 Metabolite Set Enrichment Analysis

The Metabolite Set Enrichment Analysis (MSEA) server [45] is a web-based tool designed to help researchers identify and interpret patterns of human or mammalian metabolite concentration changes in a biologically meaningful context. It is based on the concepts originally developed for gene expression or microarray analysis called Gene Set Enrichment Analysis or GSEA [8]. The central idea behind GSEA is to directly investigate the enrichment of pre-defined groups of functionally related genes (or gene sets) instead of individual genes. This group-based approach does not require pre-selection of genes with an arbitrary threshold. Instead, functionally related genes are evaluated together as gene sets, allowing additional biological information to be incorporated into the analysis process. Key to the development of GSEA has been the compilation of libraries or databases of gene expression changes that are associated with specific conditions, pathways, diseases or perturbations. Therefore in order to develop MSEA, it was necessary to extract a large body of metabolite expression changes (i.e. chemical profiles) and metabolic pathway information from a variety of databases. Fortunately, the existence of SMPDB and HMDB made the compilation of this metabolite expression library relatively easy. By downloading the freely available data in HMDB and SMPDB, the authors of MSEA were able to construct a collection of five metabolite set libraries containing ~1,000 biologically meaningful groups of metabolites. In MSEA, a group of metabolites are considered to constitute a metabolite set if they are known to be: a) involved in the same biological processes (i.e., metabolic pathways, signaling pathways); b) changed significantly under the same pathological conditions (i.e., various metabolic diseases); and c) present in the same locations

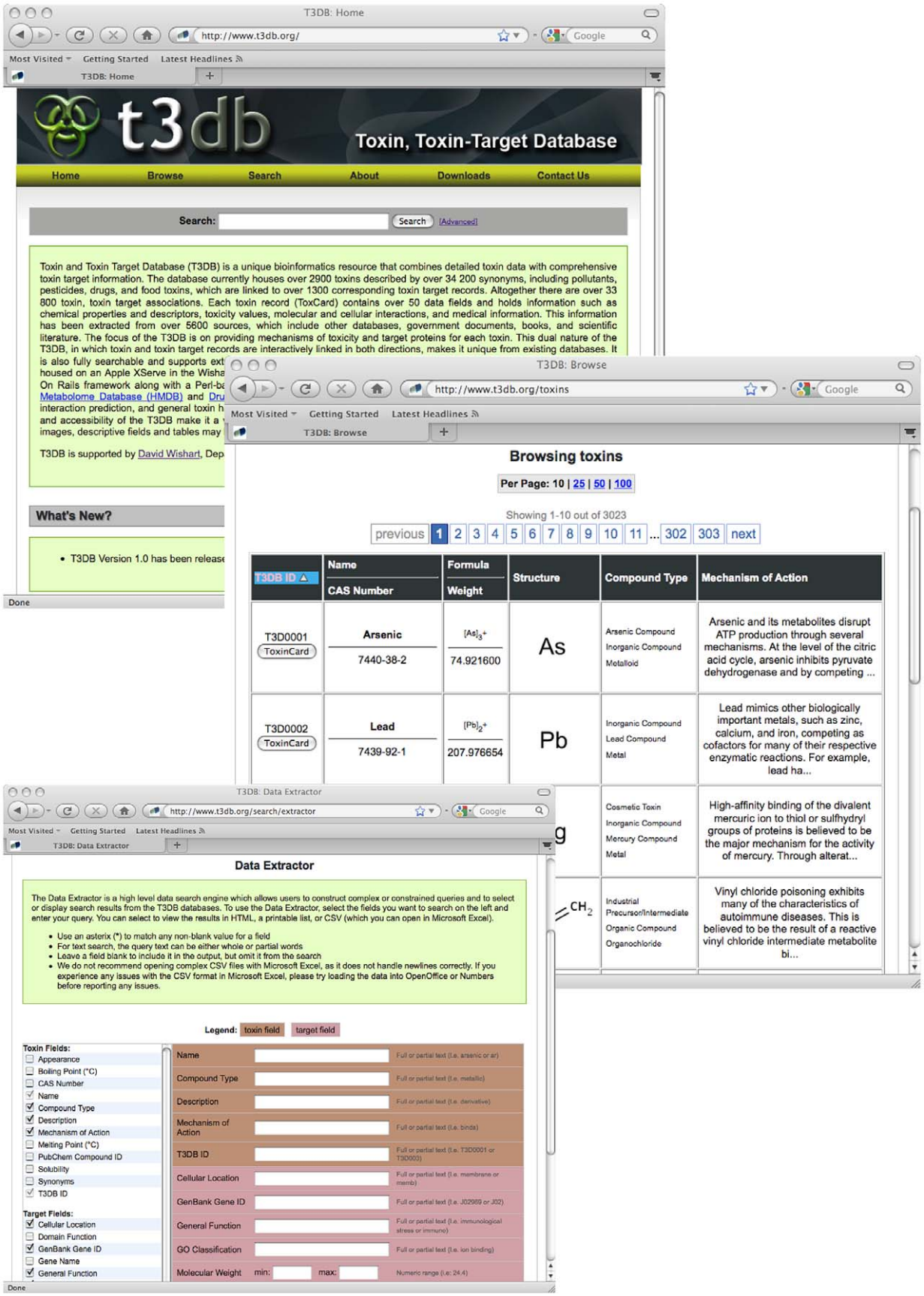

Figure 4. A screenshot montage illustrating the types of viewing and searching options available in T3DB (http://www.t3db.org).
doi:10.1371/journal.pcbi.1002805.g004

such as organs, tissues or cellular organelles. The resulting metabolite sets were organized into three categories: pathway-associated, disease-associated, and location based. MSEA's pathway-associated metabolite library contains 84 entries based on the 84 human metabolic pathways found in SMPDB. MSEA's disease-associated metabolite sets were mainly collected from information in the HMDB, the Metabolic Information Center (MIC), and SMPDB. Using these resources, a total of 851 physiologically informative metabolite sets were created. These disease-associated metabolite sets were further divided into three sub-categories based on the biofluids in which they were measured: 398 metabolite sets in blood, 335 in urine, and 118 in cerebral-spinal fluid (CSF). MSEA's location-based library contains 57 metabolite sets based on the "Cellular Location" and "Tissue Location" listed in the HMDB.

While the exact statistical or enrichment analysis methods used in MSEA are well beyond the scope of this chapter, suffice it to say that MSEA essentially allows one to take lists of metabolites and to identify which pathways, diseases or medical conditions are most likely to be associated with that metabolite set. It is also possible to do the same kind of operation with a list of metabolites and their absolute (or relative) concentrations. While disease/metabolite associations can be made through HMDB and SMPDB, these primitive search tools do not have the same statistical rigor that characterizes a full-fledged enrichment analysis. Furthermore, the MSEA pathway and disease data set is somewhat larger than what is found in the HMDB or SMPDB. This means that MSEA will be far more likely to find a useful (and statistically significant) pathway or disease than what could be done with HMDB or SMPDB.

Overall, MSEA is an example of an analytical software tool that exploits chemical-bioinformatic data to perform robust statistical analyses of metabolomic or clinical chemistry data. Given their close similarity, it is reasonable to expect MSEA could eventually be integrated with GSEA, thereby allowing a comprehensive analysis of both gene and metabolite expression changes on a single integrated program or website. No doubt this kind of integrated "omic" analysis tool is not far away from being developed.

8. Summary

With today's focus on genes and proteins as the "primary" causes or biomarkers of disease, the relationship between small molecules and human disease is often overlooked. However it is important to remember that more than 95% of all diagnostic clinical assays are designed to detect small molecules (i.e. blood glucose, serum creatinine, amino acid analysis, etc.). Likewise nearly 90% of all known drugs are small molecules, 50% of all drugs are derived from pre-existing metabolites and 30% of identified genetic disorders involve diseases of small molecule metabolism. Clearly, small molecules are important and given the rapid growth in metabolomics, pharmacogenomics and systems biology, it is likely that their role in disease diagnosis and disease treatment will continue to grow. Given these exciting growth prospects and given the importance of small molecules in medicine and translational research, scientists are now realizing that there is a critical need to link information about small molecules to their corresponding "big molecule" targets. This has led to the emergence of a new field of bioinformatics – called chemical bioinformatics.

This chapter has covered several topics related to chemical bioinformatics and the role that chemical bioinformatics can play in identifying the chemicals that cause (toxins), cure (drugs) or characterize (biomarkers) many human diseases. The first part of the chapter gave a brief overview of some of the most important or widely used chemical bioinformatic resources along with a more detailed discussion of some of the major classes of chemical-bioinformatic databases. In particular four major database classes were described: 1) small molecule (or metabolic) pathway databases; 2) metabolite or metabolomic databases; 3) drug databases; and 4) toxin or toxic substance databases. Examples of each of these databases were given and many of their strengths and limitations were discussed. While most of these chemical-bioinformatic databases provide links between small molecules and their large molecule targets, relatively few provide linkages to clinical, physiological or disease information.

The second part of this chapter focused on describing a number of recently developed databases that explicitly relate small molecules to disease. This included detailed descriptions of four databases: 1) the Small Molecule Pathway Database (SMPDB); 2) the Human Metabolome Database (HMDB); 3) DrugBank and 4) the Toxin, Toxin-Target Database (T3DB). SMPDB is a graphically oriented pathway database that contains ~450 metabolic pathways, disease pathways and drug pathways. The HMDB is a comprehensive metabolomic database that is primarily oriented to answering questions in clinical metabolomic and clinical biochemistry. DrugBank is a comprehensive drug database containing detailed information about drugs, drug targets and clinical pharmacology. The T3DB is a toxicology database containing detailed information about toxins, toxin targets and their corresponding toxicological information. Each of these databases was described in terms of its content, general design and query/search functions. Additionally, explicit examples of various translational or disease-related applications were provided for each database. The final part of this chapter provided a short discussion of some of the newly emerging software tools that exploit these databases, including PolySearch and MSEA (Metabolite Set Enrichment Analysis). PolySearch is a text-mining tool that exploits the synonym data found in these small molecule databases to allow expansive PubMed queries to be performed. MSEA is a metabolomic analysis tool that exploits the pathway and disease information found in SMPDB and HMDB to perform pathway and disease identification from raw metabolomic data.

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Glossary

Cheminformatics – a field of information technology that uses computers to facilitate the collection, storage, analysis and manipulation of large quantities of chemical data.

DrugBank – A database containing chemical and biological data on drugs and drug targets.

GSEA – Gene Set Enrichment Analysis. GSEA is a statistically based bioinformatic method designed to directly investigate the enrichment of pre-defined groups of functionally related genes (or gene sets) from gene expression data.

HMDB – The Human Metabolome Database. A database containing chemical and biological data on human metabolites aimed at clinical metabolomic studies.

MS – Mass Spectrometry. An analytical method that measures molecular weight of compounds based on their mass to charge ratio. Mass spectrometry is one of the standard methods to determine the molecular formula of new compounds and to confirm the identity of synthesized chemicals or natural products.

Metabolome – the collection of all small molecule metabolites found in a given cell, tissue, organ or organism.

Metabolomics - a branch of "omics" research that is primarily concerned with the high-throughput identification and quantification of small molecule (<1500 Da) metabolites in the metabolome.

MSEA – Metabolite Set Enrichment Analysis. MSEA is a statistically based bioinformatic method designed to directly investigate the enrichment of pre-defined groups of functionally related metabolites (or metabolite sets) from metabolomic data.

NMR – Nuclear Magnetic Resonance Spectroscopy. An analytical method that measures nuclear magnetism under very high magnetic fields. NMR is the standard method used by chemists today to identify and characterize small molecules.

Pharmacogenomics – A newly emerging field of pharmacology that integrates genotyping and gene expression data with classical pharmacological and adverse drug reaction studies.

SMPDB – The Small Molecule Pathway Database. A database containing pathway diagrams and interactive viewing tools for small molecules involved in metabolism, drug action and disease.

T3DB – The Toxin, Toxin-Target Database. A database with chemical and biological data on common toxins, poisons, household chemicals, pollutants and other harmful substances.

Toxicogenomics – A newly emerging field of toxicology that integrates genotyping and gene expression data with classical toxicological and toxicity studies.

While the sub-discipline of chemical bioinformatics is still quite young, and the number of tools for translational applications is still relatively small, it should be clear that what is now out there has considerable potential for a wide range of clinical, biomedical, pharmaceutical and toxicological applications. Certainly as more tools are developed and as more databases evolve, it is likely that chemical bioinformatics will soon be able to establish itself as one of the most medically useful sub-disciplines in the entire field of bioinformatics.

9. Exercises

1) A compound with a molecular weight of 136.053 daltons has been isolated from the urine of a 3 month-old baby with unusually light coloring of the skin, eczema (an itchy skin rash), and a musty body odor. What compound is it and what disease might this baby have?

2) Your natural product chemist neighbor has just isolated a compound from the Tanzanian periwinkle – a rare plant species found only in the highlands of Eastern Tanzania. Locals use the plant as a treatment for a variety of blood disorders. The structure of the compound is given by the following SMILES string: COC1 = CC = C2C(= C C1 = O)C(CCC1 = CC(OC) = C(OC)C(OC) = C21)NC(CO)

What compound is this similar to, what diseases could it be used to treat and what proteins might it bind?

3) A viral protein with the following sequence has been isolated from a number of dead and dying African Green Monkeys that were housed at a local zoo.

PQVTLYQRPLVTIRVGGQLKEALIDTGADD
TVLENMNLPGRWKPKMIGAIAGFIKVKQYDQI
TVEICGHKGIGTILVGPTPVNIIGRNLLTLIG
CTLNF

The illness seems to be spreading to other monkey colonies in the zoo. What drugs could be used to treat the sick monkeys and to prevent the spread of the disease?

4) A farmer who has just finished harvesting his barley field has come into the clinic complaining of skin irritation, burning and itching, a rash and a series of skin blisters. He also has eye pain, conjunctivitis, burning sensations about the eyes, and blurred vision. Other symptoms have included nausea, vomiting and fatigue. Suspecting that he may have been exposed to some toxin or pesticide a chemical analysis has been performed of his blood, urine and lacrimal (tear) fluid. MS analysis of all three fluids has identified an unusual compound with a molecular weight of 296.126 daltons. What compound might this be?

5) What kind of drugs can be used to treat breast cancer? Describe your search strategy and your rationale for this search strategy.

Answers to the Exercises can be found in Text S1.

Supporting Information

Text S1 Answers to Exercises.
(DOC)

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Chapter 4: Protein Interactions and Disease

Mileidy W. Gonzalez[1], Maricel G. Kann[2]*

1 National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Maryland, United States of America, **2** Biological Sciences, University of Maryland, Baltimore County, Baltimore, Maryland, United States of America

Abstract: Proteins do not function in isolation; it is their interactions with one another and also with other molecules (e.g. DNA, RNA) that mediate metabolic and signaling pathways, cellular processes, and organismal systems. Due to their central role in biological function, protein interactions also control the mechanisms leading to healthy and diseased states in organisms. Diseases are often caused by mutations affecting the binding interface or leading to biochemically dysfunctional allosteric changes in proteins. Therefore, protein interaction networks can elucidate the molecular basis of disease, which in turn can inform methods for prevention, diagnosis, and treatment. In this chapter, we will describe the computational approaches to predict and map networks of protein interactions and briefly review the experimental methods to detect protein interactions. We will describe the application of protein interaction networks as a translational approach to the study of human disease and evaluate the challenges faced by these approaches.

This article is part of the "Translational Bioinformatics" collection for *PLOS Computational Biology*.

1. Introduction

Early biological experiments revealed proteins as the main agents of biological function. As such, proteins ultimately determine the phenotype of all organisms. Since the advent of molecular biology we have learned that proteins do not function in isolation; instead, it is their interactions with one another and also with other molecules (e.g. DNA, RNA) that mediate metabolic and signaling pathways, cellular processes, and organismal systems.

The concept of "protein interaction" is generally used to describe the physical contact between proteins and their interacting partners. Proteins associate physically to create macromolecular structures of various complexities and heterogeneities. Proteins interact in pairs to form dimers (e.g. reverse transcriptase), multi-protein complexes (e.g. the proteasome for molecular degradation), or long chains (e.g. actin filaments in muscle fibers). The subunits creating the various complexes can be identical or heterogeneous (e.g. homodimers vs. heterodimers) and the duration of the interaction can be transient (e.g. proteins involved in signal transduction) or permanent (e.g. some ribosomal proteins). However, protein interactions do not always have to be physical [1]. The term "protein interaction" is also used to describe metabolic or genetic correlations, and even co-localizations. Metabolic interactions describe proteins involved in the same pathway (e.g. the Krebs cycle proteins), while genetically identified associations identify co-expressed or co-regulated proteins (e.g. enzymes regulating the glycolytic pathway). As the name implies, protein interactions by co-localization list proteins found in the same cellular compartment.

Whether the association is physical or functional, protein-protein interaction (PPI) data can be used in a larger scale to map networks of interactions [2,3]. In PPI network graphs, the nodes represent the proteins and the lines connecting them represent the interactions between them (Figure 1). Protein interaction networks are useful resources in the abstraction of basic science knowledge and in the development of biomedical applications. By studying protein interaction networks we can learn about the evolution of individual proteins and about the different systems in which they are involved. Likewise, interaction maps obtained from one species can be used, with some limitations, to predict interaction networks in other species. Protein interaction networks can also suggest functions for previously uncharacterized proteins by uncovering their role in pathways or protein complexes [4]. Due to their central role in biological function, protein interactions also control the mechanisms leading to healthy and diseased states in organisms. Diseases are often caused by mutations affecting the binding interface or leading to biochemically dysfunctional allosteric changes in proteins. Therefore, protein interaction networks can elucidate the molecular basis of disease, which in turn can inform methods for prevention, diagnosis, and treatment [5,6].

The study of human disease experienced extensive advancements once the biomedical characterization of proteins shifted to studies taking into account a protein's network at different functional levels (i.e. in pair-wise interactions, in complexes, in pathways, and in whole genomes). For instance, consider how our understanding of Huntington's disease (HD) has evolved from the early Mendelian single-gene studies to the latest HD-specific network-based analyses. HD is an autosomal dominant neurodegenerative disease with features recognized by Huntington in 1872 [7], and whose specific patterns of inheritance were documented in 1908 [8]. After almost a century of genetics studies, the culprit gene in HD was identified; in 1993, we learned that HD was caused by the repeat expansion of a CAG trinucleotide in the Huntingtin (*Htt*) gene [9]. This expansion causes aggrega-

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* E-mail: mkann@umbc.edu

What to Learn in This Chapter

- Experimental and computational methods to detect protein interactions
- Protein networks and disease
- Studying the genetic and molecular basis of disease
- Using protein interactions to understand disease

tion of the mutant *Htt* in insoluble neuronal inclusion bodies, which consequently leads to neuronal degeneration. Yet, even when the key disease-causing protein in HD had been identified, the mechanism for *Htt* aggregation remained unknown. In 2004, Goehler *et al.* [10] mapped all the PPIs that take place in HD and discovered that the interaction between *Htt* and GIT1, a GTPase-activating protein, mediates *Htt* aggregation. Further validation ([11,12]) confirmed GTI1's potential as a target for therapeutic strategies against HD.

In this chapter, we will describe the main experimental methods to identify protein interactions and the computational approaches to map their networks and to predict new interactions purely *in silico*. We will describe the application of protein interaction networks as a translational approach to the study of human disease and evaluate the challenges faced by these approaches.

2. Experimental Identification of PPIs

2.1 Biophysical Methods

Protein interactions are identified through different biochemical, physical, and genetic methods (Figure 2). Historically, the main source of knowledge about protein interactions has come from biophysical methods, particularly from those based on structural information (e.g. *X-ray crystallography*, *NMR spectroscopy*, *fluorescence*, *atomic force microscopy*). Biophysical methods identify interacting partners and also provide detailed information about the biochemical features of the interactions (e.g. binding mechanism, allosteric changes involved). Yet, since they are time- and resource-consuming, biophysical characterizations only permit the study of a few complexes at a time.

2.2 High-Throughput Methods

To document protein interactions at a larger scale, automated methods have been developed to detect interactions directly or to deduce them through indirect approaches (Figure 2).

2.2.1 Direct high-throughput methods. *Yeast two-hybrid* (Y2H) is one of the most-commonly used direct high-throughput method. The Y2H system tests the interaction of two given proteins by fusing each of them to a transcription-binding domain. If the proteins interact, the transcription complex is activated, which transcribes a reporter gene whose product can be detected. Since it is an *in vivo* technique, the Y2H system is highly effective at detecting transient interactions and can be readily applied to screen large genome-wide libraries (e.g. to map an organisms' full set of interactions or interactome). But, the Y2H system is limited by its biases toward non-specific interactions. Likewise, Y2H cannot identify complexes (i.e. it only reports binary interactions) or interactions of proteins initiating transcription by themselves. Although protein interactions are usually detected and studied in pair-wise form, in reality they often occur in complexes and as part of larger networks of interaction. *In vitro* direct detection methods (e.g. *mass spectrometry*, *affinity purification*) are better suited to detect macromolecular interactions, yet, they have their own limitations: interactions occurring *in vitro* do not necessarily occur *in vivo* (e.g. when proteins are compartmentalized in different cell locations) and complexes are often difficult to purify, which is a required step in the protocol [13].

2.2.2 Indirect high-throughput methods. Several high-throughput methods deduce protein interactions by looking at characteristics of the genes encoding the putative interacting partners. For instance, *gene co-expression* is based on the assumption that the genes of interacting proteins must be co-expressed to provide the products for protein interaction. Expression profile similarity is calculated as a correlation coefficient between relative expression levels and subsequently compared against a background distribution for random non-interacting proteins. *Synthetic lethality*, on the other hand, introduces mutations on two separate genes, which are viable alone but lethal when combined, as a way to deduce physically interacting proteins [14].

3. Computational Predictions of PPIs

As discussed in section 2, experimental approaches provide the means to either empirically characterize protein interactions at a small scale or to detect them at a large scale. Still, experimental detections only generate pair-wise interaction relationships and with incomplete coverage (because of experimental biases toward certain protein types and cellular localizations). Experimental identification methods also exhibit an unacceptably high fraction of false positive interactions and often show low agreement when generated by different techniques [15–17]. Experimental biophysical methods can complement the high-throughput detections by providing specific interaction details; but they are expensive, extremely laborious, and can only be implemented for a few complexes at a time.

Computational methods for the prediction of PPIs provide a fast and inexpensive alternative to complement experimental efforts. Computational interaction studies can be used to validate experimental data and to help select potential targets for further experimental screening [18]. More importantly, computational methods give us the ability to study proteins within the context of their interaction networks at different functional levels (i.e. at the complex, pathway, cell, or organismal level), thus, allowing us to convert lists of pair-wise relationships into complete network maps. Since they are based on different principles, computational techniques can also uncover functional relationships and even provide information about interaction details (e.g. domain interactions), which may elude some experimental methods.

3.1 Computational Methods for PPI Predictions

Computational interaction prediction methods can be classified into two types: methods predicting protein domain interactions from existing empirical data about protein-protein interactions and methods relying entirely on theoretical information to predict protein-protein or domain-domain interactions (Figure 2).

3.1.1 Empirical predictions. The computational techniques based on experimental data use the *relative frequency of interacting domains* [19], *maximum likelihood estimation of domain interaction probability* [20,21], *co-expression* [22], or *network properties* [23–27] to predict protein and domain interactions. The main disadvantage of empirical computations is that, by relying on an existing protein network to infer new nodes, they propagate the inaccuracies of the experimental methods.

3.1.2 Theoretical predictions. Theoretical techniques to predict PPIs

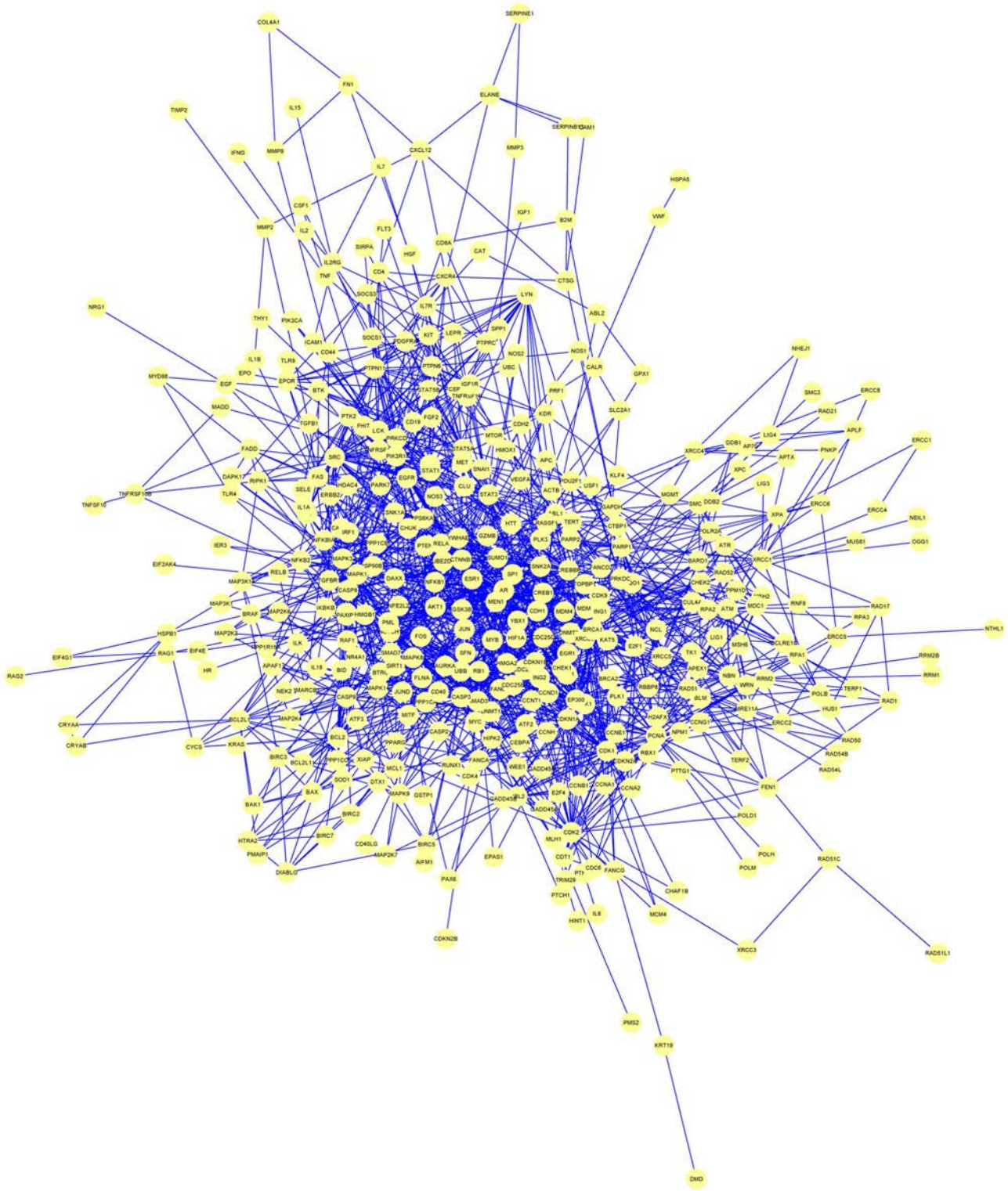

Figure 1. A PPI network of the proteins encoded by radiation-sensitive genes in mouse, rat, and human, reproduced from [89]. Yellow nodes represent the proteins and blue lines show the interactions between them. The radiation-related genes were text-mined from PubMed and the protein interaction information was obtained from HPRD.
doi:10.1371/journal.pcbi.1002819.g001

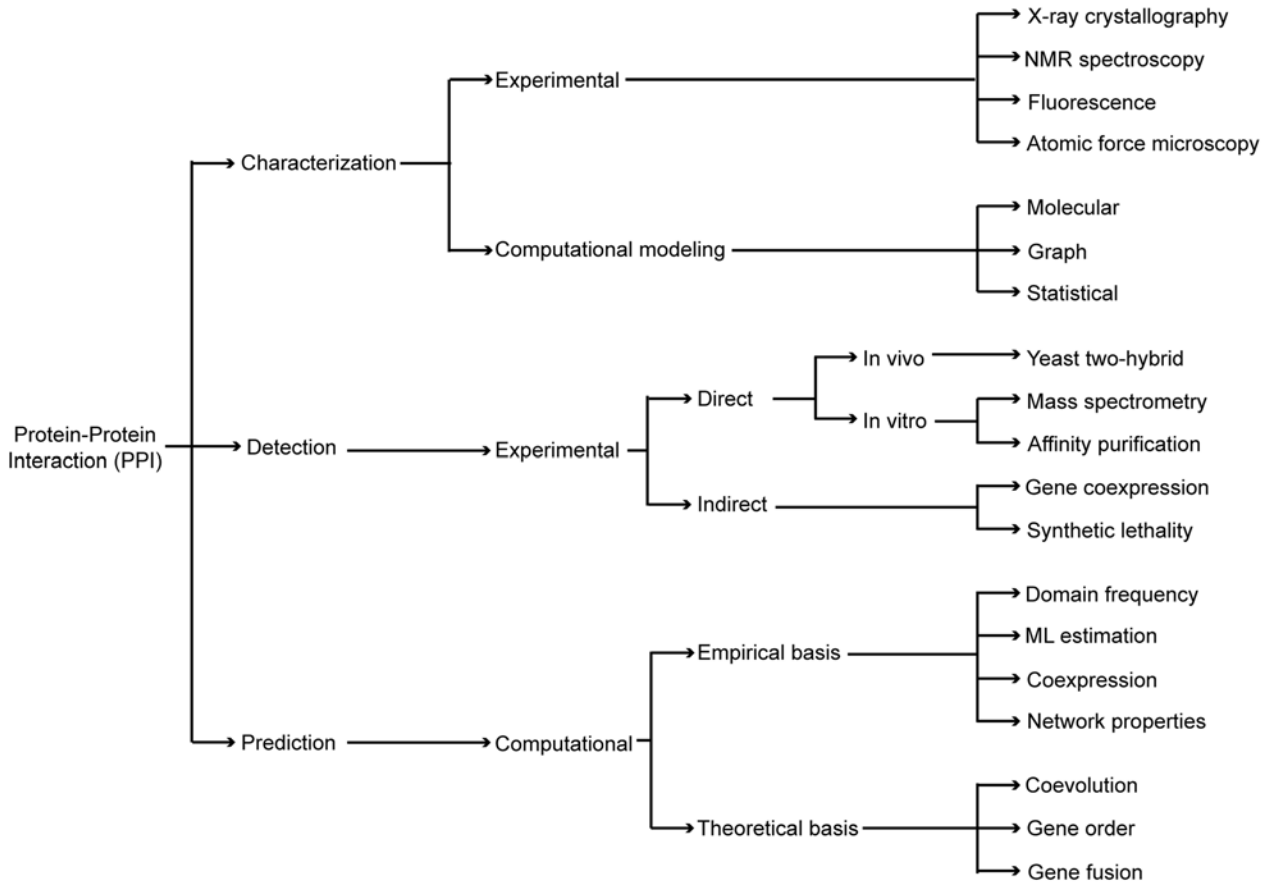

Figure 2. A diagram of the different experimental and computational methods to characterize, detect, and predict PPIs.
doi:10.1371/journal.pcbi.1002819.g002

incorporate a variety of biological considerations; they take advantage of the fact that interacting proteins coevolve to preserve their function (e.g. *mirrortree*, *phylogenetic profiling* [28–35]), occur in the same organisms (e.g. [36,37]), conserve gene order (e.g. *gene neighbors method* [38,39]) or are fused in some organisms (e.g. the *Rosetta Stone method* [40,41]).

3.2 Theoretical Predictions of PPIs Based on Coevolution

Below, we will expand on two methods generating theoretical PPI predictions through coevolutionary signal detection either at the residue or at the full-sequence level.

3.2.1 Coevolution at the residue level. Pairs of residues within the same protein can coevolve because of three-dimensional proximity or shared function [42]. The intramolecular correlations of interacting protein partners can be used to predict intermolecular coevolution. Residue-based coevolution methods measure the set of correlated pair mutations in each protein. A pair of proteins is assumed to interact if they show enrichment of the same correlated mutations [42].

3.2.2 Coevolution at the full-sequence level. Methods detecting coevolution at the full-sequence level are based on the idea that changes in one protein are compensated by correlated changes in its interacting partner to preserve the interaction [29,30,42–45]. Therefore, as interacting proteins coevolve, they tend to have phylogenetic trees with topologies that are more similar than expected by chance [46]. The coevolution of interacting proteins was first qualitatively observed for polypeptide growth factors, neurotransmitters, and immune system proteins with their respective receptors [47]. Several methodologies have been developed to measure coevolution at the full-sequence level, and among them, the mirrortree method is one of the most intuitive and accurate options. As shown in Figure 3, mirrortree measures coevolution for a given pair of proteins by i) identifying the orthologs of both proteins in common species, ii) creating a multiple sequence alignment (MSA) of each protein and its orthologs, iii) from the MSAs, building distance matrices, and iv) calculating the correlation coefficient between the distance matrices. The mirrortree correlation coefficient is used for measuring tree similarity, thereby, allowing the evaluation of whether the proteins in question coevolved [28–35].

The mirrortree method has been successfully implemented to confirm experimental interactions in *E. coli* [4], *S. cerevisiae* [48], and *H. sapiens* [49]. But, the degree of similarity between the phylogenetic trees is strongly affected by the sequence divergence driven by the underlying speciation process [4,50]. Therefore, two proteins may have similar phylogenetic trees due only to common speciation events, but they may not necessarily be interacting partners. By subtracting the signal from speciation events Pazos *et al.* [4] and Sato *et al.* [50] showed improvements for the performance of the mirrortree method. One approach creates a "speciation" vector from the distance matrices derived from

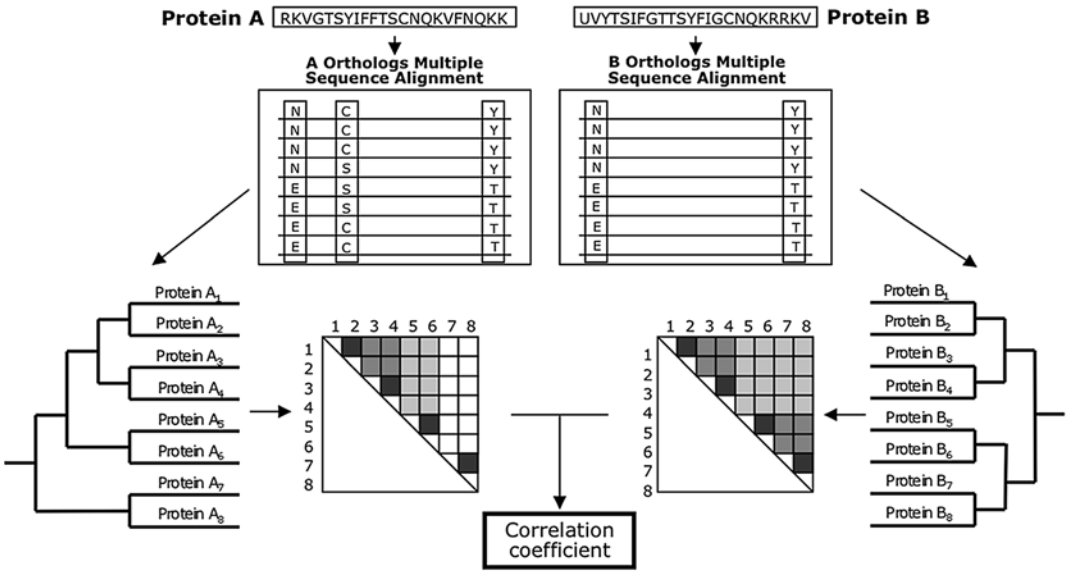

Figure 3. A schema of the mirrortree method for predicting interacting proteins. The orthologs of two proteins (A and B from the same species) are used to construct two multiple sequence alignments (MSAs). Distance matrices, which implicitly represent evolutionary trees, are constructed from the MSAs. Each matrix square represents the tree distance between two orthologs and dark colors represent closeness. The two distance matrices are compared using linear correlation. A high correlation between the distance matrices suggests interaction between proteins A and B.
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the ribosomal 16S sequences (for prokaryotes and 18S for eukaryotes), while the other uses the average distance of all proteins in a pair of organisms. Both methods subtract the speciation vector from the original distance matrix constructed for the given protein pair.

In principle, to characterize protein interactions at a systems level, all protein-protein and domain-domain interactions in a given organism must be catalogued. The mirrortree method is a suitable option to complement experimental detections because it is inexpensive and fast. Moreover, mirrortree only requires the proteins' sequences as input and thus can be used to analyze proteins for which no other information is available. Since mirrortree predictions are based on different principles than any other computational or experimental techniques, they can also uncover functional relationships eluding other methods. Still, the implementation of the mirrortree approach is under several limitations. One limitation of the mirrortree method is the minimum number of orthologs it requires. Selecting orthologs in large families with many paralogs is also a considerable challenge for mirrortree [49]. In addition, coevolution does not necessarily take place uniformly across the sequence; different sites may coevolve at different rates based on functional constraints. Thus, coevolution signals vary when measured across the entire sequence vs. at the domain level [51].

4. Protein Networks and Disease

4.1 Studying the Genetic Basis of Disease

The majority of our current knowledge about the etiology of various diseases comes from approaches aiming to uncover their genetic basis. In the near future, the ability to generate individual genome data using next generation sequencing methods promises to change the field of translational bioinformatics even more.

Since the inception of Mendelian genetics in the 1900's, great effort has gone into cataloguing the genes associated with individual diseases. A gene can be isolated based on its position in the chromosome by a process known as positional cloning [52]. A few examples of human disease-related genes identified by positional cloning include the genes associated with cystic fibrosis [53], HD [9], and breast cancer susceptibility [54,55]. Even in simple Mendelian diseases, however, the correlation between the mutations in the patient's genome and the symptoms is not often clear [56]. Several reasons have been suggested for this apparent lack of correlation between genotype and phenotype, including pleiotropy, influence of other genes, and environmental factors.

Pleiotropy occurs when a single gene produces multiple phenotypes. Pleiotropy complicates disease elucidation because a mutation on a pleiotropic gene may have an effect on some, all, or none of its traits. Therefore, mutations in a single gene may cause multiple syndromes or only cause disease in some of the biological processes the gene mediates. Establishing which genotypes are responsible for the perturbed phenotype of interest is not straightforward.

Genes can influence one another in several ways; genes can interact synergistically, (as in *epistasis*), or they can modify one another (e.g. the expression of one gene might affect the expression of another). Cystic fibrosis and Becker muscular dystrophy, previously considered classical examples of Mendelian patterns of inheritance, are now believed to be caused by a mutation of one gene which is modified by other genes [57,58]. Thus, even simple Mendelian diseases can lead to complex genotype-phenotype associations [59].

Environmental factors (e.g. diet, infection by bacteria) are also major determinants of disease phenotype expression often acting in combination with other genotype-phenotype association confounders (i.e. pleiotropy and gene modifiers). In fact, most common diseases such as cancer, metabolic, psychiatric and cardio-vascular disorders (e.g. diabetes, schizophrenia and hypertension) are believed to be caused by several genes (multigenic) and are affected by several environmental factors [60].

4.2 Studying the Molecular Basis of Disease

Much can be learned from documenting the genes associated with a particular

disease (e.g. identifying risk factors that might be used for diagnostic purposes). Yet, to understand the biological details of pathogenesis and disease progression and to subsequently develop methods for prevention, treatment and even diagnosis, it is necessary to identify the molecules and the mechanisms triggering, participating, and controlling the perturbed biological process. Deciphering the molecular mechanisms leading to diseased states is an even bigger challenge than elucidating the genetic basis of complex diseases [61]. Even when the genetic basis of a disease is well understood, not much is known about the molecular details leading to the disorders.

4.2.1 The role of protein interactions in disease. Protein interactions provide a vast source of molecular information; their interactions (with one another, DNA, RNA, or small molecules) are involved in metabolic, signaling, immune, and gene-regulatory networks. Since protein interactions mediate the healthy states in all biological processes, it follows that they should be the key targets of the molecular-based studies of biological diseased states. Disease-causing mutations affecting protein interactions can lead to disruptions in protein-DNA interactions, protein misfolds, new undesired interactions, or can enable pathogen-host protein interactions.

Protein-DNA interaction disruptions are most clearly illustrated by the p53 tumor suppressor protein and its role in cancer. Mutations on p53's DNA-binding domain destroy its ability to bind to its target DNA sequences, thus preventing transcriptional activation of several anti-cancer mechanisms it mediates (e.g. apoptosis, genetic stability, and inhibition of angiogenesis).

Protein misfolding can result in disruptions of protein-protein interactions, as occurs in the Von Hippel-Lindau syndrome (VHL)—VHL is a rare condition in which hemangioblastomas are formed in the cerebellum, spinal cord, kidney, and retina. A mutation from Tyrosine to Histidine at residue 98 on the binding site disrupts binding of the VHL protein to the hypoxia-inducible factor (HIF) protein. As a result, the VHL protein no longer degrades the HIF protein, which leads to the expression of angiogenic growth factors and local proliferation of blood vessels [62,63].

New undesired protein interactions are the main causes of several diseases, including Huntington's disease (see introduction), cystic fibrosis, and Alzheimer's disease. New interactions alter homeostasis since they can lead to the loss of vital cellular functions (due to misfolding and aggregation) and can cause cytotoxicity [11].

Pathogen-host protein interactions also play a key role in bacterial and viral infections by facilitating the hijacking of the host's metabolism for microbial need. The interaction between the Human papillomavirus (HPV) and its host provides one of the most striking examples of the centrality of protein interactions in infectious diseases. HPV infection occurs in a large fraction of the population (75–80% of Americans [64]) by generating lesions of the anogenital tract and for some it leads to cancer. Upon infection, the HPV genome is frequently integrated into the host genome, but only two viral genes (*E6* and *E7*) are retained and expressed. Remarkably, the interactions of only two viral proteins with the host's proteins are enough to cause HPV-induced carcinogenesis. E6 and E7 bypass the immune system by interacting with important negative cell regulatory proteins to target them for degradation and thus, inactivation. These two proteins also inhibit cellular terminal differentiation, induce cellular transformation and immortalization of the host cells, and direct the proliferation of the tumorigenically-transformed cells [65].

4.2.2 Using PPI networks to understand disease. *PPI networks can help identify novel pathways* to gain basic knowledge of disease. Note that pathways are different from PPI networks. PPI networks map the physical or functional interaction between protein pairs resulting in a complex grid of connections (Figure 1). Pathways, on the other hand, represent genetic, metabolic, signaling, or neural processes as a series of sequential biochemical reactions where substrates are changed in a linear fashion. For instance, the glycolysis pathway maps the conversion of glucose to pyruvate through a linear chain of ten different steps.

Pathway analysis alone cannot uncover the molecular basis of disease. When performing pathway analysis to study disease, differential expression experiments are the main source of protein candidates. However, most of the gene expression candidates are useless to pathway-based analysis of disease because the majority of human genes have not been assigned to a pathway. Protein interaction networks can be used to identify novel pathways. Protein interaction subnetworks tend to group together the proteins that interact in functional complexes and pathways [66]. Thus, new methods are being developed to accurately extract interaction subnetworks to yield pathway hypotheses that can be used to understand different aspects of disease progression [67,68]. See Table 1 for useful resources incorporating pathway and PPI information in disease elucidation.

Mapping interactomes provide the opportunity to identify disease pathways by identifying key subnetworks. In 2005, Rual *et al.* [69] mapped the human protein interactome. Below are some of the findings that have been uncovered when combining PPI and pathway analysis since then.

(i) Over 39,000 protein interactions have been identified in the human cell [70].

(ii) Disease genes are generally non-essential and occupy peripheral positions in the human interactome [71], although, in a few diseases like cancer, disease genes tend to encode highly-connected proteins (hubs) [72,73].

(iii) Disease genes tend to cluster together and co-occur in central network locations [6].

(iv) Proteins involved in similar phenotypes (e.g. all cancer proteins) are highly interconnected [73].

(v) Viral networks differ significantly from cellular networks, which raises the hypothesis that other intracellular pathogens might also have distinguishing topologies [74].

(vi) Etiologically unrelated diseases often present similar symptoms because separate biological processes often use common molecular pathways [75].

PPI networks can be used to explore the differences between healthy and diseased states. Building interaction networks for systems under different conditions (e.g. wild type vs. mutant, presence of environmental factor vs. its absence) might be the key to understanding the differences between healthy and pathological states. The work by Charlesworth *et al.* [76] on the perturbation of the canonical pathways and networks of interactions when humans are exposed to cigarette smoke illustrates the potential of such approaches. As one might expect, this study found that the smoking-susceptible genes were overrepresented in pathways involved in several aspects of cell death (cell cytotoxicity, cell lysis), cancer (e.g. tumorigenesis), and respiratory functions. A somewhat more unexpected finding, however, confirmed that exposure to the smoke environmental factor affected a large subnet-

Table 1. Pathway databases with disease information.

Resource	Featured organisms	Disease information	Website
KEGG	Yeast, mouse, human	Comprehensive	http://www.genome.jp/kegg/disease/
REACTOME	Human+20 other species	Sparse	http://reactome.org/
SMPDB	Human	Small molecules' Metabolic disease pathways	http://www.smpdb.ca
PharmGKB	Human	Gene-drug-disease relationships	http://www.pharmgkb.org/index.jsp
NetPath	Human	10 immune and 10 cancer pathways	http://www.netpath.org/index.html

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work of proteins involved in the immune-inflammatory response. This study gave new insights into how smoke causes disease: the exogenous toxicants in smoke perturb several protein interactions in the healthy cell state, thereby depressing the immune system, while disrupting the inflammation response. The study also explained why smoking cessation has some immediate health benefits; eliminating smoke exposure reverses the alterations at the transcriptomic level and restores the majority of normal protein interactions.

Protein interaction studies play a major role in the prediction of genotype-phenotype associations while also identifying new disease genes. The identification of disease-associated interacting proteins also identifies potentially interesting disease-associated gene candidates (i.e. the genes coding for the interacting proteins are putative disease-causing genes). One of the best ways to identify novel disease genes is to study the interaction partners of known disease-associated proteins [77]. Gandhi *et al.* [78] found that mutations on the genes of interacting proteins lead to similar disease phenotypes, presumably because of their functional relationship. Therefore, protein interactions can be used to prioritize gene candidates in studies investigating the genetic basis of disease [79]. Others have used the properties of protein interaction networks to differentiate disease from non-disease proteins. Based on this approach, Xu *et al.* [80] devised a classifier based on several topological features of the human interactome to predict genes related to disease. The classifier was trained on a set of non-disease and a set of disease genes (from OMIM) and applied to a collection of over 5,000 human genes. As a result, 970 disease genes were identified, a fraction of which were experimentally validated.

New diagnostic tools can result from genotype-phenotype associations established through PPIs. The genes of interacting proteins can be studied to identify the mutation(s) leading to the interaction disruptions seen in healthy individuals or to the creation of new interactions only present in the diseased states. For example, Rossin *et al.* used genome-wide association studies (GWAS) to identify regions with variations that predispose immune-mediated diseases [81]. The GWAS studies provided a list of proteins found to interact in a preferential manner. The resulting disease single-nucleotide polymorphisms identified by GWAS studies such as that by Rossin *et al.* can be eventually incorporated into genotyping diagnostic tools.

Identifying disease subnetworks, and in turn pathways that get activated in diseased states, can provide markers to create new prognostic tools. For instance, using a protein-network-based approach, Chuang *et al.* [66] identified a set of subnetwork markers that accurately classify metastatic vs. non-metastatic tumors in individual patients. Metastasis is the leading cause of death in patients with breast cancer. However, a patient's risk for metastasis cannot be accurately predicted and it is currently only estimated based on other risk factors. When metastasis is deemed likely, breast cancer patients are prescribed aggressive chemotherapy, even when it might be unnecessary. By integrating protein networks with cancer expression profiles, the authors identified relevant pathways that become activated during tumor progression, which discriminate metastasis better than markers previously suggested by studies using differential gene expression alone.

Disease networks can inform drug design by helping suggesting key nodes as potential drug targets. Drug target identification constitutes a good example of the potential of integrating structural data with high-throughput data [82]. The structural details on binding or allosteric sites can be used to design molecules to affect protein function. On the other hand, reconstruction of the different protein networks (signaling, metabolic, regulatory, etc.) in which the potential target is involved can help predict the overall impact of the disruption. If, for example, the target is a hub (a highly connected protein), its inhibition may affect many activities that are essential for the proper function of the cell and might thus be unsuitable as a drug target. On the other hand, less connected nodes (e.g. nodes affecting a single disease pathway) could constitute vulnerable points of the disease-related network, which are better candidates for drug targets. The work by Yildirim and Goh [83] illustrates the advantages of evaluating drugs within the context of cellular and disease networks. This group created a drug-target network to map the relationships between the protein targets of all drugs and all disease-gene products. The topological analysis of the human drug-target network revealed that (i) most drugs target currently known targets; (ii) only a small fraction of disease genes encodes drug-target proteins; (iii) current drugs do not target diseases equally but only address some regions of the human disease network; and (iv) most drugs are palliative—they treat the symptoms not the cause of the disease, which largely reflects our lack of knowledge regarding the molecular basis of diseases such that for many pathologies we can only treat the symptoms but not cure them.

5. Summary—Trends in the Translational Characterization of Human Disease

We are still quite far from understanding the etiology of most diseases. Further advances on relevant experimental technology (e.g. genetic linkage, protein interaction prediction), along with integrative computational tools to organize, visualize, and test hypotheses should provide a step forward in that direction. More than ten years after the completion of the human genome project, it is clear that our approach to human disease elucidation needs to change. The \$3-billion human "book of life" and the \$138-million effort to catalog the common gene variants relevant to disease have so far failed to deliver the wealth of biological knowledge of human diseases and the subsequent

personalization of medicine the scientific community expected [84].

To date, biomedical research of the etiology of disease has largely focused on identifying disease-associated genes. But, the molecular mechanisms of pathogenesis are extremely complex; gene-products interact in different pathways and multiple genes and environmental factors can affect their expression and activity. Likewise, the same proteins may participate in different pathways and mutations on their genes may or may not affect some or all of the biological processes they mediate. Thus, gene-disease associations cannot be straightforwardly deduced and their usefulness alone (in the absence of a molecular context) in elucidating the biology of healthy phenotype disruptions is questionable. Evidence is accumulating to suggest that in the majority of cases illnesses are traceable to a large number of genes affecting a network or pathway. The effects on healthy phenotype disruption may vary from one individual to another based on the person's gene variants and on how disruptive the alterations might be to the network [85].

To achieve a comprehensive genotype-phenotype understanding of disease, translational research should be conducted within a framework integrating methodologies for uncovering the genetics with those investigating the molecular mechanisms of pathogenesis. In fact, the studies yielding the most biological insight into disease to which we alluded in this chapter were those which implemented a combined genotype-phenotype approach; those studies identified the disease-susceptible genes and investigated their network of interactions and affected pathways. As a result, the combined approaches managed to explain known clinical observations while also suggesting new mechanisms of pathology.

PPI analysis provides an effective means to investigate biological processes at the molecular level. Yet, any conclusions obtained based on PPI methods must be validated since these methods are subject to limitations inherent to the nature of data collection and availability. First, one must be aware that the roles of protein interactions are context-specific (tissue, disease stage, and response). Thus, two proteins observed to interact *in vitro* might not interact *in vivo* if they are localized in different cell compartments. Even when in common cell compartments, protein abundance or presence of additional interactors might affect whether the interaction occurs at all. Second, most of the PPI methodologies use a simplistic 'static' view of proteins and their networks. In reality, proteins are continuously being synthesized and degraded. The kinetics of processes and network dynamics need to be considered to achieve a complete understanding of how the disruptions of protein interactions lead to disease. Third, human PPIs are often predicted based on homology and from studies investigating disease in other organisms. The same mechanisms of interaction might or might not exist in the organism of interest or their regulation and phenotypic effects might be different. Ideally, since network and structural approaches are complementary, the combination of network studies with a more detailed structural analysis has the potential to enhance the study of disease mechanisms and rational drug design.

Currently, in the PPI field, a large number of studies focus on the topological characterization of organisms' interactomes. Those studies have yielded valuable information regarding general trends of molecular organization and their differences across genomes. To gain a deeper understanding of individual diseases, however, the trend needs to move from global characterizations to disease-specific interactomes. Phenotype-specific interaction network analyses should help identify subnetworks mapping to pathways that can be targeted therapeutically and point to key molecules essential to the biological function under study. Since disease inferences are as good as the modeled PPI networks, the ontologies used by PPI resources need to be expanded to better describe disease phenotypes, cytological changes, and molecular mechanisms.

6. Exercises

Objective: To investigate Epstein-Barr Virus (EBV) pathogenesis using protein-protein interactions

EBV is a member of the herpesvirus family and one of the most common human viruses. According to the CDC, in the United States around 95% of adults have been infected by EBV. Upon infection in adults, EBV replicates in epithelial cells and establishes latency in B lymphocytes, eventually causing infectious mononucleosis 35%–50% of the time and sometimes cancer [86]. In the next four sections, your goal will be to study the interactions among EBV proteins and between the virus and its host (using the EBV-EBV and EBV-human interactomes respectively) as a means to investigate how EBV leads to disease at the molecular level.

Datasets:

The following datasets were adapted with permission from [87]

- Dataset S1: EBV interactome
- Dataset S2: EBV-Human interactome

Software requirements:

Download and install Cytoscape (http://www.cytoscape.org, [88]) locally.

Note:

The instructions below correspond to Cytoscape v. 2.8.0; but, should be applicable to future releases.

I. Visualize the EBV interactome using Cytoscape

A. **Import Dataset S1 into cytoscape**

- *Select File ->Import ->Network (Multiple File Types)*
- *Click the "Select" button* to browse to Dataset S1's location
- *Click "Import"*

B. **Change the network layout**

- *Click on View->Hide data panel*
- *Click the 1:1 magnifying glass icon* to zoom out to display all elements of the current network"
- *Select Layout->Cytoscape Layouts->Force-directed (unweighted) Layout*

C. **Format the nodes and edges**

- *Select View->Open Vizmapper*
- *Choose the "Default" Current Visual Style*
- *Click on the pair of connected nodes icon in the "Defaults" box*
- *Scroll down on the resulting dialog* to change the following default visual properties:

  NODE__SIZE = 20
  NODE_FONT_SIZE = 20
  NODE_LABEL_POSITION = (Node Anchor Points) SOUTH

  Note: Feel free to click and drag any nodes with labels that overlap to increase visual clarity.

D. **Print the EBV interactome**

- Select File->Export->Current Network View as Graphics

Answer the following questions:

i. How many nodes and edges are featured in this network?

Table 2. Topological properties of human proteins for exercise III.

Average topological property	ET-HP	Random human protein
Degree	15±2	5.9±0.1
Number of components	4	12.6±0.25
Nodes in largest component	1,112	521±5
Distance to other proteins	3.2±0.1	4.03±0.01

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ii. How many self interactions does the network have?

iii. How many pairs are not connected to the largest connected component?

iv. Define the following topological parameters and explain how they might be used to characterize a protein-protein interaction network: node degree (or average number of neighbors), network heterogeneity, average clustering coefficient distribution, network centrality.

II. Characterize the EBV-Human interactome

Import Dataset S2 into cytoscape to create a map of the EBV-Human interactome. Format and output the network according to steps A through D in part I.

Answer the following questions:

i. How many unique proteins were found to interact in each organism?

ii. How many interactions are mapped?

iii. How many human proteins are targeted by multiple (i.e. how many individual human proteins interact with >1) EBV proteins?

iv. How does identifying the multi-targeted human proteins help you understand the pathogenicity of the virus? —Hint: Speculate about the role of the multi-targeted human proteins in the virus life cycle.

v. How might you test the predictions you formulated above?

III. Characterize the topological properties of the human proteins that are targeted by EBV

Use the topological information provided for you in Table 2 to investigate whether the EBV-targeted Human Proteins (ET-HPs) differ from the average human protein.

Answer the following questions:

i. Based on the 'degree' property, what can you deduce about the connectedness of ET-HPs? What does this tell you about the kind of proteins (i.e. what type of network component) EBV targets?

ii. What do the number and size of the largest components tell you about the inter-connectedness of the ET-HP subnetwork?

iii. Why is distance relevant to network centrality? What is unusual about the distance of ET-HPs to other proteins and what can you deduce about the importance of these proteins in the Human-Human interactome?

iv. Based on your conclusions from questions i–iii, explain why EBV targets the ET-HP set over the other human proteins and speculate on the advantages to virus survival the protein set might confer.

IV. Integrating knowledge from three different interactomes

Answer the following questions:

i. The Rta protein is a transactivator that is central to viral replication in EBV. When Rta is co-expressed with the LF2 protein replication attenuates and the virus establishes latency. Solely based on the EBV-EBV network, formulate a hypothesis to explain how LF2 may be driving EBV to latency suggesting at least one molecular mechanism by which LF2 may inactivate Rta.

ii. Why is establishing latency (opposed to promoting rapid replication of viral particles) an effective mechanism of virus infection?

iii. Assign putative functions to EBV's SM and EBNA3A proteins based on the function of the human proteins with which they interact—Hint: Locate these proteins in the EBV-Human network. What clinical observation (see the introductory paragraph to section 6. Exercises) might these proteins' subnetworks explain?

Answers to the Exercises can be found in Text S1.

Supporting Information

Text S1 Answers to Exercises.
(DOCX)

Dataset S1 EBV Interactome Data.
(SIF)

Dataset S2 EBV-Human Interactome Data.
(SIF)

Figure S1 EBV Interactome Map.
(PDF)

Figure S2 EBV-Human Interactome Map.
(PDF)

Further Reading

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Glossary

Mendelian traits or diseases, named after Gregor Mendel, are the traits inherited and controlled by a single gene.

Positional cloning is a method to find the gene producing a specific phenotype in an area of interest in the genome. The first step of positional cloning is linkage analysis, in which the gene is mapped using a group of DNA polymorphisms from families segregating the disease phenotype.

Epistasis refers to the phenomenon in which one gene masks the phenotypic effect of another.

Angiogenesis is the physiological process leading to growth of new blood vessels. Angiogenesis is a normal and vital process in growth, development, and wound healing; but it is also a fundamental step in the transition of tumors from a dormant to a malignant state.

Hemangioblastomas are tumors of the central nervous system that originate from the vascular system.

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Chapter 5: Network Biology Approach to Complex Diseases

Dong-Yeon Cho[◔], **Yoo-Ah Kim**[◔], **Teresa M. Przytycka***

National Center for Biotechnology Information, NLM, NIH, Bethesda, Maryland, United States of America

Abstract: Complex diseases are caused by a combination of genetic and environmental factors. Uncovering the molecular pathways through which genetic factors affect a phenotype is always difficult, but in the case of complex diseases this is further complicated since genetic factors in affected individuals might be different. In recent years, systems biology approaches and, more specifically, network based approaches emerged as powerful tools for studying complex diseases. These approaches are often built on the knowledge of physical or functional interactions between molecules which are usually represented as an interaction network. An interaction network not only reports the binary relationships between individual nodes but also encodes hidden higher level organization of cellular communication. Computational biologists were challenged with the task of uncovering this organization and utilizing it for the understanding of disease complexity, which prompted rich and diverse algorithmic approaches to be proposed. We start this chapter with a description of the general characteristics of complex diseases followed by a brief introduction to physical and functional networks. Next we will show how these networks are used to leverage genotype, gene expression, and other types of data to identify dysregulated pathways, infer the relationships between genotype and phenotype, and explain disease heterogeneity. We group the methods by common underlying principles and first provide a high level description of the principles followed by more specific examples. We hope that this chapter will give readers an appreciation for the wealth of algorithmic techniques that have been developed for the purpose of studying complex diseases as well as insight into their strengths and limitations.

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1. Introduction

Complex diseases are caused, among other factors, by a combination of genetic perturbations. Thus in the case of a complex disease we do not assume that a single genetic mutation can be pinned down as a cause. Many diseases fall in this category including cancer, autism, diabetes, obesity, and coronary artery disease. Even though there are other factors involved in such diseases, this review will focus on genetic causes.

One of the fundamental difficulties in studying genetic causes of complex diseases is that different disease cases might be caused by different genetic perturbations. In addition, if a disease is caused by a combinatorial effect of many mutations, the individual effects of each mutation might be small and thus hard to discover. For example, autism is considered to be one of the most heritable complex disorders, but its underlying genetic causes are still largely unknown [1]. One of the proposed factors that contribute to this difficulty is the role of rare genetic variations in the emergence of the disease [2].

An additional difficulty in studying complex diseases relates to disease heterogeneity. Specifically, in a complex disease, disease phenotypes might vary significantly among patients. The recognition of this fact has lead, for example, to renaming "autism" to "autism spectrum disorders" (ASDs) referring in this way to a group of conditions characterized by impairments in reciprocal social interaction and communication, and the presence of restricted and repetitive behaviors [1]. Similar heterogeneity is present in other complex diseases including cancer.

Given the above challenges, how can we approach the study of complex diseases? A useful clue is provided by the fact that genes, gene products, and small molecules interact with each other to form a complex interaction network. Thus a perturbation in one gene can be propagated through the interactions, and affect other genes in the network. However, the fact that we observe similar disease phenotypes despite different genetic causes suggests that these different causes are not unrelated but rather dys-regulate the same component of the cellular system [3]. Therefore in studies of complex diseases researchers increasingly focus on groups of related/interconnected genes, referred to as modules or subnetworks.

2. Interactome

Biomoecules in a living organisim rarely act individually. Instead, they work together in a cooperative way to provide specific functions. A variety of intermolecular interactions including protein-protein interactions, protein-DNA interactions, and RNA interactions are essential to these cooperative activities. These interactions can be conveniently represented as networks (graphs) with nodes (vertices) which denote molecules, and links (edges) which denote interactions between them. Depending on the type of interaction, the corresponding edge might be directed or

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* E-mail: przytyck@ncbi.nlm.nih.gov

◔ These authors contributed equally to this work.

What to Learn in this Chapter

- Characteristics and challenges of complex diseases
- Physical and functional networks and the methods to construct them
- Different classes of algorithms that use networks to leverage genotype, gene expression, and other types of data to identify dys-regulated pathways in diseases:
  - Scoring, correlation, and set cover based methods for identification of dys-regulated network modules based on various data types including genotype and phenotype.
  - Distance and flow based methods for inferring information flow from genotype to phenotype
- Applications to disease classification and treatment

undirected. For example, a binding between two proteins is usually represented as an undirected edge while an interaction between a transcription factor and a gene whose expression is regulated by the given transcription factor is usually represented as a directed edge where the direction goes from the transcription factor to the gene.

Biological interaction networks have characteristic topological properties [4]. One of the basic properties observed in many biological networks is the scale-free property [5]. A scale free network is defined as a network whose node degree distribution follows a power law. Formally, the function $P(k)$ indicating the fraction of nodes interacting with k other nodes in the network follows $P(k) \sim \alpha k^{-\gamma}$, where α is a normalization constant and the degree exponent γ is usually in the range of $2<\gamma<3$. Obviously, in biological networks the scale free property holds only approximately and practically the most important implication of this observation is the fact that these networks are characterized by a small number of highly connected nodes while most nodes interact with only a few neighbors. These highly connected nodes, called hubs have been proposed to play important roles in biological processes [6] and shown to be related to the modular structure of the physical and functional interaction networks [7]. Therefore it might be interesting to consider disease related genes in the context of the topological properties of interaction networks such as connectivity or modularity [8,9]. With respect to connectivity, one should note that known disease genes tend to be more studied which might introduce a bias towards higher connectivity. Importantly, independently of the source of the non-uniformity of node degree distribution, this characteristic property of interaction networks needs to be kept in mind while designing proper null models for conclusions derived using these networks.

In the following subsections, we briefly describe how physical and functional interactions networks are constructed and how they are applied to analyze complex diseases. We then explore the modularity of networks – a widely accepted phenomenon in biological networks that has proven to be helpful in disease studies.

2.1 Physical Interaction Networks

Physical contacts between proteins are critical in many biological functions. In fact much of the molecular machinery responsible for transcription, translation, and degradation is made of stable protein complexes. There are two main approaches for detecting physical protein interactions [10]. The first approach is to detect physical interactions between protein pairs. The most widely used high-throughput technology for detecting pairwise interaction is yeast two-hybrid (Y2H) method. Alternatively, physical interactions among groups of proteins can be detected without explicit consideration of interacting partners. For this type of approach, interaction data is typically obtained by tandem affinity purification coupled to mass spectrometry (TAP-MS). A more detailed review on experimental methods for the detection and analysis of protein-protein interactions can be found in [11]. It is worth noting that networks obtained with various technologies often have different topological properties [7]. For example, in the case of the yeast TAP-MS network, hub nodes are enriched with essential genes (the genes without which yeast cannot survive in standard growth medium). In contrast, hubs in yeast Y2H networks are enriched with genes that are pleiotropic [12]. Finally, experimental procedures detecting protein-protein interactions have also been complemented by various computational methods using evolutionary-based approaches, statistical analysis, and/or machine learning techniques (for a review, see [13]).

While these physical interaction networks have significantly advanced our understanding of the relationships between molecules, a concern is their level of noise and incompleteness. Indeed, physical interaction networks obtained by high-throughput techniques are found to include numerous non-functional protein-protein interactions [14] and at the same time many missing true interactions. Therefore physical interactions are often complemented with functional interactions.

2.2 Functional Interaction Networks

While physical interaction networks provide information on how proteins interact with each other, sometimes we may be more interested in how proteins work together to perform a certain function. Functional networks aim to connect genes with similar or related functions even if they do not necessarily physically interact. Similarly functional regulatory networks are constructed so that the interactions depict direct or indirect regulatory relationships. Consequently, several computational methods have been proposed to derive functional interaction networks.

Since functionally related genes are likely to show mutual dependence in their expression patterns [15], gene expression data has been often used to detect functional relationships. Co-expression networks can be constructed by computing correlation coefficients or mutual information between gene expression profiles of every pair of genes in different experimental settings. To build more comprehensive functional networks, co-expression data is frequently combined with other types of data such as Gene Ontology [16,17], outcome of genetic interaction experiments, and physical interactions. Such integrated networks have been constructed for a variety of organisms including yeast [18], fly [19], mouse [20], and human [21].

Gene regulatory network reconstruction algorithms such as ARACNE [22] and SPACE [23] identify regulatory relationships building on the assumption that changes in the expression level of a transcription factor should be mirrored in the expression changes of the genes regulated by the transcription factor (TF). Causal relations among genes can also be naturally modeled using Bayesian networks which can represent conditional dependencies between expression levels (for a primer on Bayesian network analysis utilizing expression data (see [24]); for a recent review see [25]). Considering the temporal aspects of gene expression profiles, dynamic

Bayesian networks have been used to model feedback loops as well as gene regulation patterns [26,27]. While expression profiles serve as primary data sources for constructing functional regulatory networks, this data is often complemented with additional information such as experimentally derived transcription factor binding data from ChiP-seq experiments or computationally identified binding motifs.

2.3 Modules and Pathways

It is widely accepted that the cellular system is modular. Hartwell *et al.* defined a functional module as an entity, composed of many types of interacting molecules, whose function is separable from those of other modules [28]. While the precise meaning of separation is left undefined, this general description provides a good intuition behind the concept of a module. Traditionally, molecular pathways have been delineated by focused studies of particular functions such as cell growth. Typically, these pathways contain not only topological connectivity information but also the roles of molecules such as whether a given molecule is an activator or inhibitor of the activity of another molecule. However, these hand-curated pathways are often incomplete. In addition, while some functions, such as cell growth or differentaition, have been relatively well studied, studies of other pathways are less extensive. Therefore, given the availability of large scale interaction networks, it is natural to attempt to extract meaningful functional modules from such networks. While there is no unique way to mathematically define functional modules, the most common approach is to search for densely connected subgraphs or clusters [29–46]. Additionally, gene expression information can be used alone or in concert with protein interaction data to obtain gene modules by grouping co-expressed genes into one module [47–49].

It is important to keep in mind that modules identified by analysis of high-throughput data are noisy, containing both false negative and false positive edges. In addition they do not usually provide information about the nature of an interaction. Therefore, unlike hand curated pathways, computationally identified network modules typically lack a mechanistic explanation of pathway activities but rather serve as groups of genes that work together to achieve a particular function.

An important advantage of working with modules rather than individual genes relates to the fact that it is often easier to predict the function of a module than the function of a gene. In particular, while the functions of many genes are still unknown, the prediction of the functional role of a module may be possible if the module contains a sufficient number of genes of known functions. Such enrichment analysis builds on the assumption that a fraction of genes can be assigned a functional category such as Gene Otology (GO) term [17]. The question of whether the number of genes with a functional annotation in a given gene module is higher than expected by chance can be determined by statistical tests such as χ^2 or Fisher exact test. A variety of software tools have been developed to perform such an analysis [50].

3. Identifying Modules and Pathways Dys-regulated in Diseases

Since complex diseases are believed to be caused by combinations of genetic alterations affecting a common component of the cellular system, module-centirc approaches are particulalry pormissing in thier study. How can disease associated modules/subnetworks be identified? Complementing interaction data with additional data related to disease states helps in separating subnetworks perturbed in a disease of interest from the remainder of the network. Both genotypic data (e.g., SNP, copy number alteration) and molecular phenotypic data such as gene expression profiles in disease samples have been used to aid the identification of perturbed network modules and explain the connection between genotypic and phenotypic data (reviewed in [51]). Basing on the assumption that complex diseases are caused by a set of mutations which, although strongly vary among patients, are likely to dys-regulate common pathways, such dys-regulated pathways might be uncovered by mapping genes altered in the diseases onto a PPI (protein-protein interaction) network and then searching for network modules enriched with the altered genes. On the other hand, organismal level phenotypes such as diseases are directly related to molecular level changes such as gene expression. Thus an alternative group of approaches considers modules enriched with abnormally expressed genes. Finally, molecular pathways can also be considered as means of information flow. For example, the activation of the EGFR signaling pathway starts with the activation of the EGFR receptor, which in turn activates a number of signaling proteins downstream which initiate several signal transduction cascades, such the MAPK, Akt and JNK pathways and culminate in cell proliferation. Thus the third type of approaches focuses on predicting molecules and modules that mediate such information propagation.

What are the benefits of analyzing phenotypic and genotypic differences in diseases in the context of their molecular interactions? First, the integrative network based approaches can identify subnetworks that include genes that do not necessarily show a significantly different state in disease versus control but still play an important role within a module by mediating a connection between other disease associated genes. For example, in their pioneering approach, Ideker *et al.* [52] integrated yeast protein–protein and protein-DNA interactions with gene expression changes in response to perturbations of the yeast galactose utilization pathway and identified *Active Subnetworks* (sets of connected genes with significantly differential expression) which included common transcription factors showing moderate changes in their gene expression level but connecting other dys-regulated genes. Second, a module based approach increases statistical power, allowing the identification of a perturbed module even in the case when the perturbation of each individual gene in the module might not be statistically significant. For example, many cases of genetic diseases such as autism and schizophrenia are affected by rare germline variations which are difficult to distinguish from noise due to their rarity. However, recent studies showed that a significant portion of the altered genes belong to a highly interconnected protein network [53], suggesting the network approach can better detect the causal genes. Third, identified network modules can provide better understanding of the biological underpinning of the diseases and therefore more reliable markers in disease diagnosis and treatments (see Section 4 for more discussion).

3.1 Network Modules Enriched with Genetic Alterations

One way in which differing genetic variations might dys-regulate a common pathway is when the genes containing these alterations belong to the pathway. This potential explanation has led to the idea that the dys-regulated pathways might be uncovered by mapping the genes altered in the diseases to an interaction network and searching for the modules enriched with the altered genes (See Figure 1).

Following this principle, the first step to identify such modules is to select candidate genes whose alterations may have caused a disease of interest. Genes or whole geno-

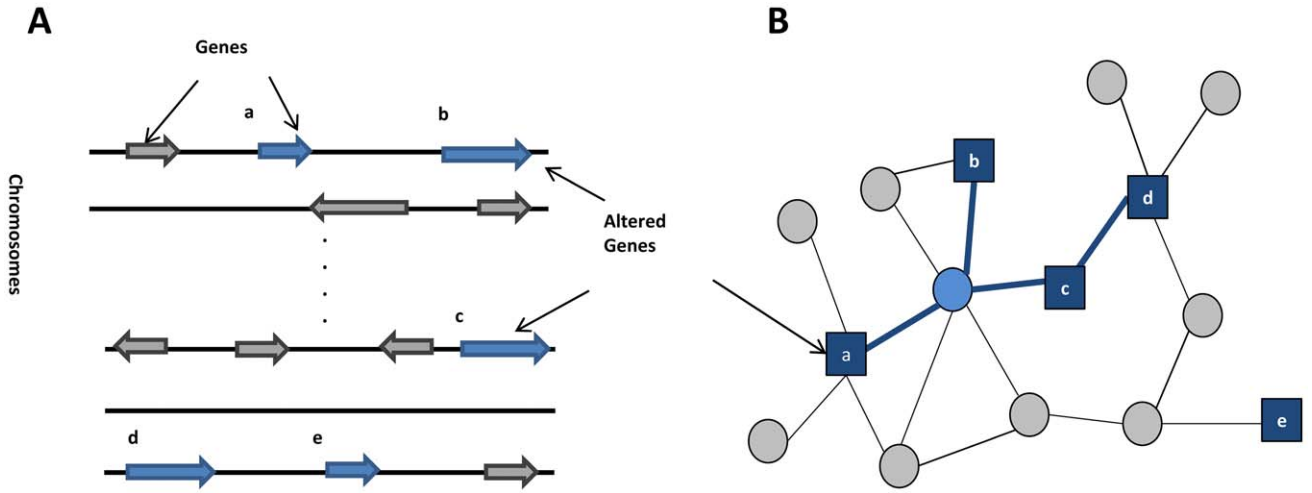

Figure 1. Identification of network modules enriched with genetic alterations. (A) Genomic regions with alterations. (B) Genes in the altered regions are mapped to the interaction network and modules enriched with such genes are identified.
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mic regions that are altered in the disease are first identified, and the genes residing in the altered regions are mapped to an interaction network. Both physical and functional interaction networks can be used, and edges might be weighted based, for example, on the likelihood of having the same phenotypes or influences between genes [54–56]. Next, modules are typically defined as subsets of genetically altered genes that are highly interconnected or within close proximity to each other in the interaction network together with non-altered genes necessary to mediate these connections. Edge weights, if given, can be used to prioritize the modules. In many cases, finding the best subnetwork is computationally expensive and search algorithms such as greedy growth heuristics or more sophisticated approximation algorithms have been proposed. Finally, rigorous statistical tests have been applied to evaluate the significance of selected modules.

Examples. The idea of finding genetically altered network modules has been utilized in various disease studies. Analyzing ovarian cancer TCGA data (The Cancer Genome Atlas), HOTNET identified subnetworks in a protein interaction network in which genes are mutated in a significant number of patients [54]. The identified networks includes the NOTCH signaling pathway which is indeed known to be significantly mutated in cancer samples [57]. The method is based on the set cover approach (see Set cover based approach section below), which is found to be effective in capturing different genetic variations across patients. In the NETBAG (NETwork-Based Analysis of Genetic associations) method, developed by Gilman *et al.* and applied to identify a biological subnetwork affected by rare de novo copy number variations (CNVs) in autism [58,59], the authors first constructed a gene network where edges were assigned the likelihood odd ratio for contributing to the same genetic phenotype. Subsequently a greedy growth algorithm was used to find clusters in this network. In another approach, Rossin *et al.* [60] considered the genomic regions found to be associated with Rheumatoid Arthritis (RA) and Crohn's disease (CD) in previous GWAS studies, and connected the genes residing in these regions based on interaction data to obtain network modules. It was also verified that those identified modules exhibited significant differences in expression level in the disease samples.

3.2 Differentially Expressed Network Modules

Another popular and successful approach to find disease associated modules is to search for subnetworks that are significantly enriched with genes whose expression levels are changed in disease samples. Building on the observation that a molecular perturbation typically affects the expression levels of genes in a whole module rather than individual genes, these approaches identify the modules which exhibit different expression patterns in disease states relative to a control. Gene expression data has been widely utilized for identifying dys-regulated modules and drug targets, inferring interactions between genes, and classifying diseases. While these approaches are based on the common idea of finding gene modules enriched with genes that have abnormal expression, several different computational techniques have been used to achieve these tasks, which we discuss shortly below. The methods are also illustrated in Figure 2.

3.2.1 Scoring based methods. Suppose that there is a subset of genes which are differentially expressed in disease samples and they are closely connected to each other in an interaction network. A subnetwork including such genes might be a good candidate for a disease associated network module (Figure 2A). Implementing this idea requires a way to score candidate modules. Various methods have been suggested for measuring the significance of the differential expression of genes in a module and their connectivity (the distances between the genes). In addition, different methods adopt different search algorithms to find high scoring candidate modules. Finally, some approaches additionally require that all genes are either up-regulated or down-regulated in the same direction.

Examples. Chuang *et al.* defined the activity score for a subnetwork by comparing gene expression profiles from two different types of samples (metastatic or non-metastatic in their study) [61]. More specifically, they first computed how well the expression of a gene discriminates between the two patient groups and then scored candidate subnetworks based on aggregate discriminative power over all genes in the subnetwork. Then they searched for the most discriminative networks in a greedy manner. While the method was used for disease classification

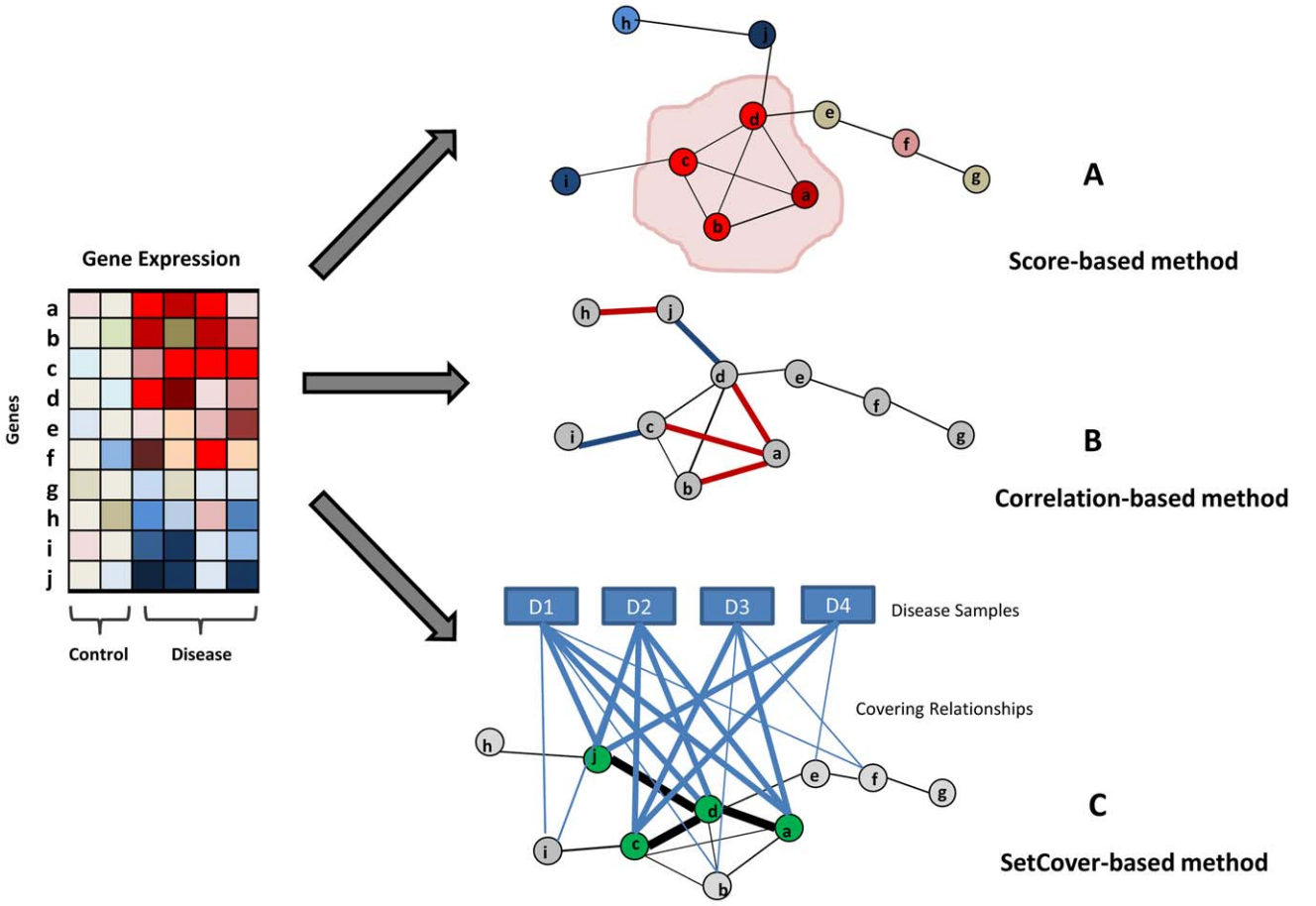

Figure 2. Finding differentially expressed modules. (A) Score based method selects the module with significant expression changes. (B) Correlation based method selects edges with correlation changes. The red and blue edges are correlated and anti-correlated edges, respectively. (C) Set cover based method selects a set of genes covering all samples. In this example, each sample has at least 2 differentially expressed genes and the genes are connected in the network.
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(see Section 4), it can readily be applied to leverage the difference between disease and non-disease cohorts.

3.2.2 Correlation based methods. Comparing expression patterns between genes is a basis for constructing a co-expression network, extracting modules exhibiting similar expression patterns, and further understanding molecular changes in diseases. Considering expression correlation of disease cases in the context of interactions can provide additional power in the identification of a disease associated module (Figure 2B). If the expression changes of two neighboring nodes are correlated with each other, this may suggest that the two interacting genes have related functional roles. With this in mind, some approaches look at connected components which show highly correlated and anti-correlated expression patterns. Other approaches search for loss and gain of correlation in disease states to identify dys-regulated edges.

Examples. Aiming to identify regulatory networks defining phenotypic classes of human cell lines, Müller *et al.* searched for Jointly Active Connected Subnetworks (connected subnetworks with high average internal expression similarity) in a human interaction network [62] and demonstrated the power of combining network and expression data.

IDEA (Interactome Dysregulation Enrichment Analysis) method [63] focused on the identification of perturbed network edges in a combined interaction network (PPI, transitional, signaling, posttranslational modifications predicted by MINDy [64]), and searched for the edges connecting genes which in a disease state show loss or gain of expression correlation. The utility of the method was demonstrated in the analysis of FL lymphoma and other cancer types. In particular, they identified BCL2 as the gene adjacent to the largest number of dys-regulated edges in FL lymphoma. This analysis also identified the SMAD1 gene, which could not be detected by differential expression analysis only.

To understand the mechanism of aging, Xue *et al.* applied a network module approach [65,66]. They utilized a PPI network and overlaid expression data obtained from various stages of aging. Two types of edges – correlated and anti-correlated – were selected. The subnetwork that includes only those edges was called the NP (negative and positive) network, is proposed to be related to the aging mechanism. Further modularizing the network with hierarchical clustering of expression patterns, they obtained a few age related modules and found some genes connecting different modules through PPIs are more likely to affect aging/longevity, which was also experimentally validated.

3.2.3. Set cover based methods. A group of methods employ a combinatorial approach named set cover. In a set cover, a gene is considered to cover a disease

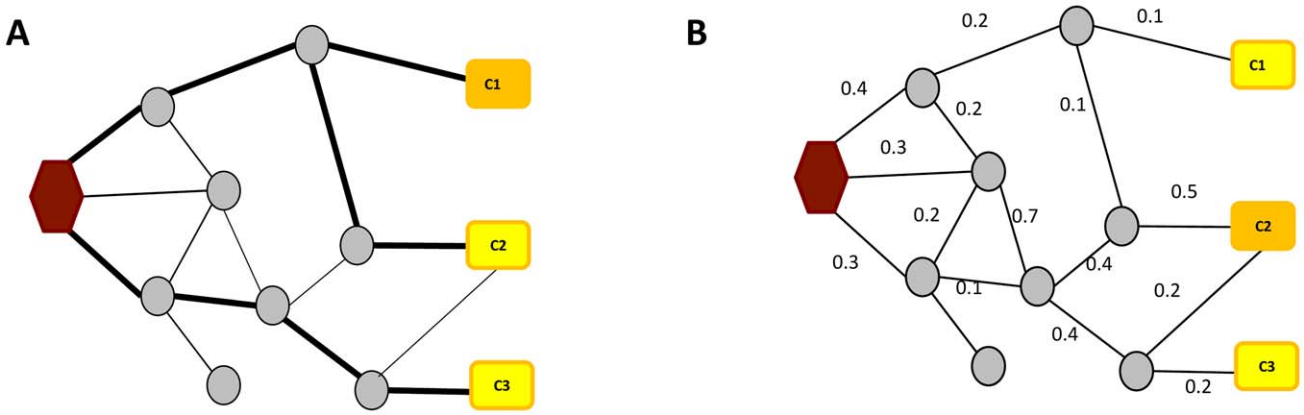

Figure 3. Finding information propagation modules. (A) Shortest path approach to uncover information propagation. The shortest paths from a target gene (with hexagon shape) to each of three candidate genes are shown. The closest gene is identified as the most probable disease causing gene. (B) Flow based approach. The gene receiving the most significant amount of flow is identified as the disease gene. The information flow methods often follow Kirchhoff's current law (the amount of incoming information equals the amount of outgoing information).
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sample if it is dys-regulated in the sample. For example, it can be decided if a gene is covering a sample or not based on the fold change of gene expression level in the sample or using a statistical test such as z-test. The main principle of the set cover approach is that each disease case has some dys-regulated (thus covering) genes but in heterogeneous diseases, different cases will typically have different covering genes. Set cover approaches provide a strategy to select a representative set of such covering genes (Figure 2C). This is usually done by defining some optimization criterion and attempting to select a set of genes which is optimal with respect to this criterion. For example, given a set of genes and disease samples along with covering relationships, a subset of genes is selected so that each sample is covered by some minimal number of genes while the total number of selected genes is minimized.

Many observed organism-level phenotypes arise in a heterogeneous way. Diseases such as cancer are now seen as a spectrum of related disorders that manifest themselves in a similar fashion. Since different samples may be covered by different genes and those genes may be connected in an interaction network, set cover approaches can be useful to identify gene modules explaining a heterogeneous set of samples [67–69].

Examples. Aiming to detect dys-regulated pathways in complex diseases, Ulitksy *et al.* extended the set cover technique by integrating expression data and interaction networks [67]. Their method, named DEGAS (de novo discovery of dys-regulated pathways) searches for a smallest set of genes forming a connected subnetwork so that each disease sample is covered by a certain minimal number of genes. They applied this approach to a Parkinson's disease dataset. Chowdhury *et al.* [68], developed an alternative network cover based algorithm and used the identified modules for disease classification in a human colorectal cancer dataset.

Set Cover approaches have also been applied to data types other than gene expression. For example, Kim *et al.* proposed a module cover approach to identify gene modules which collectively cover disease samples [70]. At the same time they required that each module is coherent, containing genes with similar genotype-phenotype mappings (see Section 4 for more discussion). The HotNet Algorithm discussed in Section 3.1 also utilized a variant of a set cover approach to find genetically altered modules. In their case, a gene is defined to cover a sample if the gene is mutated in the sample, and they looked for a fixed size connected set of genes covering as many samples as possible. The Dendrix (De novo Driver Exclusivity) algorithm was also developed to discover mutated gene modules in cancer and, though it does not utilize interaction data, it aims to find sets of genes, domains, or nucleotides whose mutations exhibit both high coverage and high exclusivity in the disease samples [71].

3.3 Uncovering Information Propagation Modules

The approaches discussed thus far have dealt with modules of genes associated with either phenotypic or genotypic information. While both approaches are helpful for predicting dys-regulated modules, a more effective way to understand disease mechanisms might be to combine both genotypic (the putative causes of diseases) and phenotypic data (their effects). Expression Quantitative trait loci (eQTL) analysis is a useful method to find the relationship between genotype and phenotype [72,73]. eQTL treats the level of gene expression as a quantitative phenotype, which is assumed to be controlled by genotypic information. Loci that putatively control the expression of a given gene are identified by determining the associations between genotype and gene expression. Given an association between a genotypic variation in a locus and expression level of a gene, the next challenge is to uncover the pathway(s) through which the genetic variation leads to the expression change. Recently, several groundbreaking pathway elucidation methods have emerged, as illustrated in Figure 3 and described below.

3.3.1 Distance based methods. A simple approach to identify a possible pathway from a genetically altered gene (putative cause) to the gene with correlated expression change (target gene) is to test if there is a path in an interaction network connecting the putative causal gene to its target gene. The shortest path connecting a causal gene and its target is often used to explain their causal relationship (Figure 3A). The intermediate nodes on such a shortest path are likely members of an affected pathway/module. Several variations of the shortest path approach have been used in extracting disease associated network modules [74–76]. For example, Carter *et al.* searched for the shortest directionally consistent paths in molecular interaction networks connecting

seed genes to their targets. The targets were inferred by linear decomposition of gene expression data [76].

When multiple target genes exist, the well-known graph-theoretical concept of a Steiner tree is often used in place of a set of shortest paths. Given a set of nodes to be connected, a Steiner tree is an acyclic subgraph (a tree) connecting all these nodes while using the minimum number of edges. In a Steiner tree, the individual path from the putative causal gene (the root of the tree) to each of the target genes does not need to be the shortest, but the size (i.e., the number of edges) of the whole tree is minimized. The Steiner tree approach has been used to find new functional associations for proteins [77]. Tuncbag *et al.* extended the approach to the Steiner forest problem (allowing multiple trees), applying it to proteomic data from glioblastoma multiforme (GBM). In their study, each tree was rooted in a different cell surface receptor and represented independent signaling pathways originated from this receptor [78].

Distance based methods, such as the shortest path approach or the Steiner tree method, have several shortcomings. In particular, they ignore the fact that a pair of genes may have multiple paths connecting them in a network. In addition they use network topology without considering additional data (e.g. gene expression) and assume that the shortest pathways are the most informative or most likely used paths, which may not always be the case.

3.3.2 Flow-based methods. In the information flow approach, genotypic variations are considered the source of perturbation, while genes with phenotypic changes are considered the targets of a perturbation pathway. Instead of finding single paths connecting source and targets, flow-based methods compute the fraction of flow going through each intermediate node/edge. Fraction of flow indicates the probability of using the given path in information propagation (Figure 3B). In the case of current flow approach, the network is modeled to mimic the behavior of current in an electronic circuit, where each edge has an associated resistance. The current flow network provides an efficient framework equivalent to a random walk, which is also often used for modeling information flow in biological networks (see discussion below). An important advantage of network flow approaches their ability to incorporate additional data (such as gene expression, confidence level of interactions, and functional associations of genes) to the probabilistic network models. By incorporating such additional data, network flow approaches can more confidently suggest information propagation pathways.

The information flow of biological networks has been used to predict protein functions, to prioritize candidate disease genes, and to find network centralities [7,79–88]. The flow-based approach is particularly useful for augmenting network information for eQTL analysis. Specifically, it can be used to pinpoint likely causal genes in genomic eQTL regions and to uncover genes involved in the propagation of information signals from such causal genes to their target genes.

There are several mathematical formulations that can be used to capture information propagation. In addition to the aforementioned current flow, other approaches include random walk and network flow. While mathematically different, many information propagation methods share a number of similar assumptions such as flow conservation (Kirchhoff's law). In the random walk method, a number of random walkers repeatedly start from a node. The likelihood of associating a gene in the network to a disease is estimated by the number of random walkers arriving at the gene. Gene expression correlation provides one way to compute the weight of a gene in the network which, in turn, provides the transition probability of the random walker. The network flow methods are closely related to the current flow approach. Unlike current flow, however, the network flow model resembles water-finding paths through pipes. Capacities are associated with pipes (edges) providing constraints on how much flow can go through each pipe.

Examples. Tu *et al.* [79] used the random walk approach to infer causal genes and underlying causal paths over a molecular interaction network for yeast knock-out experimental data. Current flow is an equivalent form of random walk that can be used in a more computationally efficient way [89]. Using this knowledge, Suthram *et al.* [80] developed the eQED method, which integrates eQTL analysis with molecular interaction information modeled as a current flow network.

Kim *et al.* further extended the eQED idea to identify causal genes and dys-regulated pathways and applied it to Glioma sample analysis [69,90].One of the challenges of eQTL analysis is a massive multiple testing problem, for which various multiple testing correction methods have been proposed. Without such corrections, eQTL analysis typically finds multiple associated regions for each target gene, many of which are simply by chance. However, simply applying a more stringent p-value cutoff for multiple testing corrections often eliminates many true causal regions. Moreover, each region may contain dozens of candidate causal genes. Current flow analysis can be applied to complement eQTL analysis and help to identify the genes whose alterations are most likely to cause abnormal expression for the target gene. Using copy number variations and gene expression profiles of the same set of cancer patients, Kim *et al.* first identified chromosomal regions where copy number variations correlated with gene expression changes. Subsequently, they used the current flow algorithm to identify potential causal genes in the associated regions. By selecting genes receiving significant amounts of current in the network, Kim *et al.* identified putative causal genes in Glioblastoma and uncovered commonly dys-regulated pathways, including insulin receptor signaling pathways and RAS signaling. The identified pathways featured several hub nodes, such as EGFR, that were known to be important players in Glioma or more generally in cancer. Compared to simple genome-wide association studies, which only identify putative associations between causal loci and target genes, the current flow based method provides increased power to predict causal disease genes and to uncover dys-regulated pathways.

A variant of the network flow approach, the minimum cost network flow, was used to model the response to increased expression of alpha-synuclein, a protein implicated in several neurodegenerative disorders, including Parkinson's disease [81]. In addition to the edge capacities, the min cost network flow approach associates weights with edges representing the cost of sending flow through an edge. These weights were computed based on the probability of the two genes interacting in a response pathway, while capacities were calculated using the transcript levels of target genes.

4. Applications of Network Modules – Disease Diagnosis and Treatment

Can network modules help facilitate a more personalized approach for disease diagnosis and treatment? Traditional approaches of clinical disease classification have been based on pathological analysis of patients and existing knowledge of diseases. However, traditional diagnostic approaches are prone to errors. Alterna-

tively, knowledge about dys-regulated pathways can be used to subtype diseases and to develop relevant treatments for individual disease subgroups. For example, network modules have been used to predict patient survival, metastasis, drug responses for various types of cancer [61,68,91–94].

4.1 Disease Classification

A supervised approach to disease classification starts with a set of samples with a known partition into disease subtypes (e.g., metastatic or not) and attempts to identify a classifying principle using specific molecular features. The general strategy for supervised disease classification is to search for subnetworks, also called subnetwork markers, whose activities best discriminate the two disease subtypes. As in the case of single-gene disease markers, a network marker will distinguish some but not all disease cases and multiple subnetworks might be necessary. Among selected candidate network markers, the best markers are selected based on a set of training samples. Some methods take an unsupervised approach, where subclasses and their features are discovered without using a known training set.

Examples. Chuang *et al.* showed how dys-regulated network modules (described in Section 3.2) provide more robust and accurate predictions than those by single gene based classifications when applied to breast cancer metastasis analysis [61]. Chuang *et al.*'s work provided the proof of principle for using network modules in disease classification. A number of subsequent extensions and improvements to Chuang *et al.*'s work were suggested. For example, Lee *et al.* incorporated curated pathways, and searched for a subset of genes with discriminative features for the disease phenotype [94]. More recently, Dao *et al.* developed alternative network based approaches for classification of cancer subtypes by identifying densely connected subnetwork and randomized algorithms [92,93]. Other techniques for best marker identification, such as set cover and bottom-up enumeration techniques, were also proposed [68,91].

Kim *et al.* identified gene modules using a module cover approach to capture disease heterogeneity in brain cancer samples from Rembrandt and Ovarian Cancer samples from TCGA [70]. Next, Kim *et al.* superimposed the selected modules onto the results from an independently proposed classification scheme [57]. As a result, Kim *et al.* uncovered which disease classes are characterized by which combinations of modules.

4.2 Disease Similarity

Network modules can also be used to explain disease similarity. Overlaps of dys-regulated network modules explain why some complex diseases share similar phenotypic traits. Suthram *et al.* used a variant of PathBlast [95] to identify dense subnetworks. Analysis of disease similarity was achieved by comparing expression patterns of various diseases in the modules [96]. Several dys-regulated modules were found to be common to many diseases, which explains why some drugs can treat many different diseases.

4.3 Response to Treatment

Modules may help determine whether a given patient will respond to a particular drug, which is valuable for treatment design. In addition, understanding molecular differences between responders and non-responders is likely to help development of alternative treatments. For example, Chu and Chen used a network approach to discover apoptosis drug targets [97]. Chu and Chen constructed a PPI network for apoptosis in normal cells and applied a nonlinear stochastic model to remove false positive interactions using microarray data. Comparing the resulting subnetworks helped to shed some light on the mechanisms leading to apoptosis and to identify potential drug targets.

5. Summary

Network biology provides powerful tools for the study of complex diseases. Network-based approaches leverage the idea that complex diseases can be better understood from the perspective of dys-regulated modules than at the individual gene level. Modularity is a widely accepted concept in molecular networks and module-based approaches provide a number of advantages including robustness in the identification of dys-regulated pathways and improved disease classification.

In addition, network based formulations allow using a wealth of methods already developed in graph theory, such as shortest paths, network flow, and Steiner trees. Network-based methods have several limitations including the lack of mechanistic explanations. Despite the limitations, network analysis has been applied successfully in many disease studies, suggesting testable hypotheses.

6. Exercises

1. Construct coexpression networks following the steps below [98].

   a. Download the three expression datasets from the following page: http://www.geneticsofgeneexpression.org/network/download
   b. Compute 3 population-specific correlations for each pair of 4238 genes with the expression data. (Hint: There are 8,978,203 pairs of genes.)
   c. For gene pairs which have similar correlations in the 3 datasets, calculate the weighted average correlation, weighted by the number of individuals in each population. Hint: In the Supplemental Table 1 published with [98] (http://genome.cshlp.org/content/suppl/2009/10/02/gr.097600.109.DC1/nayak_supplemental_material.pdf), you can find the list of gene pairs whose correlations differ significantly among the 3 datasets.
   d. Construct the correlation network by connecting gene pairs whose weighted average correlations are greater than a pre-defined threshold (e.g., 0.5).
   e. Compute specific parameters describing the network topology. (Hint: You can use the NetworkAnalyzer Cytoscape plugin http://med.bioinf.mpi-inf.mpg.de/netanalyzer/)
   f. For different correlation thresholds, compare the networks in terms of topological properties.

2. Suppose that in a co-expression network two genes are identified to have correlated expression patterns. Provide at least two possible biological explanations of this correlation.
3. Some variants of information flow approaches that identify pathways of information flow from a mutated gene to a target gene with correlated expression require that the last but one node gene on such a pathway (the node preceding the target gene) to be a transcription factor. What is a justification for such requirement? What can be advantages and disadvantages of such a design?
4. Consider a set cover approach to find a representative set of genes dys-regulated in a given set of cancer patients. The algorithm finds the smallest number of genes so that each disease case is covered at least k times. How does the number of selected genes depend on k? If you suspect that data for 5%

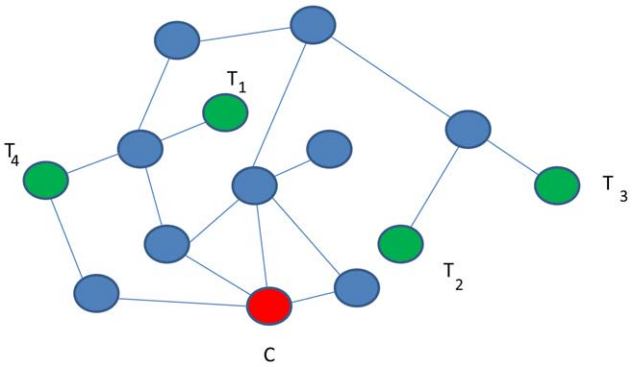

Figure 4. A hypothetical interaction network to be used with Exercises 5 and 6.
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patients might be incorrect, how would you modify the optimization problem?

5. A Steiner tree connecting a set of nodes does not need to be unique. In the graph shown in Figure 4, find two different Steiner trees connecting genes C, T_1, T_2, T_3, and T_4.
6. In the graph shown in Figure 4, find the shortest paths connecting C with each of T_1, T_2, T_3, and T_4. Do the edges used by these paths correspond to a Steiner tree? Explain why or why not.

Answers to the exercises are provided in Text S1.

Supporting Information

Text S1 Answers to Exercises.
(PDF)

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Chapter 6: Structural Variation and Medical Genomics

Benjamin J. Raphael*

Department of Computer Science and Center for Computational Molecular Biology, Brown University, Providence, Rhode Island, United States of America

Abstract: Differences between individual human genomes, or between human and cancer genomes, range in scale from single nucleotide variants (SNVs) through intermediate and large-scale duplications, deletions, and rearrangements of genomic segments. The latter class, called structural variants (SVs), have received considerable attention in the past several years as they are a previously under appreciated source of variation in human genomes. Much of this recent attention is the result of the availability of higher-resolution technologies for measuring these variants, including both microarray-based techniques, and more recently, high-throughput DNA sequencing. We describe the genomic technologies and computational techniques currently used to measure SVs, focusing on applications in human and cancer genomics.

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1. Introduction

The decade since the assembly of the human genome has witnessed dramatic advances in understanding the genetic differences that distinguish individual humans and that are responsible for specific traits. Genome-wide association studies (GWAS) in humans have identified common *germline*, or inherited, DNA variants that are associated with various common human diseases, including diabetes, heart disease, etc. At the same time, cancer genome sequencing studies have cataloged numerous *somatic* mutations that arise during the lifetime of an individual and that drive cancer progression. These successes are ushering in the era of personalized medicine, where treatment for a disease is tailored to the genetic characteristics of the individual.

Despite this progress, significant hurdles remain in achieving a comprehensive understanding of the genetic basis of human traits and disease. The germline variants discovered by GWAS thus far explain only a small fraction of the heritability of many traits, and this "missing heritability" gap [1] is a major bottleneck for future GWAS. The somatic mutations measured in cancer genomes are very heterogeneous, with relatively few mutations that are shared by large numbers of cancer patients, even those with the same (sub)type of cancer. This mutational heterogeneity complicates efforts to distinguish functional mutations that drive cancer development from random passenger mutations [2].

Comprehensive studies of the genetic basis of disease require the measurement of *all* variants that distinguish individual genomes. Until recently, GWAS focused on the measurement of single nucleotide polymorphisms (SNPs), or single nucleotide differences between individual genomes. In the past few years, it has become clear that germline variants occupy a continuum of scales ranging from SNPs to larger structural variants (SVs) – duplications, deletions, inversions, and translocations of large (>100 nucleotides) blocks of DNA sequence. Moreover, until recently GWAS focused attention on common SNPs, those whose frequency in the population was at least 5%. This restriction was part of the "common disease, common variant" hypothesis which posits that an appreciable fraction of susceptibility to common diseases results from germline variants that are common in the population. However, this restriction was also dictated by technological limitations, as it was not cost effective to measure all genetic variants in the large number of individual genomes that are necessary to perform a GWAS.

In the past five years, next-generation DNA sequencing technologies became commercially available from companies such as 454, Illumina, Life Technologies, and Complete Genomics. These and other sequencing technologies continue to advance at a breathtaking pace, and consequently the cost of DNA sequencing has declined by several orders of magnitude in the past decade. These technologies provide an unprecedented opportunity to measure all variants; germline and somatic; SNPs and SVs, in both normal and cancer genomes.

In this chapter, we discuss the application of these sequencing technologies in medical genomics, and specifically on the characterization of structural variation.

2. Germline and Somatic Structural Variation

Structural variants are important contributors to genome variation and consideration of these variants is necessary for disease association and cancer genetics studies. In this section, we briefly review current knowledge about structural variation in human and cancer genomes.

2.1 Germline Structural Variation

Characterizing the DNA sequence differences that distinguish individuals is a major challenge in human genetics. Until a few years ago, the primary focus was to identify single nucleotide polymorphisms (SNPs), and projects such as HapMap [3] provide catalogs of common SNPs in several human populations. Recent

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* E-mail: braphael@brown.edu

What to Learn in This Chapter

- Current knowledge about the prevalence of structural variation in human and cancer genomes.
- Strategies for using microarray and high-throughput DNA sequencing technologies to measure structural variation.
- Computational techniques to detect structural variants from DNA sequencing data.

whole-genome sequencing and microarray measurements have shown that structural variation, including duplications, deletions, and inversions of large blocks of DNA sequence, is common in the human genome [4]. SVs include both copy number variants – duplications and deletions – that change the number of copies of a segment of the genome, and balanced rearrangements – such as inversions and translocations – that do not alter the copy number of the genome. The Database of Genomic Variants [5] currently (winter 2011) lists apprroximately 66 thousand copy number variants and approximately 900 inversion variants in the human genome, and this number continues to increase. Some of these entries are multiple reports of the same variant due to problems in merging SV predictions across different platforms/technologies (see Section 5 below). Nevertheless, SVs are extensive in human populations.

Germline SVs account for a greater share of the total nucleotide differences between two individual human genomes than SNPs [6]. Copy number variants alone account for approximately 18% of genetic variation in gene expression, having little overlap with variation associated to SNPs [7], and can affect the expression of genes up to 300 kb away from the variant [8]. Both common and rare SVs have recently been linked to several human diseases including autism [9] and schizophrenia [10]. In addition to SVs that cause disease, SVs segregating in a population perturb patterns of linkage disequilibrium and haplotype structure [11]. Thus, it is essential to catalog SVs in order to understand their consequences for human population genetics. Incorrect identification of SVs in samples can lead to spurious genetic associations resulting from the undetected SVs, erroneous merging of distinct variants in different samples, and failure to recognize heterozygosity at a locus.

Finally, structural variants are also present in model organisms such as mouse and fruit fly. Identifying these variants is important for animal models of human diseases.

2.2 Somatic Structural Variation and Cancer

Cancer is a disease driven by somatic mutations that accumulate during the lifetime of an individual. The inheritance of mutations by daughter cells during mitosis and selection for advantageous mutations make cancer a "microevolutionary process" [12,13] within a population of cells. Decades of cytogenetic studies have shown that somatic structural variants are a feature of many cancer genomes. These early studies, particularly in leukemias and lymphoma, identified a number of *recurrent* chromosomal rearrangements that are present in many patients with the same type of cancer. For example, a significant fraction of patients with chronic myelogenous leukemia (CML) exhibit a translocation between chromosomes 9 and 22. The breakpoints of this translocation lie in two genes, BCR and ABL, and the translocation results in the BCR-ABL fusion gene that is directly implicated in the development of this cancer. In addition to fusion genes, somatic SVs can also lead to altered expression of oncogenes and tumor suppressor genes due to both genetic and epigenetic mechanisms [14]. For example, in Burkitt's lymphoma, a translocation activates the MYC oncogene by fusing it with a strong promoter.

In solid tumors, the situation is more complicated. Many solid tumors have genomes that are extensively rearranged compared to the normal healthy genome from which they were derived [14]. These highly rearranged genomes are thought to be a product of genome instability resulting from mutations in the DNA repair machinery. This complex organization of cancer genomes obscures functional driver SVs in a background of passenger mutations. However, with the availability of higher-resolution genomics technologies, recurrent fusion genes are also being found in solid tumors, such as prostate [15] and lung cancers [16]. These results suggest that additional events remain to be discovered [17]. Next-generation DNA sequencing technologies provide the opportunity to reconstruct the organization of cancer genomes at single nucleotide resolution [18,19]. Projects including The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov) and International Cancer Genome Consortium (ICGC) are using these technologies to measure somatic mutations in thousands of cancer genomes [20].

2.3 Mechanisms of Structural Variation

As additional genetic and somatic structural variants are characterized, there is increasing opportunity to characterize the mechanisms that produce these variants. A distinguishing feature of the different mechanisms is the amount of sequence similarity, or homology, at the breakpoints of the structural variant. One extreme is little or no sequence similarity. These variants are thought to result from random (or near random) double-stranded breaks in DNA. These breaks might occur due to exposure to external DNA damaging agents. For example, ultraviolet radiation or various chemotherapy drugs produce double-stranded breaks. Aberrant repair of these breaks result in structural variants. This mechanism is termed non-homologous end-joining (NHEJ) [21,22].

The opposite extreme is high sequence similarity at the breakpoints. This mechanism is termed non-allelic homologous recombination (NAHR). This mechanism is similar to the normal biological process of homologous recombination that occurs during meiosis and exchanges DNA between two homologous chromosomes. But as the name states, NAHR is a rearrangement that occurs between homologous sequences that are not the same allele on homologous chromosomes. Rather NAHR occurs between repetitive sequences on the genome (Figure 1) [23–25]. The human genome contains numerous repetitive sequences ranging from *Alu* elements of 300 bp to segmental duplications, also called low copy repeats, of tens to hundreds of kbp [26]. Thus, there are numerous substrates for NAHR in the human genome, and not surprisingly numerous reported structural variants that result from NAHR. For example, the 1000 Genomes Project, a large NIH project to survey all classes of variation – SNPs through SV – in 1000 human genomes recently reported that approximately 23% of deletions were a result of NAHR [27]. Importantly, due to technical limitations in discovering NAHR-mediated SVs (see

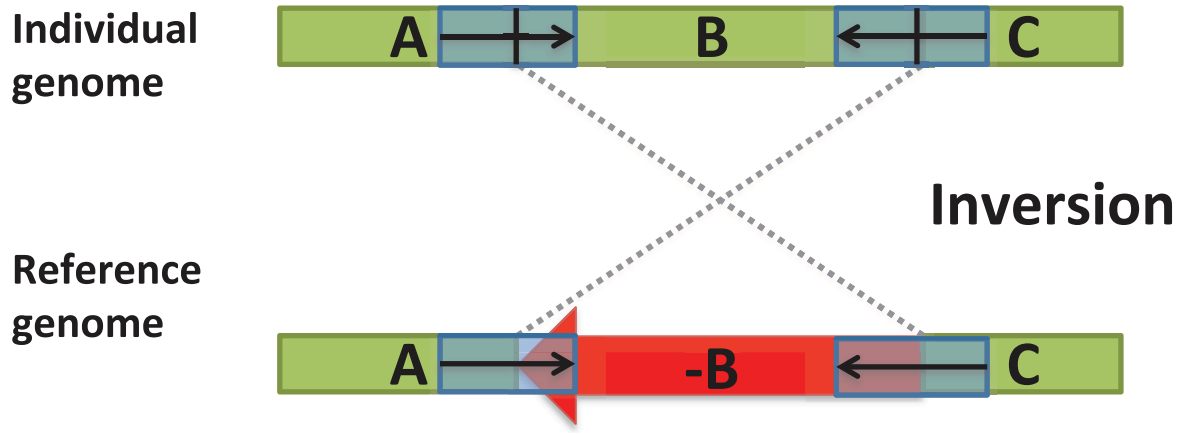

Figure 1. An inversion resulting from non-allelic homologous recombination (NAHR) between two nearly identical segmental duplications (blue boxes) with opposite orientations (arrows). The inversion flips the orientation of the subsequence, or block, *B* in one genome relative to the other genome.
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below), this percentage may be an underestimate.

There are other mechanisms for the formation of SVs. The division between homology mediated and non-homologous mechanisms may not be so strict. NHEJ events sometimes have some degree of microhomology (e.g. 2–25 bp of similarity) at their breakpoints. Other mechanisms such as fork stalling and template switching (FoSTeS) have also been proposed. Some of these are reviewed in [28]. Finally, the relative contribution of each of these mechanisms in generating germline SVs versus somatic SVs remains an active area of investigation, with conflicting reports about the importance of repetitive sequences in somatic structural variants found in cancer genomes [21,22,24,25,29].

3. Technologies for Measurement of Structural Variation

Structural variants vary widely in size and complexity, ranging from insertions/deletions of hundreds of nucleotides to large scale chromosomal rearrangements. Large structural variants can be visualized directly on chromosomes, through cytogenetic techniques such as chromosome painting, spectral karyotyping (SKY), or fluorescent in situ hybridization (FISH). In fact, Sturtevant and Dobzhansky studied inversion polymorphisms in *Drosophila* in the 1920's – well before the modern genomics era. However, SVs that are too small to be directly observed on chromosomes are generally more difficult to detect and to characterize than single nucleotide polymorphisms (SNPs). Much of the recent excitement surrounding structural variation stems from improvements in genomics technologies that allow more complete measurements of SVs of all types. These include microarrays and more recently next-generation DNA sequencing technologies. In this section, we briefly describe these technologies.

3.1 Microarrays

The first genome-wide surveys of SVs in the human genome in 2004 utilized microarray-based techniques such as array comparative genomic hybridization (aCGH). In aCGH, differentially fluorescently labeled DNA from an *individual*, or *test*, genome and a *reference* genome are hybridized to an array of genomic probes derived from the reference genome. Measurements of test:reference fluorescence ratio, called the copy number ratio, at each probe identifies locations of the test genome that are present in higher or lower copy in the reference genome. Microarrays containing hundreds of thousands of probes are available, and thus one obtains copy number ratios at hundreds of thousands of locations. Since individual copy number ratios are subject to various types of experimental error, computational techniques are needed to analyze aCGH data. For further details about aCGH and aCGH analysis, see [30].

aCGH is equally applicable for measurement of germline SVs in normal genomes and somatic SVs in cancer genomes. In fact, aCGH was originally developed for cancer genomics applications. aCGH is now very affordable making it possible to detect copy number variants in large numbers of genomes at reasonable cost. However, aCGH has two important limitations. First, because aCGH measures only differences in the number of copies of a genomic region between a test and reference genome, aCGH detects only copy number variants. Thus, aCGH is blind to copy-neutral, or balanced, variants such as inversions, or reciprocal translocations. Moreover, aCGH requires that the genomic probes from the reference genome lie in non-repetitive regions, making it difficult to detect SVs with breakpoints in repetitive regions, such as NAHR events or the insertion/deletion of repetitive sequences.

3.2 Next-generation DNA Sequencing Technologies

DNA sequencing technology has advanced dramatically in recent years, and several "next-generation" DNA sequencing technologies from companies such as Illumina, ABI, and 454 have significantly lowered the cost of sequencing DNA. However, these technologies, and the Sanger sequencing technique they are replacing, are severely limited in the length of a DNA molecule that can be sequenced. Present sequencing technologies produce short sequences of DNA, called reads, that range from 25–1000 nucleotides, or base pairs (bp), with the upper end of this range requiring technologies (e.g. Sanger and 454) that are considerably more expensive. Much of the recent excitement in DNA sequencing has been in *short read* DNA sequencers (e.g.llumina Genome Analyzer, Life Technologies SOLiD and Ion Torrent) that yield reads of only 25–150 nucleotides. These reads are much shorter than the one to two hundred million bp of a typical human chromosome. However, the large

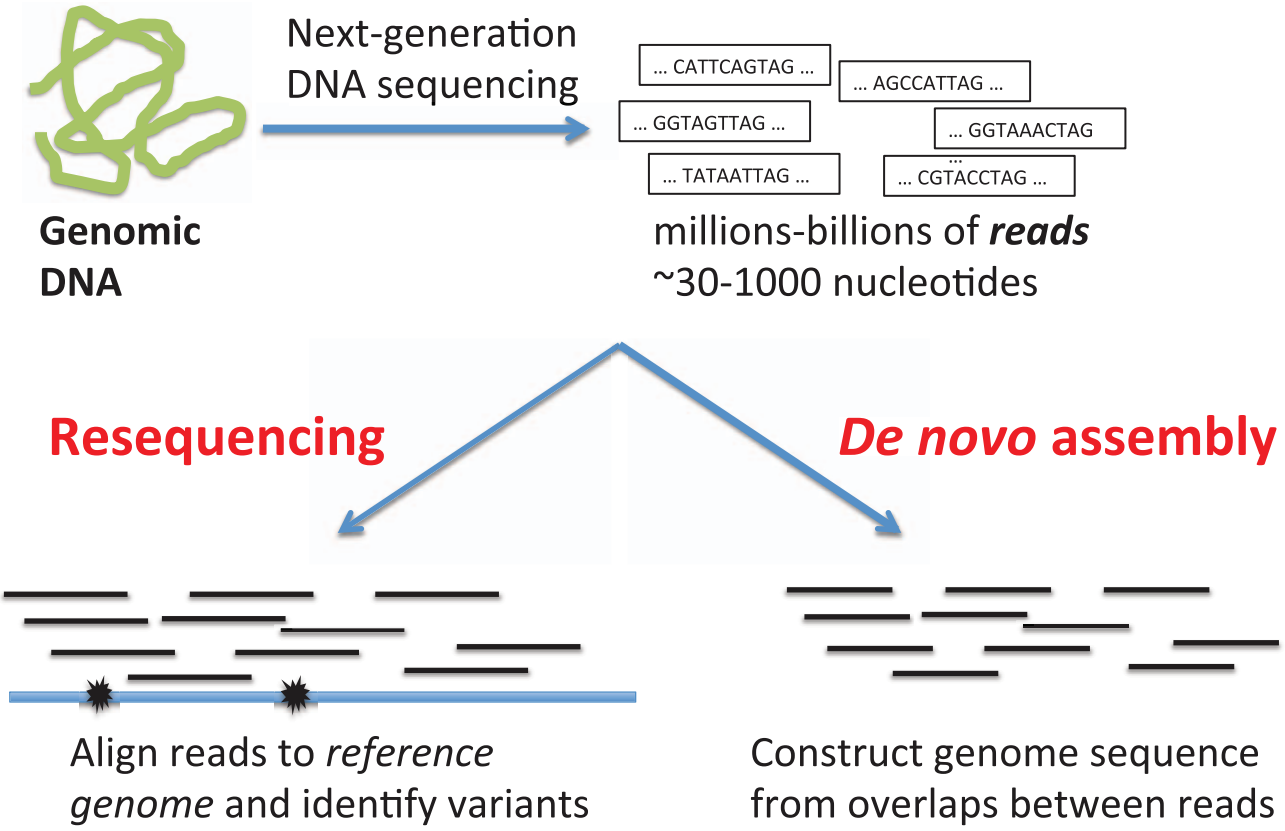

Figure 2. Two major approaches to detect structural variants in an individual genome from next-generation sequencing data are *de novo* assembly and resequencing. In *de novo* assembly, the individual genome sequence is constructed by examining overlaps between reads. In resequencing approaches, reads from the individual genome are aligned to a closely related reference genome. Examination of the resulting alignments reveals differences between the individual genome and the reference genome.
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number of reads that are produced (hundreds of millions), results in a cost per nucleotide that is several orders of magnitude lower than Sanger sequencing.

Many DNA sequencing technologies employ a paired end, or mate pair, sequencing protocol to increase the effective read length. In this protocol two reads are generated from opposite ends of a longer DNA fragment, or insert. With earlier Sanger sequencing protocols, the sizes of these DNA fragments were dictated by the cloning vector that was used. Fragment, or insert, sizes of 2 kb–150 kb could be obtained by cloning into bacterial plasmids or bacterial artificial chromosomes (BACs). With next-generation technologies, a variety of techniques have been employed to generate paired reads. At present, the most efficient and effective techniques produce paired reads from fragments of only a few hundred bp, although fragments of 2–3 kb are available. Thus, next-generation sequencing technologies have both limited read lengths and limited insert sizes compared to Sanger sequencing.

There are two approaches to detecting SVs from next-generation DNA sequencing data (Figure 2). The first is *de novo* assembly. In this approach, sophisticated algorithms are used to reconstruct the genome sequence from overlaps between reads. The assembled genome sequence is then compared to the reference genome, or the assembled genomes of other individuals, to identify all types of variants. If the genome sequence is successfully assembled, this approach is the best for characterization of SVs. Unfortunately, assembling a human genome *de novo* – i.e. with no prior information – of sufficient quality for structural variation studies remains difficult with limited read lengths. Currently, human genome assemblies are highly fragmented, consisting of tens-hundreds of thousands of contigs, intermediate sized sequences of thousands to tens of thousands of nucleotides. Moreover, the associations between some structural variants and repetitive sequences implies that assemblies of *finished* (not *draft* quality) are necessary for comprehensive coverage of structural variation. Improving *de novo* assembly is a very active research area (see [31]), but human genome assemblies of high enough quality for SV studies remain out of reach for inexpensive short-read technologies.

The second approach to detect SVs in next-generation DNA sequencing data is a "resequencing" approach that leverages the extensive finishing efforts undertaken in the Human Genome Project. In a resequencing approach, one finds differences between an individual genome and a closely related reference genome whose sequence is known by aligning reads from the individual genome to the reference genome. Differences (variants) between the genomes correspond to differences between the aligned reads and the reference sequence. In the next section, we describe how to predict SVs using a resequencing approach.

3.3 New DNA Sequencing Technologies

Many of the challenges in reliable measurement of SVs described above are related to limitations in sequencing tech-

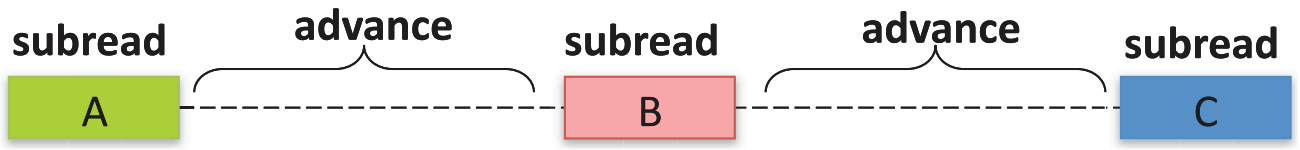

Figure 3. A strobe with 3 subreads.
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nologies. In particular, SVs with breakpoints in highly-repetitive sequences are beyond the abilities of current technologies. New "third-generation" and single-molecule technologies promise additional advantages for structural variation discovery. These advantages include longer read lengths, easier sample preparation, lower input DNA requirements, and higher throughput. For example, Pacific Biosciences recently released their Single-Molecule Real Time (SMRT) sequencing, a technology that measures in real time the incorporation of nucleotides by a single DNA polymerase molecule immobilized in a nanopore [32].

One application of this technology is *strobe sequencing*. A strobe read, or strobe, consists of multiple subreads from a single contiguous molecule of DNA. These subreads are separated by a number of "dark" nucleotides (called advances), whose identity is unknown (Figure 3). Thus far, Pacific Biosciences has demonstrated strobes of lengths up to 20 kb with 2–4 subreads each of 50–400 bp. Additional improvements are expected as technology matures. Strobes generalize the concept of paired reads by including more than two reads from a single DNA fragment. Strobes provide long-range sequence information with low input DNA requirements, a feature missing from current sequencing technologies. This additional information is useful for detection and *de novo* assembly of complex SV that lie in highly repetitive regions, or contain multiple breakpoints in a small region. However, the advantages of strobes are reduced by higher single-nucleotide error rates. Thus, realizing the advantages of strobes requires new algorithms that exploit information from multiple, spaced subreads to overcome high single-nucleotide error rates [33].

Sequencing technologies continues its rapid development. Improvements in the chemistry, imaging, and manufacture of existing technologies are increasing their read lengths, insert lengths, and throughput. Additional sequencing technologies are under active development. Nanopore-based technologies that directly read the nucleotides of long molecules of DNA hold promise for a dramatic shift in DNA sequencing where extremely long reads (tens of kb) are generated, making both *de novo* assembly and variant detection by resequencing straightforward problems.

4. Resequencing Strategies for Structural Variation

A resequencing strategy predicts SVs by alignments of sequence reads to the reference genome. There are two main steps in any resequencing strategy: (1) alignments of reads; (2) prediction of SVs from alignments. Resequencing approaches are straightforward in principle, but in practice sensitive and specific detection of structural variation in human genomes is notoriously difficult [34,35]. While some types of SVs are easy to detect with next-generation sequencing technologies, other complex SVs are refractory to detection. This is due to both technological limitations and biological features of SVs. DNA sequencing technologies produce reads with sequencing errors, have limited read lengths and insert sizes, and have other sampling biases (e.g. in GC-rich regions). Biologically, human SVs are: (i) enriched for repetitive sequences near their breakpoints [23]; (ii) may overlap, have multiple states or complex architectures; and (iii) recurrent (but not identical) variants may exist at the same locus [36,37]. These properties mean that the alignment of reads to the reference genome and the prediction of SVs from these alignments is not always an easy task. Algorithms are required to make highly *sensitive* and *specific* predictions of SVs.

In this section we review the main issues in predicting SVs using a resequencing approach. We begin with read alignment. Then we describe the three major approaches that are used to identify structural variants from aligned reads: (i) split reads; (ii) depth of coverage analysis; and (iii) paired-end mapping.

4.1 Read Alignment

Alignment of reads to a reference genome is a special case of sequence alignment, one of the most researched problems in bioinformatics. However, the specialized task of aligning millions-billions of individual short reads led to the development of new software programs tailored to this task, such as Maq, BWA, Bowtie/Bowtie2, BFAST, mrsFAST, etc. [38–43]. A key decision in read alignment for SV detection is whether to consider only reads with a single, best alignment to the reference genome, or to also include reads with multiple high-quality alignments. Some read alignment programs will output only a single alignment for each read, in some cases choosing an alignment randomly if there are multiple alignments of equal score. If one uses only reads with a unique alignment, then there is limited power to detect SVs whose breakpoints lie in repetitive regions, such as SVs resulting from NAHR. On the other hand, if one allows reads whose alignment is ambiguous, then the problem of SV prediction requires an algorithm to distinguish among the multiple possible alignments for each read. Many SV prediction algorithms analyze only unique alignments, although several recent algorithms use ambiguous alignments. A few of these are noted below.

4.2 Split Reads

A direct approach to detect structural variants from aligned reads is to identify reads whose alignments to the reference genome are in two parts. These so called split reads contain the breakpoint of the structural variant (Figure 4). To reduce false positive predictions of structural variants, one requires the presence of multiple split reads sharing the same breakpoint. Because the two parts of a split read align independently to the reference genome, these alignments must be long enough to be aligned uniquely (or with little ambiguity) to the reference. Thus, split read analysis is a feasible strategy only when the reads are sufficiently long. For example, if one has a 36 bp read containing the breakpoint of an SV at its midpoint, one must align the two 18 bp halves of the read to the reference genome. Finding unique alignments of an 18 bp sequence is often not possible. There are no reports of successful prediction of structural variants from split

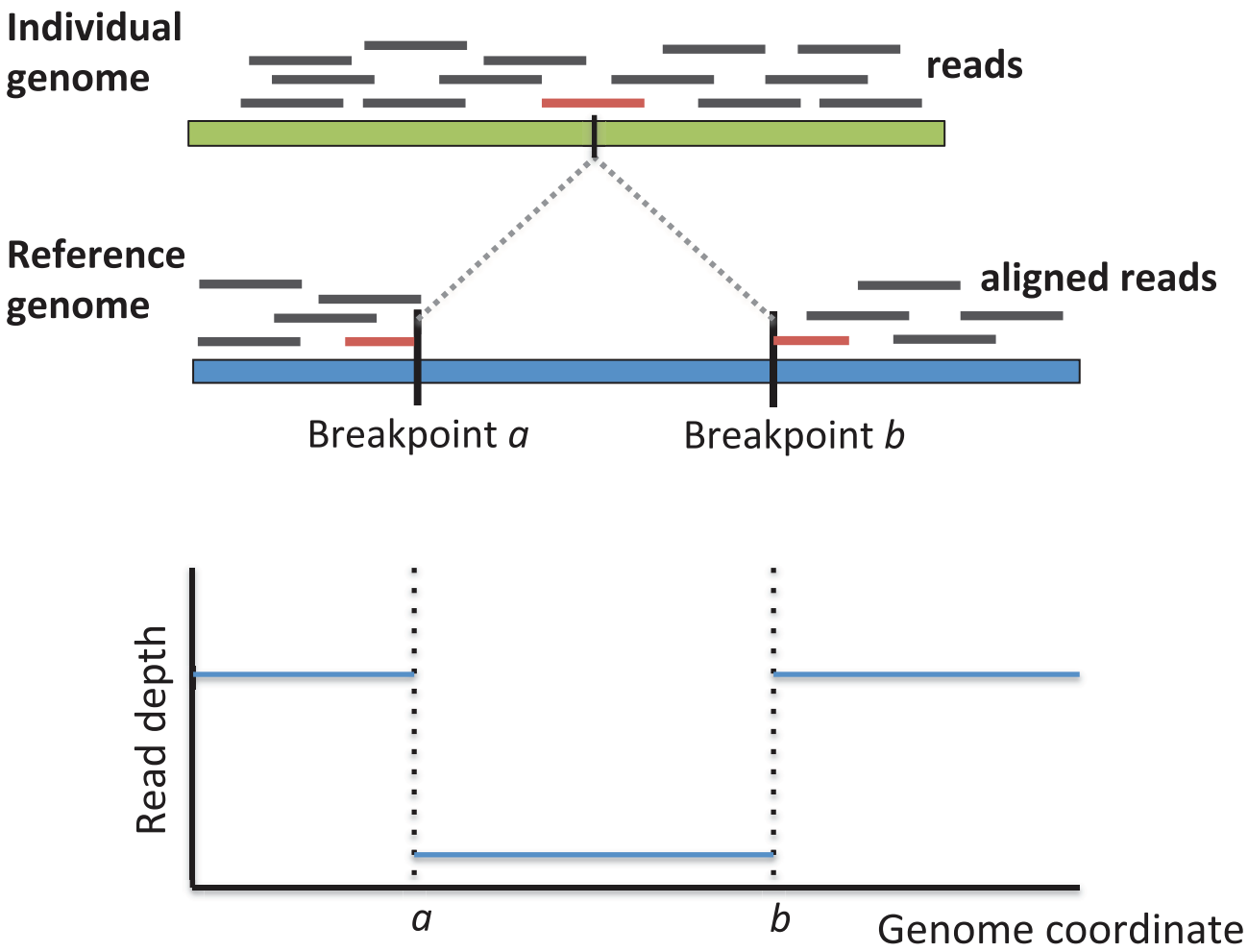

Figure 4. Identification of a deletion in an individual genome by split read analysis (middle), and by depth of coverage analysis (bottom).
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reads alone using next generation DNA sequencing reads less that 50 bp in length. Instead, split read methods have been proposed that use paired reads, and require that one read in the pair has a full length alignment to the reference. This alignment of the read from one end of the fragment is used to anchor the search for alignments of the other split read of the fragment [44–46].

4.3 Depth of Coverage

Depth of coverage (also called read depth) analysis detects differences in the number of reads that align to intervals in the reference genome. Assuming that reads are sampled uniformly from the genome sequence, the number of reads that contain a given nucleotide of the reference is, on average, $c = \frac{NL}{G}$, where N is the number of reads, L is the length of each read, and G is the length of the genome. This is the Lander-Waterman model, and the parameter c, called the coverage, is a key parameter in a sequencing experience. For example, recent cancer sequencing projects with Illumina technology have used "30X coverage" which means that the number of reads and length of reads are chosen such that $c = 30$.

Now, if the individual genome contained a deletion of a segment of the human reference genome, the coverage of this segment would be reduced by half – if the deletion was heterozygous – or reduced to zero – if the deletion was homozygous (Figure 4). Similarly, if an interval of the reference genome was duplicated, or amplified, in the individual genome, the coverage of this interval would increase in proportion to the number of copies. Thus, the observed coverage of an interval of the reference genome, the depth of coverage, gives an indication of the number of copies of this interval in the individual genome. Of course, there are numerous additional factors to consider beyond this simple analysis. For example, since reads are sampled at random from the genome, coverage is not constant, but rather follows a distribution with mean c. A Poisson distribution is typically used as an approximation to this distribution, although other distributions sometimes provide a better fit to the data. In addition, repetitive sequences in the reference genome and biases in sequencing (e.g. different coverage of GC-rich regions) also affect depth of coverage calculations. Nevertheless, there are several computational methods for depth of coverage analysis [47,48]. Many of these are largely similar to those used to analyze microarray copy number data.

4.4 Paired-end Sequencing and Mapping

The most common approach for resequencing SVs is paired-end mapping (PEM) (Figure 5). Paired-end mapping was used to identify somatic SVs in cancer

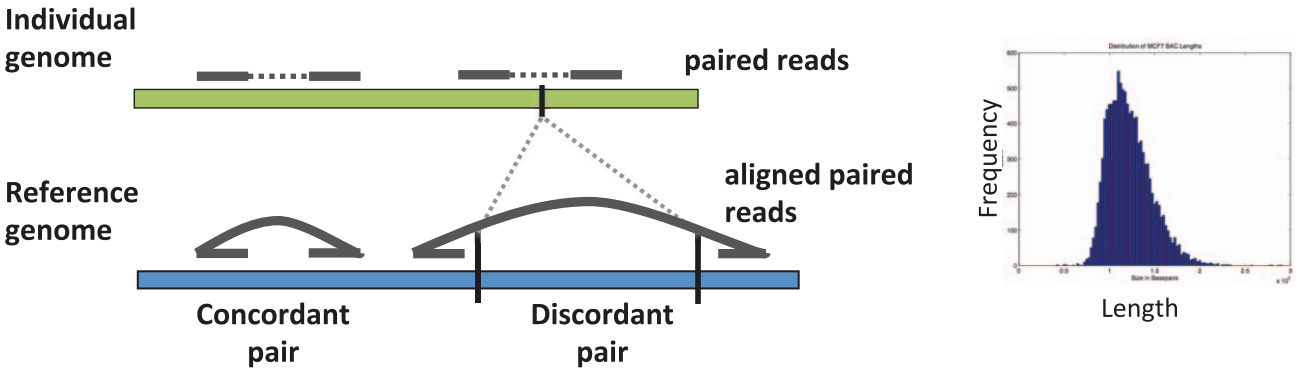

Figure 5. Paired end mapping (PEM). Fragments from an individual genome are sequenced from both ends and the resulting paired reads are aligned to a reference genome. Most paired reads correspond to concordant pairs, where the distance between the alignment of each read agrees with the distribution of fragment lengths (right). The remaining discordant pairs suggest structural variants (here a deletion) that distinguish the individual and reference genomes.
doi:10.1371/journal.pcbi.1002821.g005

genomes [49,50] and the same idea has been applied to identify germline structural variants [51,52]. While the early paired-end mapping studies used older clone-based sequencing, paired-end mapping is now possible using various next-generation sequencing technologies.

In PEM, a paired-end sequencing protocol is used to obtain paired reads from opposite ends of a larger DNA fragment, or clone, from a *individual genome*. These paired reads are then aligned to a reference genome. Most paired reads result in concordant pairs where the distance between aligned reads is equal to the fragment length. In contrast, discordant pairs have alignments with abnormal distance or that lie on different chromosomes. These suggest the presence of an SV or a sequencing error. For example, a discordant pair whose distance between alignments is too long suggests a deletion in the individual genome (Figure 5), while a discordant pair whose alignments are on different chromosomes suggests a translocation. Other types of discordant pairs identify inversions, transpositions, or duplications that distinguish the individual genome from the reference genome. Note that in general the length of any particular sequenced fragment is not known. Rather, during the preparation of genomic DNA for sequencing, the DNA is fragmented and fragments are size-selected to an appropriate target length. It is desirable for this size selection to be as strict as possible, so that only fragments near the target length are sequenced. However, in practice the size selection procedure produces fragments whose lengths vary around the target length. Typically, the distribution of fragment lengths is obtained empirically by examining the distances between all aligned paired reads, as most fragments will correspond to a concordant pair (Figure 5).

To distinguish real SVs from sequencing errors, one looks for clusters of discordant pairs that indicate the same SV. Numerous algorithms have been developed to predict SVs by finding clusters of discordant pairs. Early algorithms used only those paired reads whose alignments to the reference genome were non-ambiguous; i.e. there was only a single "best alignment" [53–55]. More sophisticated algorithms use paired reads with multiple ambiguous alignments to the reference genome and use a variety of combinatorial and statistical techniques to select among these alignments [56–58]. Finally, some approaches model the fact that the human genome is diploid to avoid making inconsistent structural variant predictions [59].

All of the approaches above rely on predicting structural variants that are supported by multiple paired reads. Some, but not all, of them are careful when determining whether a group of paired reads genuinely support the same variant. We illustrate the issue here using the Geometric Analysis of Structural Variants (GASV) method of [55]. A key feature of GASV is that it records both the information that the paired reads reveal about the boundaries (breakpoints) of the structural variant *and the uncertainty associated with this measurement*. Most types of SV, including deletions, inversions, and translocations have two breakpoints a and b where the reference genome is cut. The segments adjacent to these coordinates are then pasted together in a way that is particular to the type of SV. For example, a deletion is defined by coordinates a and b in the reference genome such that the nucleotide at position a is joined to the nucleotide at position b in the individual genome (Figure 6). Note that this is a simplification of the underlying biology, as there are sometimes small insertions or deletions at breakpoints, but these small changes have limited effect on the analysis of larger structural variants.

Now the discordant pairs that indicate an SV have the property that the locations of the read alignments are near the breakpoints a and b. However, a paired read does not give independent information about the breakpoint a and the breakpoint b. Rather, the breakpoints a and b are related by a linear inequality that defines a polygon in 2D genome space called the breakpoint region (Figure 6). For example, suppose that the pair of reads from a single fragment align to the same chromosome of the reference genome such that the read with lower coordinate starts at position x in the reference and the read with higher coordinate ends at position y in the reference. (For simplicity, we ignore the fact that the sequence of a read can align to either strand (forward or reverse) of the reference genome. The strand of an alignment gives additional information about the location of the breakpoint. See [55] for further details.) If the sequenced fragment has length L then the breakpoints a and b satisfy the equation $(a-x)+(y-b)=L$. As described above, the size of any particular fragment is typically unknown. Rather, one defines a minimum size $L_{\min}$ and maximum size $L_{\max}$ of a sequenced fragment, perhaps according to the empirical fragment length distribution. Thus, we have the inequality

$$L_{\min} \leq (a-x)+(y-b) \leq L_{\max}.$$

This equation defines the unknown breakpoints a and b in terms of the known

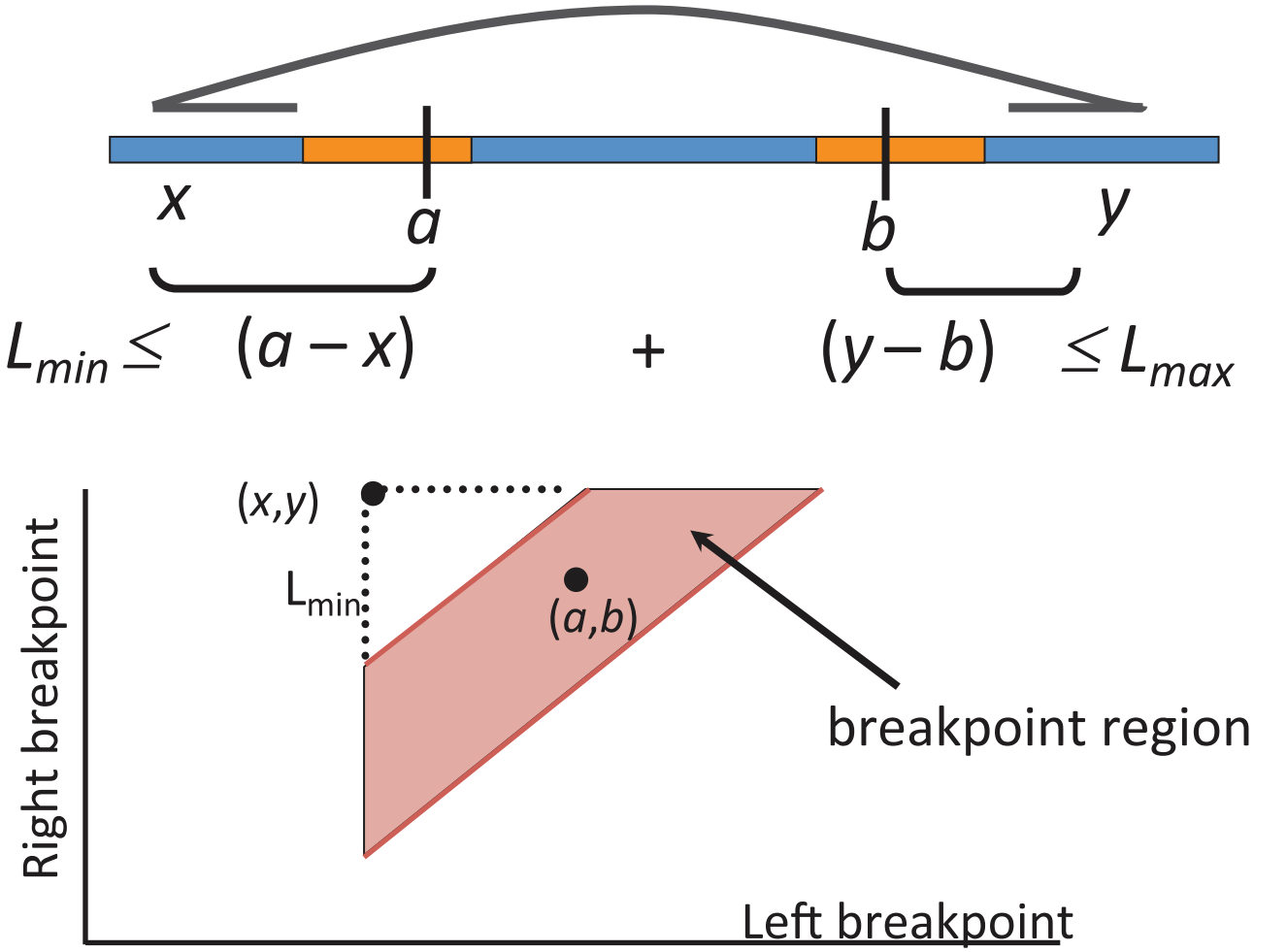

Figure 6. (Top) A discordant pair (arc) indicates a deletion with unknown breakpoints a and b located in orange blocks. Positions x, y and the minimum L_{min} and maximum L_{max} length of end-sequenced fragments constrain breakpoints (a,b) to lie within the indicated orange blocks, and are governed by the indicated linear inequalities. (Bottom) A polygon in 2D genome space expresses the linear dependency between breakpoints a and b and records the uncertainty in the location of the breakpoints.
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coordinates x and y of the aligned reads and the length of sequenced fragments. The pairs of breakpoints (a,b) that satisfy this equation form a polygon (specifically a trapezoid) in two-dimensional genome space. We define the breakpoint region B of discordant pair (x,y) to be the breakpoints (a,b) satisfying the above equation.

This geometric representation provides a principled way to combine information across multiple paired-reads: multiple paired-reads indicate the same variant if their corresponding breakpoint regions intersect. The geometric representation also provides precise breakpoint localization by multiple paired reads; separates multiple measurements of the same variant from measurements of nearby or overlapping variants; and facilitates robust comparisons across multiple samples and measurement technologies. Finally, the approach is computationally efficient as it relies on computational geometry algorithms for polygon intersection. These scale to millions of discordant pairs that result from next-generation sequencing platforms.

While the algorithms above consider many of the issues in prediction of structural variants, there remains room for improvement. Most notably, many algorithms still use only one of the possible signals of structural variants: read depth, split reads, or paired reads. Improvements in specificity are likely possible by integrating these multiple signals into a single prediction algorithm [60].

5. Representation of Structural Variants

Next generation DNA sequencing technologies are dramatically reducing the cost of sequence-based surveys of structural variants, while oligonucleotide aCGH techniques are now used in studies profiling tens of thousands of genomes. Large projects like the 1000 Genomes Project and The Cancer Genome Atlas (TCGA) are performing paired-end sequencing and aCGH of many human genomes, and matched tumor and normal genomes, respectively. At the same time, smaller or single investigator projects are using a variety of paired-end sequencing approaches and/or microarray-based techniques with different trade-offs in cost-per-sample vs. measurement resolution. Thus, in the near future there will be an enormous number of measurements of SVs, but using a wide range of technologies of varying resolution, sensitivity, and specificity. This diversity of approaches will likely continue for some time as investigators explore tradeoffs between the cost of measuring variants in one

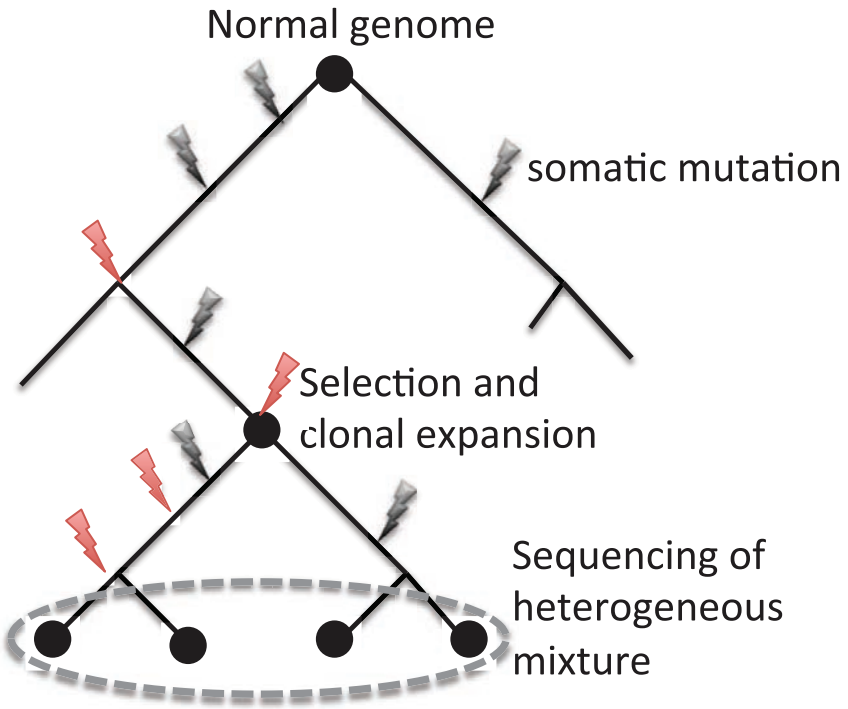

Figure 7. Mutation, selection, and clonal expansion in tumor development leads to genomic heterogeneity between cells in a tumor. Current DNA sequencing approaches sequence DNA from many cells and thus result in a heterogenous mixture of mutations, with varying numbers of both passenger mutations (black) and driver mutations (red).
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sample with high confidence versus surveying variants in many samples with lower confidence per sample. For example, in cancer genome studies the goal of finding recurrent mutations demands the survey of many genomes and thus large sample sizes might be preferred over high coverage sequencing of one sample.

The problem of comparing variants across samples and/or measurement platforms is less studied than the problem of detecting variants in a single sample. Standard practice remains to use heuristics that merge predicted structural variants into the same variant in they overlap by a significant fraction (e.g. 50–70%) on the reference genome. For example, the Database of Genomic Variations (DGV) [5], arguably the most comprehensive repository of measured human structural variants, merges structural variant predictions whose coordinates overlap by $\geq 70\%$ on the reference genome. Such heuristics are typically the only approach available to databases of human structural variants because many early studies did not report information on the uncertainty (i.e. "error bars") in the boundaries (breakpoints) of the variant. This situation makes it difficult to explicitly separate multiple measurements of the same variant from measurements of nearby variants or overlapping variants. This situation is now improving, and more recent software records both the information that the measurement reveals about the breakpoints of the structural variant and the uncertainty associated with this measurement. Software that uses this uncertainty to classify and compare SVs across samples and measurement platforms is also now available [55]. Such precision provides increased confidence in associations between a structural variant and a disease, helps separate germline from somatic structural variants in cancer genome sequencing projects, and aids in the study of rare recurrent variants that might occur on a variety of genetic backgrounds.

6. Challenges for Cancer Genomics Studies

The study of somatic structural variation in cancer genomes presents additional challenges beyond those described above for generic resequencing approaches. First, most cancer genomes are aneuploid, meaning that the number of copies of regions of the genome are variable, due to duplications and deletions of segments of the normal genome. High-resolution reconstructions of cancer genomes by paired read sequencing showed that many rearrangements were too small to be detected by cytogenetics, and identified highly rearranged genomic loci that encompass a complex intertwining of rearrangement and duplication [21,29,49,50,61–63]. Such highly rearranged loci are hypothesized to result from genome instability caused by defective DNA repair in cancer cells, or from external DNA damage. An extreme example is the phenomenon of chromothripsis that results from massive, simultaneous breakage and aberrant repair of many genomic loci [64]. Identifying all of the SVs and thereby reconstructing the organization of cancer genomes can suggest that certain regions of the genome are selected for their pathogenetic properties, and also lend insight into the mechanisms of genome instability in tumors [14].

A second challenge is that cancer tissues are a heterogeneous mixture of cells with possibly different numbers of mutations. This heterogeneity includes admixture between normal and cancer cells, as well as subpopulations of tumor cells. Some of these subpopulations might contain important driver mutations, or drug resistance mutations. Because of the amount of DNA required for current sequencing technologies, most cancer genome sequencing studies do not sequence single tumor cells but rather sequence a mixture of cells (Figure 7). Since the signal for detecting variants is proportional to the number of cells in the mixture that contain the variant, presence of normal cells will reduce the power to detect somatic mutations. Further, the ability to detect mutations that are rare in the tumor cell population will be even lower. Eventually, whole genome sequencing of single cells will provide fascinating datasets to study cancer genome evolution, with some recent hints of the discoveries to come in [65].

7. Future Prospects

This chapter described the challenges in identification and characterization of structural variants. With further improvements in sequencing technologies and algorithms over the next few years, it will be possible to systematically measure nearly all but the most complex variants in an individual genome. The most difficult cases, such as variants mediated by homologous recombination between nearly identical sequences, might remain inaccesible until significantly different types of DNA sequencing technologies become available. Nevertheless, the fact that systematic identification of nearly all germline and somatic structural variants in

Further Reading

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an individual genome is now possible will enable further progress in human and cancer genetics.

For genetic association studies, having complete lists of germline variants from many individuals means that unexplained heritability for a trait cannot readily be blamed on lack of measurement of genetic information. Unfortunately, this does not necessarily imply that finding the genetic basis for specifc traits will become easy. There remain other challenges, including the possiblity that combinations of variants, interactions between genetic and environmental factors, or other epigenetic mechanisms, may contribute to phenotype. See [66] in this collection for further discussion of these issues. Finally, translating genetic information about susceptibility to a disease or efficacy of particular treatments into improved medical outcomes will require additional work.

The opportunities and challenges are similar in cancer genetics. Systematic measurement of all somatic mutations will yield information that might guide treatments, and eventually lead to personalized oncology. Current cancer treatments are limited by the non-specificity of most cancer drugs and by the fact that cancer cells can evolve resistance to single drug treatments. Tailoring of treatment to the particular genetic mutations in a tumor promises to revolutionize cancer therapy. There are already several examples of such personalized treatments including the drug Gleevec that targets the BCR-ABL fusion gene in chronic myelogenous leukemia (CML) and Iressa that targets the EGFR gene in lung cancer. Discovery of additional cancer-specific drug targets requires not only technologies to globally survey somatic mutations in cancer genomes, but also techniques (experimental and/or computational) to classify the subset of variants that are functional, and then the further subset of these functional variants that are druggable.

The sequencing technologies and algorithms described in this chapter are laying the foundation for personalized medicine, but much work remains to translate the information revealed by genome sequencing into improved clinical practice.

8. Exercises

(1) Consider the chromosomal inversion in Figure 1. What signals in next-generation sequencing data can be used to detect a chromosomal inversion?

(2) The human genome is diploid with two copies, maternal and paternal, of each chromosome. What constraints does this place on prediction of germline structural variants?

Answers to the Exercises can be found in Text S1.

Supporting Information

Text S1 Answers to Exercises.
(PDF)

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Chapter 7: Pharmacogenomics

Konrad J. Karczewski[1,2], Roxana Daneshjou[2,3], Russ B. Altman[2,3]*

1 Program in Biomedical Informatics, Stanford University, Stanford, California, United States of America, **2** Department of Genetics, Stanford University, Stanford, California, United States of America, **3** Department of Medicine, Stanford University, Stanford, California, United States of America

Abstract: There is great variation in drug-response phenotypes, and a "one size fits all" paradigm for drug delivery is flawed. Pharmacogenomics is the study of how human genetic information impacts drug response, and it aims to improve efficacy and reduced side effects. In this article, we provide an overview of pharmacogenetics, including pharmacokinetics (PK), pharmacodynamics (PD), gene and pathway interactions, and off-target effects. We describe methods for discovering genetic factors in drug response, including genome-wide association studies (GWAS), expression analysis, and other methods such as chemoinformatics and natural language processing (NLP). We cover the practical applications of pharmacogenomics both in the pharmaceutical industry and in a clinical setting. In drug discovery, pharmacogenomics can be used to aid lead identification, anticipate adverse events, and assist in drug repurposing efforts. Moreover, pharmacogenomic discoveries show promise as important elements of physician decision support. Finally, we consider the ethical, regulatory, and reimbursement challenges that remain for the clinical implementation of pharmacogenomics.

This article is part of the "Translational Bioinformatics" collection for *PLOS Computational Biology*.

1. Introduction

A child with leukemia goes to the doctor's office to be treated. The oncologist has decided to use mercaptopurine, a drug with a narrow therapeutic range. The efficacy and toxicity of this drug lies in its ability to act as a myelosuppressant, which means it suppresses white and red blood cell production. Despite the dangers this regimen poses, the oncologist is confident with his ability to administer the drug based on his experience with prior patients. However, after the child has undergone treatment, he begins experiencing unexpected bone marrow toxicity, immunosuppression, and life-threatening infections. This type of scenario was encountered after mercaptopurine first came on the market in the 1950s. In the mid-1990s, scientists began to realize that genetics could explain a majority of the cases of life-threatening bone marrow toxicity [1]. Now, many drugs that were once noted to cause so-called "unpredictable" reactions are being re-evaluated for drug-gene interactions.

The history of medicine is full of medications with unintended consequences; the ability to understand some of the underlying causes has been a recent development. In the 1950s, succinylcholine was used by anesthesiologists as a muscle relaxant during operations. However, about 1 in 2500 individuals experienced a horrific reaction – respiratory arrest. Later research revealed that those individuals had defects in both copies of cholinesterase, the enzyme required to metabolize succinylcholine into an inactive form. During the 1980s, a drug used to treat angina, perhexiline, caused neural and liver toxicity in a subset of patients. Scientists later found that this toxicity occurred in individuals with a rare polymorphism of CYP2D6, an enzyme involved in the drug's metabolism. Genetics not only plays a role in adverse events, but also influences an individual's optimal drug dose. Two anticoagulants, warfarin and clopidogrel, have different therapeutic doses based on an individual's genetic makeup. Scientists are increasingly learning more about the interaction between drugs and human genetics in order to take modern medicine down a more personalized path.

Modern physicians prescribe medications based on clinical judgment or evidence from clinical trials. In order to select a drug and dosage, physicians take clinical factors such as gender, weight, or organ function into consideration. The personal variation that may affect drug selection or dosing, such as genetics, is not considered in many settings. Thus, while a daily 75 mg dose of clopidogrel for a 70 kg adult would obviously be inappropriate for a 20 kg child, it is less obvious that two adults with identical presentations and clinical backgrounds might require vastly different doses. However, for an increasing number of drugs, this appears to be the case. For instance, two patients with similar clinical presentations could be given the same dose of the anti-platelet drug clopidogrel, and one would be adequately protected against cardiovascular events while the other experiences a myocardial infarction due to inadequate therapeutic protection. What accounts for this difference? Genetics – the patient with the inadequate therapeutic protection likely has a polymorphism of CYP2C19 with decreased activity, so that this key enzyme cannot efficiently metabolize clopidogrel into its active metabolite. The interaction between drugs and genetics has been termed pharmacogenomics.

In general, pharmacogenomics can be defined as the sum of the word's parts: the study and application of genetic factors (often in a high-throughput, genomic fashion) relating to the body's response to drugs, or pharmacology (for the major questions in the field of pharmacoge-

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* E-mail: russ.altman@stanford.edu

What to Learn in This Chapter

- Interactions between drugs (small molecules) and genes (proteins)
- Methods for pharmacogenomic discovery
  - Association- and expression-based methods
  - Cheminformatics and pathway-based methods
- Database resources for pharmacogenomic discovery and application (PharmGKB)
- Applications of pharmacogenomics into a clinical setting

nomics, see Box 1). Once a patient takes a drug, the drug must travel through the body to its target(s), act on its target(s), and then leave the body. The first and last of these processes is facilitated by pharmacokinetic (PK) genes, which may affect a drug in the "ADME" processes: to be **a**bsorbed into and **d**istributed through the body, **m**etabolized (either to an active form or broken down into an inactive form), and **e**xcreted. The action of a drug on its targets involves pharmacodynamic (PD) genes, which include the direct targets themselves, genes affected downstream, and the genes responsible for the clinical outcome. PK and PD genes can be involved in both intentional "on-target" effects that produce the desired therapeutic response, as well as unintentional "off-target" effects that cause adverse events (side effects or other unintended consequences of the drug). Current researchers are working to tease out genes involved in both the PK and PD pathways that affect drug action in order to improve dosing and avoid adverse drug reactions.

The search for genetic factors that relate to pharmacological response begins much like the search for a genetic association of any trait. Standard association study methods (such as GWAS) search for significant associations between a binary or continuous trait and the genetic profiles of case and control sets. In a GWAS, the trait of interest can be a disease state or physical trait. Specifically, in the case of pharmacogenomics, the trait is an actual drug dose, response, or adverse event profile, though the study design should be carefully considered for the specific application (see below: Methods). Additionally, high-throughput expression analysis and cheminformatics have provided investigators with valuable tools for learning about physiological drug responses. Finally, as sequencing technologies become exponentially cheaper and the "$1,000 Genome" becomes an attainable goal, whole-genome or exome sequencing will soon become commonplace in pharmacogenomic studies. As these types of studies become less expensive and more mainstream, pharmacogenomics will transition from simply an interesting research topic to a main role player in pharmacological development and clinical application.

The applications of pharmacogenomics are of interest to industry, clinicians, academics, and patients alike. For the biopharmaceutical industry, pharmacogenomics can improve the drug development process through faster and safer drug trials and the early identification of drug responders, non-responders, and those prone to adverse events. For clinicians and patients, pharmacogenomics can aid the decision-making process in prescriptions and determination of the optimal dose of a drug.

Many significant challenges remain in the field of pharmacogenomics, beyond the simple identification of more genetic variants related to drug response. First, the transition to whole-genome sequencing will require newer analysis methods, as well as more extensive annotations, to assign meaning to novel variants. A database of the relation between genes, variants, and drugs, such as PharmGKB, will be instrumental in the aggregation of information curated from the literature. In addition, the characterization of adverse events and their underlying causes is a topic of active research. Finally, the application of pharmacogenomics to a clinical setting will require the education of physicians in the utility of genome sequencing or genotyping for the benefit of their patients.

With the dawn of human genome sequencing, especially the impending widespread availability of personal genotyping to the public, and an expanded knowledge of the clinical impact of genetics and molecular biology, physicians around the world are beginning to use patients' personal genetics in informing prescription decisions. While still in its early phases, pharmacogenomics will undoubtedly lead the way in the development of personalized medicine.

Box 1. Problem Statement

- What are the genes involved in a drug's mechanism of action?
- How are a drug's effects propagated through pathways?
- How can this information be applied to characterize "off-target" adverse events?
- How can pharmacogenomics information be utilized in prescription and dosing decisions?

2. Pharmacogenomics in Action

When a physician administers a drug, an intricate cascade of events unfolds as this molecule interacts with the physiological environment. In the simplest scenario, a drug (after interacting with a number of proteins on its way to its target) may act as an agonist or an antagonist against a receptor, which is composed of one or more proteins. At the molecular level, the metabolite can bind to the protein's active site, which can include ligand-binding sites, conformation-altering sites, or catalytic sites. This effect can then be propagated through biochemical pathways to produce a cellular and finally, systemic physiological effect. Along the way, human genetic variation can affect the way these receptors interact with drugs, leading to consequences in the efficacy of the drug and causing potential adverse events.

2.1. Drug-Receptor Interactions: Agonists and Antagonists

Agonists interact with a receptor in an activating fashion: these small molecules mimic the behavior of the receptor's natural ligand, producing a result that is either weaker than, the same as, or stronger than the natural ligand. For example, sympathomimetic drugs are a clinically important class of agonists that interact with the G-protein-coupled receptors that are endogenously stimulated by catecholamines. These drugs are given to produce responses normally elicited by the sympathetic nervous system. Some examples of sympathomimetic drug action include relaxation of bronchial smooth muscle in asthma, increasing the muscular contractions of the heart in cases of reversible heart failure due to cardiogenic or septic shock, or vasoconstriction of superficial vasculature to reduce nasal congestion. There are several subtypes of adrenoreceptors and different drugs stimulate different receptor subtypes. For instance, a very clinically relevant drug, albuterol, can be inhaled to

stimulate β2 receptors (whose natural ligand is norepinephrine) on the smooth muscle of the lungs. Its action leads to the activation of adenylyl cyclase, which ultimately leads to the dilation of bronchial smooth muscle, providing life-saving relief for asthma patients (See Chapter 9 of [2]). However, some studies have identified that the very agonists that provide relief to asthmatics can lead to asthma exacerbation or death in a subset of patients. Research has indicated that at least in some populations, this phenomenon could be related to genetic polymorphisms of the β2 receptors [3].

Antagonists, on the other hand, inhibit the receptor partially or fully, reversibly or irreversibly so that the cascade caused by normal receptor activation cannot occur. The same adrenergic receptor subclasses mentioned before can also be antagonized. β receptor blockers ("beta-blockers") are an antagonist drug class clinically indicated to treat chronic, irreversible heart failure. The mechanism of the beneficial effects of β blockers is not well understood. The prevailing theory is that since the high levels of circulating catecholamines triggered by heart failure lead to detrimental cardiac remodeling, blocking the cardiac catecholamine receptors (β1 and β2 receptors) with a β blocker can slow down additional de-compensation. The β blockers for heart failure, bisoprolol, carvedilol, and metoprolol, antagonize (that is, inhibit) β1 and β2 receptors: their action is substantially greater at the β1 receptor, which is the dominant receptor in the heart. However, some patients do not respond as well to this therapy as others, and clinical studies have suggested that this may be due to β1 receptor polymorphisms. More extensive studies of these polymorphisms are underway to definitively identify the pharmacogenetic variables affecting β blocker success [4].

Often in the literature, the discussion of drugs and proteins has involved vague notions of "interactions" without any discussion about the underlying molecular mechanisms. A drug's interaction with any receptor is dependent on how well the molecular conformation of the drug can interact with the structure of the target. Before any discussion of downstream physiological effects, a drug's mechanism of action begins with the specific molecular reaction between the drug and cellular proteins. This interaction itself can provide insight into the effect of drugs on physiology and influence potential pharmacogenomic knowledge.

2.2. Drug-Receptor Interactions: The Details

While biologists tend to represent proteins as colored ovals existing in an idealized environment, in reality, proteins are complex molecules with intricate secondary and tertiary structures: they harbor rugged landscapes on their surfaces, with charged or hydrophobic hills and valleys serving as pockets to which potential small molecules can bind. At these twists and turns, proteins contain their active sites, including structural sites, binding sites, and catalytic sites. Metabolites (drugs) that enter a protein's binding site or catalytic site can either switch on the function of the protein (agonists) or prevent further reactions (antagonists). Such an effect is especially common if the drug bears chemical similarity to the natural ligand of the protein.

Non-steroidal anti-inflammatory drugs (NSAIDs), which cause both reversible and irreversible inhibitory processes, are a familiar drug class that illustrates drug-protein interactions. In general, NSAIDs inhibit the action of cyclooxygenases (coded by the COX genes), which mediate inflammation (see below: Molecular and Physiological Effects; reviewed in [5]). For instance, ibuprofen inhibits cyclooxygenases in a reversible fashion, by localizing to its critical catalytic site and competing with arachidonic acid to prevent the modification of the substrate [6].

Alternatively, a drug can react covalently with a protein's critical structural, binding, or catalytic site to affect the structure of the site or the protein as a whole. As mentioned previously, drugs can covalently modify their protein targets, causing protein inactivation. In the case of NSAIDs, aspirin irreversibly inactivates cyclooxygenases by acetylating critical serine resides (e.g. Serine 530 of COX-2): the bulky sidechain renders the catalytic sites unable to modify arachidonic acid [7]. Irreversible reactions can also work in the opposite direction, where the protein modifies the structure of the drug, potentially altering its activity (see below: PK Interactions).

Often, such an interaction occurs because a drug bears structural similarity to the molecule's natural ligand. For instance, methotrexate is an antifolate drug used to treat a number of diseases, including cancers and autoimmune diseases. Methotrexate is structurally similar to dihydrofolate (Figure 1A) and as such, binds to the same region of DHFR (Figure 1B). Dihydrofolate typically fits into DHFR in a known conformation (Figure 1C), but a phenylalanine to arginine mutation changes this binding conformation (Figure 1D–E). This mutation is hypothesized to confer methotrexate resistance in individuals with this variant [8].

All such drug-protein interactions are often associated with the "intended" action of the drug, whether they involve "what the body does to the drug" (pharmacokinetics, PK) or "what the drug does to the body" (pharmacodynamics, PD). However, drug-protein interactions may also lead to "off-target" interactions, which can cause adverse events. Along the way, variants in genes can affect these interactions, which influence the pharmacological effect of the drug (See Figure 2 of [9]).

2.2.1. Pharmacokinetic (PK) interactions. On the way to its target and on its way out, a drug may interact with many proteins that aid or hinder its progress. These interactions define a drug's pharmacokinetics, which encompass absorption, distribution, metabolism, and excretion (ADME) processes. These parameters determine how quickly a drug reaches its target and how long its action can last.

When a drug is administered, it must first be absorbed by the body and distributed to the relevant organs and cells. One important parameter, bioavailability, involves the fraction of the dose of the drug that ends in systemic circulation, much of which is based on mode of administration: intravenous delivery would provide 100% bioavailability, while an orally ingested tablet or capsule may be incompletely absorbed by the gastrointestinal tract or metabolized before it reaches systemic circulation. For non-injection methods (as most prescription drugs are administered), bioavailability often depends on absorption and enzymatic action. If the drug is administered orally, bioavailability is influenced by gastric emptying (i.e. transit time), gastrointestinal enzymatic action, gastrointestinal absorption, and liver metabolism. Since drugs absorbed from the gastrointestinal system are taken to the liver via the portal vein prior to entering systemic circulation, the liver can exert a tremendous effect on first pass metabolism. Once a drug has entered systemic circulation, issues of molecular transport affects the drug's ability to distribute (or reach its target). Genetic variation in the proteins that mediate these processes can affect the absorption and distribution of certain drugs. For instance, the class of ABC (ATP binding cassette) transporters is involved in many of the transport processes in the circulation of drugs and metabolites, especially in the gut and across the blood-brain barrier: polymorphisms in these genes is associated with altered bioavailability of certain drugs, such as the cardiac drug digoxin (digitalis; reviewed in [10]).

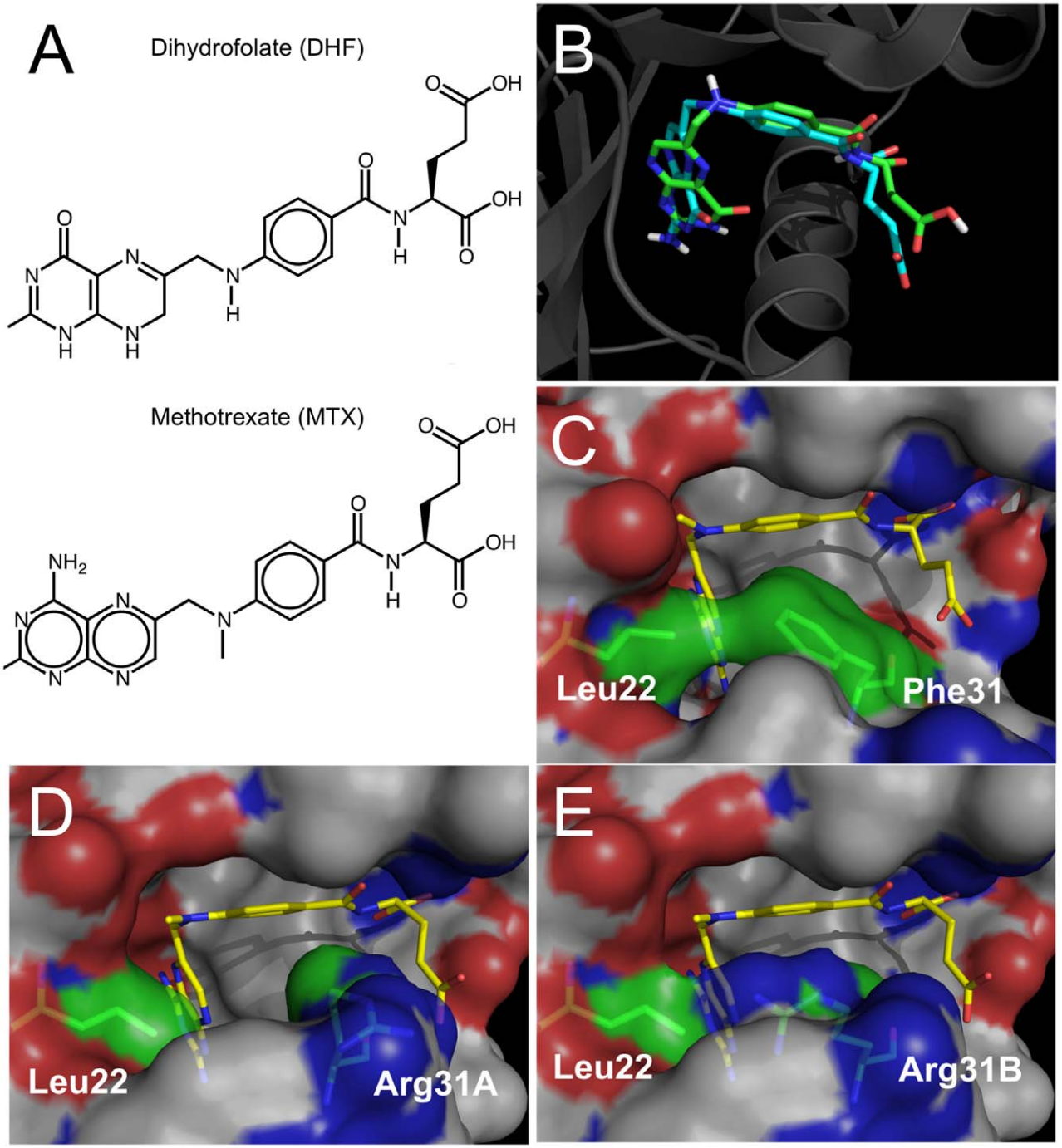

Figure 1. Methotrexate binds to the folate-binding region of DHFR. (A) Structural similarity between methotrexate and dihydrofolate. (B) Methotrexate (green) and dihydrofolate (blue) fit into the same binding pocket of DHFR. (C) The conformation of dihydrofolate bound to the reference version of the receptor. (D–E) Two possible conformations of dihydrofolate bound to the F31R/Q35E variants of the receptor. These variants have decreased affinity to methotrexate, relative to dihydrofolate. Reprinted with permission from [8].
doi:10.1371/journal.pcbi.1002817.g001

The body's metabolism of a drug can lead to the conversion of a precursor drug into an active metabolite or the breakdown of the active form into an inactive form for excretion. As with absorption and distribution, inter-individual variation in metabolism can often be explained by genetics (specifically, changes in the proteins that interact with the drug). Perhaps the most famous drug-metabolizing proteins are members of the cytochrome P450 family ("CYP" genes), which are involved in the phase I metabolism of the majority of known drugs [11]. Polymorphisms in these genes have been implicated in human drug response variation, affecting up to 25% of all drug therapies (reviewed in [12]). For instance, CYP2C9 plays a major role in the metabolism of warfarin to the inactive hydroxylated forms, including 7-hydroxywarfarin ([13], reviewed in [14]). As such, CYP2C9 is the second greatest contributor to the variation in warfarin dosage discovered thus

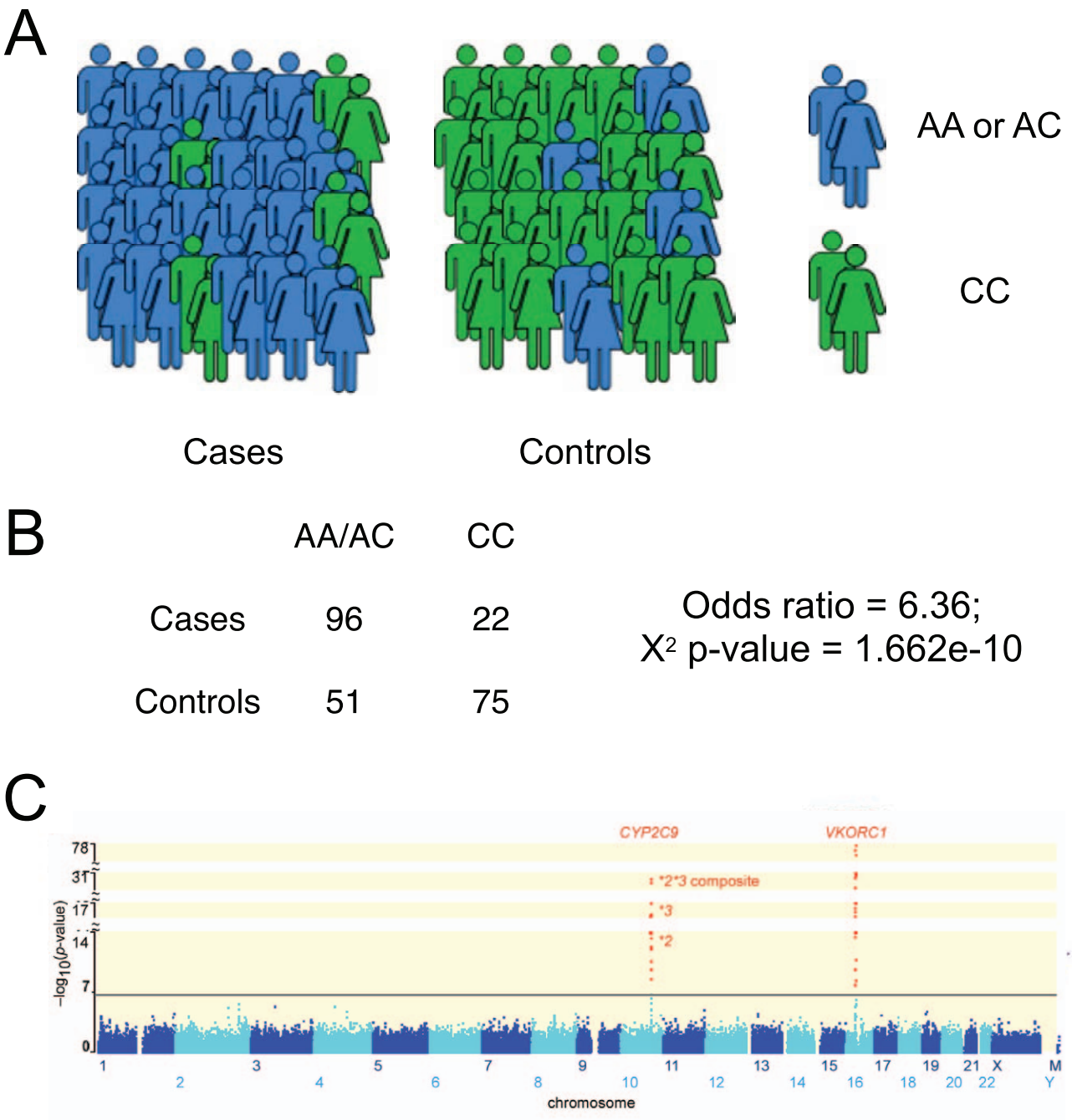

Figure 2. Association methods. (A) An association study with cases and controls. Millions of genetic loci are probed to ascertain "association," or separation between genotypes in cases and controls. (B) Each SNP is tested independently using a 2×2 contingency table and a χ^2 test or Fisher's exact test. (C) Each SNP is assessed for "genome-wide" significance, after Bonferroni correction. Reprinted from [64].
doi:10.1371/journal.pcbi.1002817.g002

far, which has led to its inclusion in pharmacogenetic dosing equations [15].

Finally, the body constantly cycles through the gamut of small molecules that flow through it. For example, the kidney is involved in finely regulating ionic concentrations and purging out unwanted metabolites. As small molecules, drugs are not exempt from these processes and are also excreted from the body, purging what was brought in and circulated by absorption and distribution. For instance, one member of the ABC family, P-glycoprotein (P-gp or ABCB1) is a transporter protein that actively pumps drugs and other metabolites out of cells (a detailed view into the mechanism of P-gp can be found in [16]). Upregulation of P-gp causes increased efflux of small molecules, which causes multi-drug resistance. For example, resistance to statins and chemotherapeutic drugs occurs because the drugs are pumped out before achieving their therapeutic effect (reviewed in [17]). Thus, inhibition of P-gp has remained an active area of research for augmenting cancer treatment [18]. Additionally, upregulation of elimination mediators such as P-gp should be considered for pharmacogenomic dose adjustments, with the caveat that increasing a drug's dose may have other potential detrimental effects.

2.2.2. Pharmacodynamic (PD) interactions. Pharmacodynamics (PD) encapsulates the specific effect of the drug on its targets and downstream pathways. The drug-target interactions can be "on-target", where interactions lead to a therapeutic effect, or "off-target", where interactions lead to undesired effects. PD also deals with how a drug concentration affects the target – what concentration is needed to reach the maximum effect, beyond which additional drug does not increase response (maximal effect) and what concentration is required to reach half of this maximal effect (sensitivity).

In many cases, structurally similar molecules (e.g. a drug that is similar to a protein's natural ligand) can bind and affect the same region of a protein and produce a pharmacological effect. For instance, vitamin K and warfarin both interact with VKORC1 (Vitamin K ep-Oxide Reductase Complex subunit 1), an enzyme that typically converts the inactive epoxidized form of Vitamin K back to the active reduced form [19]. Warfarin binds to VKORC1 near its catalytic site (See Figure 3 of [20]), inhibiting the reduction reaction; the ensuing lack of active Vitamin K results in the downstream anti-coagulant effects of warfarin (See Figure 2 of [21] and below: Molecular and Physiological Effects). Polymorphisms in VKORC1 are intensely linked to the efficacy of warfarin [22] by affecting warfarin's ability to bind to VKORC1 and displace vitamin K. As such, sensitivity to warfarin varies significantly in individuals, leading to twenty-fold dose differences. Warfarin's optimal dose can be better estimated by including VKORC1 polymorphisms in a dosing equation rather than using clinical factors alone [15].

Often, a drug's mechanism of action involves its localization to some binding pocket that then disrupts (or enhances) the function of the protein. For example, hydrocortisone is a lipid-soluble drug that diffuses across the cell membrane and interacts with the glucocorticoid receptors. These receptors reside in an inactive conformation because they are bound to heat shock proteins, which hold the glucocorticoid receptors in the inactive state. The binding of hydrocortisone causes the dissociation of the heat shock protein and allows the DNA-binding and transcription-activating binding domains of the glucocorticoid receptor to enter an active conformation. Now, target genes can be transcribed, and the many anti-inflammatory downstream effects of hydrocortisone can occur (See Chapter 2 of [2]).

2.3. Propagation through Pathways

As in the example of hydrocortisone, once a drug affects a gene (whether "on-target" or "off-target"), the effects can propagate through multiple proteins in the same pathway. Biology does not occur in a vacuum: proteins are dynamic and interact with many other proteins to produce a physiological function.

In the simplest cases, if the direct effect of a drug is the inhibition of a functional protein, all downstream effects of that protein will be affected. For instance, if a drug disrupts a kinase's active site, all downstream factors in a kinase cascade would not be phosphorylated. As in the case of hydrocortisone, a drug's activation of a transcription factor's DNA-binding domain will switch on the expression of the transcription factor's targets. These downstream targets lead to many of the biological effects of a given drug. Thus, a variant in a pharmacogene may be considerably upstream or downstream of the drug's direct protein interactions, but still affect the action of the drug.

For instance, suppose protein A is known to interact with proteins B and C. When a drug is used to block protein A in order to inhibit protein B's downstream effects, the interaction between proteins A and C may also be affected. If protein A and C's interaction is essential for healthy cellular function, administration of the drug could lead to severe adverse events. Most of the interactions discussed so far comprise "on-target" effects (A and B), while "innocent bystander" interactions (A and C) are known as "off-target" events. In other cases, the drug may exert an effect on an unrelated protein D (that may, for example, bear structural resemblance to protein A).

2.4. Adverse Events ("Off-Target")

Drugs are designed for their therapeutic effects, which require the molecule to bind to one or more targets that then produce downstream effects. Adverse events, however, can occur when the "on-target" interaction produces a potentially related, but unintended effect, or when drugs bind to "off-target" proteins to produce an unrelated, unintended effect. Such effects may be harmful to the patient, but may occasionally be inadvertently helpful (see below: Drug Repurposing). For instance, this adverse event can occur due to the intended interaction in an unintended tissue: the β blockers used to treat heart failure can also block β receptors in the bronchial smooth muscle, causing bronchial spasm, a dangerous event for asthmatics (See Chapter 13 of [2]). Another example is tamoxifen, the selective estrogen receptor modulator (SERM), which has improved outcomes in patients with estrogen receptor positive breast cancers. This drug antagonizes the estrogen receptor in the breast, blocking one of the signals that the cancer cells rely on. However, tamoxifen also has agonist activity at the estrogen receptors in endometrial tissue. This off target action can lead to a 2- to 7-fold increased risk of endometrial cancer [23].

Alternatively, a drug may interact with a protein (unrelated to the intended target) to produce an "off-target" adverse event. For example, in addition to the "on-target" adverse events described above, tamoxifen is also associated with cardiac abnormalities and muscle cramping. Preliminary data (discovered by docking methods, see below: Cheminformatics) suggest that these events may be due to an "off-target" interaction with sarcoplasmic reticulum Ca^{2+} ion channel ATPase protein (SERCA) [24].

2.5. Molecular and Physiological Effects

A drug's interaction with its target and the downstream effects (through any of the target's pathways) leads to the alterations in cellular physiology. In some cases, a cellular "systemic" response may be activated or switched off, such as apoptosis or inflammation. The cell may signal to other cells to produce a larger response, which is then observed in the larger context of the body. For instance, warfarin's inhibition of VKORC1 slows the vitamin K-dependent clotting pathway. This results in decreased thrombus formation by platelets, or colloquially known as "blood thinning." In other cases, a drug may suppress a body's natural response. For instance, NSAIDs such as aspirin and ibuprofen inhibit COX proteins, preventing the conversion of arachidonic acid to prostoglandin H_2 (PGH_2) and blocking the downstream production of other prostoglandins, which mediate inflammation and pain response.

While in the case of VKORC1, pharmacogenomic variation is observed at the direct site of action of warfarin, variation in downstream receptors can also influence the effect of drugs on the body. For instance, calumenin (CALU) is an inhibitor of the vitamin K-dependent clotting pathway. While calumenin's effects are downstream of the direct interaction between VKORC1 and warfarin,

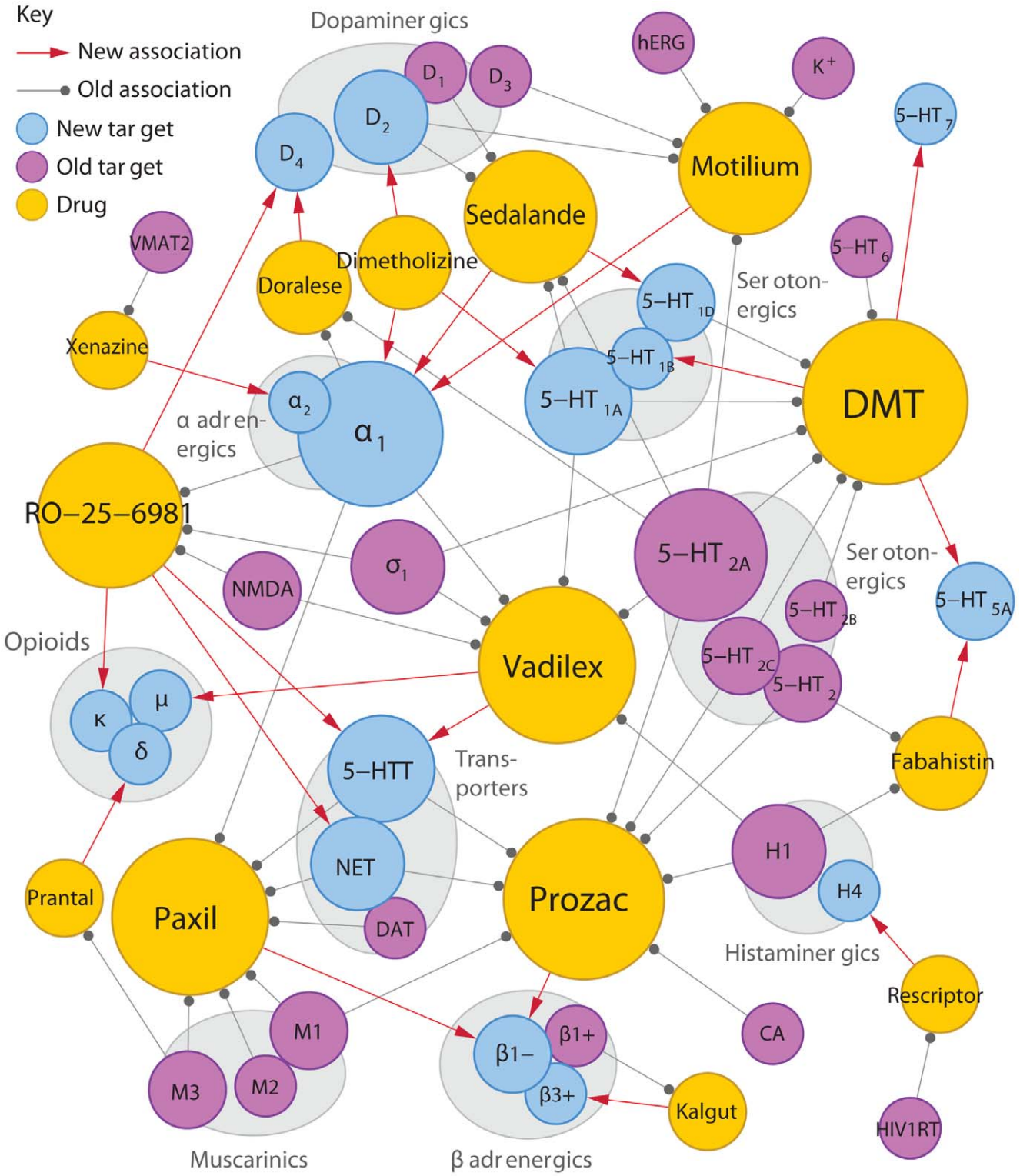

Figure 3. Cheminformatics methods. New associations discovered by cheminformatics methods. The Similarity Ensemble Approach (SEA) uses ligand similarity methods to discover potential new associations between drugs and targets. Reprinted with permission from [33].
doi:10.1371/journal.pcbi.1002817.g003

variants in calumenin are also associated with differences in warfarin dosage [25].

3. Methods for Discovery of Pharmacogenomic Genes and Variants

Pharmacogenomic research aims to identify the genes (and gene variants) involved in the interaction between a drug and the body. For any of the pharmacogenomic applications discussed below, there exist methods for discovering relevant genes and variants (typically single nucleotide polymorphisms, or SNPs) related to drug response. Traditional SNP-based methods, such as genome wide association studies (GWAS), can be used to discover candidate regions of interest. Alternatively, analysis of other sources of data, including expression or biochemical data, may provide additional gene candidates. Once candidate variants are identified, further computational and experimental follow-up may be required to fully characterize all the genes and pathways involved in the drug's progression through the body.

3.1. Association Methods

In a GWAS, hundreds of thousands or millions of SNPs (representing regions of the genome with the most inter-individual variation) are probed on a DNA microarray for each individual in a set of cases and controls (Figure 2A). For each SNP, significance of the association between a SNP and the trait is measured a chi-squared test, based on a 2×2 contingency table of alleles (or genotypes, if a dominant or recessive model is assumed) and case/control status (Figure 2B). In the case of a continuous independent variable, such as drug dose, a likelihood-ratio test or a Wald test is applied to measure whether there is a significantly different dose between the two groups of genotypes.

Each SNP is tested independently, and thus significance (p-values) must be corrected for multiple hypotheses, usually using a Bonferroni correction or False Discovery Rate (FDR). A SNP that reaches "genome-wide significance" (Figure 2C) is then a candidate for follow-up analysis, as are genes in or near the significant SNP, genes for which the SNP is an eQTL (a SNP associated with the expression of some other gene), and genes in the same pathway as these genes.

The two most important considerations for the design of any pharmacogenomic study include the selection of representative genetic markers, as well as phenotypically well-characterized patients (including cases and controls). The first of these, design of a suitable genotyping array, is technically easy and inexpensive, though the exact design can depend on the desired balance between unbiased genome-wide studies and a targeted SNP panel (see below). As in any trait-association study, the second consideration: the selection, characterization, and covariate identification of cases and controls provides a significant challenge.

Because performing a million independent tests requires stringent significance correction, large numbers of cases and controls, often in the thousands, are required to discover a SNP that will achieve "genome-wide" significance. SNP-based GWAS methods are effective when there is a strong signal from some SNP for the size of the study (that is, when there is good separation between genotypes for the cases and controls). However, under this stringent independence model, weaker signals may be lost among the noise that plagues genetic association studies. Thus, combining data from multiple SNPs in a single gene can boost power and decrease the number of hypotheses for multiple hypothesis correction [26]. Alternatively, if we have prior information about the drug's mechanism of action, we can create targeted SNP panels, limited to genes in the drug target's pathway, to decrease the hypothesis space [27].

As the price of high-throughput sequencing continues to fall, many investigators are turning to exome or whole genome sequencing to discover genetic factors of drug response. Such technologies have the advantage of remaining unbiased in SNP discovery, detecting less common (and even personal) mutations, and capturing larger-scale information, including copy number variants (CNVs) and structural variants (SVs).

Often, in major association studies, the SNP platform (DNA microarray) used is comprised of SNPs that serve as "tags" for a larger stretch of nearby SNPs. Such an approach is possible due to the presence of "linkage disequilibrium" in the genome, a phenomenon where SNPs tend to be inherited together ("linked"); the particular structure of these "haploblocks" (which SNPs are typically inherited together) is specific to each racial population. Because different populations have different linkage structures and a different series of polymorphisms, platforms that are optimized for one population may not be the best choice for another. This problem is further complicated by underlying differences in genomes: the effect a given SNP has on drug response may be different (or even the opposite) because of a hidden interaction with an alternate variant of another gene. Specifically, since many of the original genotyping platforms were developed for Caucasian populations, studies on Africans or Asians will require different approaches. Additionally, the first SNP identified is typically simply an "associated" variant, rather than the causative variant. In order to determine the specific proteins directly involved in drug response, further experimental or informatics analysis must be performed on genes and variants "linked" to the associated variant.

3.2. Expression Methods

In addition, other sources of data can be used to identify genes involved in drug response, including RNA expression data from microarrays or RNA-Seq experiments from drug-treated samples. For instance, using expression profiles from patients with a disease of interest, one can identify the genes involved in the progression of the disease and identify potential drug target candidates. Alternatively, expression profiles generated from a drug treated sample (compared to control) can be used to determine a molecular response to a drug. Ideally, such drug treatment experiments would be done on humans in order to generate organic in vivo physiological response. However, such experiments are unethical for experimental (early phase) drugs, require significant regulatory approval, and are expensive. Thus, established cell lines have provided a valuable, lower-cost resource for investigators to generate gene expression profiles.

One such effort, the connectivity map (CMAP), a publicly available resource of gene expression data of cell lines treated with various small molecules, has been used to compare expression profiles (See Figure 1 of [28]) to identify metabolite-protein interactions, small molecules with similar binding profiles, and metabolites that may mimic or suppress disease [28]. For instance, this approach predicted gedunin to be an inhibitor of HSP90 due to the similarity between gedunin's expression profile and the profile of known inhibitors. Despite the lack of structural similarity between gedunin and other HSP90 inhibitors, CMAP's predicted result was validated biochemically.

Thus, cell lines can be used as surrogates for individuals, where a cellular phenotype is used as a proxy for the individual's own physiological response based on the cellular expression response

to a drug treatment. For instance, one can search for associations between a cell line's genetic makeup and cell viability after drug treatment (the IC_{50} of drug for each cell lines) [29]. Alternatively, similar methods can be used to characterize toxicological response: treating cell lines with a drug and measuring gene expression can suggest genes involved in the drug's toxicity.

While not yet extensively employed in practice, other sources of high-throughput experimental "omics" data, such as metabolomics or proteomics data could be used for similar analyses.

3.3. Cheminformatics/NLP (Other Discovery Methods)

While not strictly "pharmacogenomics" methods, cheminformatics has provided a valuable tool for investigators in the initial stages of drug discovery. For instance, combining information about protein structure and small molecule structure, docking methods predict the best fit of a molecule (or all molecules in a database such as PubChem or ChEMBL) by minimizing the conformation energy of the molecule-protein "fit". Such methods are computationally expensive, as they explore a large search space for each pair of molecule and protein and use molecular dynamics or genetic algorithms to optimize fits. Therefore, molecule docking can be limited to the active site of a protein with a group of molecules to decrease the search space. Alternatively, if a given molecule is previously known to interact with a protein, molecule similarity metrics can be included to suggest similar molecules as protein-binding candidates. In this way, a search limited to ligands that score above a similarity threshold to the known ligand would be much faster than a search through all of PubChem. While such predictions must still be confirmed through biochemistry (such as binding assays), these methods can be used to limit the hypothesis space for drug discovery, prioritizing the expensive, lower-throughput biochemical assays.

For a potential drug target, cheminformatics methods can be used to identify new "hits" or optimize "leads" by suggesting molecules that may disrupt the function of the protein. For instance, docking methods were used to successfully identify novel molecules that could serve as inhibitors of CTX-M β-lactamase at millimolar binding affinities [30]. Various algorithms have been developed for screening ligand-target fits using docking (reviewed in [31]). Additionally, methods that incorporate the structure of the ligand along with known interactions can identify patterns of related drug targets [32]. Such an approach can suggest new functions for known drugs, explain "off-target" adverse events, and importantly, predict "polypharmacology," or the action of a single drug on multiple targets [33] (Figure 3). These methods leverage small molecule databases such as PubChem and ChEMBL, which maintain structures and properties of small molecules and ligands, as well as bioassay results of these compounds.

Additionally, a wealth of scientific information is available in the biomedical literature as lower-throughput free text. Thus, text mining techniques such as natural language processing (NLP), which exploits sentence syntax to pull structured knowledge from the literature, can be used to mine PubMed and other sources of published information to discover new drug-protein interactions [34].

3.4. Pathway Discovery

Once a candidate gene is identified, studying the gene's known genetic networks, cascades, and pathways can help identify other possible candidates that affect drug action. For instance, if a kinase is identified as a drug target, the proteins it phosphorylates (and any proteins affected downstream) may be relevant to the study of the drug. Additionally, knowledge of biological pathways influencing a disease can aid in the drug discovery process (see below: Drug Discovery).

Numerous online or downloadable resources exist for pathway and network analysis, such as Biocarta, Ingenuity, KEGG, and PharmGKB. For a gene-drug relationship of interest, information on the gene's network or pathway can be used to limit the hypothesis space of other analyses and experiments. Pathway analysis can "connect the dots" between known gene-drug interactions to generate new hypotheses of key genes that may also contribute to the pharmacogenomics of the drug. Additionally, a mechanism of action can be formalized by closing the loop between all the genes involved.

3.5. Validation and Application

The methods discussed thus far provide only computational evidence for potential drug-protein interactions. In order to prove drug-protein interactions and effects, follow-up biochemical methods, such as measuring binding affinity or functional assays, are required to demonstrate a molecule's potential therapeutic activity or to definitively prove an interaction.

Ideally, multiple sources of evidence can be integrated to fully characterize the physiological response to a drug. Once sufficient confidence is generated for a potential pharmacogenomic mechanism, the first step towards clinical application involves the storage and dissemination of the information in a curated database, such as PharmGKB. Combining information from multiple analyses will allow for more powerful characterization of the pharmacogenomic response. For instance, dosing equations for sensitive drugs such as warfarin can be developed by multiple linear regression of variants (as well as clinical covariates) on observed doses [15]. Finally, a centralized resource such as PharmGKB will allow for systematic pharmacogenomic analysis: such as for automated annotation of an input of genomic variants.

4. Pharmacogenomics in Drug Discovery

Pharmacogenomics can impact how the pharmaceutical industry develops drugs, as early as the drug discovery process itself (Figure 4). First, cheminformatics and pathway analysis can aid in the discovery of suitable gene targets, followed by small molecules as "leads" for potential drugs. Additionally, discovery of pharmacogenomic variants for the design of clinical trials can allow for safer, more successful passage of drugs through the pharmaceutical pipeline.

4.1. Small Molecule Candidate Identification

A key starting point in developing a drug for illness or disease involves finding a suitable gene to target. Typically, genes implicated in a disease can be discovered by GWAS, exome sequencing, analysis of RNA expression profiles, or other biochemical methods. These genes and others in the same pathway can be considered as candidate drug targets. The potential target space could be limited by excluding genes on the basis of their similarity to other genes (possibly due to paralogy) to avoid "off-target" effects.

Once potential gene or pathway targets are identified, cheminformatics methods can be used to generate predictions for potential "leads" (or drug candidates) for a high-throughput drug screen. For instance, protein structure information can be combined with small molecule structure information to predict favorable drug-gene interactions. After such predictions are generated, follow-up biochemical experiments would be required to confirm the interaction before the small molecules are considered further.

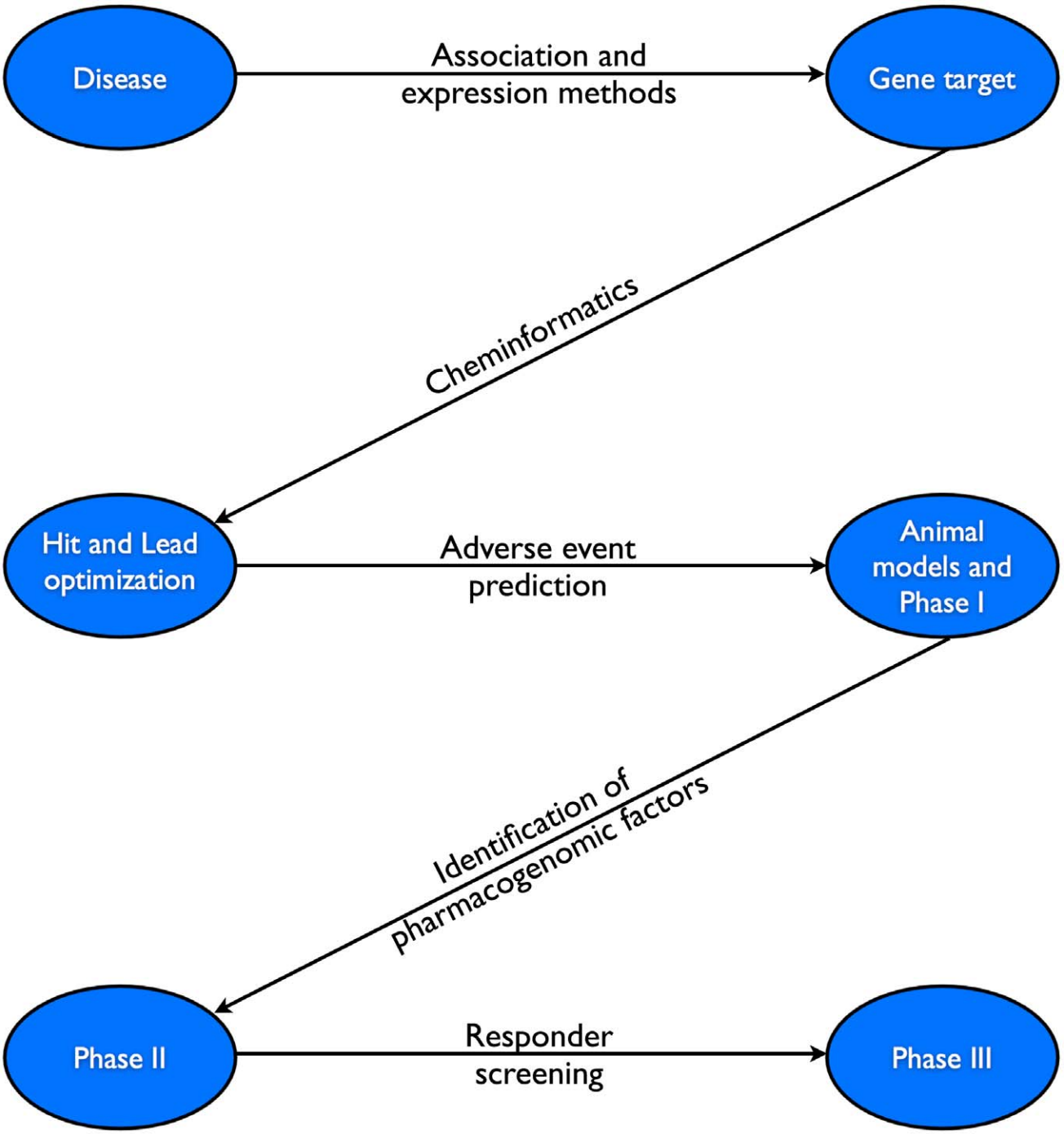

Figure 4. Drug discovery. Pharmacogenomics can be used at multiple steps along the drug discovery pipeline to minimize costs, as well as increase throughput and safety. First, association and expression methods (as well as pathway analysis) can be used to identify potential gene targets for a given disease. Cheminformatics can then be used to narrow the number of targets to be tested biochemically, as well as identifying potential polypharmacological factors that could contribute to adverse events. After initial trials (including animal models and Phase I trials), pharmacogenomics can identify variants that may potentially affect dosing and efficacy. This information can then be used in designing a larger Phase III clinical trial, excluding "non-responding" and targeting the drug towards those more likely to respond favorably.
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In a similar vein, pathway analysis can be used to select new, potentially safer drug targets. Namely, if a drug (which targets some gene) is initially discovered as effective, but found to cause adverse events, safer alternatives might be found by searching for drugs that target genes in the same pathway as the original gene.

4.2. Clinical Trial Pipeline

Once a small molecule has been biochemically identified as a "lead" and a lack of toxicity verified in animal models, the small molecule goes through a series of increasingly larger phases of clinical trials. Basic efficacy and relative safety are demonstrated before and during Phase II clinical trials, on the path to Phase III.

However, Phase III trials often require thousands of patients, and thus, a pharmaceutical company would ideally be confident that the drug will successfully pass and be profitable before investing in such an expensive endeavor.

Most of the time, patient response to a drug is variable during the initial Phase II trials and as this response is often related to genetic factors (PK or PD protein variability), pharmacogenomics can be used to limit the cohort for Phase III trials. Specifically, if a protein variant is identified that separates drug responders from non-responders, individuals with the "non-responder" variant could be excluded from the next phase of the trial (reviewed in [35]). While this would limit the scope and usability of the drug, it would ensure the passage of the drug through the trial. As such, pharmaceutical companies would need to balance the loss in revenue of a less globally applicable drug with the risk of FDA rejection of the drug.

4.3. Drug Repurposing

As mentioned previously, cheminformatics methods can be used to identify novel drug-protein interactions. While these predicted interactions can be used to discover new small molecules for therapeutic purposes, any new drug must still go through the significant regulatory hurdles of safety and efficacy testing. However, drugs already on the market for some therapeutic purpose are FDA-approved for safe use in humans, and their "repurposing" would simply involve demonstrating that the drug can be used effectively for a different indication. In general, any method that can be used to characterize "off-target" effects can be used in drug repurposing, by finding effects that are salubrious.

For instance, docking methods have been used in discovering novel functions for already-established small molecules (or drug "repurposing" or "repositioning"). The similarity between a drug target for Parkinson's disease, catchol-O-methyltransferase (COMT) and a bacterial protein in Mycobaterium tuberculosis (the enoyl-acyl carrier protein reductase, InhA) narrowed down an investigation of potential drug targets for M. tuberculosis infections (Figure 5). From this result, entacapone, a drug already approved to treat Parkinson's by inhibiting COMT, was predicted to bind to InhA, which was then validated biochemically and shown to have antibacterial activity [36]. Thus, while full efficacy for treatment of tuberculosis must still be demonstrated in larger studies, studies on a known safe drug are significantly cheaper and carry much lower risk.

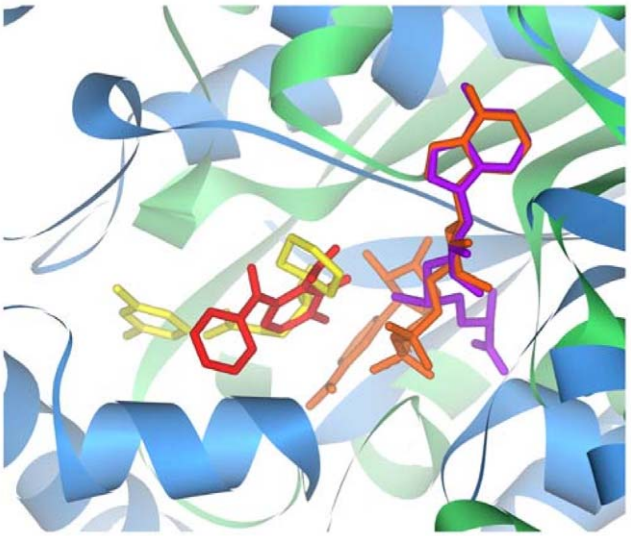

Figure 5. Drug repurposing. Docking methods suggest binding site similarity between COMT (green) and InhA (blue). The overlap between the predicted locations of their cofactors (purple and orange, respectively) and ligands (red and yellow, respectively) suggest potential similarity in their functions. Thus, the same drug that has been used to inhibit COMT (entacapone) was predicted to inhibit the M. tuberculosis protein InhA for potential treatment of tuberculosis. Reprinted from [36].
doi:10.1371/journal.pcbi.1002817.g005

5. Applying Pharmacogenomic Knowledge

Pharmacogenomics has the potential to transform the way medicine is practiced, by replacing broad methods of screening and treatment with a more personalized approach that takes into account both clinical factors and the patient's genetics. As demonstrated previously, genetic variation can greatly influence the nature of the effects a drug will have on an individual (whether it will work or cause an adverse event), as well as the amount of drug required to produce the desired effect. To this end, pharmacogenomics will impact the way drugs are prescribed, dosed, and monitored for adverse reactions.

On an individual scale, the derivation of clinically actionable pharmacological information from the genome is already a reality: the clinical annotation of a patient's full genome sequence has suggested the patient's likely resistance to clopidogrel, positive response to lipid-lowering drugs, and lower initial dose requirement of warfarin [37]. Thus, physicians will use pharmacogenomics alongside traditional clinical practices to predict which drugs are more or less likely to work, which patients will require more or less medication to achieve therapeutic response, and which drugs should be avoided on basis of adverse events. In order to achieve these goals, the findings of the research lab needs to be translated into the clinic, and the practice of using pharmacogenomics must be integrated into the existing medical system (Figure 6).

5.1. Prescribing

When a physician treats a condition, there can be multiple approaches to that treatment. Currently, a physician considers clinical and social factors when choosing an approach, asking questions such as, "how is the patient's organ function?", "have their been any past problems with this type of treatment?", "how compliant will the patient be with one treatment versus another?", and "for this kind of patient, what is the best evidence-based treatment?". Based on his or her clinical experience, the physician then chooses a drug to use. If there are multiple treatments available, the physician will choose one and monitor the patient's progress. Having the ability to know which drugs will work best beforehand can improve care, because a physician will administer the best treatment and not waste time on a treatment that is likely to fail for a particular individual.

One area where gene-based prescribing is steadily advancing is in the area of cancer genomics. Cancer drugs generally have

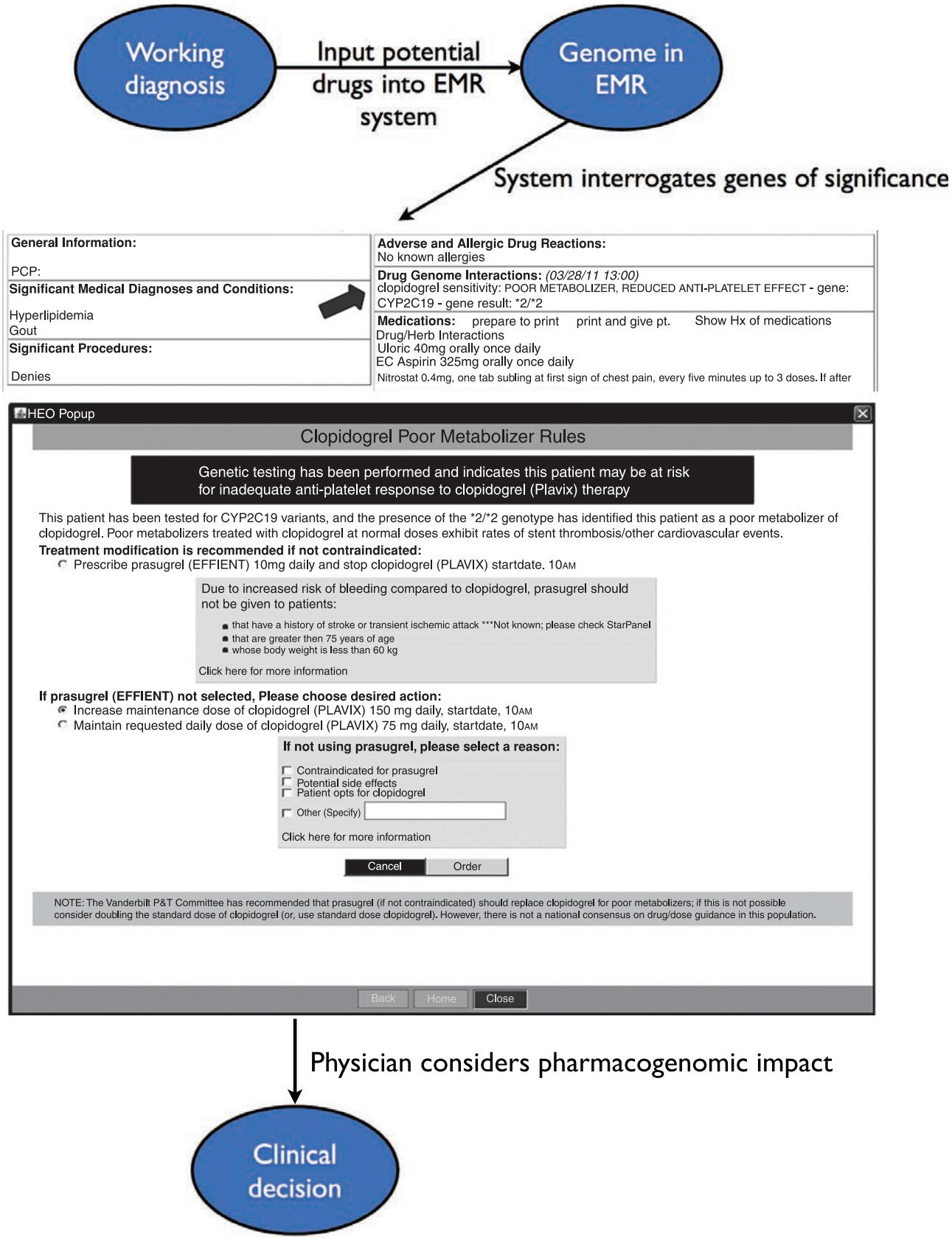

Figure 6. Applying pharmacogenomics in the clinic. A proposed clinical workflow including pharmacogenomic information. A physician considers the patient's current presentation and past history when coming up with a working diagnosis and based on his or her clinical judgment, decides what drugs the patient may need. For example, if the physician wanted to add clopidogrel to the patient's regimen, the physician would

input it into the electronic medical record (EMR). The EMR would interrogate the genome and present a message such as "clopidogrel sensitivity: POOR METABOLIZER, REDUCED ANTI-PLATELET EFFECT - gene: CYP2C19 - gene result *2/*2." Based on this recommendation, the physician may adjust the dose accordingly or choose another drug. In this case, the physician will likely increase the dose of clopidogrel in order to achieve therapeutic effect. Reprinted with permission from [65].
doi:10.1371/journal.pcbi.1002817.g006

many toxic side effects, and in many cases of advanced cancers, physicians "guess and test" medications by prescribing them and monitoring progress. In addition, the very nature of cancer is "personal," insofar as each specific cancer is caused by the unique sum of individual somatic mutations (that is, mutations that occur in the individual after birth and are not inherited or passed on). Certain "signatures" of cancers, or mutations that produce similar cancer phenotypes, allow for the grouping of cancers into distinctions, such as leukemia or lymphoma, but even these exhibit significant variability among classifications.

Thus, the ability to sequence and study the genomes of cancer cells of an individual can help identify the driving somatic mutations and provide a tool for rational drug choice. For example, the median survival for advanced or recurrent endometrial cancer is very poor, due to the fact that physicians treat empirically with chemotherapy, which may have no therapeutic benefits. Researchers studying mutations in the pathways of endometrial cancer cell lines found that response to doxorubicin, a chemotherapy used to treat endometrial cancer, was related to mutations in the Src pathways, which are involved in cell proliferation, motility, and survival. By pinpointing mutations in this pathway, the researchers were able to rationalize supplementing the drug regimen with the addition of SU6656, a drug that competitively inhibits the Src pathway, which increased the sensitivity of some of the cell lines to doxorubicin [38]. As cancers are typically characterized by a lack of error-correction mechanisms and inhibited apoptosis, such an approach is particularly important, as the initial failure of a chemotherapeutic drug allows time for a cancer to develop further mutations and spread further. In the future, interrogating cancer genomes could allow rational drug prescribing, decreasing the amount of time spent on ineffective therapies and increasing the number of successful cures.

Pharmacogenomics can also play a role in drug decisions for prevalent conditions, allowing physicians to predict when a commonly successful therapy may fail. For instance, there is an arsenal of drugs doctors can use to combat the co-morbidities of type II diabetes. These co-moribidities are usually cardiac risk factors, such as lipid abnormalities and high blood pressure: the cardiac risk factor conferred by type II diabetes is equivalent to that of a prior myocardial infarction in a nondiabetic individual. Presently, the physician chooses drugs based on his best clinical judgment and then monitors the outcome of the treatment. However, as the tolerance and efficacy of certain popularly prescribed drugs has been shown to be tied to genetics, such information could be used in prescription decisions. For instance, statins are a class of drugs that are inhibitors of HMG-CoA reductase, an enzyme that helps produce cholesterol in the liver. Thus, statins are given in an effort to lower cholesterol, particularly low-density lipoprotein (LDL) cholesterol, whose increased levels are a cardiac risk factor. Statins are often prescribed to patients with type II diabetes and high cholesterol in order to help them reach a more healthy cholesterol range. Even though studies have suggested genetic influences on statin efficacy and tolerance, such findings are not yet widely applied in clinical medicine.

One study found that in individuals with diabetes, variation in the HMG-coA reductase gene was associated with a decreased response to statin therapy. In this study, a significantly greater percentage of individuals heterozygous for the G minor allele of rs17238540 were unable to reach target cholesterol and triglyceride goals when compared to individuals homozygous for the major allele. Additionally, these individuals had a 13% smaller reduction in total cholesterol and a 27% smaller reduction in triglycerides. This is an example of just one variation in the HMG-coA gene; other variations certainly exist and can impact how well a patient responds to statins [39]. Another gene that has been found to affect response to statins is the APOE gene, which is associated with the regulation of total cholesterol and LDL cholesterol. There are several variants in this gene, and there are differences between how type II diabetic individuals carrying these variants respond to statins. For instance, the individuals homozygous for the E2 variant were all able to reach their target LDL cholesterol; however 32% of individuals homozygous for the E4 variant failed to reach target LDL cholesterol. Moreover, E2 variant homozygotes had a significantly greater lipid lower response to statins than some of the other variants. Thus, APOE is another gene that may be predictive of statin resistance or reduced efficacy. Knowledge of these genes could play a role in the future of drug prescribing, as physicians would be able to predict a priori if a drug was going to succeed or if another drug would be a better choice [40].

One major caveat of gene-based prescription decisions (as well as dosing, as discussed below) involves the applicability of a finding in one population to other populations (see above: Association Methods). While a pharmacogenomic effect may be true for a given population (with a certain genetic background, in animal model parlance), it may not directly apply to other populations due to unknown genetic factors, especially combinatorial effects. Because there is no current standard for translating a result between ethnicities, follow-up work is required for each specific pharmacogenomic interaction before it is applied in a clinical setting.

5.2. Adverse Drug Reactions

Another factor physicians need to consider when choosing a drug is the risk of adverse events, or any detrimental, unintended consequence of administering a drug at indicated clinical doses. In a milder form, an adverse event could be an allergic rash from penicillin. These events can also be much more intense: severe adverse drug reactions (SADRs) are those that can cause significant injury or even death, and are estimated to occur in about 2 million patients a year in the United States. In fact, SADRs are the fourth leading cause of death in the United States, with about 100,000 yearly deaths. Because of the impact of SADRs, scientists and physicians hope that the application of pharmacogenomics can help predict which patients are most susceptible to experiencing an SADR to a given drug. With this knowledge in hand, a physician can either more closely monitor these patients or choose an alternative therapy [41].

For instance, statins have been associated with a rare but incredibly severe adverse reaction: myopathy and rhabdomyolysis. A study looking at the possible genetic influences of this reaction found a SNP in the SLCO1B1 gene associated with this severe adverse drug reaction, with an odds ratio of 4.5 [42]. However, there are also cases of individuals who experience milder symptoms and develop statin intolerance. Some of these individuals experience an elevation in creatine kinase or alanine aminotransferase while on statins, indicating possible muscle or liver damage. A recent study found that the functional variants V174A and N130D in the SLCO1B1 gene, which encodes the

organic anion transporting polypeptide OATP1B1, are predictive of statin intolerance [43]. OATP1B1 in these individuals has reduced maximal transport ability, possibly leading to higher levels of statins in the patient's blood. Currently, studies are underway to determine if there is a difference between the available statin drugs with regards to these pharmacogenetic components, in order to better inform physicians about the drug choices they make.

In the effort of applying pharmacogenetics in the clinic, trials have already shown that screening tests have clinical utility. For instance, abacavir, a nucleoside reverse-transcriptase inhibitor used to treat AIDS, causes a hypersensitivity reaction in 5 to 8% of patients. This reaction can include fever, rash, and gastrointestinal or respiratory symptoms. Since this adverse reaction necessitates stopping therapy (and patients cannot be put on the drug again because of the risk of a more severe reaction upon re-exposure), physicians could avoid prescribing this drug if they were capable of predicting which individuals would have a reaction. Recently, it was identified that HLA-B*5701 was associated with hypersensitivity to abacavir. Armed with this information, a double-blind, randomized, prospective study in nearly 2000 patients was conducted to determine if screening for this variant could help prevent hypersensitivity reactions in AIDS patients. The results supported the use of pharmacogenetics in the clinic: prescreening eliminated immunologically confirmed hypersensitivity reactions and significantly decreased hypersensitivity symptoms, compared to the control group [44].

5.3. Dosing

Once a physician has chosen a drug based on efficacy and consideration of adverse events, the next step is to determine what the correct dose at which to administer the drug. Currently, clinical factors such as gender, weight, and kidney or liver function may be taken into account when dosing a medication. However, genetics can play a large role in how a drug is dosed as well.

As mentioned previously, a major reason drug doses differ between individuals is due to polymorphisms in proteins involved in pharmacokinetics or pharmacodynamics. Variation in enzymes involved in pharmacokinetics, such as the Cytochrome P450 metabolic enzymes (and mainly, CYP2D6, CYP2C9, and CYP3A4), can affect the availability of drugs reaching their targets. Alternatively, the targets themselves (PD genes) can respond differently based on their specific structure.

One of the emerging examples of dosing based on genetics is the anticoagulant, warfarin. Prescriptions for warfarin number about 30 million cases annually and are indicated to prevent myocardial infarction, venous thrombosis, and cardioembolic stroke. However, the dose needed to achieve adequate anticoagulation can vary by as much as twentyfold between patients. Currently, physicians start with an initial dose and titrate (adjust) over time until the target international normalized ratio (INR), an indicator of anticoagulation, is reached. However, until the therapeutic dose is reached, there is the opportunity for over-coagulation, which leads to an increased risk of thromboembolic events, or under-dosing, which can lead to ineffectiveness, and thus, hemorrhaging and bleeding. The discovery of variants affecting warfarin dosing have led to the creation of algorithms that use clinical (such as weight and other drug status) and pharmacogenetic (variants in CYP2C9 and VKORC1; see above, PK and PD Interactions, respectively) information in order to predict a patient's optimal starting warfarin dose. One such dosing algorithm, produced by the International Warfarin Pharmacogenetic Consortium, was capable of predicting doses using a pharmacogenetic algorithm at a significantly more accurate rate than an algorithm using clinical factors alone [15]. However, one of the drawbacks is that these predictions are most accurate in a Caucasian population; additional research is needed in populations of different ancestries in order to produce a more broad-spanning pharmacogenetic algorithm.

5.4. Applying Pharmacogenomics in the Clinic

Though examples exist of how pharmacogenomics could impact prescribing drugs, predicting adverse events, and dosing drugs, the actual application of pharmacogenomics is just beginning to gain traction. As pharmacogenomics knowledge steadily increases and the infrastructure for its usage continually develops, the day when all physicians regularly apply genetics to drug dosing draws closer. The challenges that remain include surmounting regulatory hurdles, developing ways to continually update known findings, delivering knowledge to physicians, and integrating genomics into medicine. However, scientists have worked to address these challenges, and pharmacogenomics will likely serve as one of the first major clinical applications of personalized genomic medicine.

In the United States, the FDA regulates drugs and drug labels. Therefore, the communication between scientists and the FDA will be critical to the adoption of pharmacogenomic information on drug labels. Evaluation will depend on the trial design, sample size, reproducibility, and effect size [45]. One benefit of pharmacogenomics is that the associations between genetics and drug effects is more concrete and immediately applicable than in other translational bioinformatics concepts such as disease risk assessment, where scientists are struggling with "missing heritability" and combinations of moderate risks. Because of this, unlike other therapies, which require a randomized clinical trial in order to prove efficacy, the application of pharmacogenomic principles may not require the same level of scrutiny. Rather than providing some novel therapy, the vast majority of pharmacogenomic findings are simply supplementing physician knowledge about previously approved drugs. Physicians already utilize the clinical backgrounds of their patients (i.e. weight, gender, presumed organ function, drug interactions, compliance) when making decisions about drugs. As long as adding the variable of genetic information is non-inferior to the current standard of care, there should not be resistance to its implementation [46].

Once a biomarker is shown to be important, other decisions will have to be made: Should testing for the biomarker be required, or should it just be recommended? Socio-economic considerations along with the predictive value of the biomarker will need to be considered. At first pass, the use of pharmacogenomic data may be completely left to the clinician's judgment until the FDA has formalized its role in their application. Once a pharmacogenetic biomarker is approved, the drug's label will need to reflect the genetic components involved: biomarkers identifying the patient population that should receive the drug would be printed under "indication," biomarkers related to drug mechanism may appear under the "clinical pharmacology" section, and biomarkers related to safety may be indicated in "adverse events." The challenge for the FDA and clinicians alike will require vigilance about updating new information as the onslaught of pharmacogenetic associations continues to pour in [45].

Pharmacogenetic research continues to discover new drug-gene interactions. The volume of new findings exceeds the capabilities of any individual to parse. Thus, bioinformatics will have to play an integral role in the translation of the data to the bedside. Text mining (see Methods: Cheminformatics/NLP) will be instrumental to extracting structured data from the literature in order to update knowledge bases, such as PharmGKB. Ultimately, this knowledge will be integrated into a centralized database to make the information accessible to all.

In order to fully translate pharmacogenomics into the clinic, this information must be well integrated with the electronic medical

Table 1. Examples of pharmacogenomics used in this chapter. Additional examples can be found at PharmGKB.

Drug	Gene (Selected Examples)	SNPs/Genotypes (Selected Examples)	Sources
Mercaptopurine	Inosine triphosphate, pyrophosphatase (ITPA), Thiopurine methyltransferase (TMPT)	rs41320251, rs1800584	[63], [62]
Succinylcholine	Butyrylcholinesterase (BCHE)	rs28933390, rs28933389	[61]
Perhexiline	Cytochrome P450 2D6 (CYP2D6)	CYP2D6 *4/*5, *5/*6, *4/*6	[60]
Clopidogrel	Cytochrome P450 2C19 (CYP2C19)	rs4244285	[59]
Albuterol	Beta-2 adrenergic receptor (ADRB2)	rs1042713	[58]
Metoprolol	Beta-1 adrenergic receptor (ADRB1)	rs1801252	[57]
Methotrexate	Methylenetetrahydrofolate reductase (MTHFR)	rs4846051	[56]
Warfarin	Cytochrome P450 2C9 (CYP2C9), Vitamin K expodide reductase (VKORC1), Calumenin (CALU)	rs1799853, rs1057910, rs7294, rs9934438, rs9923231, rs339097	[55], [54], [53], [52], [25], [51]
Atorvastatin	P-glycoprotein (ABCB1)	rs1045642, rs2032582	[50]
Statins	HMG-coA reductase (HMGCR), Apolipoprotein E (APOE), Solute carrier organic anion transporter family, member 1B1 (SLCO1B1)	rs17238540, APOE - E2, E4, rs4149056, rs2306283	[39], [40], [49], [43]
Abacavir	HLA-B*5701 genes	rs2395029, rs3093726	[48]

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system (Figure 6). Full adoption will require a curated, updated database with FDA or evidence-based approved drug-gene interactions that would be available for physicians to use in their medical practice. For example, PharmGKB is primarily used as a scientific tool for identifying drug-gene interactions. However, its clinical utility was shown when it was used to generate drug recommendations based on an individual's fully sequenced genome [37]. Such resources serve as the precursor to the systems that will be in place when all individuals have sequenced genomes readily available for physician use.

Finally, for pharmacogenomics to be widely applied, personal genomics needs to become ingrained into modern medicine. Physicians and patients must be educated as to the benefits of genomic medicine, in order to dispel any myths and to avoid ethical issues. Moreover, genetic testing facilities meeting the U.S. government's Clincial Laboratory Improvement Amendments (CLIA) certification requirements need to be established in order to provide patients with genomic data that is considered acceptable for clinical use. Finally, insurance companies must be on board to reimburse genetic testing. Since sequencing costs continue to drastically fall, the debates surrounding cost will soon become moot [46]. Thus, we are rapidly entering an age where every patient can have his or her genome available. With the availability of an individual's genome, a physician looking to administer a drug such as a statin can check to see whether or not the statin would be expected to work and if any possible adverse events might be expected (Figure 6).

Pharmacogenetics is a rapidly developing field; however, some challenges remain in implementing scientific findings from the bench to the bedside. Because of the continued development and work in this field, these challenges will be addressed, ushering in an age of personalized drug treatments.

6. Summary

Pharmacogenomics encompasses the interaction between human genetics and drugs, which can be affected by variation in genes involved in pharmacokinetics (PK) and pharmacodynamics (PD). Thus, a major goal of pharmacogenomics is to elucidate which genes affect drug action, using cheminformatics, expression studies, and genome-wide association studies (GWAS). Association methods can be used to discover novel associations by comparing the genetic differences between cases with a certain phenotype and controls. Expression analysis and cheminformatics can be used to expand knowledge about drug-gene interactions by comparing gene expression or interaction profiles among drugs and genes. Analysis of these studies can yield information about how these genes affect drug action. Because of differences in haplotype structure between populations, studies validated in one population may not be directly applicable to a different population. However, as knowledge accumulates about drug-gene interactions, scientists can contribute to databases, such as PharmGKB, documenting known relationships (Table 1). As the volume of knowledge grows, text mining methods may become instrumental in interrogating the literature and collecting relevant data for clinical use. The application of pharmacogenomics in the clinic can help inform physicians in drug prescribing, drug dosing, and prediction of adverse events. Because many of the drugs undergoing pharmacogenomic study are already FDA-approved, adoption of pharmacogenomics in the clinic is mostly dependent on the availability of genome sequencing and the development of implementation infrastructure. Moreover, pharmacogenomics can also aid in drug development, providing pharmaceutical companies with an additional tool to design more successful, cheaper trials. Thus, pharmacogenomics promises to help launch medicine and drug development into the realm of personalized care.

7. Exercises

1. (A) Download a genotype and phenotype dataset of your choosing. Using PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/) or a statistical program such as R (http://www.r-project.org/), calculate the association (using a Fisher's exact test) between <Trait> and each SNP. After Bonferroni correction, does any SNP reach genome-wide significance? (B) Does using a different correction method such as Benjamini or False Discovery Rate (FDR) result in any more significant SNPs?
2. (A) Use a pharmacogenomic database (such as PharmGKB) to find genes that may interact with metformin. (B) Are any of these genes known to interact

with other drugs? Which drugs? (C) Bonus question: Are any of these drugs related (by structure or function) to metformin?

3. (A) Implement a warfarin dosing equation (e.g. the one found in [15]). If you have a personal genotype, input your information and calculate your optimal starting warfarin dose; otherwise, calculate the optimal dose (as predicted by both the clinical and pharmacogenetic algorithms) for a 66-year old Caucasian (175 cm, 75 kg), not taking amiodarone or enzyme inhibitors, who is rs9923231 TT and CYP2C9 *2/*2? (B) Would the clinical algorithm have over- or under-estimated his (or your) dose and what are the potential consequences of such an error?
4. You are a physician and would like to prescribe simvastatin. What parts of the genome would you want interrogated to know about prescribing this drug and why?
5. Read about the clinical uses of a whole genome or exome in healthy [37] and diseased [47] individuals. How can pharmacogenomics be directly applied in a clinical setting?

Answers to the Exercises can be found in Text S1.

Supporting Information

Text S1 Answers to Exercises.
(DOCX)

Further Reading

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Glossary

- Adverse event - A "side effect," or unintended consequence of taking a drug.
- Cheminformatics - Methods that utilize chemical structures of metabolites and/or protein structure to discover potential drug-gene interactions.
- Drug Target - The specific protein whose interaction with a drug constitutes that drug's mechanism of action.
- (Gene) Expression - The relative amount of RNA from a gene in a cell at a given snapshot in time, often used as a proxy for activity of the gene in the condition in which the experiment was performed.
- Hit - A small molecule that disrupts the function of a potential drug target (for treatment of a disease).
- Lead - An optimized (often chemically modified) "hit" with high specificity for its target and reasonable pharmacogenomic properties.
- Linkage - The property that multiple SNPs are often inherited together. When a SNP is associated with a trait or disease, it is not necessarily the causal SNP, but may be "linked" to other variation that is the molecular and physiological cause of the association.
- (DNA) Microarray - An experimental method that probes hundreds of thousands or millions of regions of the genome to determine the genotype at each locus.
- "Off-target" effect - The effects of a drug propagated by interactions with proteins other than the drug target ("innocent bystanders").
- "On-target" effect - The effects of a drug propagated by the intended interaction with the drug target.
- Pharmacodynamics - The mechanisms that relate to "what the drug does to the body," including "on-target" and "off-target" effects, intended and unintended, beneficial or harmful.
- Pharmacogenomics - The study and application of genetic factors relating to the body's response to drugs.
- Pharmacokinetics - The range of mechanisms that relate to "what the body does to the drug," including absorption, distribution, metabolism, and elimination of a drug.
- Polymorphism - A mutation in the genome that varies among individuals in a sizable fraction (often, minor allele frequency >0.01) of the population.
- Polypharmacology - The interaction of a drug with multiple targets.
- SADR - Severe Adverse Drug Reaction. An adverse event that results in significant injury or death.
- SNP - Single Nucleotide Polymorphism (see Polymorphism)

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Chapter 8: Biological Knowledge Assembly and Interpretation

Ju Han Kim[1,2,3]*

1 Division of Biomedical Informatics, Seoul National University College of Medicine, Seoul, Korea, **2** Seoul National University Biomedical Informatics (SNUBI), Seoul National University College of Medicine, Seoul, Korea, **3** Systems Biomedical Informatics National Core Research Center (SBI-NCRC), Seoul National University College of Medicine, Seoul, Korea

Abstract: Most methods for large-scale gene expression microarray and RNA-Seq data analysis are designed to determine the lists of genes or gene products that show distinct patterns and/or significant differences. The most challenging and rate-liming step, however, is to determine what the resulting lists of genes and/or transcripts biologically mean. Biomedical ontology and pathway-based functional enrichment analysis is widely used to interpret the functional role of tightly correlated or differentially expressed genes. The groups of genes are assigned to the associated biological annotations using Gene Ontology terms or biological pathways and then tested if they are significantly enriched with the corresponding annotations. Unlike previous approaches, Gene Set Enrichment Analysis takes quite the reverse approach by using pre-defined gene sets. Differential co-expression analysis determines the degree of co-expression difference of paired gene sets across different conditions. Outcomes in DNA microarray and RNA-Seq data can be transformed into the graphical structure that represents biological semantics. A number of biomedical annotation and external repositories including clinical resources can be systematically integrated by biological semantics within the framework of concept lattice analysis. This array of methods for biological knowledge assembly and interpretation has been developed during the past decade and clearly improved our biological understanding of large-scale genomic data from the high-throughput technologies.

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1. Introduction

One of the challenges in DNA microarray and RNA-Seq data analysis is to extract biological meanings from the massive amounts of transcriptome expression data. Most of the microarray and RNA-Seq data analysis methods are designed to determine the lists of genes or gene products that show distinct patterns and/or significant differences. Clustering and differential expression analysis, for example, typically generate lists of 'significantly' clustered and Differentially Expressed Genes (DEGs), respectively. The most challenging and rate-liming step, however, is to determine what the resulting lists of genes or gene products biologically mean.

The first analytic approach for the biological interpretation of obtained gene lists was to manually collect and put down all available descriptive information concerning each gene next to it and to try to infer the collective meaning of the textual descriptors for the group of genes under the biological systems context. The assumption here is that if a certain keyword is significantly over-represented or a meaningful pattern is found among the textual descriptors for a gene group, then the keyword or the pattern can be regarded as the semantic interpretation of the gene group.

It seems that Tavazoie *et al.* [1] was first to formally analyze the over-representation of 'functional annotations' for the lists of genes with semantic interpretations. By means of partitional clustering and motif discovery, given genome-wide gene-expression clusters, he analyzed significantly over-represented regulatory motifs in the upstream sequences of clustered yeast genes for uncovering new 'regulons' (i.e., sets of co-regulated genes) and their putative *cis*-regulatory elements. Here, the discovered motifs seem to be regarded as functional annotations to the corresponding genes. Many Functional Annotation Analysis (FAA) methods have been developed to test whether certain Gene Ontology (GO) terms [2] or biological pathways are significantly enriched within a particular list of genes. Many GO and biological pathway-based tools for gene expression analysis have been developed and proven to be useful [3–9].

FAA is an attempt to extract biological semantics from given lists of genes that are determined without considering any biological meaning but by a quantitative statistical analysis like clustering and DEG analysis methods. Gene Set Enrichment Analysis (GSEA) [10,11], however, takes quite the reverse way. GSEA uses pre-defined gene sets with a priori established biological meanings like biological pathways. For each pre-defined gene set, GSEA tries to determine if it shows significant expression change. Therefore, what GSEA essentially tests is if the pre-defined 'biological meaning' assigned to the gene set shows significant change or not. It has been successfully demonstrated that GSEA can successfully detect subtle but set-wise coordinated expression changes that cannot be detected by individual gene tests [10].

The gene-set approach greatly improves biological interpretability by using pre-defined gene sets with established biological meanings. The same strategy can be applied

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* E-mail: juhan@snu.ac.kr

What to Learn in This Chapter

- How to find genes associated with a particular disease (or condition) from microarray or RNA-Seq data
- How to find biological pathways and/or biomedical ontology terms for the interpretation of particular gene groups associated with a particular disease
- How to characterize biological properties of a particular list of genes
- Which data resources are useful for interpreting large-scale gene expression profiles
- What are the limitations of individual gene-based analysis for determining differentially expressed genes (even with multiple hypothesis correction)
- How to identify gene groups that are differentially expressed or differentially co-expressed between normal and disease samples
- Compare in terms of semantic interpretation the functional annotation analysis methods for co-expressed genes as in clustering and for pre-defined gene sets as in GSEA
- How to organize and visualize a massive and redundant annotation list of genes or gene sets into a unified framework of biological understanding

for the analysis of differential co-expression analysis. Cho *et al.* proposed dCoxS algorithm that determines if a pair of gene sets' coordinated co-expression patterns shows significant changes across different conditions [12]. If a pair of gene sets (or pathways) shows a positive co-expression pattern in normal tissue but a negative co-expression pattern in cancer cells, then it can be assumed that the pair of gene sets may play an important role in the cancerous transformation. This dyadic relation can easily be extended to create a network of gene sets showing differential co-expression patterns across different conditions.

Sometimes, given the genomic scale, even the extracted list of biological meanings and significant functional annotations are too big and complex such that they need to be systematically organized. Ordering of obtained semantics using concept lattice analysis improves biological interpretation of microarray gene-expression data. BioLattice considers gene expression clusters as objects and annotations as attributes and provides a graphical 'executive summary' (i.e. the context of the whole experiment) of the order relations by arranging them on a concept lattice in an order based on the set inclusion theory [13].

A wide range of tools and resources in microarray and RNA-Seq data analysis have a potential impact on personalized medicine and are invaluable in biomedical research. Integrative analysis of heterogeneous biological and clinical data is essential to discover meaningful knowledge. The construction of semantic relationships of biological resources makes it possible to unify multi-layered and heterogeneously formatted data from genome to phenome. Semantic analysis integrating gene expression profiles and annotations into a unified framework enables us to interpret complex biomedical data in a comprehensive and organized fashion.

The outline for this chapter is as follows. In Section 2, a comprehensive survey of biomedical annotation resources will be given with major ontology and biological pathway-based analysis methods. Section 3 describes gene set-wise differential expression analysis methods with its semantic interpretation power. Section 4 describes differential co-expression analysis. Finally, in Section 5, application of formal concept analysis for systematic semantic interpretation of gene expression profiles will be introduced with the following summary in section 6.

2. Pathway and Ontology-Based Analysis

GO and biological pathway-based analysis is one of the most powerful methods for inferring the biological meanings of observed expression changes (Figure 1). It enables us to analyze a list of interesting genes resulting from microarray and RNA-Seq experiments, without molecular biologist's help. The genes in the list may be the ones statistically significantly up or down regulated between conditions (i.e. DEGs), where the number of the genes belong to a list depends on the threshold of significance. Another method is to perform a co-expression (or clustering) analysis grouping genes with similar expression patterns across different experimental conditions.

Many genome databases provide GO annotations to their genes and gene products, which are also members of biological pathways. FAA determines which biological pathways or GO terms are significantly overrepresented in a given list of genes. GO annotation and pathway membership frequencies for a list of genes obtained by differential expression analysis (Figure 1 (a)) or co-expression analysis (or clustering) (Figure 1 (b)) are input to statistical analyses to test if they are significantly over-represented. For example, in Figure 1, the genes in the gene list (i.e. selected genes) are significantly enriched with a GO term, GO:000123, but not with GO:000126. It means that the genes are significantly associated with the biological meaning of the GO term, GO:000123.

In principle, any attribute of a gene can be applied for FAA including transcription factor binding sites [1], clinical phenotypes like disease associations, MeSH (Medical Subject Heading) terms, microRNA binding sites, protein family memberships, chromosomal bands, etc. as well as GO terms and biological pathways. Moreover, these features may in turn have their own ontological structures as illustrated in Figure 2. GO and MeSH have a 'tree-ish' graph structure, which is more formally a DAG (Directed Acyclic Graph), in which each term may be a child of one or more parents. Pathways have directed graph structures. Clusters may also be organized into a hierarchical tree or a graph structure. ArrayXPath [6,9] provides one of the most comprehensive collections of these structured features for annotation analysis [14].

Differential expression analysis determines significantly down- or up-regulated genes (or DEGs) between two conditions, i.e. control and treatment groups to explore the effect of a drug. Student's t-test, Wilcoxon's rank sum test and ANOVA may be applied to detect DEGs. Given the huge number of genes to be tested, multiple-hypothesis-testing problem should be properly managed. Co-expression analysis puts similar expression profiles together and different ones apart, returning lists of co-expressed genes that are assumed to be tightly co-regulated. Clustering algorithms can be classified into hierarchical-tree clustering and partitional clustering. While some partitional clustering algorithms do not impose a structure to the clusters, others like Self Organizing Feature Maps (SOM) organize clusters into a grid structure. Imposing a structure based on cluster similarity may be performed after clustering.

Although DEGs are different from clusters, biological interpretation of the resulting lists of significantly up- or down-regulated DEGs (Figure 1(a)) may also be benefited by the same ontology and pathway-based annotation analysis. Clus-

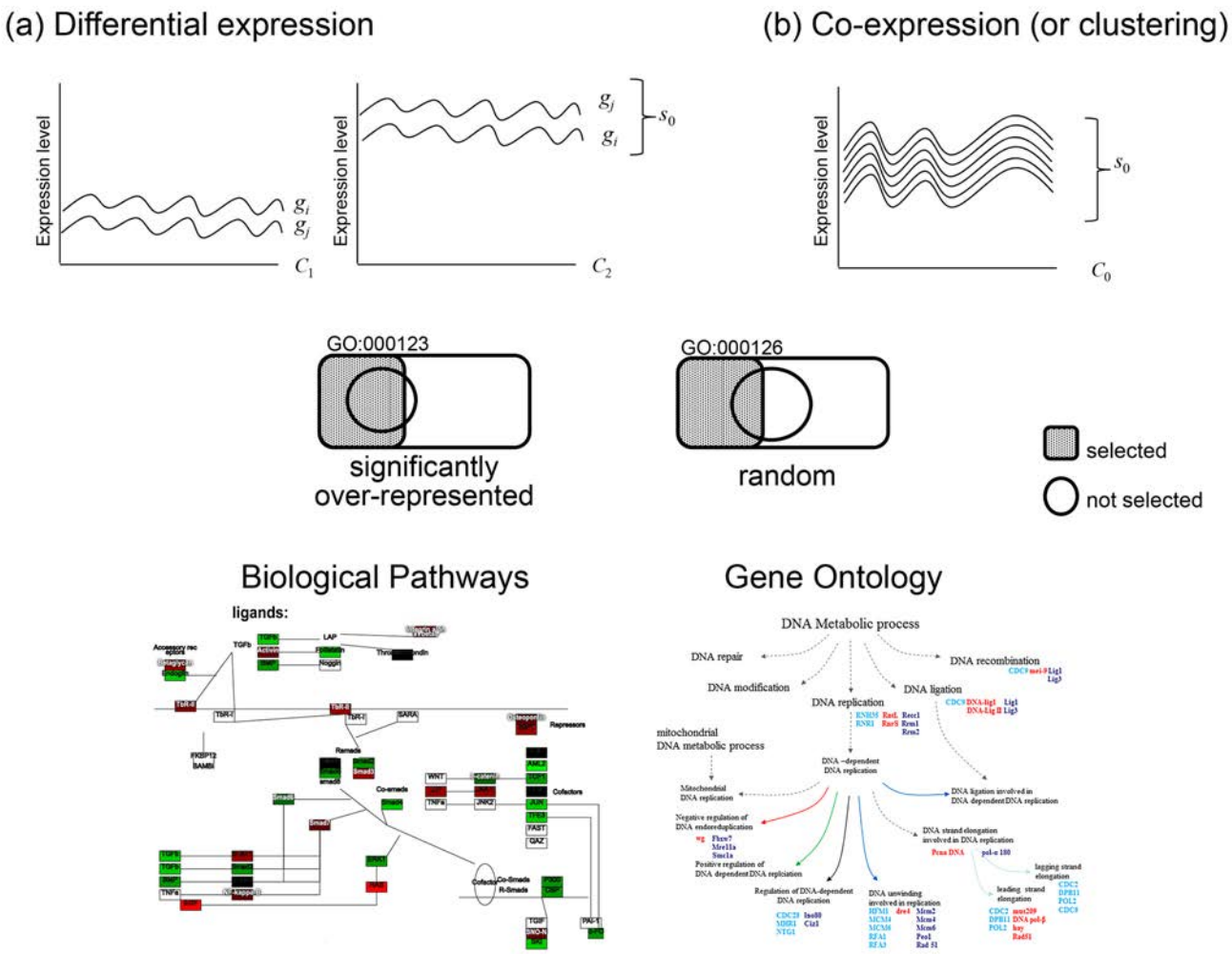

Figure 1. Functional annotation analysis based on biological pathways and GO terms. Annotation frequencies for a list of genes obtained by differential expression and co-expression analyses of microarray and RNA-Seq data are input to a statistical analysis of significant over-representation within the selected group. *C*: conditions, *g*: genes, *s*: gene groups.
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tering is classified as an unsupervised method. Results from supervised methods for a variety of classification tasks can sometimes be organized into a list based on, for example, their contributions to the task. In principle any list of genes can be carefully applied to ontology and pathway-based annotation analysis.

Metabolic pathways like KEGG and MetaCyc and signaling pathways like BioCarta are very powerful resources for the understanding of shared biological processes of a group of genes. Pathways are commonly presented as directed graphs, where nodes mainly represent molecules such as proteins and compounds, and edges represent relation types between two nodes. MetaCyc is an experimentally determined non-redundant metabolic pathway database. It is the largest collection containing over 1400 metabolic pathways [15]. It is a part of the BioCyc collection of pathways and genome databases developed by SRI International. The pathway figures of MetaCyc are not static diagrams so that it can be updated and expanded while KEGG provides static collections of pathway diagrams.

One major goal of ontology is to provide a shared understanding of a certain domain of information. GO was first created as controlled vocabularies for standardized annotation of genome databases. Genes and gene products are annotated by GO as well as free text input by curators. DAG structures are imposed to the three controlled vocabularies of GO; Molecular Function (MF), Cellular Compartment (CC), and Biological Process (BP). To each node (or GO term), a set of genes are annotated. MIPS began as a source for data on yeast biology, and now provides an integrated source for experimental, literature and computationally-predicted protein properties for a variety of complete genomes as well. MeSH has many clinical terms including disease names. Other knowledge resources like OMIM (Online Mendelian Inheritance in Man) Morbid Map can also be used to associate genes to MeSH disease names. GO and MeSH are now parts of UMLS (Unified Medical Language System) which has a semantic network structure. In principle, any biomedical ontology can be systematically applied for improving biomedical understanding of gene expression microarray and RNA-Seq data.

Once the genes of interest are successfully associated with correct functional annotations, the next step is to examine if there are any GO terms that have a larger than expected subset of listed genes in their annotation list. For example, if 20% of the genes in a gene list are annotated with a GO term '*apoptosis*' while

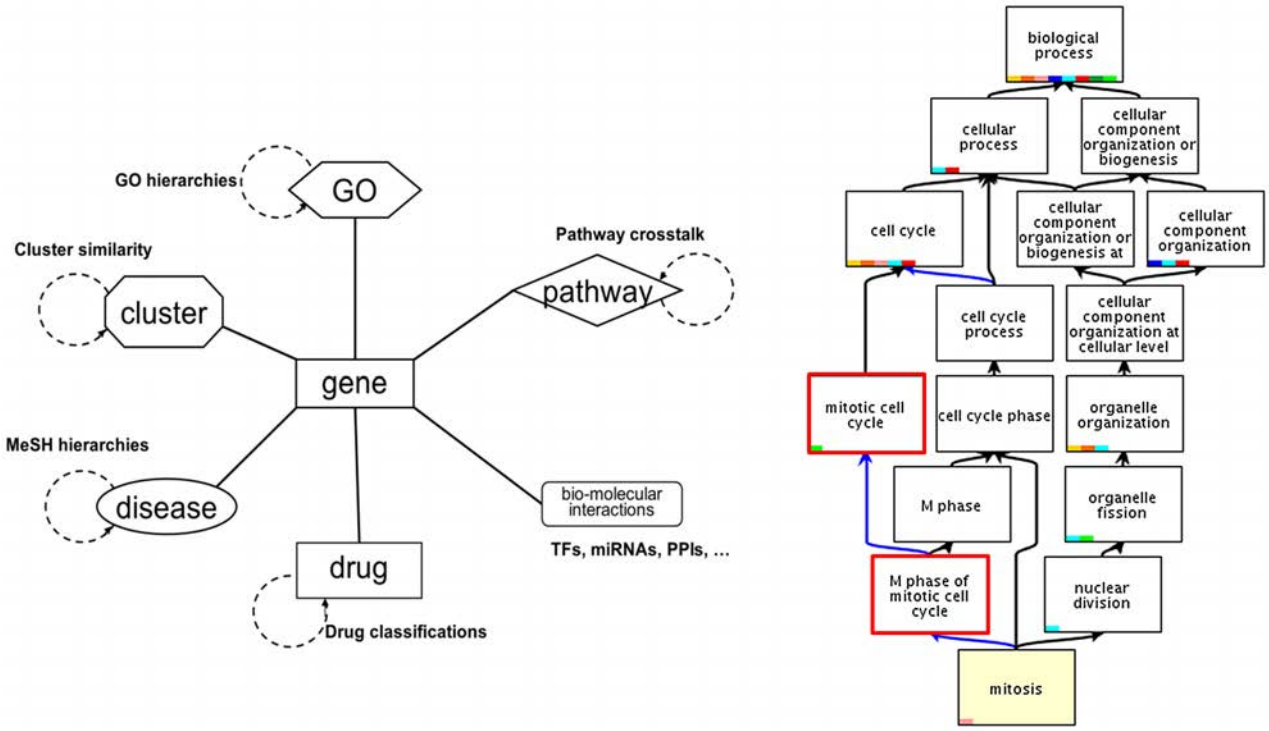

Figure 2. Collection of biological knowledge-based annotation resources for genes and gene clusters. The right panel shows an example of GO enrichment analysis result for a yeast cell division experiment.
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only 1% of the genes in the whole human genome fall into this functional category, then the gene list can be regarded as strongly related with the functional annotation. Most statistical tests like Chi-square, binomial and hypergeometric tests can be applied. Chi-square test cannot be used to test data of small sample size. Hypergeometric test is widely used for functional enrichment analysis of gene lists, but it is computationally more intensive.

Suppose we have a total of N genes with n genes belonging to a group of interest (cluster or DEGs). Among them M genes are annotated to a specific GO term and k genes belong to the interest group and are annotated to the specific GO term. The probability of having at most k genes can be calculated by hypergeometric distribution according to the following:

$$P(X \le k) = \sum_{y=0}^{k} h(y|N;M;n) = \sum_{y=0}^{k} \frac{\binom{M}{y}\binom{N-M}{n-y}}{\binom{N}{n}}$$

Hypergeometric distribution is a discrete probability distribution describing the number of successes by a serial sampling from a finite population. It is equivalent to a one-tailed Fisher's exact test. One should consider the choice of universe (or background), that makes substantial impact on the result. All genes having at least one GO annotation, all genes ever known in genome databases, all genes on the microarray, or all transcripts of RNA-Seq data that pass non-specific filters can be candidate universe. One more problem comes from the hierarchical tree (or graphical) structure of GO categories (or pathways) while the hypergeometric test assumes independence of categories. A parent term can simply be rated as significant because of the influence from its significant children. Moreover, more general statements require stronger evidence that is required to prove more specific statements. Conditional hypergeometric testing methods [16,17] exclude GO terms if there is no evidence beyond that provided by its significant children. Because many tests are performed, p-values must be interpreted with caution.

Pathway and ontology-based analysis consist of database mapping, statistical testing, and presentation steps [18]. Mapping gene lists to GO terms or pathways requires resolving gene name ambiguities and inconsistencies (not discussed here) using a wide range of genomic resources and techniques. Visual and textual presentation helps users to understand biological semantics and contexts. A number of analysis tools with these steps have been introduced: ArrayXPath, Pathway Miner, EASE in pathway analysis, GOFish, GOTree Machine, FatiGO, GOAL, GOMIner, FuncAssociate in ontology analysis and GeneMerge, MAPPFinder, DAVID, GFINDer, OntoTools in both analyses [14].

3. Gene Set-Wise Differential Expression Analysis

Researcher's primary interest with DNA microarray and RNA-Seq data is to identify differentially expressed genes (DEGs). To this aim, a number of statistical methods have been introduced, evaluating statistical significance of individual genes between two conditions. Gene set-wise differential expression analysis method, however, evaluates coordinated differential expression of gene groups, the meaning of which are previously defined as those of biological pathways. The first developed in this category is the Gene Set Enrichment Analysis (GSEA) that evaluates for each a priori defined gene set the significant association with phenotypic classes in DNA microarray experiments [10].

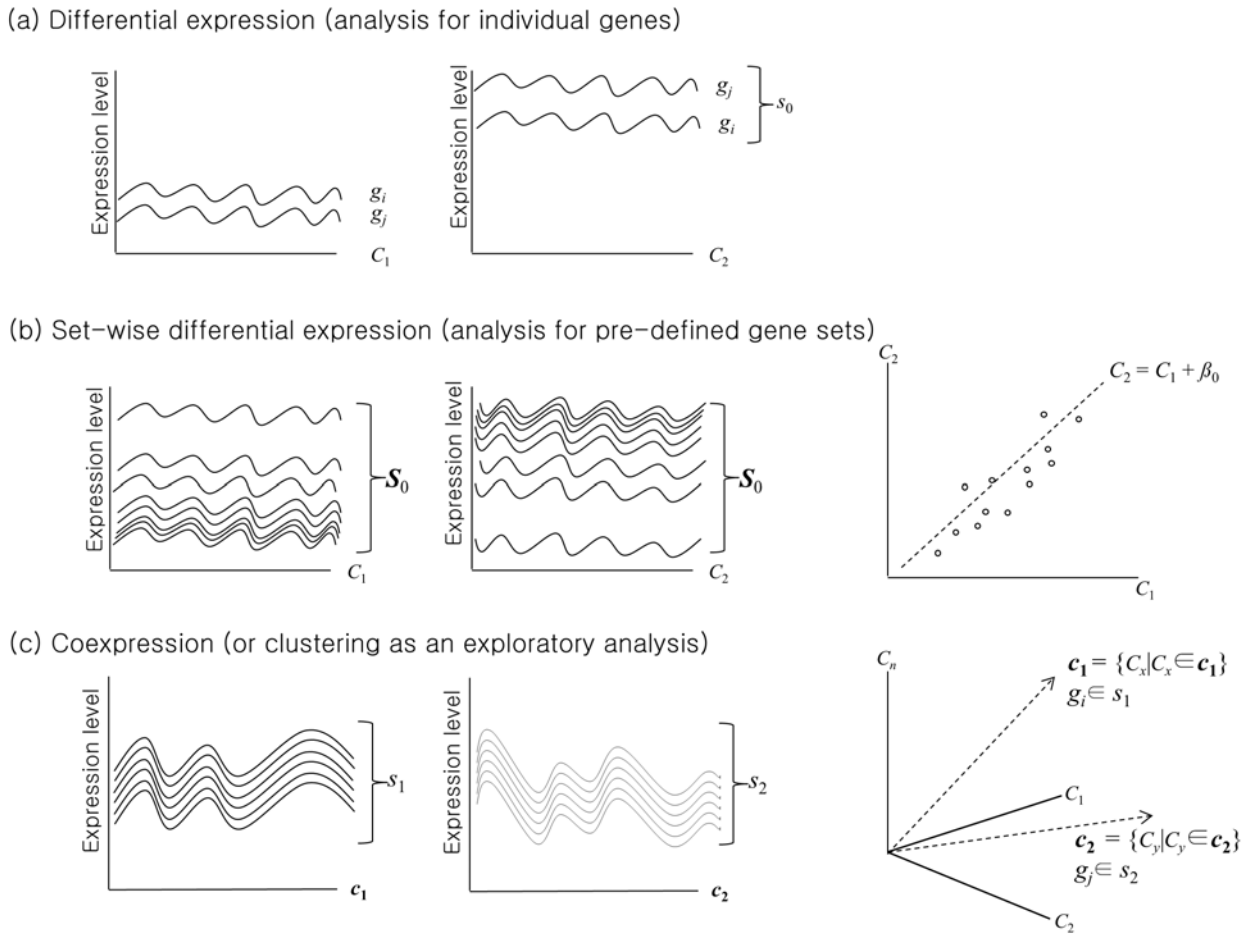

Figure 3. Differential expression analysis for individual genes and predefined gene sets. *C*: conditions, ***c***: condition sets, *g*: genes, *s*: gene groups, ***S***: predefined gene sets. (Modified from [30]).
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While FAA tries to determine over-represented GO terms or biological pathways after determining significant co-expression clusters or DEG lists (Figure 3(a) and (c)), GSEA takes the 'reverse-annotation' or 'gene set-wise' approach (Figure 3(b)). This gene set-wise differential expression analysis method successfully identified modest but coordinated changes in gene expression that might have been missed by conventional 'individual gene-wise' differential expression analysis. Moreover, gene set-wise approach provides straightforward biological interpretation because the gene sets are defined by biological knowledge. GSEA's success clearly demonstrates that many tiny expression changes can collectively create a big change that is statistically significant. Another advantage is that utilizing pre-defined and well-established gene sets rather than finding or creating novel lists of genes markedly improves semantic interpretability and computational feasibility. It is believed that functionally related genes often show a coordinated expression pattern to accomplish their functional role.

GSEA first creates a ranked list of genes according to their differential expression between experimental conditions and then determines, for each a priori defined gene set, whether members of a gene set tend to occur toward the top (or bottom) of the ranked list, in which case the gene set is correlated with the phenotypic class distinction. With the interesting gene set, *S*, Enrichment Score (ES) is calculated by evaluating the fractions of genes in *S* ("hits") weighted by their correlation and the fractions of genes not in *S* ("misses") present up to a given position i in the ranked gene list, L, where N genes are ordered according to the correlation, $r(g_j) = r_j$ of their expression profiles with interest gene set:

$$P_{\mathrm{hit}}(S,i) = \sum_{\substack{g_j \in S \\ j \le i}} \frac{|r_j|^p}{N_R}, \text{ where } N_R = \sum_{g_j \in S} |r_j|^p$$

$$P_{\mathrm{miss}}(S,i) = \sum_{\substack{g_j \notin S \\ j \le i}} \frac{1}{(N - N_H)}$$

where N_H indicates the number of genes in *S* and is an exponent to control the weight of the step. The ES is the maximum deviation from zero of $P_{\mathrm{hit}} - P_{\mathrm{miss}}$. It corresponds to a weighted Kolmogorov-Smirnov-like statistic.

GSEA assesses the significance by permuting the class labels. Concerning the definition of the null hypothesis, methods can be classified into competitive and self-contained tests [19]. A competitive test compares differential expression of the gene set to a standard defined by the complement of that gene set. A self-contained test, in contrast, compares the gene set to a fixed standard that does not depend on the measurements of genes outside the gene set. The competitive test is more popular than the self-contained test.

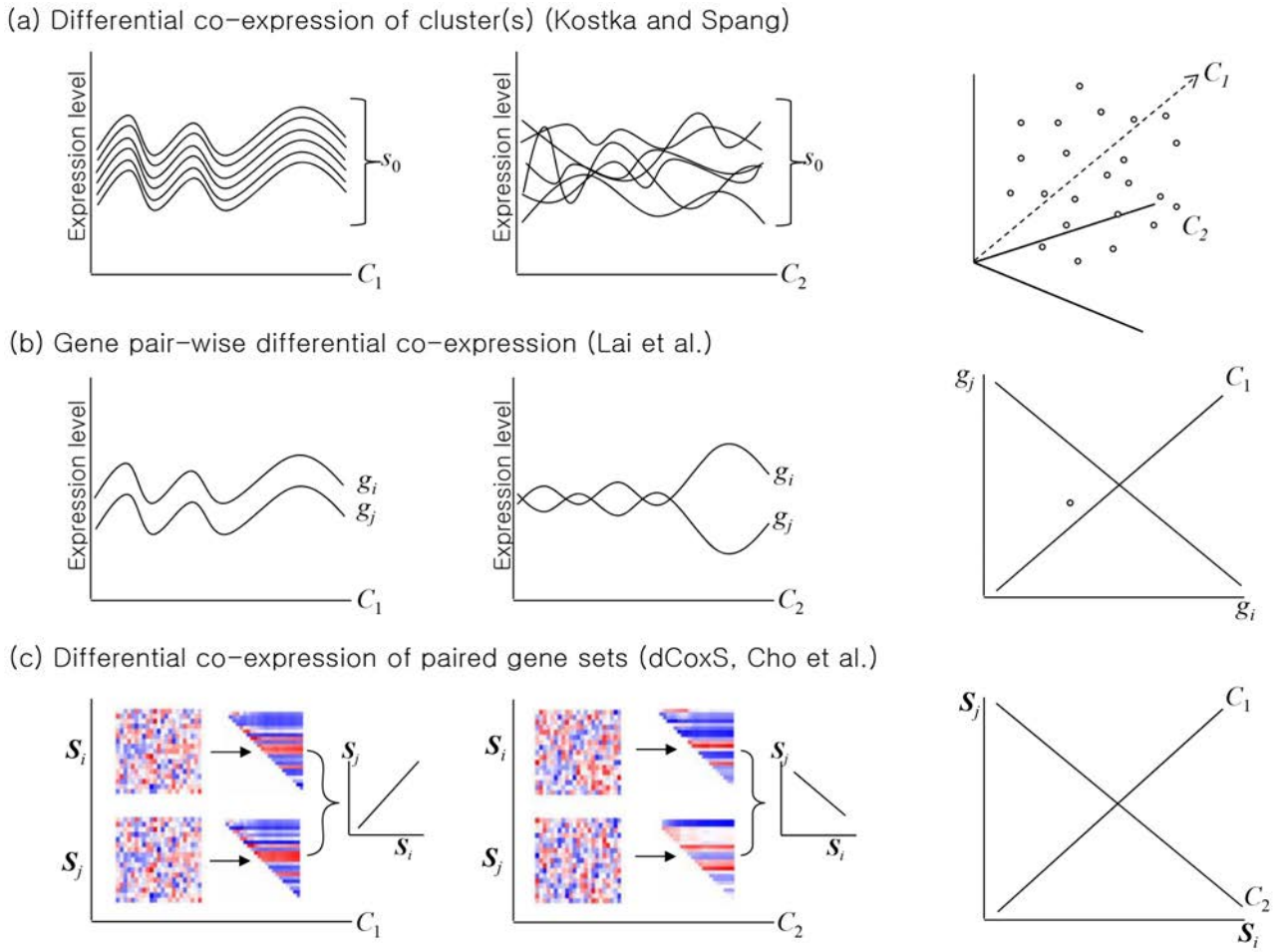

Figure 4. Differential co-expression anslyses. Differential co-expression (a) of clusters can be detected by a method proposed by Kostka and Spang [26], (b) of gene pairs can be detected by a method proposed by Lai *et al.* [24], and (c) of paired gene sets by a method proposed by Cho *et al.* [12]. *C*: conditions, *g*: genes, *s*: gene clusters, *S*: a priori defined gene sets. (Modified from [30]).
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Typical gene sets are regulatory-motif, function-related, and disease-related sets. MSigDB (Molecular Signatures Database) is one of leading gene set databases (http://www.broadinstitute.org/gsea/msigdb) containing a total of 6769 gene sets which are classified into five different collections (positional, curated, motif, computational and GO gene sets). Several interesting extensions were proposed in terms of sample level applications. For example, researchers developed genomic signatures to identify the activation status of oncogenic pathways and predict the sensitivity to individual chemotherapeutic drugs [20,21]. Significance Analysis of Function and Expression (SAFE) [22] extends GSEA to cover multiclass, continuous and survival phenotypes. It also provides more options for the test statistic, including Wilcoxon rank sum, Kolmogorov-Smirnov and Hypergeometric statistic.

4. Differential Co-Expression Analysis

Co-expression analysis determines the degree of co-expression of a group (or cluster) of genes under a certain condition. Unlike co-expression analysis, differential co-expression analysis determines the degree of co-expression difference of a gene pair or a gene cluster across different conditions, which may relate to key biological processes provoked by changes in environmental conditions [12,23–25]. Differential co-expression analysis methods can be categorized into three major types (Figure 4): (a) differential co-expression of gene cluster(s) [26], (b) gene pair-wise differential co-expression [24] and (c) differential co-expression of paired gene sets [12].

To identify differentially co-expressed gene cluster(s) between two conditions, (C_1 and C_2 in Figure 4 (a)), a method determines whether a cluster shows significant conditional difference in the degree of co-expression. An additive model-based scoring can be used based on the mean squared residual [26]. Let conditions and genes be denoted by $\mathcal{J}$ and I, respectively. The mean squared residual of model is a measurement of co-expression of genes:

$$S'(I,J) = \frac{1}{(|I|-1)(|J|-1)} \sum_{I,J} \left(a_{ij} - a_{i\cdot} - a_{\cdot j} - a_{\cdot\cdot}\right)^2$$

where an entry a_{ij} is the expression level of gene i in condition j, $a_{i\cdot}$ is the mean expression level of gene i in conditions, $a_{\cdot j}$ is the mean expression level of genes in condition j, $a_{\cdot\cdot}$is the mean expression levels of genes in conditions. A group of gene with a low score S' means high correlation of genes. Given two groups $\mathcal{J}_1$ and $\mathcal{J}_2$, e.g.

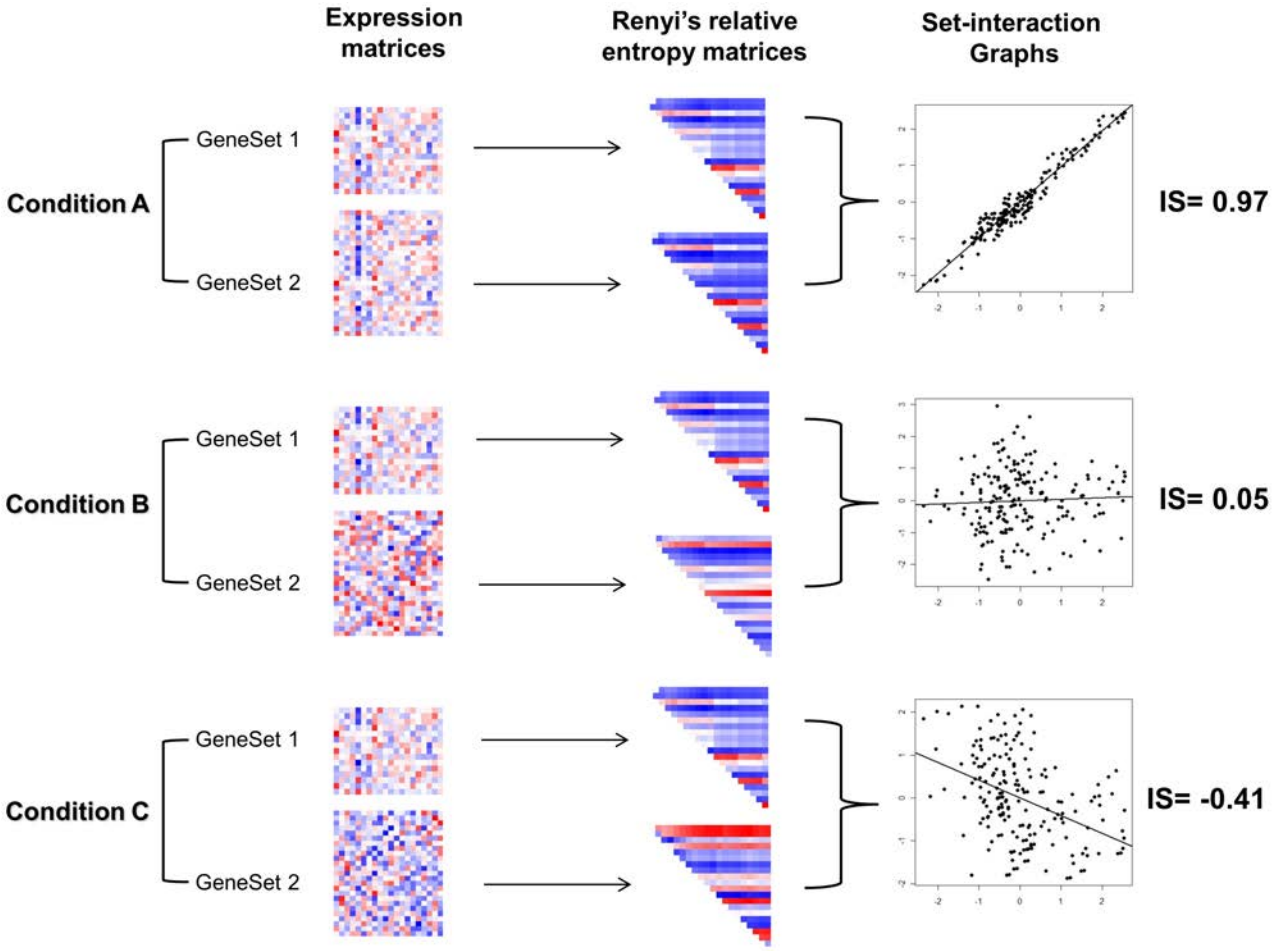

Figure 5. The dCoxS algorithm. Expression matrices of two gene sets (upper panel) are transformed into Renyi relative entropy matrices by all sample pair-wise comparisons (middle panel). For each condition, Interaction Score (*IS*), a kind of correlation coefficients, between a pair of entropy matrices is obtained. Upper diagonal heat maps in the middle panel are transformed into scatter plots in the lower panel where *IS*s are depicted as fitted lines. (Modified from [12]).
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disease and control, the method minimizes the score, $S(I)$ of a set of genes, I:

$$S(I) = \frac{S'(I,J_1)}{S'(I,J_2)} = \frac{|J_2|-1}{|J_1|-1} \cdot \frac{\sum_{I,J_1}\left(a_{ij} - a_{i\cdot}^{(1)} - a_{j}^{(1)} - a_{\cdot\cdot}^{(1)}\right)^2}{\sum_{I,J_2}\left(a_{ij} - a_{i\cdot}^{(2)} - a_{j}^{(2)} - a_{\cdot\cdot}^{(2)}\right)^2}$$

A greedy downhill approach finds local minima of the score. Another approach uses t-statistic for each cluster to evaluate the difference of the degree of co-expression between conditions, after creating gene expression clusters [27]. These methods can be viewed as an attempt to find gene clusters that are tightly co-regulated (i.e. highly co-expressed) in one condition (i.e. normal) but not in another (i.e. cancer).

To identify differentially co-expressed gene pairs in Figure 4(b), *F*-statistic can be calculated as expected conditional *F*-statistic (ECF), a modified *F*-statistic, for all pair of genes between two conditions [24]. A meta-analytic approach can also detect gene pairs with significant differential co-expression between normal and cancer samples [25]. These methodologies can be regarded as an attempt to discover gene pairs that are, in principle, positively correlated in one condition (i.e. normal) and negatively correlated in another (i.e. cancer). Identification of differentially co-expressed gene clusters or gene pairs usually do not use a priori defined gene sets or pairs but try to find the best ones among all possible combinations without considering prior knowledge. Thus the biological interpretation of the clusters or pairs may also be improved by ontology and pathway-based annotation analysis.

The idea of finding gene clusters that show positive correlation in one condition and negative correlation in another condition sounds very interesting. However, it seems that there is very little chance for such a cluster to exist. Similarly, one can hardly find such a set among a priori defined gene sets (i.e. biological pathways). It is even difficult to expect a biological pathway whose members are all highly positively (or negatively) co-expressed in a condition because a biological pathway is a complex functional system with interacting positive and negative feedback loops. Thus, members of a biological pathway may not be contained in a single co-expression cluster, especially when the cluster is not very big, but be split into different clusters.

The dCoxS (differential co-expression of gene sets) algorithm identifies (a priori defined or semantically enriched) gene set pairs differentially co-expressed across different conditions (Figure 4 (c) and Figure 5) [12]. Biological pathways can be used as pre-defined gene sets and the differential co-expression of the biological

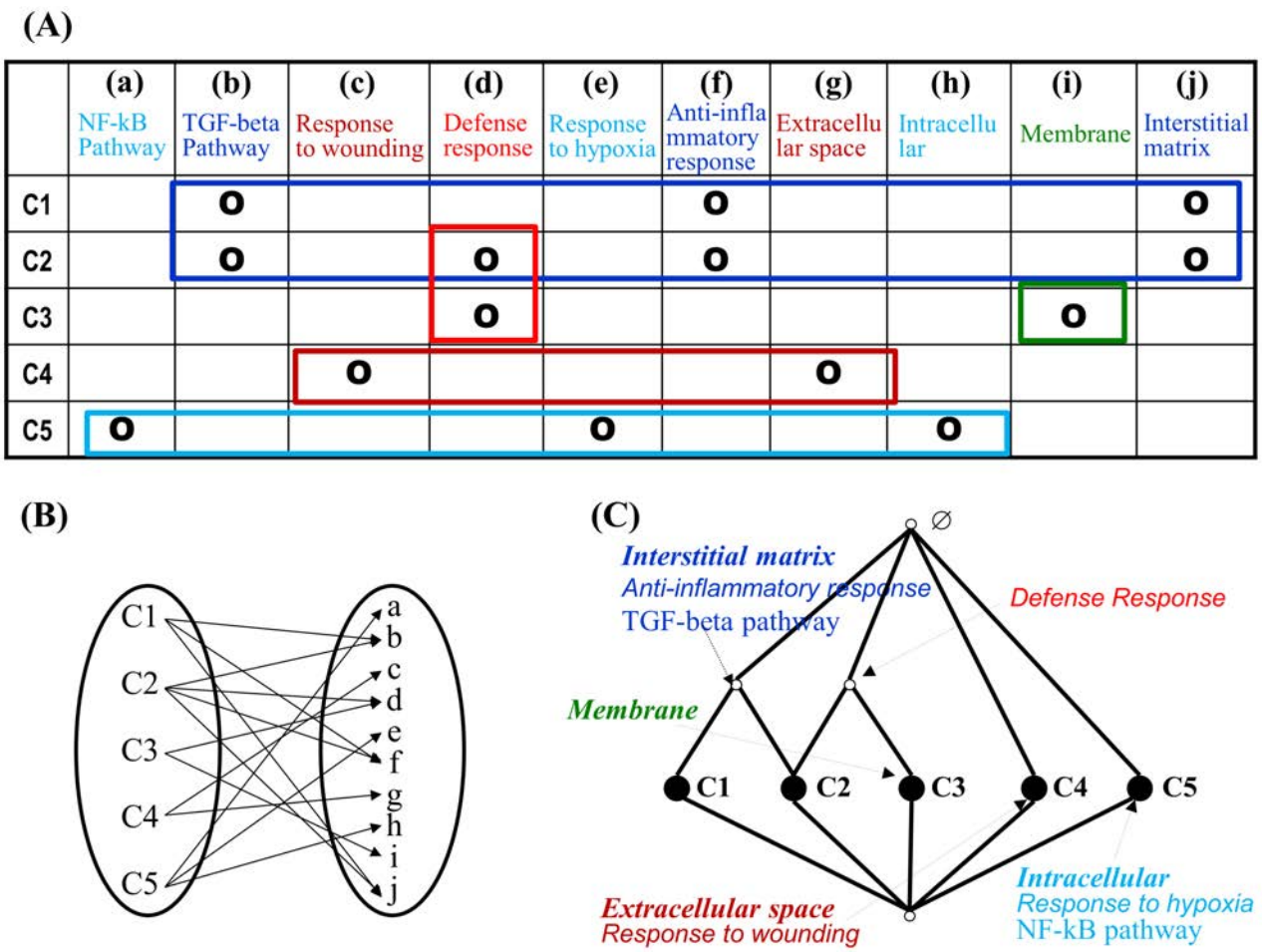

Figure 6. Concept lattice. The binary relation set $R = \{(C1,b), (C1,f), (C1,j), (C2,b), (C2,d), \ldots, (C5,e), (C5,h)\}$ can be represented as (a) a relation matrix, (b) a directed bipartite graph, and (c) a concept lattice. Colored rectangles in the relation matrix represent concepts. The same color represents the same concept in (a) and (c). (Modified from [13]).
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pathway pairs between conditions is analyzed. To measure the expression similarity between paired gene-sets under the same condition, dCoxS defines the interaction score (*IS*) as the correlation coefficient between the sample-wise entropies. Even when the numbers of the genes in different pathways are different, *IS* can always be obtained because it uses only sample-wise distances regardless of whether the two pathways have the same number of genes or not.

$$IS = \frac{\sum_{i<j}(RE^{G_1} - \overline{RE^{G_2}})(RE^{G_1} - \overline{RE^{G_2}})}{\sqrt{\sum_{i<j}(RE^{G_1} - \overline{RE^{G_1}})^2}\sqrt{\sum_{i<j}(RE^{G_2} - \overline{RE^{G_2}})^2}}$$

where RE^{Si} and RE^{Sj} are the matrices of the Renyi relative entropy of gene sets, S_i and S_j. When estimating the relative entropy, multivariate kernel density estimation was used to model gene-gene correlation structure.

For example, when we compute the *IS* of a pair of pathway expression matrices with dimensions 20 (genes) by 25 and by 15 (samples) for a condition, we calculate 190 (=(20*19)/2) sample pair-wise entropy distances for each pathway expression matrix. The *IS* is obtained by calculating the correlation coefficient between the two entropy vectors. Finally, the statistical significance of the difference of the Fisher's Z-transformed *IS*s between two conditions is tested for each pathway pair.

$$Zf = \frac{1}{2} \times ln\left(\frac{1+IS}{1-IS}\right)$$

The p-value of the difference in the $\mathcal{Z}f$ values is calculated using the standard normal distribution in equation.

$$P(Z \geq |\frac{(Zf_1 - Zf_2)}{\sqrt{1/(N_1-3)+1/(N_2-3)}}$$

$\mathcal{Z}f_1$ and $\mathcal{Z}f_2$ are the Fisher's Z-transformed values of the IS under two different conditions and N_1 and N_2 are the numbers of upper-diagonal elements, which is calculated by $n(n-1)/2$ (n = number of samples) for each condition.

For the purpose of comparison, all gene pair-wise $\mathcal{Z}f$ values are calculated for each condition and the conditional difference of the Fisher's Z-transformed correlation coefficients is tested for each gene pair as follows,

$$Zf = \frac{1}{2} \times ln\left(\frac{1+CC}{1-CC}\right)$$

$$p(Z \geq |\frac{(Zf_1 - Zf_2)}{\sqrt{1/(N_1-3)+1/(N_2-3)}}$$

where CC indicates the correlation coefficient of a gene pair, $\mathcal{Z}f_i$ Fisher's Z-transformed correlation coefficient and N_i the number of samples in conditions i. The p value for differential co-expression is obtained according to the difference

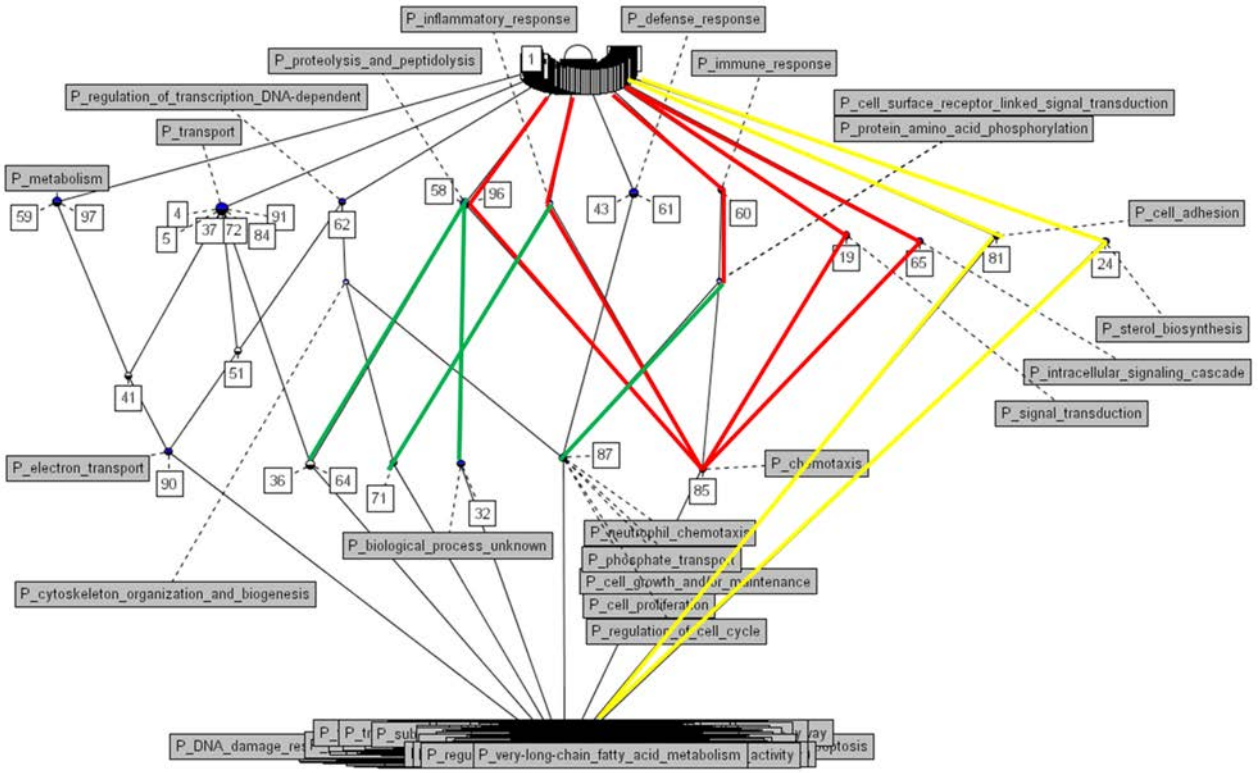

Figure 7. BioLattice of mouse renal inflammation induced by glomerular basement membrane (GBM) antibody.
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between the Z values from the normal distribution. For each gene pair, three p values are obtained, one from each condition and another from the difference between the conditions. Bonferroni correction is applied.

5. Biological Interpretation and Biological Semantics

Biological interpretation of genomic data requires a variety of semantic knowledge. Biomedical semantics provides rich descriptions for biomedical domain knowledge. Biomedical semantics is a valuable resource not only for biological interpretation but also for multi-layered heterogeneous data integration and genotype-phenotype association. Symbolic inference algorithms may add further values.

Although GO and pathway-based analysis of co-expressed gene groups is one of the most powerful approaches for interpreting microarray experiments, they have limitations. The result, for example, is typically a long unordered list of annotations for tens or hundreds of gene clusters. Most of the analysis tools evaluate only one cluster at a time in a sequential manner without considering the informative association network of clusters and annotations. It is very time-consuming to read the massive annotation lists for a large number of clusters. It is unthinkably hard to manually assemble the 'puzzle pieces' (i.e., the cluster-annotation sets) into an 'executive summary' (i.e., the context of the whole experiment). Many annotations are redundant such that many clusters share the same annotations in a very complex manner. Ideally, the assembly should involve eliminating redundant attributes and organizing the pieces in a well-defined order for better biological understanding and insight into the underlying 'context' of the experiment under investigation.

BioLattice is a mathematical framework based on concept lattice analysis to organize traditional clusters and associated annotations into a lattice of concepts for better biological interpretation of microarray gene-expression data [13]. BioLattice considers gene expression clusters as objects and annotations as attributes and provides a graphical summary of the order relations by arranging them on a concept lattice in an order based on set inclusion relation. Complex relations among clusters and annotations are clarified, ordered and visualized. Redundancy of annotation is completely removed. It also has an advantage that heterogeneous biological knowledge resources (such as transcription factor binding, chromosomal co-location and protein–protein interaction networks) can be added to better explore the underlying structures. The representation of relationship between clusters can give more insight to interpret functions of interesting genes.

Figure 6 demonstrates a context (or a gene expression dataset) with clusters and annotations. Note that the relation matrix between objects (i.e., rows or clusters) and attributes (i.e., columns or annotations) can be represented by a bipartite graph (Figure 6(b)) or a concept lattice (Figure 6(c)). A concept lattice organizes all clusters and annotations of a relation matrix into a single unified structure with no 'redundancy' and no loss of information. It is worth noting that the cluster labels, *C1* to *C5*, and the annotation labels appear once and only once in the lattice diagram (Figure 6(c)). Now one can interpret the whole experimental context (Figure 6(a)) by reading the ordered concepts with clusters and annotations.

Structural analyses methods like prominent sub-lattice analysis and core-periphery structure analysis may help further understanding [13]. Figure 7 shows a BioLattice for a mouse anti-GBM glomerulonephritis model [28]. Genes showing significant time-dose effect were clustered

into 100 clusters and annotated with GO terms. The whole complex clusters and annotations are organized into a single unified lattice graph, providing an 'executive summary.' The Ganter algorithm [29] can be used to construct BioLattice. A web-based tool using Perl, JavaScript and Scalable Vector Graphics are available at http://www.snubi.org/software/biolattice/. Prominent sub-lattice analysis reveals a meaningful sub-structure converging into cluster 85, which has the GO term '*chemotaxis*' and inherits '*proteolysis and peptidolysis*' (clusters 58 and 96), '*inflammatory response*', '*immune response*', '*protein amino acid phosphorylation*', and '*cell surface receptor linked signal transduction*' (cluster 60), '*signal transduction*' (cluster 19), '*intracellular signaling cascade*' (cluster 65). It is clearly visualized that cellular immune response system activation is the core pathological process in the IgA nephropathy model of kidney and clusters 19, 58, 60, 65, 5 and 96 are within those concepts.

Context in concept lattice analysis is a triplet (G, M, I) consisting of two sets G and M and a relation I between G and M. The elements of G and M are called objects and attributes, respectively. We denote gIm or $(g, m) \in I$ to show that object g has attribute m. For a set $A \subseteq G$ of objects, we define $A' := \{ m \in M \mid gIm \text{ for all } g \in A \}$ (i.e., the set of attributes common to the objects in A). Correspondingly, for a set $B \subseteq M$ of attributes, we define $B' := \{ g \in G \mid gIm \text{ for all } m \in B \}$ (i.e., the set of objects that have all attributes in B).

Concept lattice analysis models concepts as units of thought, consisting of two parts. A concept of the context (G, M, I) is a pair (A, B) with $A \subseteq G$, $B \subseteq M$, $A' = B$ and $B' = A$. We call A and B the extent and the intent, respectively, of concept (A, B). The extent consists of all objects belonging to the concept while the intent contains all attributes shared by the objects. The set of all concepts of the context (G, M, I) is denoted by $C(G, M, I)$. A concept lattice is drawn by ordering (A, B), which are defined as concepts of the context (G, M, I). The set of all concepts of a context together with the partial order $(A1, B1) \leq (A2, B2) :\Leftrightarrow A1 \subseteq A2$ (which is equivalent to $B1 \supseteq B2$) is called a concept lattice.

We regard A as defining gene expression clusters that share common knowledge attributes and B as defining the knowledge terms that are annotated to the clusters. The concepts are arranged in a hierarchical order so that the order of $C1 \leq C2 \Leftrightarrow A1 \subseteq A2 \Leftrightarrow B1 \supseteq B2$ is defined at $C1 = (A1, B1)$, $C2 = (A2, B2)$. The top element of a lattice is a unit concept, representing a concept that contains all objects. The bottom element is a zero concept having no object.

6. Summary

This chapter has shown major computational approaches to facilitate biological interpretation of high-throughput microarray and RNA-Seq experiments. The enrichment analysis with ontologies, biological pathways or external resources is widely used to interpret the functional role of correlated genes or differentially expressed genes. In analysis steps, the groups of genes are assigned to the associated biological annotation terms using GO terms or biological pathways. Then it is necessary to examine whether gene members are statistically enriched in each of the annotation terms or pathway by comparing background set by measuring statistical test such as Chi-square, Fisher's exact, binomial and hypergeometric test. Unlike previous approaches identifying a set of significant genes, Gene Set Enrichment Analysis uses pre-defined sets to search for groups of functionally related genes with coordinated expression across a list of genes ranked by differentially expression. Differential co-expression analysis determines the degree of co-expression difference of a gene set pair across different conditions. The dCoxS algorithm identifies differentially co-expressed gene set under different conditions. Outcomes in microarray and RNA-Seq data can be transformed into the graphical structure that represents biological semantics. A number of biomedical annotation and external repositories including clinical resources can be integrated by biological semantics analysis tools such as BioLattice.

7. Exercises

1) Select significantly DEGs from the train dataset of AML (Acute Myelocytic Leukemia) and ALL (acute lymphoblastic leukemia) expression data (http://www.broadinstitute.org/cgi-bin/cancer/publications/pub_paper.cgi?mode=view&paper_id=43) and find enriched GO terms from an ontology analysis tool. Dataset and analysis functions are also included in R statistical package, golubEsets in Bioconductor.
2) List significantly enriched pathways using a pathway analysis tool with the dataset in Exercise 1.
3) Find KEGG pathways significantly associated with leukemia subtype in the 2-sample comparison of AML and ALL by GSEA through the Kolmogorov-Smirnoff test. Analysis and data set are provided by SAFE R (http://bioconductor.org/packages/2.0/bioc/html/safe.html).
4) Identify the differentially co-expressed gene set pairs using dCoxS with simulated data in (http://www.snubi.org/publication/dCoxS). Compute interaction score between matrix M and M1 using ias function. And, compute interaction score between M and M2. Finally, using compcorr function, estimate significance of difference of ias. Note that in compcorr function, n1 and n2 is the number of all possible sample pairs.
5) Report semantic relationships of pathways and GO terms using BioLattice (http://www.snubi.org/software/biolattice/). Use the result of k-means clustering ($k = 10$) with

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Glossary

Bioconductor: a free, open source and open development software project for the analysis and comprehension of genomic data generated by wet lab experiments in molecular biology written in R Statistical Package.

Clustering: algorithm that puts similar things together and different things apart.

Gene expression profiling: the measurement of the activity (or expression) of thousands of genes at once to create a global picture of cellular function using DNA microarray technology.

Gene set: a meaningful grouping of genes like biological pathways, genes sharing certain regulatory-motifs, genes sharing certain functional annotations, and certain disease-related gene sets.

Gene Set Enrichment Analysis: an algorithm to determine whether an a priori defined set of genes shows statistically significant coordinated differential expression between conditions.

Gene Ontology: a set of controlled vocabularies in molecular function, biological process and cellular component for the standardized annotations of genes and gene products across all species.

Hypergeometric distribution: a discrete probability distribution that describes the number of successes in a sequence of *n* draws from a finite population without replacement, just as the binomial distribution describes the number of successes for draws with replacement.

Kolmogorov–Smirnov test (K–S test): a nonparametric test for the equality of continuous, one-dimensional probability distributions that can be used to compare a sample with a reference probability distribution (one-sample K–S test), or to compare two samples (two-sample K–S test).

DEG in Exercise 1. Select Category as 'biological process,' p-value$<$0.05.

Answers to the Exercises can be found in Text S1.

Supporting Information

Text S1 Answers to Exercises
(DOCX)

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Chapter 9: Analyses Using Disease Ontologies

Nigam H. Shah*, Tyler Cole, Mark A. Musen

Center for Biomedical Informatics Research, Stanford University, Stanford, California, United States of America

Abstract: Advanced statistical methods used to analyze high-throughput data such as gene-expression assays result in long lists of "significant genes." One way to gain insight into the significance of altered expression levels is to determine whether Gene Ontology (GO) terms associated with a particular biological process, molecular function, or cellular component are over- or under-represented in the set of genes deemed significant. This process, referred to as enrichment analysis, profiles a gene-set, and is widely used to makes sense of the results of high-throughput experiments. The canonical example of enrichment analysis is when the output dataset is a list of genes differentially expressed in some condition. To determine the biological relevance of a lengthy gene list, the usual solution is to perform enrichment analysis with the GO. We can aggregate the annotating GO concepts for each gene in this list, and arrive at a profile of the biological processes or mechanisms affected by the condition under study. While GO has been the principal target for enrichment analysis, the methods of enrichment analysis are generalizable. We can conduct the same sort of profiling along other ontologies of interest. Just as scientists can ask "Which biological process is over-represented in my set of interesting genes or proteins?" we can also ask "Which disease (or class of diseases) is over-represented in my set of interesting genes or proteins?". For example, by annotating known protein mutations with disease terms from the ontologies in BioPortal, Mort et al. recently identified a class of diseases—blood coagulation disorders—that were associated with a 14-fold depletion in substitutions at O-linked glycosylation sites. With the availability of tools for automatic annotation of datasets with terms from disease ontologies, there is no reason to restrict enrichment analyses to the GO. In this chapter, we will discuss methods to perform enrichment analysis using *any* ontology available in the biomedical domain. We will review the general methodology of enrichment analysis, the associated challenges, and discuss the novel translational analyses enabled by the existence of public, national computational infrastructure and by the use of disease ontologies in such analyses.

This article is part of the "Translational Bioinformatics" collection for *PLOS Computational Biology*.

1. Introduction

Advanced statistical methods are most often used to perform the analysis of high-throughput data such as gene-expression assays [1–5], the result of which is a long list of "significant genes." Extracting biological meaning from such lists is a nontrivial and time-consuming task, which is exacerbated by the inconsistencies in free-text gene annotations. The Gene Ontology (GO) offers a taxonomy that provides a mechanism to determine statistically significant functional subgroups within gene groups. One way to gain insight into the biological significance of alterations in gene expression levels is to determine whether the GO terms associated with the particular biological process, molecular function, or cellular component are over- or under-represented in the set of genes deemed significant by the statistical analysis [6]. This process, often referred to as "enrichment analysis," can be used to summarize a gene-set [7], although it can also be relevant for other high-throughput measurement modalities including proteomics, metabolomics, and studies using tissue-microarrays [8].

With the availability of tools for automatic ontology-based annotation of datasets with terms from biomedical ontologies besides the GO, we need not restrict enrichment analysis to the GO. In this chapter, we outline the methodology of enrichment analysis, the associated challenges, and discuss novel analyses enabled by performing enrichment analysis using disease ontologies. We first review the current methods of GO based enrichment analysis to provide a foundation for discussing analyses using Disease Ontologies. Note that there is also research underway on the use of "pathways" for enrichment analyses as well as comparing statistically significant, concordant differences between two biological states as in Gene Set Enrichment Analysis [9], which are not discussed here.

1.1 Gene Ontology Enrichment Analysis

The goal of enrichment analysis is to determine which biological processes (or molecular function) might be predominantly affected in the set of genes that were deemed interesting or significantly changed. The simplest approach is to calculate functional 'enrichment/depletion' for each GO term—a higher (or lower) proportion of genes with certain annotations among the significantly changed genes than among all of the genes measured in the experiment. The finding of enrichment should not be interpreted as evidence implicating the GO term in the process studied without an appropriate statistical test.

The calculation of GO based functional enrichment involves two sets of items (usually genes or proteins): 1) The reference set, which is the set of items with which the "significant-set" is to be compared; the reference set may comprise all of the genes in the genome or all of the genes for which there were probes in the high throughput experiment; 2) The set of interest, which is the subset or list of significant genes that is to be analyzed for

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* E-mail: nigam@stanford.edu

What to Learn in This Chapter

- Review the commonly used approach of Gene Ontology based enrichment analysis
- Understand the pitfalls associated with current approaches
- Understand the national infrastructure available for using alternative ontologies for enrichment analysis
- Learn about a generalized enrichment analysis workflow and its application using disease ontologies

enrichment (or depletion) of GO terms in their annotations.

The analysis process (Figure 1) counts the GO annotations for both gene lists to calculate the number of genes (n and m) annotated with a particular GO term in each list and then calculates the probability (p-value) of the occurrence of at least n genes belonging to that category among the N genes in the set of interest, given that m genes are annotated with that term among the M genes in the reference set.

There are multiple ways to calculate the probability of observing a specific enrichment value. The simplest approach is to use a binomial model. For example, if one assumes that the probability of picking a gene annotated with the GO term 'apoptosis' is fixed and is equal to the proportion of genes annotated with 'apoptosis' in the reference set, then the binomial distribution provides the probability of obtaining a particular proportion of apoptosis genes among the genes in the set of interest by chance [10]. Such an approximation is quite reasonable for large reference sets (e.g. the whole genome) because the probability of selecting a gene annotated with the term 'apoptosis' into the set of interest does not change significantly after each selection.

However, when a gene or protein is picked from a smaller reference set, then the probability that the next picked gene is annotated to apoptosis is affected by whether the previously picked genes were annotated to apoptosis. Under these circumstances, the hypergeometric distribution—a discrete probability distribution that describes the number of successes in a sequence of n draws from a finite population without replacement—is a better statistical model. Another option is the Fisher's exact test or the chi-squared distribution, both of which take into consideration how the probabilities change when a gene is picked. The hypergeometric p-value is calculated using the following formula:

$$P(n) = \frac{\binom{m}{n}\binom{M-m}{N-n}}{\binom{N}{n}}$$

The p-value reports the likelihood of finding n genes annotated with a particular GO term in the set of interest by chance alone, given the number of genes annotated with that GO terms in the reference set. A biological process, molecular function or cellular location (represented by a GO term) is called enriched if the p-value is less than 0.05. GO annotations form the corner-stone of enrichment analysis in sets of differentially expressed genes. The GO project's Web site lists over 50 tools that can be used in this process [11].

Enrichment analysis can be done as a hypothesis-generating task, such as asking which GO terms are significant in a particular set of genes or a hypothesis-driven task such as asking whether apoptosis is significantly enriched or depleted in a particular set of genes.

In the hypothesis-driven setting, the analysis can include all of the genes that are annotated both directly to apoptosis and to its child nodes to maximize the statistical power because no correction for multiple comparisons is required. The hypothesis-generating approach allows an unbiased search for significant GO annotations. The analysis can be done with a bottom-up approach where for every leaf term the genes annotated with that GO term are also assigned to its immediate parent term. One can propagate the annotations recursively up along parent nodes until a significant node is found or until the root is reached. (Note: this upward propagation of annotations is

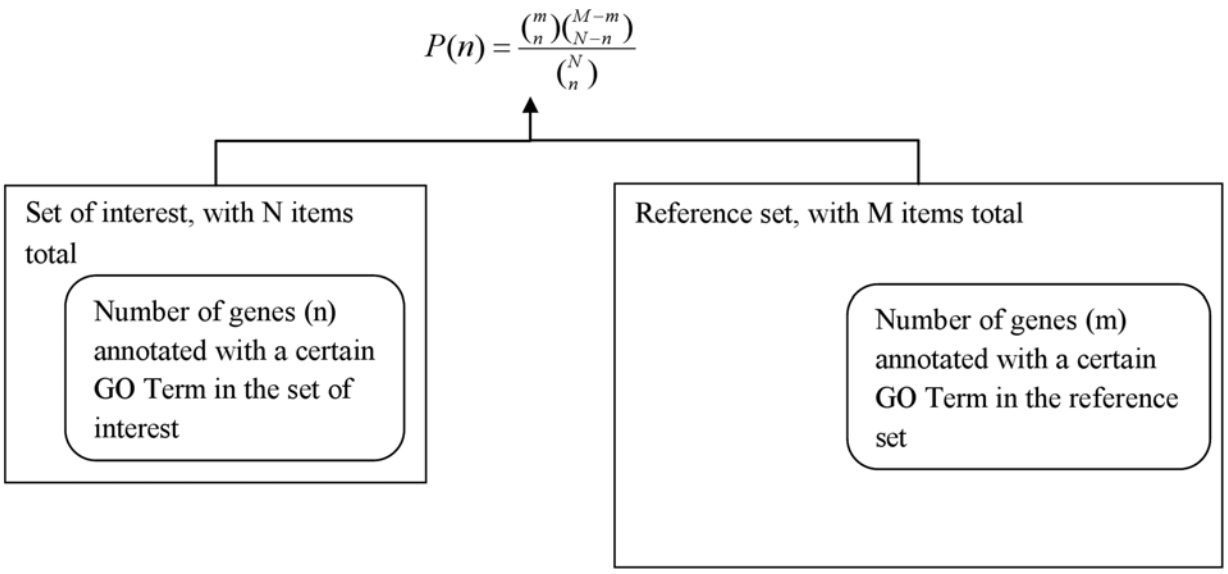

Figure 1. An overview of the process to calculate enrichment of GO categories. The steps usually followed are: (1) Get annotations for each gene in reference set and the set of interest. (2) Count the occurrence (n) of each GO term in the annotations of the genes comprising the set of interest. (3) Count the occurrence (m) of that same GO term in the annotations of the reference set. (4) Assess how "surprising" is it to find n, given m, M and N.
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referred to as computing the transitive closure of the annotation set over the graph of the Gene Ontology). Newer approaches can also perform the enrichment analysis accounting for the position of the term in the GO hierarchy [12–14].

1.1.1 Interpretation of p-values. The p-values should be interpreted with caution because the choice of the reference set to which the set of interest is compared affects the p-value. For whole genome arrays, using the list of all genes on the array as the reference set is equivalent to using the complete list of genes in the genome. However, for arrays containing a selected subset of genes associated with a biological process, the choice of the gene set to use as the reference set is not obvious. Moreover, the p-value calculation using the hypergeometric distribution assumes the independence of the GO annotation categories, an assumption that may not be justified.

Another difficulty in determining significance using the calculated p-value and a cutoff of 0.05, especially in the hypothesis-generating approach mentioned above, is that multiple testing increases the likelihood of obtaining what appears to be a statistically significant value by chance. Multiple testing occurs because the GO term to be tested for enrichment is not pre-selected, but each term is tested. This allows multiple opportunities (equal to the number of terms tested) to obtain a statistically significant p-value by chance alone in a given gene list. However, correcting for multiple testing by using a Bonferroni correction in which the critical p-value cut-off is divided by the number of tests performed is too restrictive—especially when annotations are propagated up to the root node via a transitive closure; then the number of tests is equal to the number of terms in the GO hierarchy.

In this situation, calculation of the false discovery rate (FDR), which provides an estimate of the percentage of false positives among the categories considered enriched at a certain p-value cutoff, allows for a more informed choice of the p-value cutoff. One can estimate the false discovery rate (FDR) for the enriched categories by performing simulations which generate a user-specified number of random gene sets of the same size as the set of interest and calculate the average number of categories that are considered enriched in the random gene sets, at a p-value cutoff of 0.05. If the FDR is above the desired threshold, we can lower the p-value cutoff in order to re-duce the FDR to acceptable levels. Multiple hypothesis testing is a general problem that is not specific to GO (see [15] for a general review).

A related issue arising from performing the transitive closure—the propagation of annotations along the parent-child paths—is that the parallel tests performed for nodes in a given path will be correlated because the same genes can appear several times on each path. Correction methods that assumes independence of categories might not function well in this situation and might preclude identification of some categories that are indeed enriched [6]. It is possible to use the structure of the GO to decorrelate the analysis of various terms [12–14] or to use corrections methods such as a Benjamini–Yekutieli correction, which accounts for the dependency between the multiple tests [16].

1.2 Summary of Existing Limitations

In 2005, Khatri and Draghici noted that, despite widespread adoption, GO-based enrichment analysis has intrinsic drawbacks [17] and scientists must still rely on literature searches to understand a set of genes fully. These drawbacks represent conceptual limitations of the current state of the art and include:

- Incomplete annotations—even today, roughly 20% of genes lack any GO annotation
- Annotation bias because of inter-relationships between annotations (e.g. annotation with certain GO terms is not conditionally independent).
- Lack of a systematic mechanism to define a level of abstraction, to compensate for differing levels of granularity.

The remainder of the chapter discusses approaches to using existing, public bioinformatics tools to address these limitations and use disease ontologies in such analyses.

2. Using Disease Ontologies—Going beyond GO Annotations

As we have discussed, enrichment analysis provides a means of understanding the results of high-throughput datasets [17,18]. Conceptually, enrichment analysis involves associating elements in the results of high-throughput data analysis to concepts in an ontology of interest, using the ontology hierarchy to create a summarization of the result, and computing statistical significance for any observed trend. The canonical example of enrichment analysis is in the interpretation of a list of differentially expressed genes in some condition. The usual approach is to perform enrichment analysis with the GO [17]. There are currently over 400 publications on methods and tools for GO-based enrichment, but (to the best of our knowledge) only a single tool, *Genes2Mesh*, uses something other than the GO (i.e. the Medical Subject Headings or MeSH), to calculate enrichment [19].

While GO has been the principal target for enrichment analysis, we can carry out the same sort of profiling using Disease Ontologies. Just as scientists can ask "*Which biological process is over-represented in my set of interesting genes or proteins?*", they also should be able to ask "*Which disease (or class of diseases) is over-represented in my set of interesting genes or proteins?*" For example, by annotating known protein mutations with disease terms from the ontologies in BioPortal, Mort et al. recently identified a class of diseases—blood coagulation disorders—that were associated with a 14-fold depletion in substitutions at O-linked glycosylation sites [20].

There are several resources that can be used as disease ontologies for enrichment analysis. We use the term "disease ontology" to refer to artifacts—terminologies, vocabularies as well as ontologies—that can provide a hierarchy of parent-child terms for disease conditions. One of the most elaborate ontology for diseases is the Systematized Nomenclature for Medicine-Clinical Terms (SNOMED CT) is considered to be the most comprehensive, multilingual clinical healthcare terminology in the world [21]. SNOMED CT was a joint development between the NHS in England and the College of American Pathologists (CAP). It was formed in 1999 by the convergence of SNOMED RT and the United Kingdom's Clinical Terms Version 3 (formerly known as the Read Codes). As of 2007, SNOMED CT is maintained and distributed by the International Health Terminology Standards Development Organization (IHTSDO). Currently, SNOMED CT contains more than 311,000 active concepts with unique meanings and formal logic-based definitions organized into multiple hierarchies. The disease hierarchy is available under the clinical finding root node (analogous to the "biological process" root node in the Gene Ontology). Another widely used disease ontology is the National Cancer Institute thesaurus (NCIt), which is an ontology that provides terms for clinical care, translational and basic research, and public information and administrative

activities. NCIt is a widely recognized standard for biomedical coding and reference, used by a variety of public and private institutions including the Clinical Data Interchange Standards Consortium Terminology (CDISC), the U.S. Food and Drug Administration (FDA), the Federal Medication Terminologies (FMT), and the National Council for Prescription Drug Programs (NCPDP). The disease hierarchy is available under the root node of "Diseases, Disorders and Findings". The most widely *used* disease ontology is the International Classification of Diseases (ICD), which is part of the WHO Family of International Classifications. Version 9 of ICD is widely used in the United States for billing purposes in the health care system. Finally, there is effort to create an ontology of Human Diseases (available at http://diseaseontology.sourceforge.net) that conforms to the principles of the Open Biomedical Ontologies Foundry [22]. The Human Disease ontology is under review by the OBO Foundry since 2006. For the purpose of the current discussion, and enrichment analysis in general, pretty much disease ontology that provides a clear hierarchy of parent-child for diseases would be suitable for use.

Enrichment analysis owes its popularity to the fact that the process is methodologically straightforward and yields these easily interpretable results. Apart from analyzing results of high throughput experiments, enrichment analysis can also be used as an exploratory tool to generate hypotheses for clinical research. Computationally generated annotations (from multiple ontologies) on patient cohorts can provide a foundation for enrichment analysis for risk-factor determination. For example, enrichment analysis can identify general classes of drugs, diseases, and test results that are commonly found in readmitted transplant patients but not in healthy recipients. As noted, the GO has been the principal target for such analysis and despite widespread adoption, GO-based enrichment analysis has intrinsic drawbacks—the primary ones being incompleteness of and bias among available manually created annotations. Below, we discuss recent advances in the use of ontologies for automated creation of annotations that allow us to address these drawbacks and apply enrichment analysis using disease ontologies.

2.1 Advances in Ontology Access and Automated Annotation

There are several recent advances that enable us to use disease ontologies in enrichment analysis. The most obvious advancement is that almost all biomedical ontologies are now available in public repositories such as BioPortal [23]—built as a part of the NIH's Biomedical Information Science and Technology Initiative—which enables the use of terms from multiple ontologies in data analysis workflows. As of this writing, the BioPortal library contains more than 204 publicly accessible biomedical ontologies and their metadata, ranging in domains from genomics to clinical medicine to biomedical software resources, and comprising nearly 1.5 million terms. BioPortal's ontology library includes ontologies that individual investigators submit directly to BioPortal, terminologies drawn from both the Unified Medical Language System (UMLS) and the WHO Family of International Classifications (WHO-FIC). The BioPortal library also includes the ontologies that are candidates to the OBO Foundry, which is an initiative to create a set of well-documented and well-defined reference ontologies that are designed to work with one another to form a single, non-redundant system [22]. In addition to ontologies, BioPortal contains more than 1 million mappings between similar terms in different ontologies and 16.4 billion automatically created annotations on records from 22 public databases of biomedical data. Resources such as BioPortal provides a unified view of all its ontologies, which may be encoded in different formats, each of which has its own purpose, scope, and use. The unified view of the content enables uniform programmatic access to all ontologies and terminologies in the library for use in data analysis workflows.

The availability of automated annotation tools, such as the Annotator Web service from the NCBO and MetaMap from the National Library of Medicine allows the creation ontology-based annotations from free-text descriptions of gene and protein functions (such as GeneRIFs); as a result the lack of preexisting, manually assigned annotations is no longer a bottleneck. For example, the Annotator Web service enables users to provide a textual metadata of an item of interest—such as a GeneRIF describing a gene's function or an abstract corresponding to a PubMed record—to computationally generate ontology-based annotations for the item of interest. The user specifies which ontologies to use, and whether also to use mappings to other ontologies or transitive closure of hierarchy relations to extend the annotations. The service returns the ontology terms that it recognizes from the text—the annotations—and their position in the submitted record.

Finally the availability of large annotation repositories such as the Resource Index, which is a large repository of automatically created annotations by the NCBO, and the NIF database index, which is another large repository of computationally generated annotations on public data sources relevant to neuroscience, provide a source of co-occurrence statistics among ontology-terms in annotations. The availability of such annotation corpora makes the dependence between annotations with different ontology terms explicit.

Given these publicly available sources for ontologies, tools for creating ontology-based annotations and large repositories (or corpora) of annotations, it is now feasible to use disease ontologies in enrichment analysis in a manner similar to the Gene Ontology.

As we have discussed, one key aspect of calculating statistical enrichment is the choice of a reference-term frequency. It is not clear what the appropriate reference-term frequency should be when calculating enrichment of ontology-terms for which a "background set" is not defined. For example, in the case of Gene Ontology annotations, the background set is usually the GO annotations of the set of genes on which the data were collected or the GO annotations of a set of genes known in the genome for the species on which the data were collected. A background set is not available, however, when calculating enrichment using disease ontologies that have not been used for manual annotation in a way the Gene Ontology has. For this situation, there are two main options: 1) to construct a reference set programmatically (discussed in Section 2.3); or 2) use the frequency of particular terms in a large corpus, such as the Resource Index, Medline abstracts or on Web pages indexed by Internet search engines such as Google.

Multiple hypothesis testing—because each term is tested for enrichment individually—is also unavoidable when performing enrichment analysis with disease ontologies. However, methods of correcting the resultant increase in false discovery rates that work in the case of GO based enrichment analyses are directly applicable when using disease ontologies for such analyses.

Several researchers have noted that enrichment analysis is more meaningful when performed for combinations for terms [24]. For example, it is biologically more meaningful to know that a certain

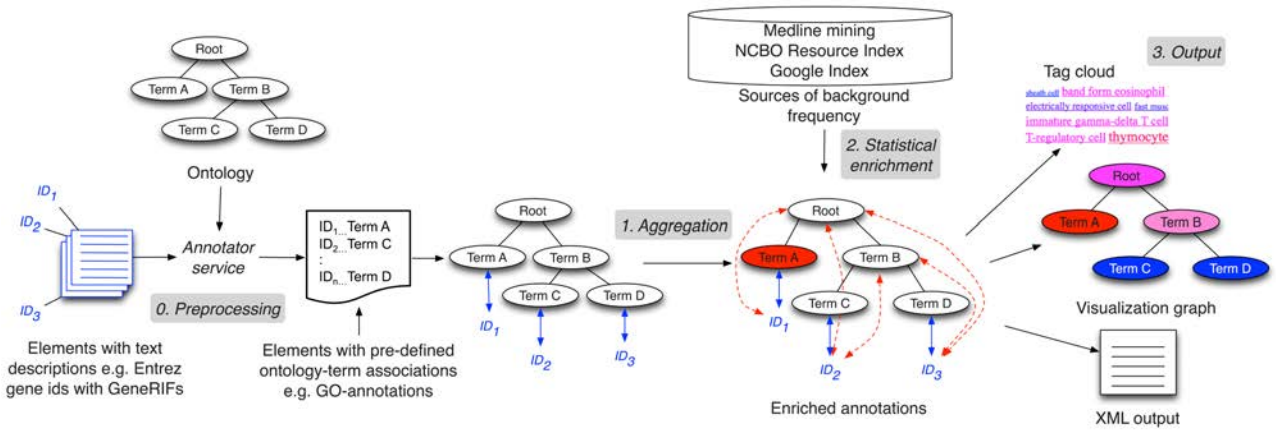

Figure 2. Workflow schematic of enrichment analysis. If the input set has only textual annotations, we first run the Annotator service to create ontology-term annotations. The annotation counts in the input set are first aggregated along the ontology hierarchy and then compared with a background set for a statistically significant difference in the frequency of each ontology term. If a significant difference in the term frequency is found, that term is called "enriched" in the input set of entities. The results of the analysis are returned either as a tag-cloud, a graph, or as an XML output that users can process as required.
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molecular function in a certain *biological process* at a certain *cellular location* is enriched than it is to know about each of the terms separately. Similarly, when using ontologies other than GO, it is more meaningful to look for enrichment of combinations such as certain adverse reactions in a given disease when treated by a particular drug. However, exhaustively examining all possible 3-term combinations of ontology terms is computationally expensive and most of the random term combinations make no biological sense. The identification of combinations that are meaningful and appear at a high enough frequency to justify their use in enrichment computations is an exciting and fruitful area of research.

2.2 DIY Disease Ontology-based Enrichment Analysis Workflow

We have seen that the progress in the current state of the art in storing, accessing and using ontologies for annotation provides components that allow enrichment analysis when preexisting annotations do not exist; as in the case of disease ontologies. We now discuss a workflow to conduct enrichment analysis in domains beyond just expression analysis. A schematic of the workflow is shown in Figure 2.

A user can start with two principal types of inputs. In the first case, the user already has the elements of the dataset of interest annotated with specific ontology terms—i.e. the user already has a file associating element identifiers (gene names, patient ID numbers, etc.) with ontology term identifiers. In the second case, the user has associations of identifiers to textual descriptions instead of ontology terms. For example, a user might have a file associating gene IDs with their GeneRIF descriptions from NCBI. In this situation a user can invoke the NCBO Annotator service [25,26] to process these textual descriptions and assign ontology terms to the element identifiers (*Step 0*). Given the user's selection of an ontology, the annotator processes the input text (say GeneRIFs) to identify concepts that match ontology terms (based on preferred names or synonyms). The implementation details and accuracy of the Annotator service are described in [25]. The result is a list of computationally annotated element identifiers based on the input textual description, and this output is equivalent to the first input type. Using this step, we're able to create ontology-based annotations from free-text descriptions. Thus, we are no longer reliant on the availability of exhaustive manually-curated annotations, such as those required with GO-based analyses.

Step 1 After this optional preprocessing step, for each ontology term in the input dataset one can programmatically traverse the ontology structure and retrieve the complete listing of paths from the concept to the root(s) of the ontology using Web services [27]. A traversal through each of these paths, essentially recapitulates the ontology hierarchy. Each term along the path is associated as an annotation to that element identifier in the input dataset to which the starting term was associated with. This procedure of tracing terms back to the graph's root performs the transitive closure of the annotations over the ontology hierarchy. In essence, for each child-parent (IS_A) relationship, we generate the complete set of implied (indirect) annotations based on child-parent relationships, by traversing and aggregating along the ontology hierarchy.

Step 2 Once the ontology terms and their aggregate frequencies in the input dataset are calculated, we arrive at the step of determining the meaning or significance of the results. Enrichment analysis with GO has benefited from the existence of a natural and easily defensible choice for a background set—all of the given organism's genes, all genes measured on the platform, etc. For most of the disease ontologies we consider, no such comprehensive distribution exists [28]; and as discussed before, for calculating statistical enrichment, we need the background term frequency to determine if the aggregate annotation counts after step 1 are "surprising" given the background. By leveraging existing projects and resources, there are several methods by which a user can address this problem. We discuss a couple of heuristic approaches to address this problem, and in Section 2.3 discuss a systematic process to create custom reference sets.

In the first approach, one can access a database of automatically created annotations over the entirety of MEDLINE abstracts and use these annotations source as an approximate proxy for the true "background distribution" frequency of a specific term. To generate the background frequency, for a given term X, we retrieve the text strings corresponding to its preferred name and all of its synonyms, and then add up the MEDLINE occurrence counts for each of these strings. We

Figure 3. Tag cloud output: An example for the annotations of grants from FY1981 using SNOMEDCT. Blue denotes low-frequency terms and red denotes highly frequent terms. Many concepts, such as "neoplasm of digestive tract", occur at high frequencies in most years, possibly denoting the constant focus on cancer research. An appropriate background term frequency distribution is necessary to determine significance of the high frequency.
doi:10.1371/journal.pcbi.1002827.g003

return this number (m) as well as the total number of entries in the MEDLINE annotation database (M). The fraction m/M then represents the background frequency of the term X in the annotated corpus. Using this frequency we can compute significant comparative over- or under-representation in the input dataset.

The second approach uses NCBO's Resource Index, which is a repository of automatically-created annotations. Access to the Resource Index allows a user to make the same sort of calculations as with the MEDLINE term frequencies, but also offers information on the co-occurrence of ontological terms in textual descriptions and annotations of datasets; enabling the user to quantify the degree to which terms are independent or correlated in the annotation space.

Step 3 There are several possible output mechanisms to such an analysis workflow. The simplest is a tag cloud, which intuitively summarizes the results of the analysis (Figure 3). The sizes and colors of terms in the cloud indicate the relative frequency of the terms offering a high-level overview. However, a tag cloud's representative ability is limited because there is no easy way to show significance relative to some expectation, or to show the elements in the input associated with some term.

The second output format is in XML, which is amenable to postprocessing by the user, as needed. The result for each term contains its respective frequency information in the input data along with the counts on which the frequency is based. The results on each term can also contain the list of identifiers that mapped to that term. Each node includes information on the level in the ontology at which the term is found. Using such an output, it is straight forward to create graphical visualizations similar to those that most GO based enrichment analysis tools provide [29]; see example in Figure 4.

2.2.1. Ensuring quality. For any such custom analysis workflow it is essential to set up tests that ensure technical accuracy before interpreting the results for scientific significance. To evaluate technical accuracy, we suggest that users create benchmark data sets similar to those of Toronen and colleagues [30], who created gene lists with a selected enrichment level and a selected number of independent, over-represented classes to compare different GO-based enrichment methods. In the case of analyses using disease ontologies, the benchmark data sets would comprise gene lists enriched for specific disease terms, clinical-trial lists enriched for a specific drug being studied; lists of research publications that are enriched for known NCIt terms, and so on. A sample benchmark list of *aging* related genes and their annotations is provided in Section 5. Exercises. This dataset was compiled by computationally creating disease term annotations on 261 human genes designated to be related to aging according to the GeneAge database [31]. The annotations of this gene list are enriched for disorders, such as atherosclerosis, that are known to be associated with aging. Such benchmark data sets can be used to ensure accuracy of the enrichment statistics as well as to evaluate the appropriateness of different sources of reference-term frequencies for computing enrichment.

The inconsistency of abstraction levels in ontologies is an often discussed stumbling block for enrichment analysis [17]. Two terms at equal depths may not represent concepts of similar granularity, creating a bias in the reported term enrichment. By comprehensively analyzing the frequencies of terms in MEDLINE and the NCBO Resource Index, a user can perform a thorough analysis of dependencies among ontology-term annotations to make existing biases explicit as well as to define custom abstraction levels using methods developed by Alterovitz et al. [32]. The development of methods to reliably identify the appropriate level of abstraction at which to report the results of enrichment analysis is another exciting and fruitful area of research.

2.3 Creating Reference Sets for Custom Enrichment Analysis

As discussed before, a key pre-requisite for performing enrichment analysis is the availability of an appropriate reference dataset to compare against when looking for over- or under-represented terms. In this section, we describe: (i) a general method that uses hand-curated GO annotations as a starting point, for creating reference datasets for enrichment analysis using other ontologies; and (ii) a gene–disease reference annotation dataset for performing disease-based enrichment.

GO annotations are unique because highly trained curators associate GO terms to gene products manually, based on literature review. We describe how, with the availability of tools for automatic ontology-based annotation with terms from disease ontologies, it is possible to create reference annotation datasets for enrichment analysis using ontologies other than the GO—for example, the Human Disease Ontology.

Unlike GO terms, which actually appear in the text with low frequency, or gene identifiers, which are ambiguous, disease terms are amenable to automated, term extraction techniques. Therefore, using tools which recognize mentions of ontology terms in user submitted text, we can automatically recognize occurrences of terms from the Human Disease Ontology (DO) from a given corpus of text [28]; the key is to identify the text source that can be relied upon to recognize disease terms to associate with genes.

Unlike other natural-language techniques for finding gene–disease associations, our proposed method uses manually curated GO annotations as the starting basis to identify the text source from which to recognize disease terms. Basically, we use manually curated GO annotations to identify those publications that were the

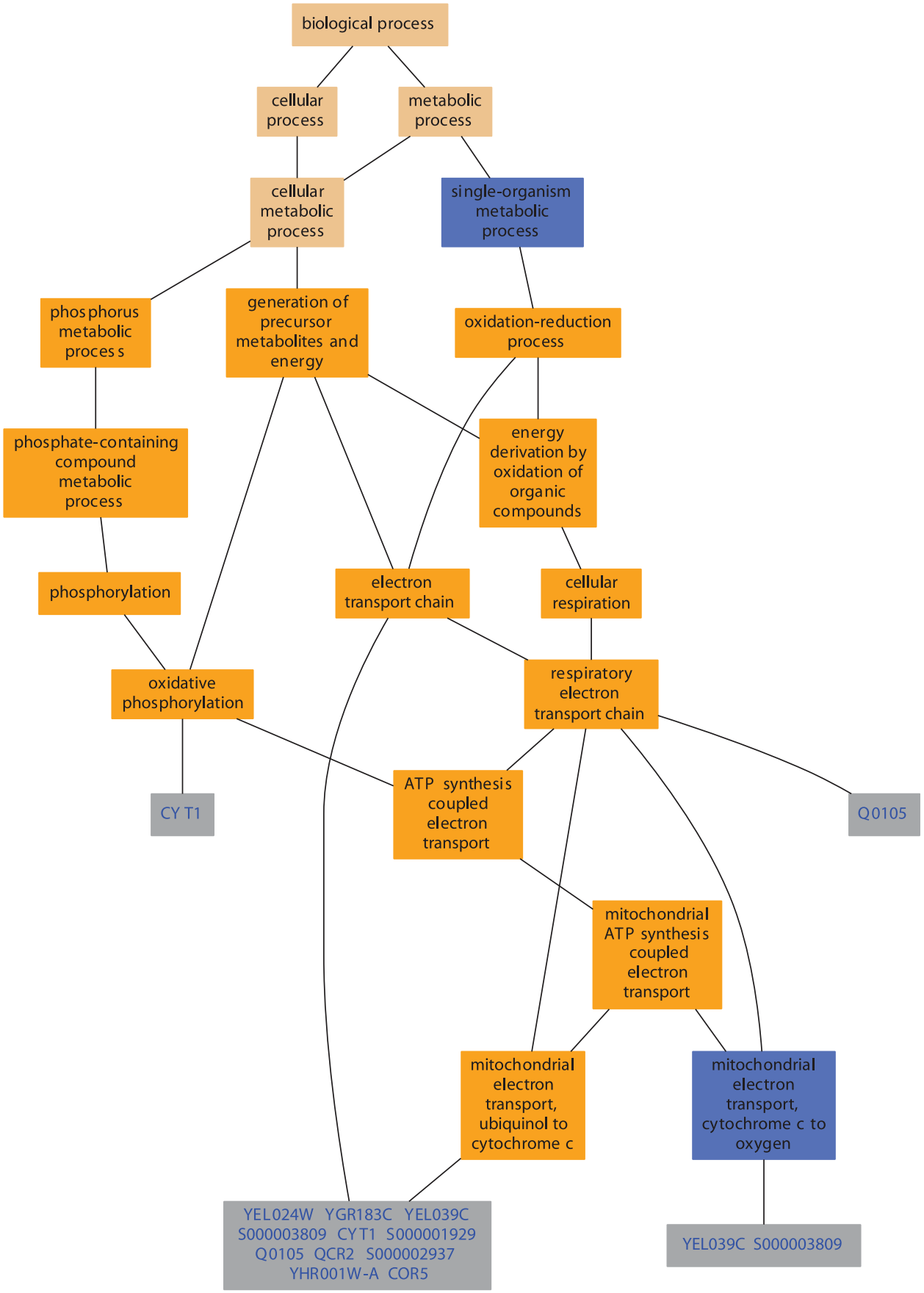
biological process
cellular process
metabolic process
cellular metabolic process
single-organism metabolic process
phosphorus metabolic process
generation of precursor metabolites and energy
oxidation-reduction process
phosphate-containing compound metabolic process
energy derivation by oxidation of organic compounds
phosphorylation
electron transport chain
cellular respiration
oxidative phosphorylation
respiratory electron transport chain
CYT1
ATP synthesis coupled electron transport
Q0105
mitochondrial ATP synthesis coupled electron transport
mitochondrial electron transport, ubiquinol to cytochrome c
mitochondrial electron transport, cytochrome c to oxygen
YEL024W YGR183C YEL039C S000003809 CYT1 S000001929 Q0105 QCR2 S000002937 YHR001W-A COR5
YEL039C S000003809

Figure 4. The figure shows a visualization generated using the GO TermFinder tool. The GO graph layout shows the significantly enriched GO terms in the annotations of the analyzed gene set. The color of the nodes is an indication of their Bonferroni corrected P-value (orange $<=$ 1e-10; yellow 1e-10 to 1e-8; green 1e-8 to 1e-6; cyan 1e-6 to 1e-4; blue 1e-4 to 1e-2; tan $>$0.01).
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basis for associating a GO term with a particular gene.

Figure 5 summarizes our method. First, we start with GO annotations, which provide the PubMed identifiers of papers based on which gene products are associated with specific GO terms by a curator. The annotations essentially give us a link between gene identifiers and PubMed articles and only those PubMed articles that were deemed to be relevant for GO annotation curation. Next, we recognize terms from an ontology of interest (e.g. Human Disease) in the title and abstracts of those articles. Finally, we associate the recognized ontology terms with the gene identifiers to which the article analyzed was associated.

In order to demonstrate feasibility of the proposed workflow and to provide a sample reference annotation set for performing disease ontology based analyses in the exercises of this chapter, we download GO annotation files for human gene products from geneontology.org. These files are tab-delimited text files that contain, among other things, a list of gene identifiers, associated GO terms, and the publication source (a PubMed identifier) on the basis of which that GO annotation was created. We removed all electronically inferred annotations (IEA) from the annotation file. We also removed all qualified annotations, such as negated (NOT) ones. As a result, we obtain a list of publications and the genes they describe, gene–publication tuples. In the next step, using the PubMed identifiers obtained from the GO annotation files, we fetch each article's title and abstract using the National Library of Medicine eUtils. We save each article's title and abstract as a file and annotate it via the Annotator service using the disease ontology as the target. Once we have the publication–disease tuples, we cross-reference them with the gene–publication tuples resulting in gene–disease associations for 7316 human genes.

Out of 25,000 currently estimated human genes, we are able to annotate 7316 genes (29.2%) with at least one disease term from the Human Disease Ontology. Previous methods that use advanced text mining have been able to annotate 4408 genes (17.7%) [33]. A study based on OMIM associated 1777 genes (7.1%) with disease terms to create a human "diseasome" [34] and an automated approach using MetaMap as the concept recognizer and GeneRIFs as well as descriptions from OMIM as the input textual descriptions annotated roughly 14.9% of the human genome with disease terms [28]. Because the number of human genes known at the time of each study varies, we make the comparisons loosely.

In order to validate our background annotation set, we evaluated our gene–disease association dataset in several ways described in [35]. First, we examined a set of genes related specifically to aging from the GenAge database [31] for their coherence in terms of the assigned disease annotations. Next, we performed disease-based enrichment analysis on the same aging related gene set using our newly created reference annotation set. The results of the enrichment analysis are shown in Figure 6 and the analysis itself is offered as an exercise for the reader in Section 6. Exercises. What differentiates our suggested method from other approaches [28,36] for finding gene–disease associations is the use of GO annotations as a basis for identifying reliable gene–publication records that serve as the foundation for generating automated annotations. Furthermore, researchers can reuse our method to examine function along other dimensions. For example, researchers can use the Pathway ontology to generate gene–pathway associations.

2.3.1 Ensuring quality. When using an automated annotation process to create a reference annotation set, there are some caveats to consider. First, not all ontologies are equally suited for creating automated annotations. Second, automated annotation depends highly on the quality of the input text corpus. Third, some errors in annotation are inevitable in an automated process. We discuss these issues below.

Using other ontologies. Although we specifically focus on creating annotations with terms from the human disease ontology, the method we have devised (Figure 6) can create annotations with terms from other ontologies. In the presented workflow, to obtain a background dataset for enrichment for some ontology other than DO, researchers would simply configure a parameter for the Annotator Web service to use their ontology of choice from BioPortal. In fact, other researchers have used a similar annotation workflow to

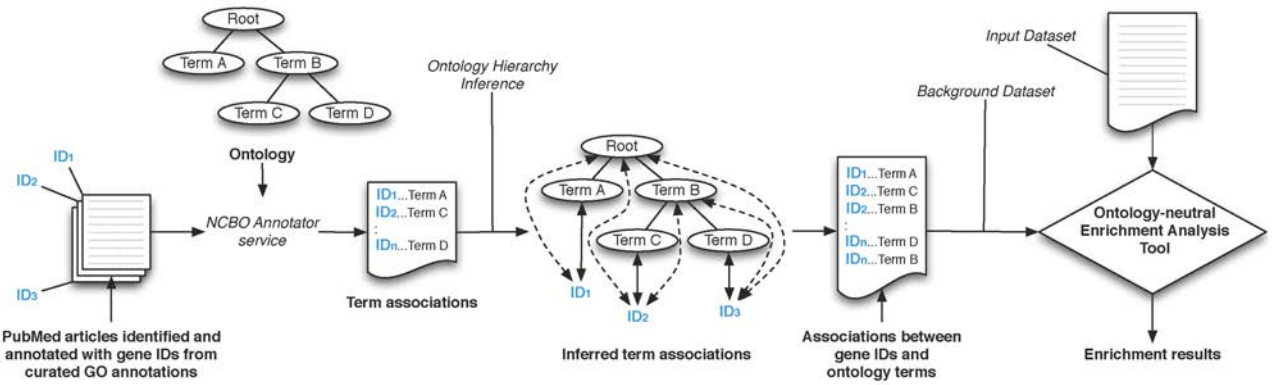

Figure 5. Workflow for generating background annotation sets for enrichment analysis: We obtain a set of PubMed articles from manually curated GO annotations, which we process using the NCBO Annotator service.
doi:10.1371/journal.pcbi.1002827.g005

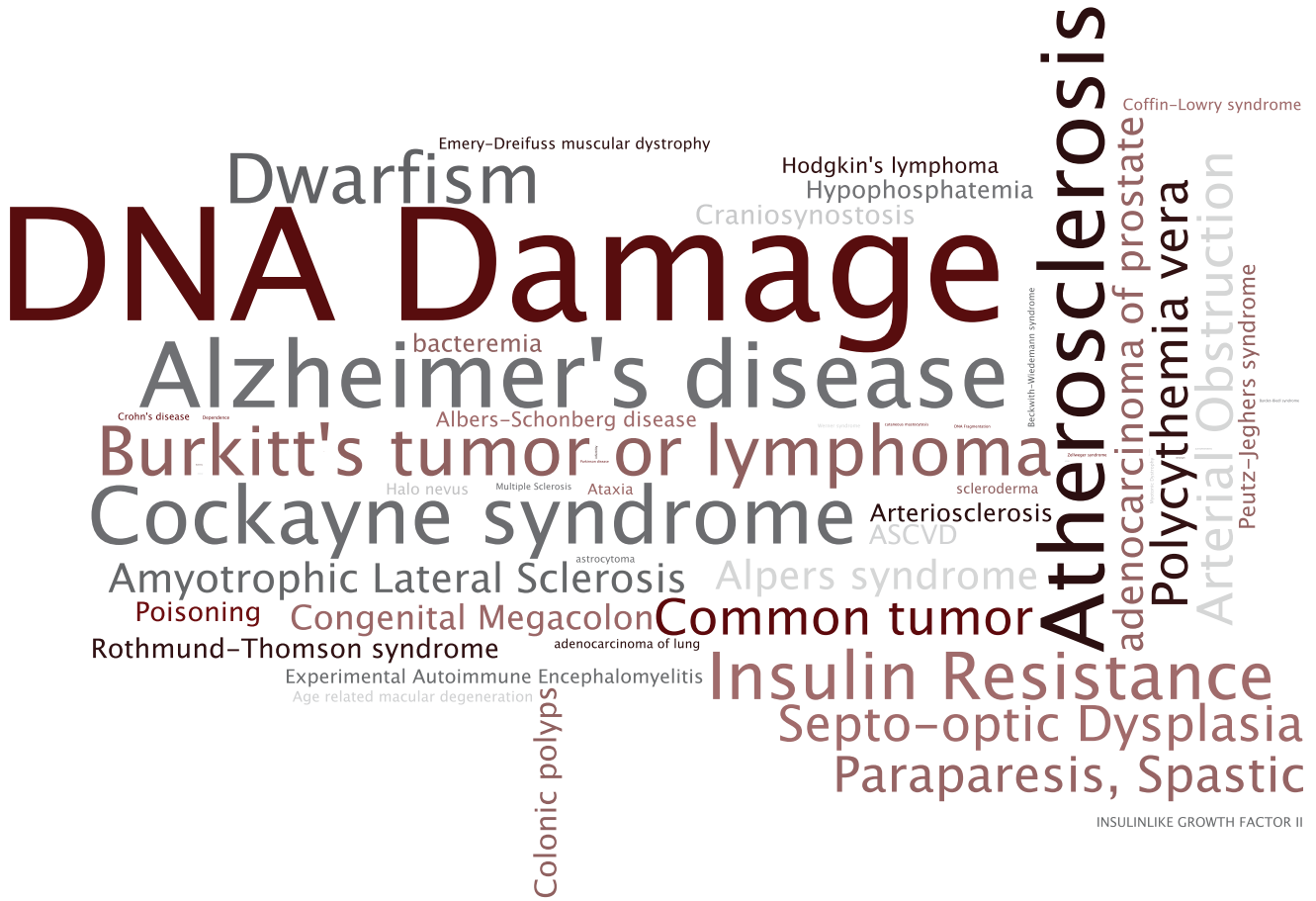

Figure 6. Disease terms significantly enriched in annotations of aging-related genes: The tag cloud shows those disease terms in the annotations of the 261 aging related genes that are statistically enriched given our gene–disease background annotation dataset. Terms that are significantly enriched appear larger. We used a binomial test to detect enriched disease terms in the aging related gene set. Note that mis-annotated terms (such as Recruitment) and non-informative terms (such as Disease) are not deemed enriched by the statistical analysis.
doi:10.1371/journal.pcbi.1002827.g006

recognize morphological features in textual descriptions of fish species [37].

Not all ontologies are viable candidates for automatic annotation because not all ontology terms appear in the text of a MEDLINE abstract. For example, using term–frequency counts in MEDLINE abstracts [38], we calculated that disease terms are mentioned 46% more often than GO terms in MEDLINE abstracts. As another example, only 10% of the manually assigned GO terms can be detected directly in the paper abstract supporting that particular GO annotation. Because disease terms are mentioned significantly more often than GO terms, the automated annotation process works well for annotating genes with disease ontology terms.

Missing annotations. Out of the 261 aging-related genes in our evaluation subset, the Annotator left out 24 genes (9%), for which we have no disease terms associated with those genes in our gene–disease association dataset. These missed annotations provide an opportunity for refining the annotation workflows to use sources of text beyond just the papers referenced in GO annotations.

Annotation errors. Some errors in annotation are inevitable in an automated process. For example, in the reference annotation set we created, TP53 was also annotated, wrongly, to "Recruitment". Papers that were the basis of creating GO annotations for TP53 certainly mention the term "Recruitment"; however that term is not a *disease*. The term "Recruitment" is in the Human Disease Ontology and is declared to be a synonym of "auditory recruitment", which does not have an asserted superclass, or a place in the hierarchy indicating a possible error in the ontology. However, because such errors will affect annotation of both the set of interest and the reference set equally, the errors will most likely cancel each other out when computing statistical enrichment (Figure 6)—though that is not guaranteed. Advanced text mining can potentially provide checks against such kinds of errors by analyzing the context in which a potential disease term is mentioned.

3. Novel Use Cases Enabled

We believe that extending the current enrichment-analysis methods to ontologies beyond GO and to extending the method beyond analyzing gene and protein annotations to any set of entities for term enrichment will enable several novel use cases. For example, a user might analyze a set of papers published in the last three years in a particular domain (say, signal transduction) and identify which pathway was mentioned most frequently. Similarly, a user could analyze descriptions of genes controlled by a particular ultra-conserved region of DNA to generate hypotheses about the region's function in

specific disease processes. We discuss the potential of some of the novel use cases enabled by disease ontology based enrichment analysis.

Analysis of protein annotations To demonstrate the feasibility of performing enrichment analysis and recovering known GO annotations as well as to demonstrate enrichment analysis with multiple ontologies, we analyzed a list of 261 known aging related genes from the GenAge database [31]. We started by collecting textual descriptions for UniProt protein entries corresponding to each human gene in the GenAge database. The textual descriptions included the protein name, gene name, general descriptions of the function and catalytic activity as well as keywords and GO terms. We processed this text as described in the workflow in Figure 6 and created annotations from Medical Subject Heading (MSH), Online Mendelian Inheritance in Man (OMIM), UMLS Metathesaurus (MTH) and Gene Ontology (GO).

We created a background set of annotations on 19671 proteins by applying the same protocol to manually annotated and reviewed proteins from SwissProt (Jan 2010 version). We calculated enrichment and depletion of specific terms, corrected for multiple hypotheses and obtained a list of significant terms for all four ontologies. Not surprisingly, 'aging' is an enriched term. There were several other terms enriched such as 'electron transport' (2.79e-10), 'protein kinase activity' (2.8e-10) and 'nucleotide excision repair' (8.78e-07) which appeared in MSH, MTH, and GO. The enriched terms also included aging associated diseases such as 'Alzheimer's disease' (0.01), 'Werner syndrome' (5.3e-05), 'Diabetes Mellitus' (1.5e-04) and 'neurodegeneration' (2.5e-03) from OMIM.

This case study demonstrate that enrichment analysis with multiple ontologies is feasible and it enables a comprehensive characterization of the biological "signal" present in gene/protein lists [39]. For example, by annotating known protein mutations with disease terms from the ontologies in BioPortal, Mort et al. recently identified a class of diseases—blood coagulation disorders—that were associated with a 14-fold depletion in substitutions at O-linked glycosylation sites [20].

Analysis of funding trends To demonstrate the feasiblity of such analyses in a novel domain, we processed the funding allocations of the NIH in fiscal years 1980–1989. We aimed to identify trends in institutional funding priorities over time, as represented by changes in the relative frequencies of ontology concepts from year-to-year. Using a database containing the complete set of grants in this interval—with their titles, amounts, recipient institutions, etc.—we selected grants worth over $1,250,000 (in constant 2008 dollars). We annotated the titles of these grants with SNOMEDCT terms and used the annotation sets to generate tag clouds for each year, such as the one shown in Figure 3 for year 1981, to create a visual summary of funding trends on a per year basis. Further analysis cross-linking annotation on grants with annotations on publications from specific institutions can enable comparative analysis of the research efficacy at different institutions.

Hypothesis generation for Clinical Research Finally, enrichment analysis can also be used as an exploratory tool to generate hypotheses for clinical research by analyzing annotations on medical records in conjunction with annotation of molecular datasets. For example, in the case of kidney transplants, extended-criteria donor (ECD) organs have a 40% rate of delayed graft function and a higher incidence of rejection compared to standard-criteria donor (SCD) kidneys. Identifying causes of this difference is crucial to identify patients in which an ECD transplant has a high chance of working.

At several research sites, the datasets collected to address this question comprise immunological metrics beyond the standard clinical risk factors, including multiparameter flow-cytometric analysis of the peripheral immune-cell repertoire, genomic analysis, and donor-specific functional assessments. These patient data sets can be annotated using automated methods [8,26] to enable enrichment analysis for risk-factor determination.

For example, simple enrichment analysis might allow identification of classes of drugs, diseases, and test results that are commonly found only in readmitted transplant patients. Enrichment analysis to identify common *pairs* of terms of different semantic types can identify combinations of drug classes and comorbidities, or test risk-factors and comorbidities that are common in this population.

4. Summary

Because enrichment analysis with GO is widely accepted and scientifically valuable, we argue that the logical next step is to extend this methodology to other ontologies—specifically disease ontologies.

Given the recent advances in ontology repositories and methods of automated annotation, we argue that enrichment analysis based on textual descriptions is possible.

We have systematically discussed how to accomplish enrichment analysis using ontologies other than the Gene Ontology as well as address some of the limitations of existing analysis methods. For example, the roughly 20% of genes that lack annotations can now be associated, via their GeneRIFs, with terms from disease ontologies. We have outlined possible directions of research for overcoming other limitations such as inconsistent abstraction levels in ontologies, performing the analysis using combinations of ontology terms, and accounting for annotation bias.

In order to perform enrichment analysis using ontologies other than the GO, a key pre-requisite is the availability of a background set of annotations to enable the enrichment calculation. We have described a general method, which uses hand-curated GO annotations as a starting point, for creating background datasets for enrichment analysis using other ontologies—such as the Human Disease Ontology, for which hand-curated annotations are not available.

To demonstrate the feasibility and utility of our proposals, we have created a background set of annotations to enable enrichment analysis with the Human Disease Ontology and validated that background set by using the created annotations to examine the coherence of known aging related genes and by performing enrichment analysis on an aging related gene set from the GeneAge database [31]. We make the set of aging related genes and the reference annotation set available for reader exercises in enrichment analysis.

We argue that enrichment analysis using computationally created ontology-based annotations from textual descriptions is possible, thus introducing enrichment analysis as a research methodology in new domains such as hypothesis generation for clinical research; without requiring manually created annotations.

5. Exercises

(1) For the 260 aging related genes in Dataset S1, perform enrichment analysis using the Human Disease ontology, using Dataset S2 as the reference annotation set. Some considerations while working through the problem:

- The genes are listed with their UniprotIDs.
- Using the notation in Section 1.1, the values of N and M are the total number of unique genes in the aging set and total set, respectively, and *not* the number of unique terms. The values of n and m are the unique genes that are annotated with a given term in the corresponding set.
- When performing the hypergeometric test, if the test calculates the p value based on finding a value of n greater than or less than what was observed (instead of equal to what was observed), remember to add or subtract 1 from the number of genes annotated with a given term when calculating. If you are using a function to calculate, refer to the documentation to understand the input required.
- Consider from which tail of the hypergeometric distribution you wish to calculate the p value.

(2) For the 260 aging related genes, perform enrichment analysis using SNOMED-CT (Systematized Nomenclature of Medicine-Clinical Terms). Use the GeneRIF (Gene Reference into Function) database as the source text to annotate with disease terms from SNOMED-CT. Choose an appropriate reference annotation set and justify the choice. Some considerations while working through the problem:

- An index of GeneRIFs, maintained by the National Center for Biotechnology Information (NCBI) and the National Institutes of Health (NIH), can be downloaded from here: ftp://ftp.ncbi.nih.gov/gene/GeneRIF/
- Mapping from UniprotIDs to GeneIDs, which are used in the GeneRIF database, can be done here: http://www.uniprot.org/help/mapping. Note that you will get 261 GeneIDs for the 260 UniprotIDs.
- Annotation using the National Center for Biomedical Ontology's BioPortal Annotator Service requires obtaining an API key. This can be done after registration and going to "Account" where your API key will be displayed: http://bioportal.bioontology.org/
- Information on the programmatic use of the BioPortal Annotator as a client can be found here: http://www.bioontology.org/wiki/index.php/Annotator_Web_service. Example code from numerous languages, including Java, R, Python, Ruby, Excel, HTML, and Perl, can be found here: http://www.bioontology.org/wiki/index.php/Annotator_Client_Examples. All NCBO REST Web services require the parameter "apikey = YourApiKey". It is strongly encouraged that all users of the NCBO Annotator Web service use only the **virtual ontology identifier**. To do so, set the "isVirtualOntolgyId" parameter to "true". This will ensure that you access the version of the ontology that is actually in the database. Failure to do this will result in your code breaking every time the database is updated.
- Output from the annotation service can be conveniently parsed in XML. To see an example of what this might look like, visit http://bioportal.bioontology.org/annotator. Insert the sample text or use text of your choice, makes selection(s) under 'Select Ontologies' and 'Select UMLS Semantic Types' and click 'Get Annotations'. At the bottom by 'Format Results As' you can select XML to see the XML tree structure of the Annotator output.
- The suggested ontology for this exercise is SNOMED-CT (ontology ID: 1353) and semantic types Anatomical Structure (T017), Disease or Syndrome (T047), Neoplastic Process (T191), and NCBO BioPortal concept (T999).
- Some processing of the GeneRIF text may be necessary to prevent errors in annotation. It is suggested to remove GeneRIFs with new line characters ('\n') and replace single or double quotes with white space.
- Many GeneIDs have multiple GeneRIF entries. The user will find more efficient annotation if all of the GeneRIF entries for a given gene are concatenated and passed to the annotator instead of annotating individual GeneRIF entries for the same gene.
- Due to the large number of GeneRIFs, the BioPortal Annotator may timeout while the user is looping through genes to annotate. It is suggested that the annotation is done incrementally and joined or intermittent saves of the annotations is done to prevent timely re-annotation.
- The given set of aging genes will have considerably more annotations terms per gene than the set of all genes in the GeneRIF database. This bias should be a consideration when deciding on an appropriate M. There are numerous approaches to address this, and a simple method may be to limit the reference set of genes M to only those with at least a given number of annotated terms. You may also want to limit the results to only those terms that appear at least a given amount of times in the aging gene annotations.

Answers to the Exercises can be found in Text S1.

Further Reading

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Supporting Information

Dataset S1 Data file for Exercise 1 (TXT)

Dataset S2 Data file for Exercise 1 (TXT)

Dataset S3 Data file for Exercise 1 (OBO)

Dataset S4 Additional info on genes mentioned in S1 and S2. Can be used in lieu of GeneRIFs in Exercise 2. (TXT)

Text S1 Answers to Exercises (DOCX)

Table S1 Exercise 1 analysis results (CSV)

Table S2 Exercise 2 analysis results (CSV)

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Education

Chapter 10: Mining Genome-Wide Genetic Markers

Xiang Zhang[1], Shunping Huang[2], Zhaojun Zhang[2], Wei Wang[3]*

1 Department of Electrical Engineering and Computer Science, Case Western Reserve University, Ohio, United States of America, **2** Department of Computer Science, University of North Carolina at Chapel Hill, North Carolina, United States of America, **3** Department of Computer Science, University of California at Los Angeles, California, United States of America

Abstract: Genome-wide association study (GWAS) aims to discover genetic factors underlying phenotypic traits. The large number of genetic factors poses both computational and statistical challenges. Various computational approaches have been developed for large scale GWAS. In this chapter, we will discuss several widely used computational approaches in GWAS. The following topics will be covered: (1) An introduction to the background of GWAS. (2) The existing computational approaches that are widely used in GWAS. This will cover single-locus, epistasis detection, and machine learning methods that have been recently developed in biology, statistic, and computer science communities. This part will be the main focus of this chapter. (3) The limitations of current approaches and future directions.

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1. Introduction

With the advancement of genotyping technology, genome-wide high-density single nucleotide polymorphisms (SNPs) of human and other organisms are now available [1,2]. The goal of genome-wide association studies (GWAS) is to seek strong associations between phenotype and genetic variations in a population that represent (genomically proximal) causal genetic effects. As the most abundant source of genetic variation, millions of SNPs have been genotyped across the entire genome. Analyzing such large amount of markers poses great challenges to traditional computational and statistical methods. In this chapter, we introduce the basic concept of genome-wide association study, and discuss recently developed methods for GWAS.

Genome-wide association study is an inter-discipline problem of biology, statistics and computer science [3,4,5,6]. In this section, we will first provide a brief introduction to the necessary biological background. We will then formalize the problem and discuss both traditional and recently developed methods for genome-wide analysis of associations.

A human genome contains over 3 billion DNA base pairs. There are four possible nucleotides at each base in the DNA: adenine (A), guanine (G), thymine (T), and cytosine (C). In some locations in the genome, a genetic variation may be found which involves two or more nucleotides across different individuals. These genetic variations are known as *single-nucleotide polymorphism* (SNPs), i.e., a variation of a single nucleotide in the DNA sequence. In most cases, there are two possible nucleotides for a variant. We denote the more frequent one as "0", and the less frequent one as "1". For bases on autosomal chromosomes, there are two parallel nucleotides, which leads to three possible combinations, "00", "01" and "11". These genotype combinations are known as "major homozygous site", "heterozygous site" and "minor heterozygous site" respectively. These genetic variations contribute to the phenotypic differences among the individuals. (A phenotype is the composite of an organism's observable characteristics or traits.) Genome-wide association study (GWAS) aims to find strong associations between SNPs and phenotypes across a set of individuals.

More formally, let $X = \{X_1, X_2, \cdots, X_N\}$ be the set of N SNPs for M individuals in the study, and Y be the phenotype of interest. The goal of GWAS is to find SNPs (markers) in X, that are highly associated with Y. There are several challenging issues that need to be addressed when developing an analytic method for GWAS [7,8].

Scalability Most GWAS datasets consist of a large number of SNPs. Therefore the algorithms for GWAS need to be highly scalable. For example, for a typical human GWAS, the dataset may contain up to millions SNPs and involve thousands of individuals. Inefficient methods may consume a large amount of computational resources and time to find highly associated SNPs.

Missing markers Even with the current dense genotyping technique, many genetic variants are still not genotyped. Current methods usually assume genetic linkage to enhance the power. Imputation, which tries to impute the unknown markers by using existing SNPs databases, is another popular approach to handle missing markers. The well known related projects include the International HapMap project [9] and the 1000 Genomes Project [10].

Complex traits One approach in GWAS is to test the association between the trait and each marker in a genome, which is successful in detecting a single gene related disease. However, this approach may have problems in finding markers associated with complex traits. This is because that complex traits are affected by multiple genes, and each gene may only have a weak association with the phenotype. Such markers with low marginal effects are hard to detect by the single-locus methods.

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* E-mail: weiwang@cs.ucla.edu

What to Learn in This Chapter

- The background of Genome-wide association study (GWAS).
- The existing computational approaches that are widely used in GWAS. This will cover single-locus, epistasis detection, and machine learning methods.
- The limitations of current approaches and future directions.

In the remainder of the chapter, we will first discuss the single-locus methods. We will then study epistasis detection (multi-locus) approaches which are designed for association studies of complex traits. For epistasis detection, we will mainly focus on exact two-locus association mapping methods.

2. Single-Locus Association Mapping

As the rapid development of high-throughput genotyping technology, millions of SNPs are now available for genome-wide association studies. Single-locus association test is a traditional way for association studies. Specifically, for each SNP, a statistical test is performed to evaluate the association between the SNP and the phenotype. A variety of tests can be applied depending on the data types. The phenotype involved in a study can be case-control (binary), quantitative (continuous), or categorical. We categorize the statistical tests based on what kind of phenotypes they can be applied on.

2.1 Problem Formalization

Let $\{X_1,\cdots,X_N\}$ be a set of N SNPs for M individuals and $X_n=\{X_{n1},\cdots,X_{nM}\}$ $(1\le n\le N)$. We use 0, 1, 2 to represent the homozygous major allele, heterozygous allele, and homozygous minor allele respectively. Thus we have that $X_{nm}\in\{0,1,2\}$ $(1\le n\le N, 1\le m\le M)$. Let $Y=\{y_1,\cdots,y_M\}$ be the phenotype. Note that the values that Y can take depend on its type.

2.2 Case-Control Phenotype

In a case-control study, the phenotype can be represented as a binary variable with 0 representing controls and 1 representing cases.

A contingency table records the frequencies of different events. Table 1 is an example contingency table. For a SNP X_n and a phenotype Y, and we use O_{ij} to denote the number of individuals whose X_n equals i and Y equals j. Also, we have $O_{i\cdot}=\sum_j O_{ij}$ and $O_{\cdot j}=\sum_i O_{ij}$. The total number of individuals $S=\sum_{i,j} O_{ij}$.

Many tests can be used to assess the significance of the association between a single SNP and a binary phenotype. The test statistics are usually based on the contingency table. The null hypothesis is that there is no association between the rows and columns of the contingency table.

2.2.1 Pearson's χ^2 test. Pearson's χ^2 test can be used to test a null hypothesis stating that the frequency distribution of certain events observed in a sample is consistent with a particular theoretical distribution [11].

The value of the test statistic is

$$X^2=\sum_i\sum_j\frac{(O_{ij}-E_{ij})^2}{E_{ij}},$$

where $E_{ij}=\frac{O_{i\cdot}O_{\cdot j}}{S}$. The degree of freedom is 2.

2.2.2 G-test. G-test is an approximation of the log-likelihood ratio. The test statistic is

$$G=2\sum_i\sum_j O_{ij}\cdot ln(\frac{O_{ij}}{E_{ij}}),$$

where $E_{ij}=\frac{O_{i\cdot}O_{\cdot j}}{S}$.

The null hypothesis is that the observed frequencies result from random sampling from a distribution with the given expected frequencies. The distribution of G is approximately that of χ^2, with the same degree of freedom as in the corresponding χ^2 test. When applied to a reasonable size of samples, the G-test and the χ^2 test will lead to the same conclusions.

2.2.3 Fisher exact test. When the sample size is small, the Fisher exact test is useful to determine the significance of the association. The p-value of the test is the probability of the contingency table given the fixed margins. The probability of obtaining such values in Table 1 is given by the hypergeometric distribution:

$$p=\frac{\binom{O_{\cdot 0}}{O_{00}}\binom{O_{\cdot 1}}{O_{01}}\binom{O_{\cdot 2}}{O_{02}}}{\binom{S}{O_{0\cdot}}}=\frac{(O_{\cdot 0}!O_{\cdot 1}!O_{\cdot 2}!)(O_{0\cdot}!O_{1\cdot}!)}{S!(O_{00}!O_{01}!O_{02}!O_{10}!O_{11}!O_{12}!)}$$

Most modern statistical packages can calculate the significance of Fisher tests. The actual computation performed by the existing software packages may be different from the exact formulation given above because of the numerical difficulties. A simple, somewhat better computational approach relies on a gamma function or log-gamma function. How to accurately compute hypergeometric and binomial probabilities remains an active research area.

2.2.4 Cochran-Armitage test. For complex traits, contributions to disease risk from SNPs are widely considered to be roughly additive. In other words, the heterozygous alleles will have an intermediate risk between two homozygous alleles. Cochran-Armitage test can be used in this case [12,5]. Let the test statistic of U be the following:

$$U=O_{1\cdot}O_{0\cdot}\sum_{i=0}^{2} i\cdot(\frac{O_{1i}}{O_{1\cdot}}-\frac{O_{0i}}{O_{0\cdot}})$$

After substitution, we get

$$U=S\cdot(O_{11}+2O_{12}-O_{1\cdot}\cdot(O_{\cdot 1}+2O_{\cdot 2})$$

The variance of U under the null hypothesis can be computed as

$$Var(U)=\frac{(S-O_{1\cdot})O_{1\cdot}}{S}[S(O_{\cdot 1}+4O_{\cdot 2})-(O_{\cdot 1}+2O_{\cdot 2})^2]$$

Notice that for a large sample size S, we have $\frac{U}{\sqrt{Var(U)}}\sim N(0,1)$, hence $\frac{U^2}{Var(U)}\sim\chi_1^2$.

2.2.5 Summary. There is no overall winner of the introduced tests. Cochran-Armitage test may not be the best if the risks are deviated from the additive model. Meanwhile, χ^2 test, G-test, and Fisher exact test can handle the full range of risks, but they will unavoidably lose some power in the detection of additive ones. Different tests may be applied on the same data to detect different effects.

Table 1. Contingency table for a single SNP X_n and a phenotype Y.

	$X_n=0$	$X_n=1$	$X_n=2$	**Totals**
$Y=0$	O_{00}	O_{01}	O_{02}	$O_{0\cdot}$
$Y=1$	O_{10}	O_{11}	O_{12}	$O_{1\cdot}$
Totals	$O_{\cdot 0}$	$O_{\cdot 1}$	$O_{\cdot 2}$	S

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2.3 Quantitative Phenotype

In addition to case-control phenotypes, many complex traits are quantitative. This type of study is also often referred to as the quantitative trait locus (QTL) analysis. The standard tools for testing the association between a single marker and a continuous outcome are analysis of variance (ANOVA) and linear regression.

2.3.1 One-way ANOVA. The F-test in one-way analysis of variance is used to assess whether the expected values of a quantitative variable within several predefined groups differ from each other.

For each SNP X_n, we can divide all the individuals into three groups according to their genotypes. Let $Y_i'(i\in\{0,1,2\})$ be a subset of phenotypes of which the individuals have the genotypes equal to i. We represent the number of phenotypes in Y'_i as M_i, and we have $Y_i'=\{y_{i1},\cdots,y_{iM_i}\}$. Notice that $\bigcup_{i=0}^{2} Y_i'=Y$ and $\sum_{i=0}^{2} M_i=M$

The total sum of squares (SST) can be divided into two parts, the between-group sum of squares (SSB) and the within-group sum of squares (SSW):

$$SST=\sum_{m=1}^{M}(y_m-\overline{Y})^2=\sum_{i=0}^{2}\sum_{m=1}^{M_i}(y'_{im}-\overline{Y})^2,$$

$$SSB=\sum_{i=0}^{2}(\overline{Y_i'}-\overline{Y})^2,\quad and$$

$$SSW=SST-SSB=\sum_{i=0}^{2}\sum_{m=1}^{M_i}(y'_{im}-\bar{Y}_i^{'})^2,$$

where

$$\overline{Y}=\frac{1}{M}\sum_{m=1}^{M}y_m\quad and\quad \bar{Y}_i^{'}=\frac{1}{M_i}\sum_{m=1}^{M_i}y'_{im}.$$

The formula of F-test statistic is $F=\frac{SSB}{SSW}$, and F follows the F-distribution with 2 and S-3 degrees of freedom under the null hypothesis, i.e., $F\sim F_{(2,S-3)}$.

2.3.2 Linear regression. In the linear regression model, a least-squares regression line is fit between the phenotype values and the genotype values [11]. For simplicity, we denote the genotypes of a single SNP to be $x_1,x_2,\cdots,x_M$. Based on the data $(x_1,y_1),\cdots,(x_M,y_M)$, we need to fit a line in the form of $Y=a+bx$.

We have the sums of squares as follows:

$$SS_{xx}=\sum_{i=1}^{M}(x_i-\overline{x})^2, SS_{yy}=\sum_{i=1}^{M}(Y_i-\overline{Y})^2,$$

$$and\quad SS_{xy}=\sum_{i=1}^{M}(x_i-\overline{x})(Y_i-\overline{Y})$$

where $\overline{x}=\frac{1}{M}\sum_{i=1}^{M}x_i\quad and\quad \overline{Y}=\frac{1}{M}\sum_{i=1}^{M}y_i$

To achieve least squares, the estimator of b is $\frac{SS_{xy}}{SS_{xx}}$. To evaluate the significance of the obtained model, a hypothesis testing for $b=0$ is then applied.

2.4 Multiple Testing Problem

In a typical GWAS, the test needs to be performed many times. We should pay attention to a statistical issue known as the multiple testing problem. In the remainder of this section, we will discuss the multiple testing problem and how to effectively control error rate in GWAS.

Type 1 error rate, is the possibility that a null hypothesis is rejected when it is actually true. In other words, it is the chance of observing a positive (significant) result even if it is not. If a test is performed multiple times, the overall Type 1 Error rate will increase. This is called the multiple testing problem.

Let α be the type 1 error rate for a statistical test. If the test is performed n times, the experimental-wise error rate α' is given by

$$\alpha'=1-(1-\alpha)^n.$$

For example, if $\alpha=0.05$ and $n=20$, then $\alpha'=1-(1-0.05)^{20}=0.64$. In this case, the chance of getting at least one false positive is 64%.

Because of the multiple testing problem, the test result may not be that significant even if its p-value is less than a significant level α. To solve this problem, the nominal p-value need to be corrected/adjusted.

2.5 Family-Wise Error Rate Control

For the single-locus test, we denote the p-value for a association test of a SNP X_i and a phenotype Y to be $p(X_i,Y)$, and the corrected p-value to be $p'(X_i,Y)$. Family-wise error rate (FWER), or the experiment-wise error rate, is the probability of at least one false association. We use α' to denote family-wise error rate, and it is given by

$$\alpha'=P(\text{reject } H_0|H_0)=P(\text{reject at least one of } H_i(1\le i\le n)|H_0),$$

where n is the total number of tests and H_0 is the hypothesis that all the $H_i(1\le i\le n)$ are true.

Many methods can be used to control FWER. Bonferroni correction is a commonly used method, in which p-values need to be enlarged to account for the number of comparisons being performed. Permutation test [13] is also widely used to correct for multiple testing in GWAS.

2.5.1 Bonferroni correction. In Bonferroni correction, the p-value of a test is multiplied by the number of tests in the multiple comparison.

$$p'(X_i,Y)=p(X_i,Y)*N$$

Here the number of tests is the number of SNPs N in a study. Bonferroni correction is a single-step procedure, in which each of the p-values is independently corrected.

2.5.2 Permutation tests. In the permutation test, data are reshuffled. For each permutation, p-values for all the tests are re-calculated, and the minimal p-value is retained. After K permutations, we get totally K minimal p-values. The corrected p-value is given by the proportion of minimal p-values which is less than the original p-value.

Let $\{Y_1,\cdots,Y_k\}$ be the set of K permutations. For each permutation $Y_k(1\le k\le K)$, the minimal p-value p_{Y_k} is given by

$$p_{Y_k}=min\{p(X_i,Y_k)|1\le i\le n\}.$$

Then we have the corrected p-value

$$p'(X_i,Y)=\frac{\#\{p_{Y_k}<p(X_i,Y)|1\le k\le K\}}{K}.$$

The permutation method takes advantage of the correlation structure between SNPs. It is less stringent than Bonferroni correction.

2.6 False Discovery Rate Control

False discovery rate (FDR) controls the expected proportion of type 1 error among all significant hypotheses. It is less conservative than the family-wise error rate. For example, if 100 observed results are claimed to be significant, and the FDR is 0.1, then 10 of results are expected to be false discoveries.

One way to control the FDR is as follows [14]. The p-values of SNPs and the phenotype are ranked from smallest to largest. We denote the ordered p-values to be $p_1,\cdots,p_N$. Starting from the largest p-value to the smallest, the original p-value is multiplied by the total number of SNPs and divided by its rank. For the i^{th} p-value p_i, its corrected p-value p_i' is given by

$$p_i'=p_i*(\frac{N}{i}).$$

In this section, we have discussed commonly used methods in single-locus study, the multiple testing problem and how to control error rate in GWAS. In the next section, we will introduce methods used for two-locus association studies. We will focus on one class work that finds exact solution when searching for SNP-SNP interactions in GWAS.

3. Exact Methods for Two-Locus Association Study

The vast number of SNPs has posed great computational challenge to genome-wide association study. In order to understand the underlying biological mechanisms of complex phenotype, one needs to consider the joint effect of multiple SNPs simultaneously. Although the idea of studying the association between phenotype and multiple SNPs is straightforward, the implementation is nontrivial. For a study with total N SNPs, in order to find the association between n SNPs and the phenotype, a brute-force approach is to exhaustively enumerate all $\binom{N}{n}$ possible SNP combinations and evaluate their associations with the phenotype. The computational burden imposed by this enormous search space often makes the complete genome-wide association study intractable. Moreover, although permutation test has been considered the gold standard method for multiple testing correction, it will dramatically increase the computational burden because the process needs to be performed for all permuted data.

In this section, we will focus on the recently developed exact method for two-locus epistasis detection. Different from the single-locus approach, the goal of two-locus epistasis detection is to identify interacting SNP-pairs that have strong association with the phenotype. FastANOVA [15] is an algorithm for two-locus ANOVA (analysis of variance) test on quantitative traits and FastChi [16] for two-locus chi-square test on case-control phenotypes. COE [17] is a general method that can be applied in a wide range of tests. TEAM [18] is designed for studies involving a large number of individuals such as human studies. In this subsection, we will discuss these algorithms, and their strengths and limitations.

3.1 The FastANOVA Algorithm

FastANOVA utilizes an upper bound of the two-locus ANOVA test to prune the search space. The upper bound is expressed as the sum of two terms. The first term is based on the single-SNP ANOVA test. The second term is based on the genotype of the SNP-pair and is independent of permutations. This property allows to index SNP-pairs in a 2D array based on the genotype relationship between SNPs. Since the number of entries in the 2D array is bound by the number of individuals in the study, many SNP-pairs share a common entry. Moreover, it can be shown that all SNP-pairs indexed by the same entry have exactly the same upper bound. Therefore, we can compute the upper bound for a group of SNP-pairs together. Another important property is that the indexing structure only needs to be built once and can be reused for all permutated data. Utilizing the upper bound and the indexing structure, FastANOVA only needs to perform the ANOVA test on a small number of candidate SNP-pairs without the risk of missing any significant pair. We discuss the algorithm in further detail in the following.

Let $\{X_1, X_2, \cdots, X_N\}$ be the set of SNPs of M individuals ($X_i \in \{0,1\}, 1 \le i \le N$) and $Y = \{y_1, y_2, \cdots, y_M\}$ be the quantitative phenotype of interest, where y_m ($1 \le m \le M$) is the phenotype value of individual m.

For any SNP X_i ($1 \le i \le N$), we represent the F-statistic from the ANOVA test of X_i and Y as $F(X_i, Y)$. For any SNP-pair (X_iX_j), we represent the F-statistic from the ANOVA test of (X_iX_j) and Y as $F(X_iX_j, Y)$.

The basic idea of ANOVA test is to partition the total sum of squared deviations SS_T into between-group sum of squared deviations SS_B and within-group sum of squared deviations SS_W:

$$SS_T = SS_B + SS_W.$$

In our application of the two-locus association study, Table 2 and Table 3 show the possible groupings of phenotype values by the genotypes of X_i and (X_iX_j) respectively.

Let A, B, a_1, a_2, b_1, b_2 represent the groups as indicated in Table 2 and Table 3. We use $SS_B(X_i, Y)$ and $SS_B(X_iX_j, Y)$ to distinct the one locus (i.e., single-SNP) and two locus (i.e., SNP-pair) analyses. Specifically, we have

$$SS_T(X_i, Y) = SS_B(X_i, Y) + SS_W(X_i, Y),$$

$$SS_T(X_iX_j, Y) = SS_B(X_iX_j, Y) + SS_W(X_iX_j, Y).$$

The F-statistics for ANOVA tests on X_i and (X_iX_j) are:

$$F(X_i, Y) = \frac{M-2}{2-1} \times \frac{SS_B(X_i, Y)}{SS_T(X_i, Y) - SS_B(X_i, Y)}, \quad (1.1)$$

$$F(X_iX_j, Y) = \frac{M-g}{g-1} \times \frac{SS_B(X_iX_j, Y)}{SS_T(X_iX_j, Y) - SS_B(X_iX_j, Y)}, \quad (1.2)$$

where g in Equation (1.2) is the number of groups that the genotype of (X_iX_j) partitions the individuals into. Possible values of g are 3 or 4, assuming all SNPs are distinct: If none of groups A, B, a_1, a_2, b_1, b_2 is empty, then $g = 4$. If one of them is empty, then $g = 3$.

Let $T = \sum_{y_m \in Y} y_m$ be the sum of all phenotype values. The total sum of squared deviations does not depend on the groupings of individuals:

$$SS_T(X_i, Y) = SS_T(X_iX_j, Y) = \sum_{y_m \in Y} y_m^2 - \frac{T^2}{M}.$$

Let $T_{group} = \sum_{y_m \in group} y_m$ be the sum of phenotype values in a specific group, and n_{group} be the number of individuals in that group. $SS_B(X_i, Y)$ and $SS_B(X_iX_j, Y)$ can be calculated as follows:

$$SS_B(X_i, Y) = \frac{T_A^2}{n_A} + \frac{T_B^2}{n_B} - \frac{T^2}{M},$$

Table 2. Grouping of Y by X_i.

$X_i = 1$	$X_i = 0$
group A	group B

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Table 3. Grouping of Y by X_iX_j.

	$X_i = 1$	$X_i = 0$
$X_j = 1$	group a_1	group b_1
$X_j = 0$	group a_2	group b_2

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$$SS_B(X_iX_j,Y)=\frac{T_{a_1}^2}{n_{a_1}}+\frac{T_{a_2}^2}{n_{a_2}}+\frac{T_{b_1}^2}{n_{b_1}}+\frac{T_{b_2}^2}{n_{b_2}}-\frac{T^2}{M}.$$

Note that for any group of A, B, a_1, a_2, b_1, b_2, if $n_{group}=0$, then $\frac{T_{group}^2}{ngroup}$ is defined to be 0.

Let $\{y_m|y_m\in A\}=\{y_{A_1},y_{A_2},\cdots,y_{A_{n_A}}\}$ be the phenotype values in group A. Without loss of generality, assume that these phenotype values are arranged in ascending order, i.e.,

$$y_{A_1}\le y_{A_2}\le\cdots\le y_{A_{n_A}}.$$

Let $\{y_m|y_m\in B\}=\{y_{B_1},y_{B_2},\cdots,y_{B_{n_B}}\}$ be the phenotype values in group B. Without loss of generality, assume that these phenotype values are arranged in ascending order, i.e.,

$$y_{B_1}\le y_{B_2}\le\cdots\le y_{B_{n_B}}.$$

We have the overall upper bound on $SS_B(X_iX_j,Y)$:

Theorem 1 *(Upper bound of $SS_B(X_iX_j,Y)$)*

$$SS_B(X_iX_j,Y)\le SS_B(X_i,Y)+R_1(X_iX_jY)+R_2(X_iX_jY).$$

The notations in the bound can be found in Table 4. The upper bound in Theorem 1 is tight. The tightness of the bound is obvious from the derivation of the upper bound, since there exists some genotype of SNP-pair (X_iX_j) that makes the equality hold.

We now discuss how to apply the upper bound in Theorem 1 in detail. The set of all SNP-pairs is partitioned into non-overlapping groups such that the upper bound can be readily applied to each group. For every X_i $(1\le i\le N)$, let $AP(X_i)$ be the set of SNP-pairs

$$AP(X_i)=\{(X_iX_j)|i+1\le j\le N\}.$$

For all SNP-pairs in $AP(X_i)$, n_A, T_A, n_B, T_B and $SS_B(X_i,Y)$ are constants. Moreover, l_{a_1}, u_{a_1} are determined by n_{a_1}, and l_{b_1}, u_{b_1} are determined by n_{b_1}. Therefore, in the upper bound, n_{a_1} and n_{b_1} are the only variables that depend on X_j and may vary for different SNP-pairs (X_iX_j) in $AP(X_i)$.

Table 4. Notations for the bounds.

Symbols	Formulas
l_{a_1}	$\sum_{i=1}^{n_{a_1}} y_{A_i}$
u_{a_1}	$\sum_{i=n_A-n_{a_1}+1}^{n_A} y_{A_i}$
$R_1(X_iX_jY)$	$\frac{\max\{(n_Al_{a_1}-n_{a_1}T_A)^2,(n_Au_{a_1}-n_{a_1}T_A)^2\}}{n_{a_1}(n_A-n_{a_1})n_A}$
l_{b_1}	$\sum_{i=1}^{n_{b_1}} y_{B_i}$
u_{b_1}	$\sum_{i=n_B-n_{b_1}+1}^{n_B} y_{B_i}$
$R_2(X_iX_jY)$	$\frac{\max\{(n_Bl_{b_1}-n_{b_1}T_B)^2,(n_Bu_{b_1}-n_{b_1}T_B)^2\}}{n_{b_1}(n_B-n_{b_1})n_B}$

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Note that n_{a_1} is the number of 1's in X_j when X_i takes value 1, and n_{b_1} is the number of 1's in X_j when X_i takes value 0. It is easy to prove that switching n_{a_1} and n_{a_2} does not change the F-statistic value and the correctness of the upper bound. This is also true if we switch n_{b_1} and n_{b_2}. Therefore, without loss of generality, we can always assume that n_{a_1} is the smaller one between the number of 1's and number of 0's in X_j when X_i takes value 1, and n_{b_1} is the smaller one between the number of 1's and number of 0's in X_j when X_i takes value 0.

If there are m 1's and $(M-m)$ 0's in X_i, then for any $(X_iX_j)\in AP(X_i)$, the possible values that n_{a_1} can take are $\{0,1,2,\cdots,\lfloor m/2\rfloor\}$. The possible values that n_{b_1} can take are $\{0,1,2,\cdots,\lfloor (M-m)/2\rfloor\}$.

To efficiently retrieve the candidates, the SNP-pairs (X_iX_j) in $AP(X_i)$ are grouped by their (n_{a_1},n_{b_1}) values and indexed in a 2D array, referred to as $Array(X_i)$.

Suppose that there are 32 individuals, and the genotype of X_i consists of half 0's and half 1's. Thus for the SNP-pairs in $AP(X_i)$, the possible values of n_{a_1} and n_{b_1} are $\{0,1,2,\cdots,8\}$. Figure 1 shows the 9×9 array, $Array(X_i)$, whose entries represent the possible values of (n_{a_1},n_{b_1}) for the SNP-pairs $(X_iX_j)\in AP(X_i)$. The entries in the same column have the same n_{a_1} value. The entries in the same row have the same n_{b_1} value. The n_{a_1} value of each column is noted beneath each column. The n_{b_1} value of each row is noted left to each row. Each entry of the array is a pointer to the SNP-pairs $(X_iX_j)\in AP(X_i)$ having the corresponding (n_{a_1},n_{b_1}) values.

For any SNP X_i, the maximum number of the entries in $Array(X_i)$ is $(\lceil\frac{M}{4}\rceil+1)^2$. The proof of this property is straightforward and omitted here. In order to find candidate SNP-pairs, we scan all entries in $Array(X_i)$ to calculate their upper bounds. Since the SNP-pairs indexed by the same entry share the same (n_{a_1},n_{b_1}) value, they have the same upper bound. In this way, we can calculate the upper bound for a group of SNP-pairs together. Note that for typical genome-wide association studies, the number of individuals M is much smaller than the number of SNPs N. Therefore, the additional cost for accessing $Array(X_i)$ is minimal compared to performing ANOVA tests for all pairs $(X_iX_j)\in AP(X_i)$.

For multiple tests, permutation procedure is often used in genetic analysis for controlling family-wise error rate. For genome-wide association study, permutation is less commonly used because it often entails prohibitively long computation times. Our FastANOVA algorithm makes permutation procedure feasible in genome-wide association study.

Let $Y'=\{Y_1,Y_2,\cdots,Y_K\}$ be the K permutations of the phenotype Y. Following the idea discussed above, the upper bound in Theorem 1 can be easily incorporated in the algorithm to handle the permutations. For every SNP X_i, the indexing structure $Array(X_i)$ is independent of the permuted phenotypes in Y'. The correctness of this property relies on the fact that, for any $(X_iX_j)\in AP(X_i)$, n_{a_1} and n_{b_1} only depend on the genotype of the SNP-pair and thus remain constant for different phenotype permutations. Therefore, for each X_i, once we build $Array(X_i)$, it can be reused in all permutations.

3.2 The FastChi Algorithm

As our initial attempt to develop scalable algorithms for genome-wide association study, FastANOVA is specifically designed for the ANOVA test on quantitative phenotypes. Another category of phenotypes is generated in case-control study, where the phenotypes are binary variables representing disease/non-disease individuals. Chi-square test is one of the most commonly used statistics in binary phenotype association

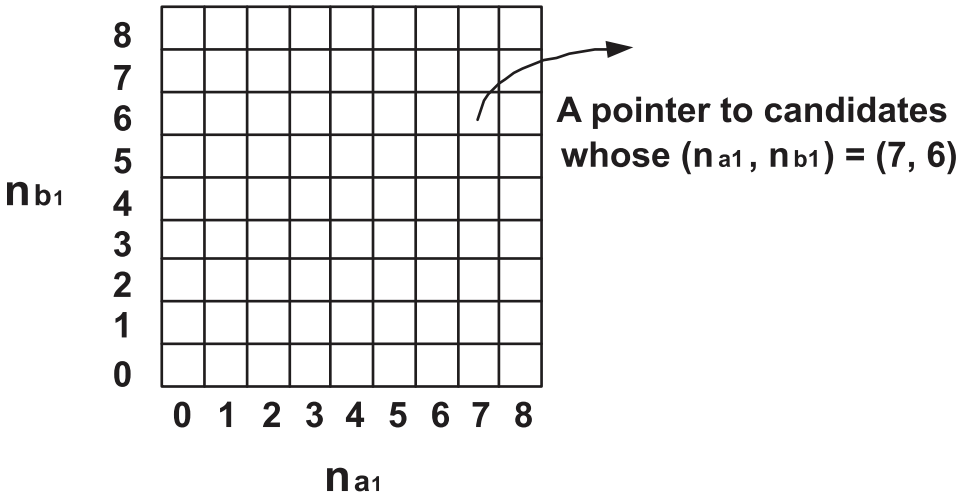

Figure 1. The index array $Array(X_i)$ for efficient retrieval of the candidate SNP-pairs.
doi:10.1371/journal.pcbi.1002828.g001

study. We can extend the principles in FastANOVA for efficient two-locus chi-square test. The general idea of FastChi is similar to that of FastANOVA, i.e., re-formulating the chi-square test statistic to establish an upper bound of two-locus chi-square test, and indexing the SNP-pairs according to their genotypes in order to effectively prune the search space and reuse redundant computations. Here we briefly introduce the FastChi algorithm.

For SNP X_i, we represent the chi-square test value of X_i and the binary phenotype Y as $\chi^2(X_i,Y)$. For any SNP-pair X_i and X_j, we use $\chi^2(X_iX_j,Y)$ to represent the chi-square test value for the combined effect of (X_iX_j) with Y. Let A,B,C,D represent the following events respectively: $Y=0\wedge X_i=0$; $Y=0\wedge X_i=1$; $Y=1\wedge X_i=0$; $Y=1\wedge X_i=1$. Let O_{event} denote the observed value of an event. T_1, T_2, S_1, S_2, $\mathcal{R}_1$, and $\mathcal{R}_2$ represent the formulas shown in Table 5. We have the upper bound of $\chi^2(X_iX_j,Y)$ stated in Theorem 2.

Theorem 2 *(Upper bound of $\chi^2(X_iX_j,Y)$)*

$$\chi^2(X_iX_j,Y)\le\chi^2(X_i,Y)+T_1S_1\mathcal{R}_1+T_2S_2\mathcal{R}_2.$$

For given phenotype Y and SNPX_i, $\chi^2(X_i,Y)$, T_1, S_1, T_2, and S_2 are constants. $\mathcal{R}_1$ and $\mathcal{R}_2$ are the only variables that depend on X_j and may vary for different SNP-pairs $(X_iX_j)\in AP(X_i)$. (Recall that $AP(X_i)=\{(X_iX_j)|i+1\le j\le N\}$.) Thus for a given X_i, we can treat equation $\chi^2(X_i,Y)+T_1S_1\mathcal{R}_1+T_2S_2\mathcal{R}_2=\theta$ as a *straight line* in the 2-D space of $\mathcal{R}_1$ and $\mathcal{R}_2$. The ones whose $(\mathcal{R}_1(X_iX_j),\mathcal{R}_2(X_iX_j))$ values fall below the line can be pruned without any further test.

Suppose that there are 32 individuals, X_i contains half 0's, and half 1's. For the SNP-pairs in $AP(X_i)$, the possible values of $\mathcal{R}_1$ (and $\mathcal{R}_2$) are $\{\frac{0}{16},\frac{1}{15},\frac{2}{14},\frac{3}{13},\frac{4}{12},\frac{5}{11},\frac{6}{10},\frac{7}{9},\frac{8}{8}\}$. Figure 2 shows the 2-D space of $\mathcal{R}_1$ and $\mathcal{R}_2$. The blue stars represent the values that $(\mathcal{R}_1,\mathcal{R}_2)$ can take. The line $\chi^2(X_i,Y)+T_1S_1\mathcal{R}_1+T_2S_2\mathcal{R}_2=\theta$ is plotted in the figure. Only the SNP-pairs whose $(\mathcal{R}_1,\mathcal{R}_2)$ values are in the shaded region are subject to two-locus Chi-square test.

Similar to FastANOVA, in FastChi, we can index the SNP-pairs in $AP(X_i)$ according to their genotype relationships, i.e., by the values of $(\mathcal{R}_1,\mathcal{R}_2)$. Experimental results demonstrate that FastChi is an order of magnitude faster than the brute force alternative.

3.3 The COE Algorithm

Both FastANOVA and FastChi rework the formula of ANOVA test and Chi-square test to estimate an upper bound of the test value for SNP pairs. These upper bounds are used to identify candidate SNP pairs that may have strong epistatic effect. Repetitive computation in a permutation test is also identified and performed once those results are stored for use by all permutations. These two strategies lead to substantial speedup, especially for large permutation test, without compromising the accuracy of the test. These approaches guarantee to find the optimal solutions. However, a common drawback of these methods is that they are designed for specific tests, i.e., chi-square test and ANOVA test. The upper bounds used in these methods do not work for other statistical tests, which are

Table 5. Notations used in the derivation of the upper bound for two-locus Chi-square test.

Symbols	Formulas
T_1	$\frac{M^2}{(O_A+O_B)(O_A+O_C)(O_C+O_D)}$
S_1	$\max\{O_A^2,O_C^2\}$
$\mathcal{R}_1$	$\min\{\left[\frac{O_{X_j=1}}{O_{X_j=0}}\|X_i=0\right],\left[\frac{O_{X_j=0}}{O_{X_j=1}}\|X_i=0\right]\}$
T_2	$\frac{M^2}{(O_A+O_B)(O_B+O_D)(O_C+O_D)}$
S_2	$\max\{O_B^2,O_D^2\}$
$\mathcal{R}_2$	$\min\{\left[\frac{O_{X_j=1}}{O_{X_j=0}}\|X_i=1\right],\left[\frac{O_{X_j=0}}{O_{X_j=1}}\|X_i=1\right]\}$

doi:10.1371/journal.pcbi.1002828.t005

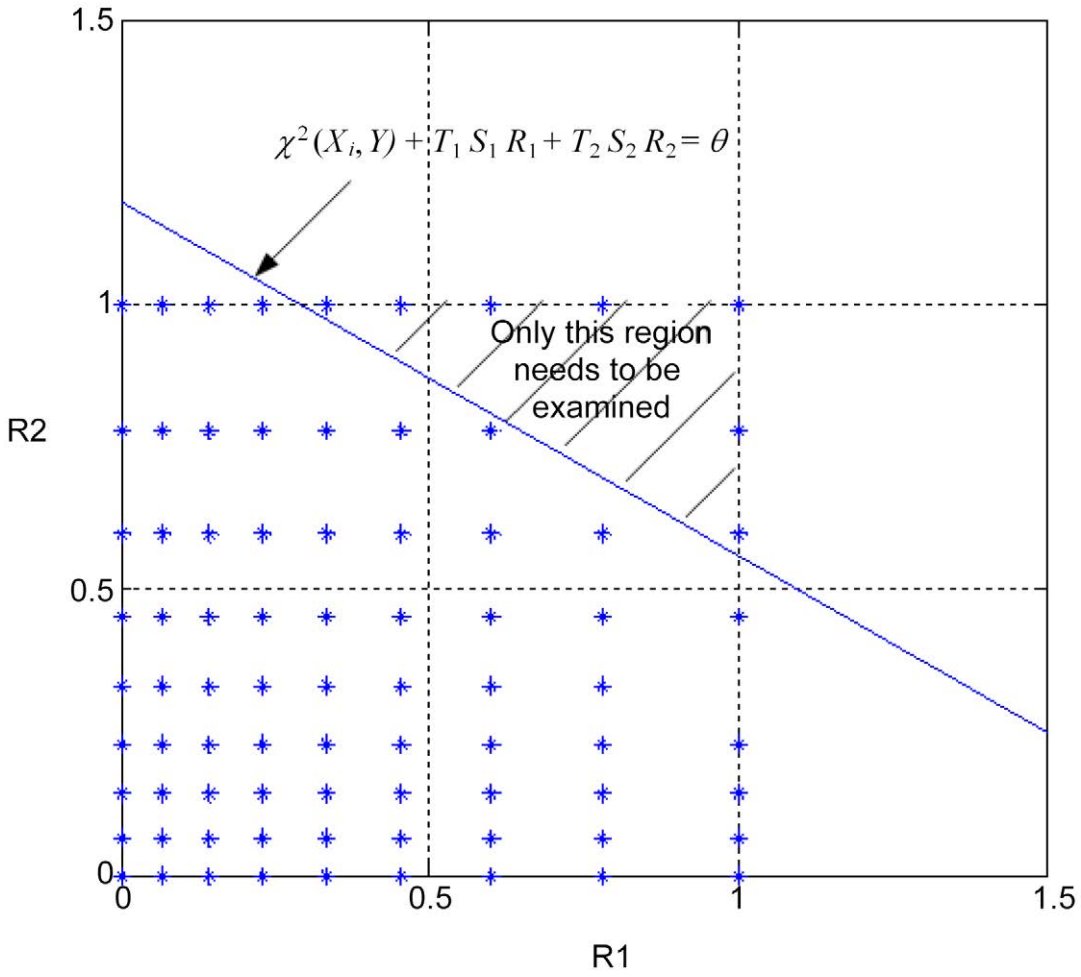

Figure 2. Pruning SNP-pairs in $AP(X_i)$ using the upper bound.
doi:10.1371/journal.pcbi.1002828.g002

also routinely used by researchers. In addition, new statistics for epistasis detection are continually emerging in the literature. Therefore, it is desirable to develop a general model that supports a variety of statistical tests.

The COE algorithm takes the advantage of convex optimization. It can be shown that a wide range of statistical tests, such as chi-square test, likelihood ratio test (also known as G-test), and entropy-based tests are all convex functions of observed frequencies in contingency tables. Since the maximum value of a convex function is attained at the vertices of its convex domain, by constraining on the observed frequencies in the contingency tables, we can determine the domain of the convex function and get its maximum value. This maximum value is used as the upper bound on the test statistics to filter out insignificant SNP-pairs. COE is applicable to all tests that are convex.

3.4 The TEAM Algorithm

The methods we have discussed so far provide promising alternatives for GWAS. However, there are two major drawbacks that limit their applicability. First, they are designed for relatively small sample size and only consider homozygous markers (i.e., each SNP can be represented as a $\{0,1\}$ binary variable). In human study, the sample size is usually large and most SNPs contain heterozygous genotypes and are coded using $\{0,1,2\}$. These make previous methods intractable. Second, although the family-wise error rate (FWER) and the false discovery rate (FDR) are both widely used for error controlling, previous methods are designed only to control the FWER. From a computational point of view, the difference in the FWER and the FDR controlling is that, to estimate FWER, for each permutation, only the maximum two-locus test value is needed. To estimate the FDR, on the other hand, for each permutation, all two-locus test values must be computed.

To address these limitations, TEAM is proposed for efficient epistasis detection in human GWAS. TEAM has several advantages over previous methods. It supports to both homozygous and heterozygous data. By exhaustively computing all two-locus test values in permutation test, it enables both FWER and FDR controlling. It is applicable to all statistics based on the contingency table. Previous methods are either designed for specific tests or require the test statistics satisfy certain property. Experimental results demonstrate that TEAM is more efficient than existing methods for large sample studies.

TEAM incorporates the permutation test for proper error controlling. The key idea is to incrementally update the contingency tables of two-locus tests. We show that only four of the eighteen observed frequencies in the contingency table need to be updated to compute the test value. In the algorithm, we build a minimum spanning tree [19] on the SNPs. The nodes of the tree are SNPs. Each edge represents the genotype difference between the two connected SNPs. This tree structure can be utilized to speed up the updating process for the contingency tables. A majority of the individuals are pruned and only a small portion are scanned to update the contingency tables. This is advantageous in human study, which usually involves

thousands of individuals. Extensive experimental results demonstrate the efficiency of the TEAM algorithm.

As a summary of the exact two-locus algorithms, FastANOVA and FastChi are designed for specific tests and binary genotype data. The COE algorithm is a more general method that can be applied to all convex tests. The TEAM algorithm is more suitable for large sample human GWAS.

4. Multifactor Dimensionality Reduction

Multifactor dimensionality reduction (MDR) [20] is a data mining method to identify interactions among discrete variables for binary outcomes. It can be used to detect high-order gene-gene and gene-environment interactions in case-control studies. By pooling multi-locus SNPs into two groups, one classified as high-risk and the other classified as low risk, MDR effectively reduces the predictors from n dimensions to one dimension. Then, the one-dimensional variable is evaluated through cross-validation. The steps are repeated for all other n factor combinations, and the factor model which has the lowest prediction error is chosen as the 'best' n factor model. Its detailed steps are as follows:

- Divide the set of factors into 10 equal subsets.
- Select a set of n factors from the pool of all factors in the training set
- Create a contingency table for these n factors by counting the number of cases and controls in each combination.
- Compute the case-control ratio in each combination. Label them as "high-risk if it is greater than a certain threshold, and otherwise, it is marked as "low-risk".
- Use the labels to classify individuals. Compute the misclassification rate.
- Repeat previous steps for all combinations of n factors across 10 training and testing subsets.
- Choose the model whose average misclassification rate is minimized and cross-validation consistency is maximized as the "best" model.

MDR designs a constructive induction method that combines two or more SNPs before testing for association. The power of the MDR approach is that it can be combined with other methodologies including the ones described in this chapter.

5. Logistic Regression

Logistic regression is a statistical method for predicting binary and categorical outcome. It is widely used in GWAS [21,22]. The basic idea is to use linear regression to model the probability of the occurrence of a specific outcome. Logistic regression is applicable to both single-locus and multi-locus association studies and can incorporate covariates and other factors in the model.

Let $Y \in \{0,1\}$ be a binary variable representing disease status (diseased verses non diseased), and $X \in \{0,1,2\}$ be a SNP. The conditional probability of having the disease given a SNP is $\theta(X) = P(Y = 1|X)$. We define the logit function to convert the range of the probability from [0,1] to $(-\infty, +\infty)$:

$$logit(X) = ln \frac{\theta(X)}{1-\theta(X)}.$$

The logit can be considered as a latent continuous variable that will be fit to a linear predictor function:

$$logit(X) \sim \beta_0 + \beta * X.$$

To cope with multiple SNP loci and potential covariates, we can modify the above model. For example, in the following model the logit is fit with predictors of SNPs (X_1, X_2) and covariates (Z_1, Z_2):

$$logit(X) \sim \beta_0 + \beta_1 * X_1 + \beta_2 * X_2 + \beta_3 * X_1 * X_2 + \beta_4 * Z_1 + \beta_5 * Z_2.$$

Although logistic regression can handle complicated models, it may be computationally demanding when the number of predictors is large [23].

6. Summary

The potential of genome-wide association study for the identification of genetic variants that underlying phenotypic variations is well recognized. The availability of large SNP data generated by high-throughput genotyping methods poses great computational and statistical challenges. In this chapter, we have discussed serval computational approaches to detect associations between genetic markers and the phenotypes. For further readings, the readers are encouraged to refer to [11,7,24,25] for discussions about current progress and challenges in large-scale genetic association studies.

7. Exercises

Question 1: The table below contains binary genotype and case-control phenotype data from ten individuals. Give the contingency table and use χ^2 test to compute the association test score.

Genotype	Phenotype
0	1
0	0
1	1
0	0
1	0
0	0
1	1
0	0
1	1
0	0

Question 2: Assuming that we have the following SNP and phenotype data, is the SNP significantly associated with the phenotype? Here, we represent each SNP site as the number of minor alleles on that locus, so 0 and 2 are for major and minor homozygous sites, respectively, and 1 is for the heterozygous sites. We also assume that minor alleles contribute to the phenotype and the effect is additive. In other words, the effect from a minor homozygous site should be twice as large as that from a heterozygous site. You may use any test methods introduced in the chapter. How about permutation tests?

Genotype	Phenotype
1	0.53
2	0.78
2	0.81
1	−0.23
1	−0.73
0	0.81
2	0.27
0	2.59
1	1.84
0	0.03

Question 3: Categorize the following methods in the table. The methods are χ^2 test, G-test, ANOVA, Student's T-test, Pearson's correlation, linear regression, logistic regression.

case − control phenotype	quantitative phenotype

Question 4: Why is it important to study multiple-locus association? What are the challenges?

Answers to the Exercises can be found in Text S1.

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Supporting Information

Text S1 Answers to Exercises
(PDF)

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Chapter 11: Genome-Wide Association Studies

William S. Bush[1]*, **Jason H. Moore**[2]

1 Department of Biomedical Informatics, Center for Human Genetics Research, Vanderbilt University Medical School, Nashville, Tennessee, United States of America, **2** Departments of Genetics and Community Family Medicine, Institute for Quantitative Biomedical Sciences, Dartmouth Medical School, Lebanon, New Hampshire, United States of America

Abstract: Genome-wide association studies (GWAS) have evolved over the last ten years into a powerful tool for investigating the genetic architecture of human disease. In this work, we review the key concepts underlying GWAS, including the architecture of common diseases, the structure of common human genetic variation, technologies for capturing genetic information, study designs, and the statistical methods used for data analysis. We also look forward to the future beyond GWAS.

This article is part of the "Translational Bioinformatics" collection for *PLOS Computational Biology*.

1. Important Questions in Human Genetics

A central goal of human genetics is to identify genetic risk factors for common, complex diseases such as schizophrenia and type II diabetes, and for rare Mendelian diseases such as cystic fibrosis and sickle cell anemia. There are many different technologies, study designs and analytical tools for identifying genetic risk factors. We will focus here on the genome-wide association study or GWAS that measures and analyzes DNA sequence variations from across the human genome in an effort to identify genetic risk factors for diseases that are common in the population. The ultimate goal of GWAS is to use genetic risk factors to make predictions about who is at risk and to identify the biological underpinnings of disease susceptibility for developing new prevention and treatment strategies. One of the early successes of GWAS was the identification of the *Complement Factor H* gene as a major risk factor for age-related macular degeneration or AMD [1–3]. Not only were DNA sequence variations in this gene associated with AMD but the biological basis for the effect was demonstrated. Understanding the biological basis of genetic effects will play an important role in developing new pharmacologic therapies.

While understanding the complexity of human health and disease is an important objective, it is not the only focus of human genetics. Accordingly, one of the most successful applications of GWAS has been in the area of pharmacology. Pharmacogenetics has the goal of identifying DNA sequence variations that are associated with drug metabolism and efficacy as well as adverse effects. For example, warfarin is a blood-thinning drug that helps prevent blood clots in patients. Determining the appropriate dose for each patient is important and believed to be partly controlled by genes. A recent GWAS revealed DNA sequence variations in several genes that have a large influence on warfarin dosing [4]. These results, and more recent validation studies, have led to genetic tests for warfarin dosing that can be used in a clinical setting. This type of genetic test has given rise to a new field called *personalized medicine* that aims to tailor healthcare to individual patients based on their genetic background and other biological features. The widespread availability of low-cost technology for measuring an individual's genetic background has been harnessed by businesses that are now marketing genetic testing directly to the consumer. Genome-wide association studies, for better or for worse, have ushered in the exciting era of personalized medicine and personal genetic testing. The goal of this chapter is to introduce and review GWAS technology, study design and analytical strategies as an important example of translational bioinformatics. We focus here on the application of GWAS to common diseases that have a complex multifactorial etiology.

2. Concepts Underlying the Study Design

2.1 Single Nucleotide Polymorphisms

The modern unit of genetic variation is the *single nucleotide polymorphism* or SNP. SNPs are single base-pair changes in the DNA sequence that occur with high frequency in the human genome [5]. For the purposes of genetic studies, SNPs are typically used as *markers* of a genomic region, with the large majority of them having a minimal impact on biological systems. SNPs can have functional consequences, however, causing amino acid changes, changes to mRNA transcript stability, and changes to transcription factor binding affinity [6]. SNPs are by far the most abundant form of genetic variation in the human genome.

SNPs are notably a type of *common* genetic variation; many SNPs are present in a large proportion of human populations [7]. SNPs typically have two alleles, meaning within a population there are two commonly occurring base-pair possibilities for a SNP location. The frequency of a SNP is given in terms of the *minor allele frequency* or the frequency of the less common allele. For example, a SNP with a minor allele (G) frequency of 0.40 implies that 40% of a population has the G allele versus the more common allele (the major allele), which is found in 60% of the population.

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* E-mail: william.s.bush@vanderbilt.edu

What to Learn in This Chapter

- Basic genetic concepts that drive genome-wide association studies
- Genotyping technologies and common study designs
- Statistical concepts for GWAS analysis
- Replication, interpretation, and follow-up of association results

Commonly occurring SNPs lie in stark contrast to genetic variants that are implicated in more rare genetic disorders, such as cystic fibrosis [8]. These conditions are largely caused by extremely rare genetic variants that ultimately induce a detrimental change to protein function, which leads to the disease state. Variants with such low frequency in the population are sometimes referred to as *mutations*, though they can be structurally equivalent to SNPs - single base-pair changes in the DNA sequence. In the genetics literature, the term SNP is generally applied to *common* single base-pair changes, and the term mutation is applied to *rare* genetic variants.

2.2 Failures of Linkage for Complex Disease

Cystic fibrosis (and most rare genetic disorders) can be caused by multiple different genetic variants within a single gene. Because the effect of the genetic variants is so strong, cystic fibrosis follows an autosomal dominant inheritance pattern in families with the disorder. One of the major successes of human genetics was the identification of multiple mutations in the *CFTR* gene as the cause of cystic fibrosis [8]. This was achieved by genotyping families affected by cystic fibrosis using a collection of genetic markers across the genome, and examining how those genetic markers segregate with the disease across multiple families. This technique, called *linkage analysis*, was subsequently applied successfully to identify genetic variants that contribute to rare disorders like Huntington disease [9]. When applied to more common disorders, like heart disease or various forms of cancer, linkage analysis has not fared as well. This implies the genetic mechanisms that influence common disorders are different from those that cause rare disorders [10].

2.3 Common Disease Common Variant Hypothesis

The idea that common diseases have a different underlying genetic architecture than rare disorders, coupled with the discovery of several susceptibility variants for common disease with high minor allele frequency (including alleles in the *apolipoprotein E* or *APOE* gene for Alzheimer's disease [11] and *PPARg* gene in type II diabetes [12]), led to the development of the *common disease/common variant* (CD/CV) hypothesis [13].

This hypothesis states simply that common disorders are likely influenced by genetic variation that is also common in the population. There are several key ramifications of this for the study of complex disease. First, if common genetic variants influence disease, the effect size (or penetrance) for any one variant must be small relative to that found for rare disorders. For example, if a SNP with 40% frequency in the population causes a highly deleterious amino acid substitution that directly leads to a disease phenotype, nearly 40% of the population would have that phenotype. Thus, the allele frequency and the population prevalence are completely correlated. If, however, that same SNP caused a small change in gene expression that alters risk for a disease by some small amount, the prevalence of the disease and the influential allele would be only slightly correlated. As such, common variants almost by definition cannot have high penetrance.

Secondly, if common alleles have small genetic effects (low penetrance), but common disorders show heritability (inheritance in families), then multiple common alleles must influence disease susceptibility. For example, twin studies might estimate the heritability of a common disease to be 40%, that is, 40% of the total variance in disease risk is due to genetic factors. If the allele of a single SNP incurs only a small degree of disease risk, that SNP only explains a small proportion of the total variance due to genetic factors. As such, the total genetic risk due to common genetic variation must be spread across multiple genetic factors. These two points suggest that traditional family-based genetic studies are not likely to be successful for complex diseases, prompting a shift toward population-based studies.

The frequency with which an allele occurs in the population and the risk incurred by that allele for complex diseases are key components to consider when planning a genetic study, impacting the technology needed to gather genetic information and the sample size needed to discover statistically significant genetic effects. The spectrum of potential genetic effects is sometimes visualized and partitioned by effect size and allele frequency (figure 1). Genetic effects in the upper right are more amenable to smaller family-based studies and linkage analysis, and may require genotyping relatively few genetic markers. Effects in the lower right are typical of findings from GWAS, requiring large sample sizes and a large panel of genetic markers. Effects in the upper right, most notably *CFH*, have been identified using both linkage analysis and GWAS. Effects in the lower left are perhaps the most difficult challenge, requiring genomic sequencing of large samples to associate rare variants to disease.

Over the last five years, the common disease/common variant hypothesis has been tested for a variety of common diseases, and while much of the heritability for these conditions is not yet explained, common alleles certainly play a role in susceptibility. The National Human Genome Institute GWAS catalog (http://www.genome.gov/gwastudies) lists over 3,600 SNPs identified for common diseases or traits, and in general, common diseases have multiple susceptibility alleles, each with small effect sizes (typically increasing disease risk between 1.2–2 times the population risk) [14]. From these results we can say that for most common diseases, the CD/CV hypothesis is true, though it should not be assumed that the *entire* genetic component of any common disease is due to common alleles only.

3. Capturing Common Variation

3.1 The Human Haplotype Map Project

To test the common disease/common variant hypothesis for a phenotype, a systematic approach is needed to interrogate much of the common variation in the human genome. First, the location and density of commonly occurring SNPs is needed to identify the genomic regions and individual sites that must be examined by genetic studies. Secondly, population-specific differences in genetic variation must be cataloged so that studies of phenotypes in different populations can be conducted with the proper design. Finally, correlations among common genetic variants must be determined so that genetic studies do not collect redundant information. The International HapMap Project was designed to identify variation

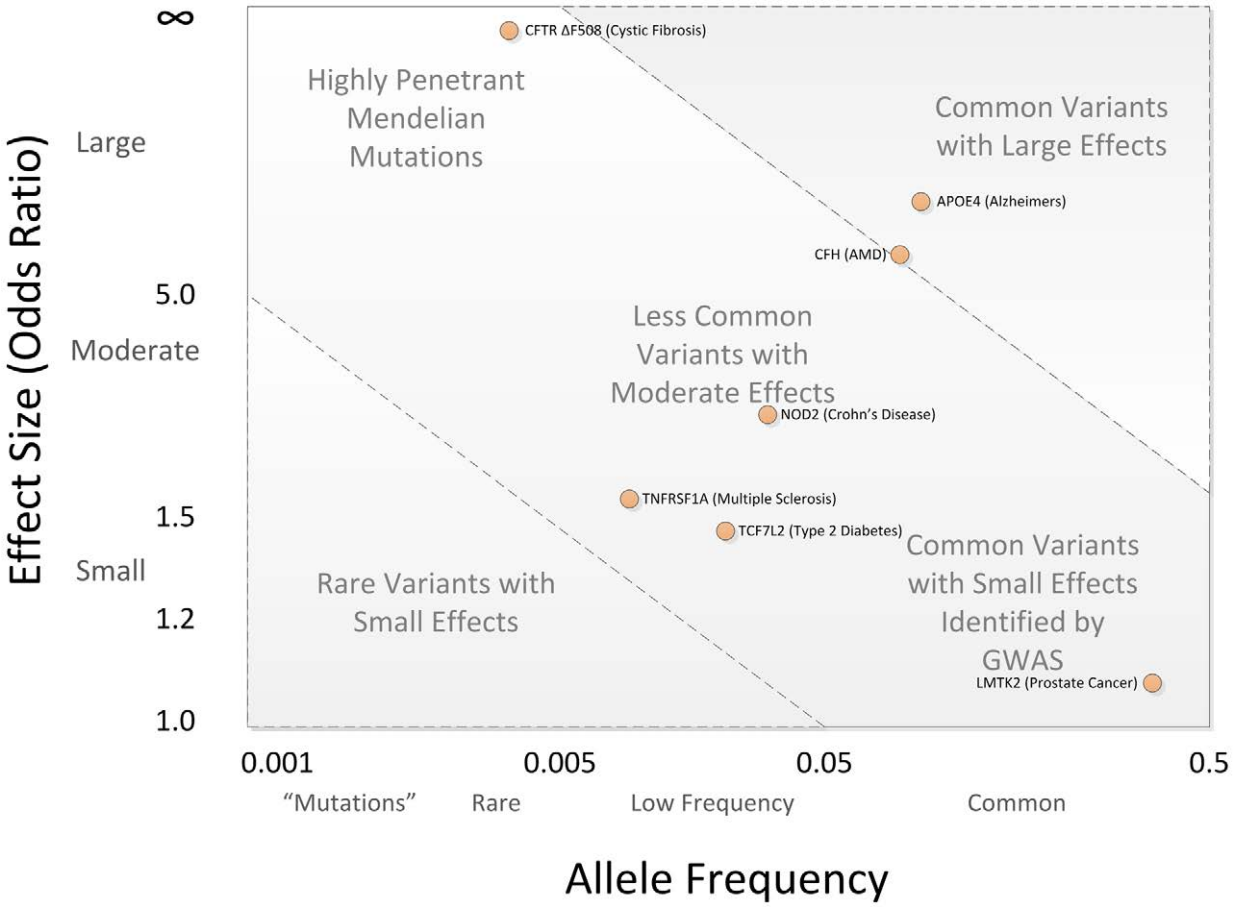

Figure 1. Spectrum of Disease Allele Effects. Disease associations are often conceptualized in two dimensions: allele frequency and effect size. Highly penetrant alleles for Mendelian disorders are extremely rare with large effect sizes (upper left), while most GWAS findings are associations of common SNPs with small effect sizes (lower right). The bulk of discovered genetic associations lie on the diagonal denoted by the dashed lines.
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across the genome and to characterize correlations among variants.

The International HapMap Project used a variety of sequencing techniques to discover and catalog SNPs in European descent populations, the Yoruba population of African origin, Han Chinese individuals from Beijing, and Japanese individuals from Tokyo [15,16]. The project has since been expanded to include 11 human populations, with genotypes for 1.6 million SNPs [7]. HapMap genotype data allowed the examination of *linkage disequilibrium*.

3.2 Linkage Disequilibrium

Linkage disequilibrium (LD) is a property of SNPs on a contiguous stretch of genomic sequence that describes the degree to which an allele of one SNP is inherited or correlated with an allele of another SNP within a population. The term linkage disequilibrium was coined by population geneticists in an attempt to mathematically describe changes in genetic variation within a population over time. It is related to the concept of *chromosomal linkage*, where two markers on a chromosome remain physically joined on a chromosome through generations of a family. In figure 2, two founder chromosomes are shown (one in blue and one in orange). Recombination events within a family from generation to generation break apart chromosomal segments. This effect is amplified through generations, and in a population of fixed size undergoing random mating, repeated random recombination events will break apart segments of contiguous chromosome (containing linked alleles) until eventually all alleles in the population are in *linkage equilibrium* or are independent. Thus, linkage between markers on a population scale is referred to as *linkage disequilibrium*.

The rate of LD decay is dependent on multiple factors, including the population size, the number of founding chromosomes in the population, and the number of generations for which the population has existed. As such, different human subpopulations have different degrees and patterns of LD. African-descent populations are the most ancestral and have smaller regions of LD due to the accumulation of more recombination events in that group. European-descent and Asian-descent populations were created by founder events (a sampling of chromosomes from the African population), which altered the number of founding chromosomes, the population size, and the generational age of the population. These populations on average have larger regions of LD than African-descent groups.

Many measures of LD have been proposed [17], though all are ultimately related to the difference between the observed frequency of co-occurrence for two alleles (i.e. a two-marker haplotype) and the frequency expected if the two markers are independent. The two commonly used measures of linkage disequilibrium are D' and r^2 [15,17] shown in equations 1 and 2. In these equations, π_{12} is the frequency of the *ab* haplotype, $\pi_{1\cdot}$ is

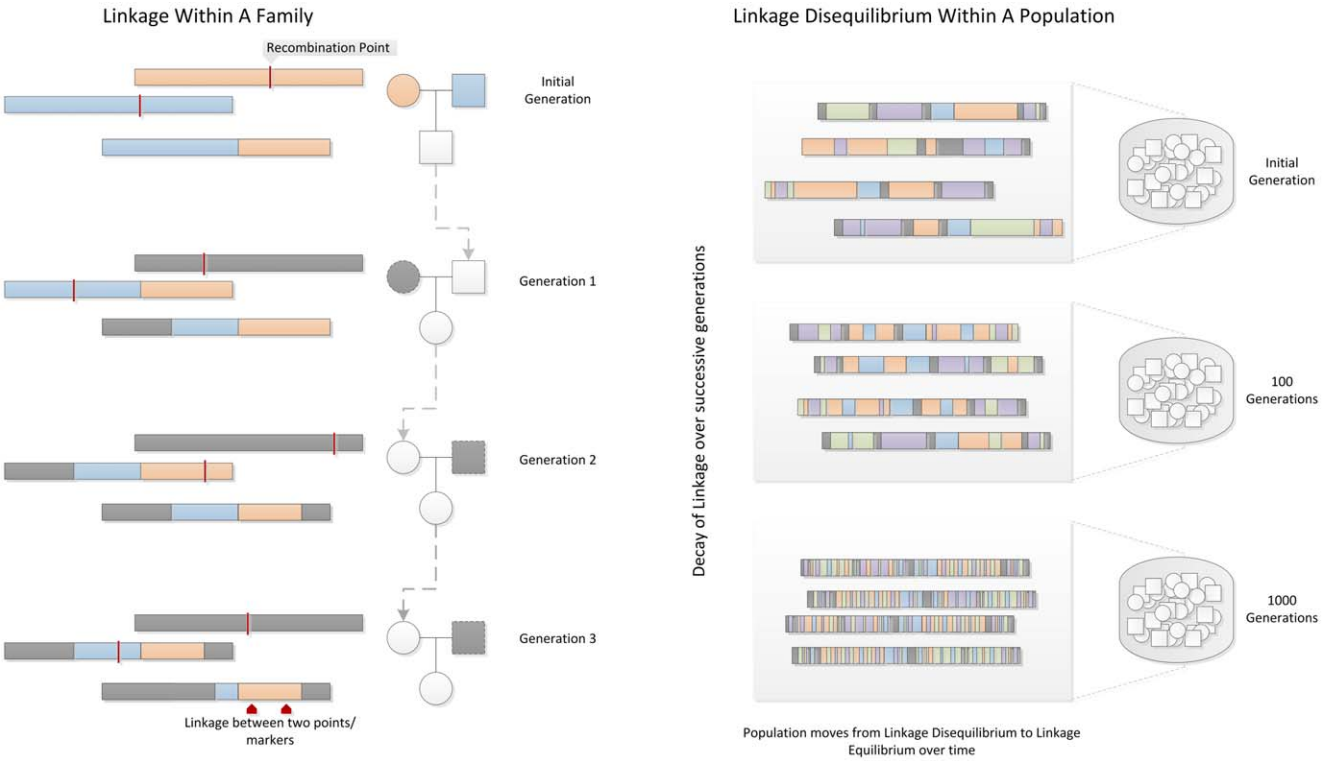

Figure 2. Linkage and Linkage Disequilibrium. Within a family, linkage occurs when two genetic markers (points on a chromosome) remain linked on a chromosome rather than being broken apart by recombination events during meiosis, shown as red lines. In a population, contiguous stretches of founder chromosomes from the initial generation are sequentially reduced in size by recombination events. Over time, a pair of markers or points on a chromosome in the population move from linkage disequilibrium to linkage equilibrium, as recombination events eventually occur between every possible point on the chromosome.
doi:10.1371/journal.pcbi.1002822.g002

the frequency of the a allele, and $\pi_{2\cdot}$ is the frequency of the b allele.

$$D' = \left\{ \begin{array}{l} \frac{\pi_{AB}\pi_{ab} - \pi_{Ab}\pi_{aB}}{\min(\pi_A\pi_b, \pi_a\pi_B)} \; if \; \pi_{AB}\pi_{ab} - \pi_{Ab}\pi_{aB} > 0 \\ \frac{\pi_{AB}\pi_{ab} - \pi_{Ab}\pi_{aB}}{\min(\pi_A\pi_B, \pi_a\pi_b)} \; if \; \pi_{AB}\pi_{ab} - \pi_{Ab}\pi_{aB} < 0 \end{array} \right\} (1)$$

$$r^2 = \frac{(\pi_{AB}\pi_{ab} - \pi_{Ab}\pi_{aB})^2}{\pi_A\pi_B\pi_a\pi_b} \quad (2)$$

D′ is a population genetics measure that is related to recombination events between markers and is scaled between 0 and 1. A D′ value of 0 indicates complete linkage equilibrium, which implies frequent recombination between the two markers and statistical independence under principles of Hardy-Weinberg equilibrium. A D′ of 1 indicates complete LD, indicating no recombination between the two markers within the population. For the purposes of genetic analysis, LD is generally reported in terms of r^2, a statistical measure of correlation. High r^2 values indicate that two SNPs convey similar information, as one allele of the first SNP is often observed with one allele of the second SNP, so only one of the two SNPs needs to be genotyped to capture the allelic variation. There are dependencies between these two statistics; r^2 is sensitive to the allele frequencies of the tow markers, and can only be high in regions of high D′.

One often forgotten issue associated with LD measures is that current technology does not allow direct measurement of haplotype frequencies from a sample because each SNP is genotyped independently and the *phase* or chromosome of origin for each allele is unknown. Many well-developed and documented methods for inferring haplotype phase and estimating the subsequent two-marker haplotype frequencies exist, and generally lead to reasonable results [18].

SNPs that are selected specifically to capture the variation at nearby sites in the genome are called *tag SNPs* because alleles for these SNPs tag the surrounding stretch of LD. As noted before, patterns of LD are population specific and as such, tag SNPs selected for one population may not work well for a different population. LD is exploited to optimize genetic studies, preventing genotyping SNPs that provide redundant information. Based on analysis of data from the HapMap project, >80% of commonly occurring SNPs in European descent populations can be captured using a subset of 500,000 to one million SNPs scattered across the genome [19].

3.3 Indirect Association

The presence of LD creates two possible positive outcomes from a genetic association study. In the first outcome, the SNP influencing a biological system that ultimately leads to the phenotype is directly genotyped in the study and found to be statistically associated with the trait. This is referred to as a *direct association*, and the genotyped SNP is sometimes referred to as the *functional SNP*. The second possibility is that the influential SNP is not directly typed, but instead a tag SNP in high LD with the influential SNP is typed and statistically associated to the phenotype (figure 3). This is referred to as an *indirect association* [10]. Because of these two possibilities, a significant SNP association from a GWAS should not be assumed as the causal variant and may require

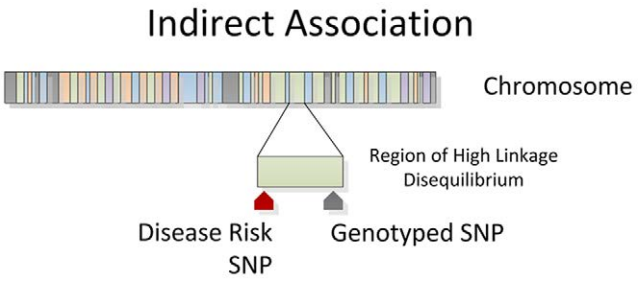

Figure 3. Indirect Association. Genotyped SNPs often lie in a region of high linkage disequilibrium with an influential allele. The genotyped SNP will be statistically associated with disease as a surrogate for the disease SNP through an indirect association.
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additional studies to map the precise location of the influential SNP.

Conceptually, the end result of GWAS under the common disease/common variant hypothesis is that a panel of 500,000 to one million markers will identify common SNPs that are associated to common phenotypes. To conduct such a study practically requires a genotyping technology that can accurately capture the alleles of 500,000 to one million SNPs for each individual in a study in a cost-effective manner.

4. Genotyping Technologies

Genome-wide association studies were made possible by the availability of chip-based microarray technology for assaying one million or more SNPs. Two primary platforms have been used for most GWAS. These include products from Illumina (San Diego, CA) and Affymetrix (Santa Clara, CA). These two competing technologies have been recently reviewed [20] and offer different approaches to measure SNP variation. For example, the Affymetrix platform prints short DNA sequences as a spot on the chip that recognizes a specific SNP allele. Alleles (i.e. nucleotides) are detected by differential hybridization of the sample DNA. Illumina on the other hand uses a bead-based technology with slightly longer DNA sequences to detect alleles. The Illumina chips are more expensive to make but provide better specificity.

Aside from the technology, another important consideration is the SNPs that each platform has selected for assay. This can be important depending on the specific human population being studied. For example, it is important to use a chip that has more SNPs with better overall genomic coverage for a study of Africans than Europeans. This is because African genomes have had more time to recombine and therefore have less LD between alleles at different SNPs. More SNPs are needed to capture the variation across the African genome.

It is important to note that the technology for measuring genomic variation is changing rapidly. Chip-based genotyping platforms such as those briefly mentioned above will likely be replaced over the next few years with inexpensive new technologies for sequencing the entire genome. These next-generation sequencing methods will provide all the DNA sequence variation in the genome. It is time now to retool for this new onslaught of data.

5. Study Design

Regardless of assumptions about the genetic model of a trait, or the technology used to assess genetic variation, no genetic study will have meaningful results without a thoughtful approach to characterize the phenotype of interest. When embarking on a genetic study, the initial focus should be on identifying precisely *what* quantity or trait genetic variation influences.

5.1 Case Control versus Quantitative Designs

There are two primary classes of phenotypes: categorical (often binary case/control) or quantitative. From the statistical perspective, quantitative traits are preferred because they improve power to detect a genetic effect, and often have a more interpretable outcome. For some disease traits of interest, quantitative disease risk factors have already been identified. High-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol levels are strong predictors of heart disease, and so genetic studies of heart disease outcomes can be conducted by examining these levels as a quantitative trait. Assays for HDL and LDL levels, being already useful for clinical practice, are precise and ubiquitous measurements that are easy to obtain. Genetic variants that influence these levels have a clear interpretation – for example, a unit change in LDL level per allele or by genotype class. With an easily measurable ubiquitous quantitative trait, GWAS of blood lipids have been conducted in numerous cohort studies. Their results were also easily combined to conduct an extremely well-powered massive meta-analysis, which revealed 95 loci associated to lipid traits in more than 100,000 people [21]. Here, HDL and LDL may be the primary traits of interest or can be considered intermediate quantitative traits or endophenotypes for cardiovascular disease.

Other disease traits do not have well-established quantitative measures. In these circumstances, individuals are usually classified as either affected or unaffected – a binary categorical variable. Consider the vast difference in measurement error associated with classifying individuals as either "case" or "control" versus precisely measuring a quantitative trait. For example, multiple sclerosis is a complex clinical phenotype that is often diagnosed over a long period of time by ruling out other possible conditions. However, despite the "loose" classification of case and control, GWAS of multiple sclerosis have been enormously successful, implicating more than 10 new genes for the disorder [22]. So while quantitative outcomes are preferred, they are not required for a successful study.

5.2 Standardized Phenotype Criteria

A major component of the success with multiple sclerosis and other well-conducted case/control studies is the definition of rigorous phenotype criteria, usually presented as rule list based on clinical variables. Multiple sclerosis studies often use the McDonald criteria for establishing case/control status and defining clinical subtypes [23]. Standardized methods like the McDonald criteria establish a concise, evidence-based approach that can be uniformly applied by multiple diagnosing clinicians to ensure that consistent pheno-

type definitions are used for a genetic study.

Standardized phenotype rules are particularly critical for multi-center studies to prevent introducing a site-based effect into the study. And even when established phenotype criteria are used, there may be variability among clinicians in how those criteria are used to assign case/control status. Furthermore, some quantitative traits are susceptible to bias in measurement. For example, with cataract severity lens photographs are used to assign cases to one of three types of lens opacity. In situations where there may be disagreement among clinicians, a subset of study records is often examined by clinicians at multiple centers to assess interrater agreement as a measure of phenotyping consistency [24]. High interrater agreement means that phenotype rules are being consistently applied across multiple sites, whereas low agreement suggests that criteria are not uniformly interpreted or applied, and may indicate a need to establish more narrow phenotype criteria.

5.3 Phenotype Extraction from Electronic Medical Records

The last few years of genetic research has seen the growth of large clinical bio-repositories that are linked to electronic medical records (EMRs) [25]. The development of these resources will certainly advance the state of human genetics research and foster integration of genetic information into clinical practice. From a study design perspective, identifying phenotypes from EMRs can be challenging. Electronic medical records were established for clinical care and administrative purposes – not for research. As such, idiosyncrasies arise due to billing practices and other logistical reasons, and great care must be taken not to introduce biases into a genetic study.

The established methodology for conducting ''electronic phenotyping'' is to devise an initial selection algorithm (using structured EMR fields, such as billing codes, or text mining procedures on unstructured text), which identifies a record subset from the bio-repository. In cases where free text is parsed, natural language processing (NLP) is used in conjunction with a controlled vocabulary such as the Unified Medical Language System (UMLS) to relate text to more structured and uniform medical concepts. In some instances, billing codes alone may be sufficient to accurately identify individuals with a particular phenotype, but often combinations of billing and procedure codes, along with free text are necessary. Because every medical center has its own set of policies, care providers, and health insurance providers, some algorithms developed in one clinical setting may not work as well in another.

Once a manageable subset of records is obtained by an algorithm, the accuracy of the results is examined by clinicians or other phenotype experts as gold-standard for comparison. The positive predictive value (PPV) of the initial algorithm is assessed, and based on feedback from case reviewers, the selection algorithm is refined. This process of case-review followed by algorithmic refinement is continued until the desired PPV is reached.

This approach has been validated by replicating established genotype-phenotype relationships using EMR-derived phenotypes [16], and has been applied to multiple clinical and pharmacogenomic conditions [26–28].

6. Association Test

6.1 Single Locus Analysis

When a well-defined phenotype has been selected for a study population, and genotypes are collected using sound techniques, the statistical analysis of genetic data can begin. The *de facto* analysis of genome-wide association data is a series of single-locus statistic tests, examining each SNP independently for association to the phenotype. The statistical test conducted depends on a variety of factors, but first and foremost, statistical tests are different for quantitative traits versus case/control studies.

Quantitative traits are generally analyzed using *generalized linear model* (GLM) approaches, most commonly the Analysis of Variance (ANOVA), which is similar to linear regression with a categorical predictor variable, in this case genotype classes. The null hypothesis of an ANOVA using a single SNP is that there is no difference between the trait means of any genotype group. The assumptions of GLM and ANOVA are 1) the trait is normally distributed; 2) the trait variance within each group is the same (the groups are homoskedastic); 3) the groups are independent.

Dichotomous case/control traits are generally analyzed using either contingency table methods or *logistic regression*. Contingency table tests examine and measure the deviation from independence that is expected under the null hypothesis that there is no association between the phenotype and genotype classes. The most ubiquitous form of this test is the popular chi-square test (and the related Fisher's exact test).

Logistic regression is an extension of linear regression where the outcome of a linear model is transformed using a logistic function that predicts the probability of having case status given a genotype class. Logistic regression is often the preferred approach because it allows for adjustment for clinical covariates (and other factors), and can provide adjusted odds ratios as a measure of effect size. Logistic regression has been extensively developed, and numerous diagnostic procedures are available to aid interpretation of the model.

For both quantitative and dichotomous trait analysis (regardless of the analysis method), there are a variety of ways that genotype data can be encoded or shaped for association tests. The choice of data encoding can have implications for the statistical power of a test, as the degrees of freedom for the test may change depending on the number of genotype-based groups that are formed. *Allelic* association tests examine the association between one allele of the SNP and the phenotype. *Genotypic* association tests examine the association between genotypes (or genotype classes) and the phenotype. The genotypes for a SNP can also be grouped into genotype classes or models, such as dominant, recessive, multiplicative, or additive models [29].

Each model makes different assumptions about the genetic effect in the data – assuming two alleles for a SNP, A and a, a dominant model (for A) assumes that having one or more copies of the A allele increases risk compared to a (i.e. Aa or AA genotypes have higher risk). The recessive model (for A) assumes that two copies of the A allele are required to alter risk, so individuals with the AA genotype are compared to individuals with Aa and aa genotypes. The multiplicative model (for A) assumes that if there is $3\times$ risk for having a single A allele, there is a $9\times$ risk for having two copies of the A allele: in this case if the risk for Aa is k, the risk for AA is k^2. The additive model (for A) assumes that there is a uniform, linear increase in risk for each copy of the A allele, so if the risk is $3\times$ for Aa, there is a $6\times$ risk for AA - in this case the risk for Aa is k and the risk for AA is $2k$. A common practice for GWAS is to examine additive models only, as the additive model has reasonable power to detect both additive and dominant effects, but it is important to note that an additive model may be underpowered to detect some recessive effects [30]. Rather than

choosing one model *a priori*, some studies evaluate multiple genetic models coupled with an appropriate correction for multiple testing.

6.2 Covariate Adjustment and Population Stratification

In addition to selecting an encoding scheme, statistical tests should be adjusted for factors that are known to influence the trait, such as sex, age, study site, and known clinical covariates. Covariate adjustment reduces spurious associations due to sampling artifacts or biases in study design, but adjustment comes at the price of using additional degrees of freedom which may impact statistical power. One of the more important covariates to consider in genetic analysis is a measure of population substructure. There are often known differences in phenotype prevalence due to ethnicity, and allele frequencies are highly variable across human subpopulations, meaning that in a sample with multiple ethnicities, ethnic-specific SNPs will likely be associated to the trait due to *population stratification*.

To prevent population stratification, the ancestry of each sample in the dataset is measured using STRUCTURE [31] or EIGENSTRAT [32] methods that compare genome-wide allele frequencies to those of HapMap ethnic groups. The results of these analyses can be used to either exclude samples with similarity to a non-target population, or they can be used as a covariate in association analysis. EIGENSTRAT is commonly used in this circumstance, where *principle component analysis* is used to generate principle component values that could be described as an "ethnicity score". When used as covariates, these scores adjust for minute ancestry effects in the data.

6.3 Corrections for Multiple Testing

A p-value, which is the probability of seeing a test statistic equal to or greater than the observed test statistic if the null hypothesis is true, is generated for each statistical test. This effectively means that lower p-values indicate that if there is no association, the chance of seeing this result is extremely small.

Statistical tests are generally called significant and the null hypothesis is rejected if the p-value falls below a predefined alpha value, which is nearly always set to 0.05. This means that 5% of the time, the null hypothesis is rejected when in fact it is true and we detect a *false positive*. This probability is relative to a single statistical test; in the case of GWAS, hundreds of thousands to millions of tests are conducted, each one with its own false positive probability. The cumulative likelihood of finding one or more false positives over the entire GWAS analysis is therefore much higher. For a somewhat morbid analogy, consider the probability of having a car accident. If you drive your car today, the probability of having an accident is fairly low. However if you drive every day for the next five years, the probability of you having one or more accidents over that time is much higher than the probability of having one today.

One of the simplest approaches to correct for multiple testing is the Bonferroni correction. The Bonferroni correction adjusts the alpha value from $\alpha = 0.05$ to $\alpha = (0.05/k)$ where k is the number of statistical tests conducted. For a typical GWAS using 500,000 SNPs, statistical significance of a SNP association would be set at 1e-7. This correction is the most conservative, as it assumes that each association test of the 500,000 is independent of all other tests – an assumption that is generally untrue due to linkage disequilibrium among GWAS markers.

An alternative to adjusting the false positive rate (alpha) is to determine the false discovery rate (FDR). The false discovery rate is an estimate of the proportion of significant results (usually at alpha = 0.05) that are false positives. Under the null hypothesis that there are no true associations in a GWAS dataset, p-values for association tests would follow a uniform distribution (evenly distributed from 0 to 1). Originally developed by Benjamini and Hochberg, FDR procedures essentially *correct* for this number of expected false discoveries, providing an estimate of the number of true results among those called significant [33]. These techniques have been widely applied to GWAS and extended in a variety of ways [34].

Permutation testing is another approach for establishing significance in GWAS. While somewhat computationally intensive, permutation testing is a straightforward way to generate the empirical distribution of test statistics for a given dataset when the null hypothesis is true. This is achieved by randomly reassigning the phenotypes of each individual to another individual in the dataset, effectively breaking the genotype-phenotype relationship of the dataset. Each random reassignment of the data represents one possible sampling of individuals under the null hypothesis, and this process is repeated a predefined number of times N to generate an empirical distribution with resolution N, so a permutation procedure with an N of 1000 gives an empirical p-value within $1/1000^{th}$ of a decimal place. Several software packages have been developed to perform permutation testing for GWAS studies, including the popular PLINK software [35], PRESTO [36], and PERMORY [37].

Another commonly used approach is to rely on the concept of *genome-wide significance*. Based on the distribution of LD in the genome for a specific population, there are an "effective" number of independent genomic regions, and thus an effective number of statistical tests that should be corrected for. For European-descent populations, this threshold has been estimated at 7.2e-8 [38]. This reasonable approach should be used with caution, however, as the only scenario where this correction is appropriate is when hypotheses are tested on the genome scale. Candidate gene studies or replication studies with a focused hypothesis do not require correction to this level, as the number of effective, independent statistical tests is much, much lower than what is assumed for genome-wide significance.

6.4 Multi-Locus Analysis

In addition to single-locus analyses, genome-wide association studies provide an enormous opportunity to examine interactions among genetic variants throughout the genome. *Multi-locus analysis*, however, is not nearly as straightforward as conducting single-locus tests, and presents numerous computational, statistical, and logistical challenges [39].

Because most GWAS genotype between 500,000 and one million SNPs, examining all pair-wise combinations of SNPs is a computationally intractable approach, even for highly efficient algorithms. One approach to this issue is to reduce or filter the set of genotyped SNPs, eliminating redundant information. A simple and common way to filter SNPs is to select a set of results from a single-SNP analysis based on an arbitrary significance threshold and exhaustively evaluate interactions in that subset. This can be perilous, however, as selecting SNPs to analyze based on main effects will prevent certain multi-locus models from being detected – so called "purely epistatic" models with statistically undetectable marginal effects. With these models, a large component of the heritability is concentrated in the interaction rather than in the main effects. In other words, a specific combination of markers

(and only the combination of markers) incurs a significant change in disease risk. The benefits of this analysis are that it performs an unbiased analysis for interactions within the selected set of SNPs. It is also far more computationally and statistically tractable than analyzing all possible combinations of markers.

Another strategy is to restrict examination of SNP combinations to those that fall within an established biological context, such as a biochemical pathway or a protein family. As these techniques rely on electronic repositories of structured biomedical knowledge, they generally couple a bioinformatics engine that generates SNP-SNP combinations with a statistical method that evaluates combinations in the GWAS dataset. For example, the Biofilter approach uses a variety of public data sources with logistic regression and multifactor dimensionality reduction methods [40,41]. Similarly, INTERSNP uses logistic regression, loglinear, and contingency table approaches to assess SNP-SNP interaction models [42].

7. Replication and Meta-Analysis

7.1 Statistical Replication

The gold standard for validation of any genetic study is replication in an additional independent sample. That said, there are a variety of criteria involved in defining "replication" of a GWAS result. This was the subject of an NHGRI working group, which outlined several criteria for establishing a positive replication [43]. These criteria are discussed in the following paragraphs.

Replication studies should have sufficient sample size to detect the effect of the susceptibility allele. Often, the effects identified in an initial GWAS suffer from winner's curse, where the detected effect is likely stronger in the GWAS sample than in the general population [44]. This means that replication samples should ideally be larger to account for the over-estimation of effect size. With replication, it is important for the study to be well-powered to identify spuriously associated SNPs where the null hypothesis is most likely true – in other words, to confidently call the initial GWAS result a false-positive.

Replication studies should be conducted in an independent dataset drawn from the same population as the GWAS, in an attempt to confirm the effect in the GWAS target population. Once an effect is confirmed in the target population, other populations may be sampled to determine if the SNP has an ethnic-specific effect. Replication of a significant result in an additional population is sometimes referred to as *generalization*, meaning the genetic effect is of general relevance to multiple human populations.

Identical phenotype criteria should be used in both GWAS and replication studies. Replication of a GWAS result should be thought of as the replication of a specific statistical model – a given SNP predicts a specific phenotype effect. Using even slightly different phenotype definitions between GWAS and replication studies can cloud the interpretation of the final result.

A similar effect should be seen in the replication set from the same SNP, or a SNP in high LD with the GWAS-identified SNP. Because GWAS typically use SNPs that are markers that were chosen based on LD patterns, it is difficult to say what SNP within the larger genomic region is mechanistically influencing disease risk. With this in mind, the unit of replication for a GWAS should be *the genomic region*, and all SNPs in high LD are potential replication candidates. However, continuity of effect should be demonstrated across both studies, with the magnitude and direction of effect being similar for the genomic region in both datasets. If SNPs in high LD are used to demonstrate the effect in replication, the direction of effect must be determined using a reference panel to determine two-SNP haplotype frequencies. For example, if allele A is associated in the GWAS with an odds ratio of 1.5, and allele T of a nearby SNP is associated in the replication set with an odds ratio of 1.46, it must be demonstrated that allele A and allele T carry effects in the same direction. The most straightforward way to assess this is to examine a reference panel, such as the HapMap data, for a relevant population. If this panel shows that allele A from SNP 1 and allele T from SNP 2 form a two-marker haplotype in 90% of the sample, then this is a reasonable assumption. If however the panel shows that allele A from SNP 1 and allele A from SNP 2 form the predominant two-marker haplotype, the effect has probably flipped in the replication set. Mapping the effect through the haplotype would be equivalent to observing an odds ratio of 1.5 in the GWAS and 0.685 in the replication set.

In brief, the general strategy for a replication study is to repeat the ascertainment and design of the GWAS as closely as possible, but examine only specific genetic effects found significant in the GWAS. Effects that are consistent across the two studies can be labeled *replicated effects*.

7.2 Meta-Analysis of Multiple Analysis Results

The results of multiple GWAS studies can be pooled together to perform a meta-analysis. Meta-analysis techniques were originally developed to examine and refine significance and effect size estimates from multiple studies *examining the same hypothesis* in the published literature. With the development of large academic consortia, meta-analysis approaches allow the synthesis of results from multiple studies without requiring the transfer of protected genotype or clinical information to parties who were not part of the original study approval – only statistical results from a study need be transferred. For example, a recent publication examining lipid profiles was based on a meta-analysis of 46 studies [21]. A study of this magnitude would be logistically difficult (if not impossible) without meta-analysis. Several software packages are available to facilitate meta-analysis, including STATA products and METAL [45,46].

A fundamental principle in meta-analysis is that all studies included examined the same hypothesis. As such, the general design of each included study should be similar, and the study-level SNP analysis should follow near-identical procedures across all studies (see Zeggini and Ioannidis [47] for an excellent review). Quality control procedures that determine which SNPs are included from each site should be standardized, along with any covariate adjustments, and the measurement of clinical covariates and phenotypes should be consistent across multiple sites. The sample sets across all studies should be independent – an assumption that should always be examined as investigators often contribute the same samples to multiple studies. Also, an extremely important and somewhat bothersome logistical matter is ensuring that all studies report results relative to a common genomic build and reference allele. If one study reports its results relative to allele A and another relative to allele B, the meta-analysis result for this SNP may be non-significant because the effects of the two studies nullify each other.

With all of these factors to consider, it is rare to find multiple studies that match perfectly on all criteria. Therefore, study heterogeneity is often statistically quantified in a meta-analysis to determine the degree to which studies differ. The most popular measures of study heterogeneity are the Q statistic and the I^2 index [48], with the I^2 index favored in more recent studies. Coefficients resulting from a meta-analysis have variability (or error) associated with

them, and the I^2 index represents the approximate proportion of this variability that can be attributed to heterogeneity between studies [49]. I^2 values fall into low (<25), medium (>25 and <75), and high (>75) heterogeneity, and have been proposed as a way to identify studies that should perhaps be removed from a meta-analysis. It is important to note that these statistics should be used as a guide to identifying studies that perhaps examine a different underlying hypothesis than others in the meta-analysis, much like outlier analysis is used to identify unduly influential points. Just as with outliers, however, a study should only be excluded if there is an obvious reason to do so based on the parameters of the study – not simply because a statistic indicates that this study increases heterogeneity. Otherwise, agnostic statistical procedures designed to reduce meta-analysis heterogeneity will increase false discoveries.

7.3 Data Imputation

To conduct a meta-analysis properly, the effect of the *same allele* across multiple distinct studies must be assessed. This can prove difficult if different studies use different genotyping platforms (which use different SNP marker sets). As this is often the case, GWAS datasets can be *imputed* to generate results for a common set of SNPs across all studies. Genotype imputation exploits known LD patterns and haplotype frequencies from the HapMap or 1000 Genomes project to estimate genotypes for SNPs not directly genotyped in the study [50].

The concept is similar in principle to *haplotype phasing* algorithms, where the contiguous set of alleles lying on a specific chromosome is estimated. Genotype imputation methods extend this idea to human populations. First, a collection of shared haplotypes within the study sample is computed to estimate haplotype frequencies among the genotyped SNPs. Phased haplotypes from the study sample are compared to reference haplotypes from a panel of much more dense SNPs, such as the HapMap data. The matched reference haplotypes contain genotypes for surrounding markers that were not genotyped in the study sample. Because the study sample haplotypes may match multiple reference haplotypes, surrounding genotypes may be given a score or probability of a match based on the haplotype overlap. For example, rather than assign an imputed SNP a single allele *A*, the probability of possible alleles is reported (0.85 *A*, 0.12 *C*, 0.03 *T*) based on haplotype frequencies. This information can be used in the analysis of imputed data to take into account uncertainty in the genotype estimation process, typically using Bayesian analysis approaches [51]. Popular algorithms for genotype imputation include BimBam [52], IMPUTE [53], MaCH [54], and Beagle [55].

Much like conducting a meta-analysis, genotype imputation must be conducted with great care. The reference panel (i.e. the 1000 Genomes data or the HapMap project) must contain haplotypes drawn from the same population as the study sample in order to facilitate a proper haplotype match. If a study was conducted using individuals of Asian descent, but only European descent populations are represented in the reference panel, the genotype imputation quality will be poor as there is a lower probability of a haplotype match. Also, the reference allele for each SNP must be identical in both the study sample and the reference panel. Finally, the analysis of imputed genotypes should account for the uncertainty in genotype state generated by the imputation process.

8. The Future

Genome-wide association studies have had a huge impact on the field of human genetics. They have identified new genetic risk factors for many common human diseases and have forced the genetics community to think on a genome-wide scale. On the horizon is whole-genome sequencing. Within the next few years we will see the arrival of cheap sequencing technology that will replace one million SNPs with the entire genomic sequence of three billion nucleotides. Challenges associated with data storage and manipulation, quality control and data analysis will be manifold more complex, thus challenging computer science and bioinformatics infrastructure and expertise. Merging sequencing data with that from other high-throughput technology for measuring the transcriptome, the proteome, the environment and phenotypes such as the massive amounts of data that come from neuroimaging will only serve to complicate our goal to understand the genotype-phenotype relationship for the purpose of improving healthcare. Integrating these many levels of complex biomedical data along with their coupling with experimental systems is the future of human genetics.

9. Exercises

1. True or False: Common diseases, such as type II diabetes and lung cancer, are likely caused by mutations to a single gene. Explain your answer.
2. Will the genotyping platforms designed for GWAS of European Descent populations be of equal utility in African Descent populations? Why or why not?
3. When conducting a genetic study, what additional factors should be measured and adjusted for in the statistical analysis?
4. True or False: SNPs that are associated to disease using GWAS design should be immediately considered for molecular studies. Explain your answer.

Answers to the Exercises can be found in Text S1.

Supporting Information

Text S1 Answers to Exercises
(DOCX)

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Further Reading

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Glossary

GWAS: genome-wide association study; a genetic study design that attempts to identify commonly occurring genetic variants that contribute to disease risk

Personalized Medicine: the science of providing health care informed by individual characteristics, such as genetic variation

SNP: single nucleotide polymorphism; a single base-pair change in the DNA sequence

Linkage Analysis: the attempt to statistically relate transmission of an allele within families to inheritance of a disease

Common disease/Common variant hypothesis: The hypothesis that commonly occurring diseases in a population are caused in part by genetic variation that is common to that population

Linkage disequilibrium: the degree to which an allele of one SNP is observed with an allele of another SNP within a population

Direct association: the statistical association of a functional or influential allele with a disease

Indirect association: the statistical association of an allele to disease that is in strong linkage disequilibrium with the allele that is functional or influential for disease

Population stratification: the false association of an allele to disease due to both differences in population frequency of the allele and differences in ethnic prevalence or sampling of affected individuals

False positive: from statistical hypothesis testing, the rejection of a null hypothesis when the null hypothesis is true

Genome-wide significance: a false-positive rate threshold established by empirical estimation of the independent genomic regions present in a population

Replication: the observation of a statistical association in a second, independent dataset (often the same population as the first association)

Generalization: the replication of a statistical association in a second population

Imputation: the estimation of unknown alleles based on the observation of nearby alleles in high linkage disequilibrium

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Chapter 12: Human Microbiome Analysis

Xochitl C. Morgan[1], Curtis Huttenhower[1,2]*

1 Department of Biostatistics, Harvard School of Public Health, Boston, Massachusetts, United States of America, **2** The Broad Institute of MIT and Harvard, Cambridge, Massachusetts, United States of America

Abstract: Humans are essentially sterile during gestation, but during and after birth, every body surface, including the skin, mouth, and gut, becomes host to an enormous variety of microbes, bacterial, archaeal, fungal, and viral. Under normal circumstances, these microbes help us to digest our food and to maintain our immune systems, but dysfunction of the human microbiota has been linked to conditions ranging from inflammatory bowel disease to antibiotic-resistant infections. Modern high-throughput sequencing and bioinformatic tools provide a powerful means of understanding the contribution of the human microbiome to health and its potential as a target for therapeutic interventions. This chapter will first discuss the historical origins of microbiome studies and methods for determining the ecological diversity of a microbial community. Next, it will introduce shotgun sequencing technologies such as metagenomics and metatranscriptomics, the computational challenges and methods associated with these data, and how they enable microbiome analysis. Finally, it will conclude with examples of the functional genomics of the human microbiome and its influences upon health and disease.

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1. Introduction

The question of what it means to be human is more often encountered in metaphysics than in bioinformatics, but it is surprisingly relevant when studying the human microbiome. We are born consisting only of our own eukaryotic human cells, but over the first several years of life, our skin surface, oral cavity, and gut are colonized by a tremendous diversity of bacteria, archaea, fungi, and viruses. The community formed by this complement of cells is called the human microbiome; it contains almost ten times as many cells as are in the rest of our bodies and accounts for several pounds of body weight and orders of magnitude more genes than are contained in the human genome [1,2]. Under normal circumstances, these microbes are commensal, helping to digest our food and to maintain our immune systems. Although the human microbiome has long been known to influence human health and disease [1], we have only recently begun to appreciate the breadth of its involvement. This is almost entirely due to the recent ability of high-throughput sequencing to provide an efficient and cost-effective tool for investigating the members of a microbial community and how they change. Thus, dysfunctions of the human microbiota are increasingly being linked to disease ranging from inflammatory bowel disease to diabetes to antibiotic-resistant infection, and the potential of the human microbiome as an early detection biomarker and target for therapeutic intervention is a vibrant area of current research.

2. A Brief History of Microbiome Studies

Historically, members of a microbial community were identified *in situ* by stains that targeted their physiological characteristics, such as the Gram stain [3]. These could distinguish many broad clades of bacteria but were non-specific at lower taxonomic levels. Thus, microbiology was almost entirely culture-dependent; it was necessary to grow an organism in the lab in order to study it. Specific microbial species were detected by plating samples on specialized media selective for the growth of that organism, or they were identified by features such as the morphological characteristics of colonies, their growth on different media, and metabolic production or consumption. This approach limited the range of organisms that could be detected to those that would actively grow in laboratory culture, and it led the close study of easily-grown, now-familiar model organisms such as *Escherichia coli*. However, *E. coli* as a taxonomic unit accounts for at most 5% of the microbes occupying the typical human gut [2]. The vast majority of microbial species have never been grown in the laboratory, and options for studying and quantifying the uncultured were severely limited until the development of DNA-based culture-independent methods in the 1980s [4].

Culture-independent techniques, which analyze the DNA extracted directly from a sample rather than from individually cultured microbes, allow us to investigate several aspects of microbial communities (Figure 1). These include taxonomic diversity, such as how many of which microbes are present in a community, and functional metagenomics, which attempts to describe which biological tasks the members of a community can or do carry out. The earliest DNA-based methods probed extracted community DNA for genes of interest by hybridization, or amplified specifically-targeted genes by PCR prior to sequencing. These studies were typically able to describe diversity at

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* E-mail: chuttenh@hsph.harvard.edu

What to Learn in This Chapter

- An overview of the analysis of microbial communities
- Understanding the human microbiome from phylogenetic and functional perspectives
- Methods and tools for calculating taxonomic and phylogenetic diversity
- Metagenomic assembly and pathway analysis
- The impact of the microbiome on its host

a broad level, or detect the presence or absence of individual biochemical functions, but with few details in either case.

One of the earliest targeted metagenomic assays for studying uncultured communities without prior DNA extraction was fluorescent *in situ* hybridization (FISH), in which fluorescently-labeled, specific oligonuclotide probes for marker genes are hybridized to a microbial community [5]. FISH probes can be targeted to almost any level of taxonomy from species to phylum. Although FISH was initially limited to the 16S rRNA marker gene and thus to diversity studies, it has since been expanded to functional gene probes that can be used to identify specific enzymes in communities [6]. However, it remains a primarily low-throughput, imaging-based technology.

To investigate microbial communities efficiently at scale, almost all current studies employ high-throughput DNA sequencing, increasingly in combination with other genome-scale platforms such as proteomics or metabolomics. Although DNA sequencing has existed since the 1970s [7,8], it was historically quite expensive; sequencing environmental DNA further required the additional time and expense of clone library construction. It was not until the 2005 advent of next-generation high-throughput sequencing [9] that it became economically feasible for most scientists to sequence the DNA of an entire environmental sample, and metagenomic studies have since become increasingly common.

3. Taxonomic Diversity

3.1 The 16S rRNA Marker Gene

Like a metazoan, a microbial community consists fundamentally of a collection of individual cells, each carrying a distinct complement of genomic DNA. Communities, however, obviously differ from multicellular organisms in that their component cells may or may not carry identical genomes, although substantial subsets of these cells are typically assumed to be clonal. One can thus assign a frequency to each distinct genome within the community describing either the absolute number of cells in which it is carried or their relative abundance within the population. As it is impractical to fully sequence every genome in every cell (a statement that should remain safely true no matter how cheap high-throughput sequencing becomes), microbial ecology has defined a number of molecular markers that (more or less) uniquely tag distinct genomes. Just as the make, model, and year of a car identify its components without the need to meticulously inspect the entirety of every such car, a marker is a DNA sequence that identifies the genome that contains it, without the need to sequence the entire genome.

Although different markers can be chosen for analyzing different populations, several properties are desirable for a good marker. A marker should be present in every member of a population, should differ only and always between individuals with distinct genomes, and, ideally, should differ proportionally to the evolutionary distance between distinct genomes. Several such markers have been defined, including ribosomal protein subunits, elongation factors, and RNA polymerase subunits [10], but by far the most ubiquitous (and historically significant [11]) is the small or 16S ribosomal RNA subunit gene [12]. This 1.5 Kbp gene is commonly referred to as the 16S rRNA (after transcription) or sometimes rDNA; it satisfies the criteria of a marker by containing both highly conserved, ubiquitous sequences and regions that vary with greater or lesser frequency over evolutionary time. It is relatively cheap and simple to sequence only the 16S sequences from a microbiome [13], thus describing the population as a set of 16S sequences and the number of times each was detected. Sequences assayed in this manner have been characterized for a wide range of cultured species and environmental isolates; these are stored and can be automatically matched against several databases including GreenGenes [14], the Ribosomal Database Project [15], and Silva [16].

3.2 Binning 16S rRNA Sequences into OTUs

A bioinformatic challenge that arises immediately in the analysis of rRNA genes is the precise definition of a "unique" sequence. Although much of the 16S rRNA gene is highly conserved, several of the sequenced regions are variable or hypervariable, so small numbers of base pairs can change in a very short period of evolutionary time [17]. Horizontal transfer, multicopy or ambiguous rDNA markers, and other confounding factors do, however, blur the biological meaning of "species" as well as our ability to resolve them technically [17]. Finally, because 16S regions are typically sequenced using only a single pass, there is a fair chance that they will thus contain at least one sequencing error. This means that requiring tags to be 100% identical will be extremely conservative and treat essentially clonal genomes as different organisms. Some degree of sequence divergence is typically allowed - 95%, 97%, or 99% are sequence similarity cutoffs often used in practice [18] - and the resulting cluster of nearly-identical tags (and thus assumedly identical genomes) is referred to as an Operational Taxonomic Unit (OTU) or sometimes phylotype. OTUs take the place of "species" in many microbiome diversity analyses because named species genomes are often unavailable for particular marker sequences. The assignment of sequences to OTUs is referred to as binning, and it can be performed by A) unsupervised clustering of similar sequences [19], B) phylogenetic models incorporating mutation rates and evolutionary relationships [20], or C) supervised methods that directly assign sequences to taxonomic bins based on labeled training data [21] (which also applies to whole-genome shotgun sequences; see below).

The binning process allows a community to be analyzed in terms of discrete bins or OTUs, opening up a range of computationally tractable representations for biological analysis. If each OTU is treated as a distinct category, or each 16S sequence is binned into a named phylum or other taxonomic category, a pool of microbiome sequences can be represented as a histogram of bin counts [22]. Alternately, this histogram can be binarized into presence/absence calls for each bin across a collection of related samples. Because diverse, general OTUs will always be present in related communities, and overly-specific OTUs may not appear outside of their sample of origin, the latter approach is typically most useful for low-complexity microbiomes or OTUs at an

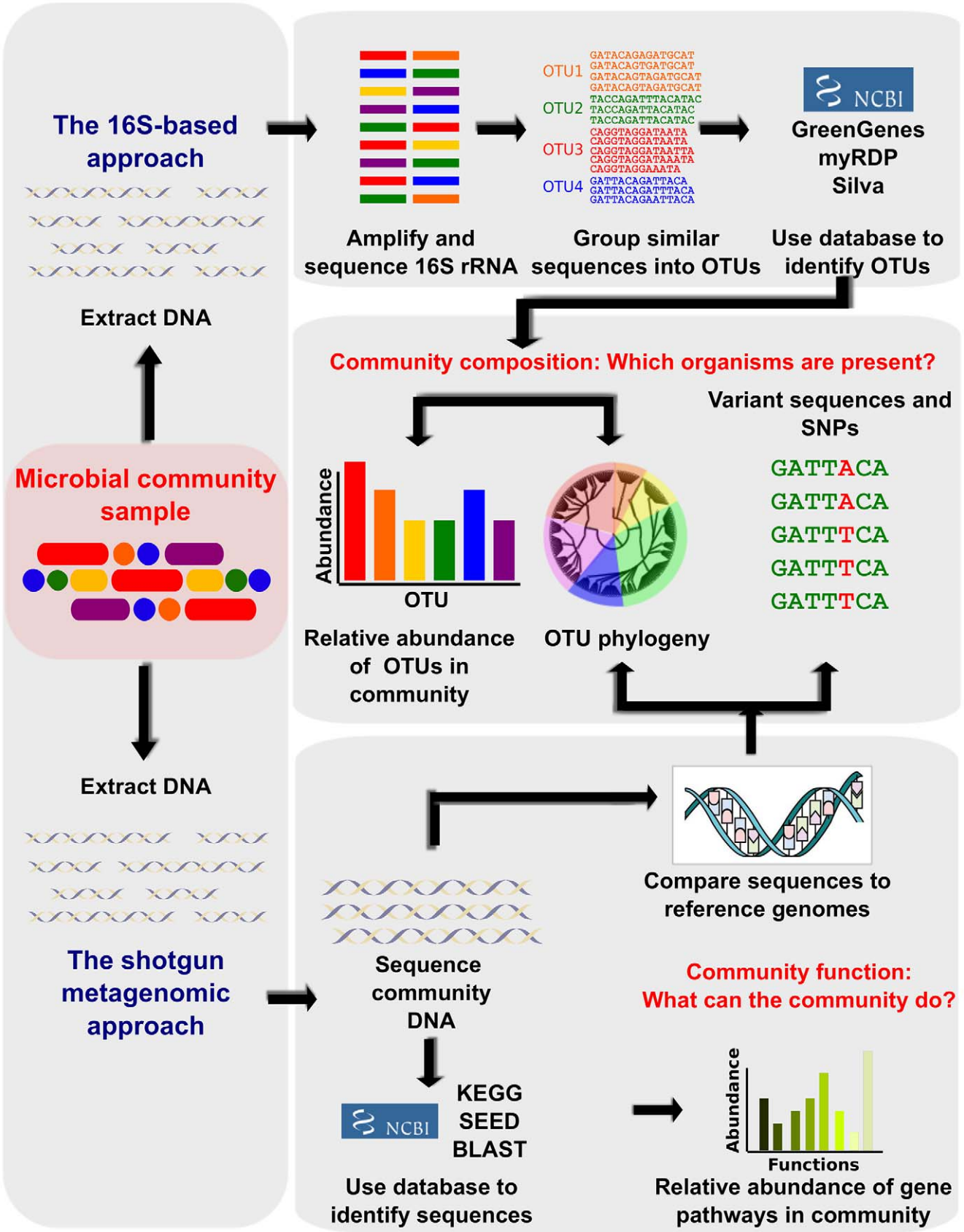

Figure 1. Bioinformatic methods for functional metagenomics. Studies that aim to define the composition and function of uncultured microbial communities are often referred to collectively as ''metagenomic,'' although this refers more specifically to particular sequencing-based assays. First, community DNA is extracted from a sample, typically uncultured, containing multiple microbial members. The bacterial taxa present in

the community are most frequently defined by amplifying the 16S rRNA gene and sequencing it. Highly similar sequences are grouped into Operational Taxonomic Units (OTUs), which can be compared to 16S databases such as Silva [16], Green Genes [14], and RDP [15] to identify them as precisely as possible. The community can be described in terms of which OTUs are present, their relative abundance, and/or their phylogenetic relationships. An alternate method of identifying community taxa is to directly metagenomically sequence community DNA and compare it to reference genomes or gene catalogs. This is more expensive but provides improved taxonomic resolution and allows observation of single nucleotide polymorphisms (SNPs) and other variant sequences. The functional capabilities of the community can also be determined by comparing the sequences to functional databases (e.g. KEGG [170] or SEED [171]). This allows the community to be described as relative abundances of its genes and pathways. Figure adapted from [172].
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appropriately tuned level of specificity. Bioinformaticians studying 16S sequences must choose whether to analyze a collection of taxonomically-binned microbiomes as a set of abundance histograms, or as a set of binary presence/absence vectors. However, either representation can be used as input to decomposition methods such as Principle Components Analysis or Canonical Correlation Analysis [23] to determine which OTUs represent the most significant sources of population variance and/or correlate with community metadata such as temperature, pH, or clinical features [24,25].

3.3 Measuring Population Diversity

An important concept when dealing with OTUs or other taxonomic bins is that of population diversity, the number of distinct bins in a sample or in the originating population. This is of critical importance in human health, since a number of disease conditions have been shown to correlate with decreased microbiome diversity, presumably as one or a few microbes overgrow during immune or nutrient imbalance in a process not unlike an algal bloom [26]. Intriguingly, recent results have also shown that essentially no bacterial clades are widely and consistently shared among the human microbiome [2]. Many organisms are abundant in some individuals, and many organisms are prevalent among most individuals, but none are universal. Although they can vary over time and share some similarity with some individuals, our intestinal contents appear to be highly personalized when considered in terms of microbial presence, absence, and abundance.

Two mathematically well-defined questions arise when quantifying population diversity (Figure 2): given that x bins have been observed in a sample of size y from a population of size z, how many bins are expected to exist in the population; or, given that x bins exist in a population of size z, how big must the sample size y be to observe all of them at least once? In other words, "If I've sequenced some amount of diversity, how much more exists in my microbiome?" and, "How much do I need to sequence to completely characterize my microbiome?" The latter is known as the Coupon Collector's Problem, as identical questions can be asked if a cereal manufacturer has randomly hidden one of several different possible prize coupons in each box of cereal [27]. Within a community, several estimators including the Chao1 [28], Abundance-based Coverage Estimator (ACE) [29], and Jackknife [30] measures exist for calculating alpha diversity, the number (richness) and distribution (evenness) of taxa expected within a single population. These give rise to figures known as collector's or rarefaction curves, since increasing numbers of sequenced taxa allow increasingly precise estimates of total population diversity [31]. Additionally, when comparing multiple popula-

A) Sequence Abundance

OTU	Sample 1	Sample 2	Sample 3
A	60	0	35
B	24	5	5
C	10	0	0
D	5	0	0
E	1	0	0
F	0	20	10
Total	100	25	50

B) Sequence Relative Abundance

OTU	Sample 1	Sample 2	Sample 3
A	0.60	0	0.70
B	0.24	0.20	0.10
C	0.10	0	0
D	0.05	0	0
E	0.01	0	0
F	0	0.80	0.20
Total	1.0	1.0	1.0

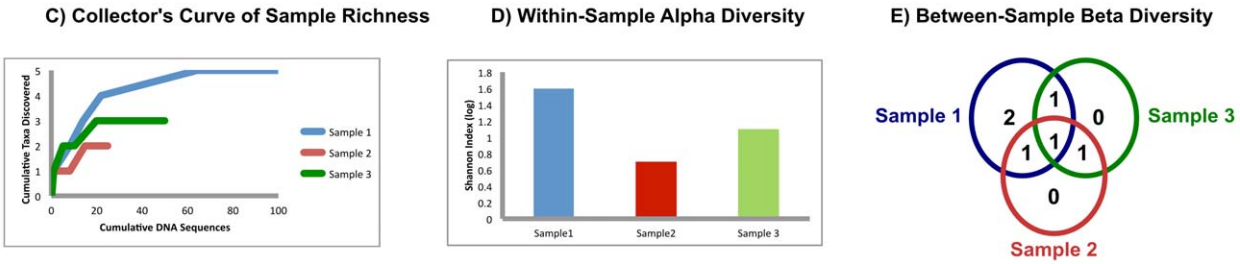

Figure 2. Ecological representations of microbial communities: collector's curves, alpha, and beta diversity. These examples describe the A) sequence counts and B) relative abundances of six taxa (A, B, C, D, E, and F) detected in three samples. C) A collector's curve, typically generated using a richness estimator such as Chao1 [28] or ACE [29], approximates the relationship between the number of sequences drawn from each sample and the number of taxa expected to be present based on detected abundances. D) Alpha diversity captures both the organismal richness of a sample and the evenness of the organisms' abundance distribution. Here, alpha diversity is defined by the Shannon index [32], $H' = -\sum_{i=1}^{S} (p_i \ln(p_i))$, where p_i is the relative abundance of taxon i, although many other alpha diversity indices may be employed. E) Beta diversity represents the similarity (or difference) in organismal composition between samples. In this example, it can be simplistically defined by the equation $\beta = (n_i - c) + (n_2 - c)$, where n_1 and n_2 are the number of taxa in samples 1 and 2, respectively, and c is the number of shared taxa, but again many metrics such as Bray-Curtis [34] or UniFrac [24] are commonly employed.
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tions, beta diversity measures including absolute or relative overlap describe how many taxa are shared between them (Figure 2). An alpha diversity measure thus acts like a summary statistic of a single population, while a beta diversity measure acts like a similarity score between populations, allowing analysis by sample clustering or, again, by dimensionality reductions such as PCA [20]. Alpha diversity is often quantified by the Shannon Index [32], $H' = -\sum_{i=1}^{S}(p_i \ln(p_i))$, or the Simpson Index [33], $D = \sum_{i=1}^{S} p_i^2$, where p_i is the fraction of total species comprised by species i. Beta diversity can be measured by simple taxa overlap or quantified by the Bray-Curtis dissimilarity [34], $BC_{ij} = \frac{S_i + S_j - 2C_{ij}}{S_i + S_j}$, where S_i and S_j are the number of species in populations i and j, and C_{ij} is the total number of species at the location with the fewest species. Like similarity measures in expression array analysis, many alpha- and beta-diversity measures have been developed that each reveal slightly different aspects of community ecology.

Alternatively, the diversity within or among communities can be analyzed in terms of its phylogenetic distribution rather than by isolating discrete bins. This method of quantifying community diversity describes it in terms of the total breadth or depth of the phylogenetic branches spanned by a microbiome (or shared among two or more). For example, consider a collection of n highly-related 16S sequences. These might be treated either as one OTU or as n distinct taxa, depending on how finely they are binned, but a phylogenetic analysis will consider them to span a small evolutionary distance no matter how large n becomes. Conversely, two highly-divergent binned OTUs are typically no different than two similar OTUs, but a phylogenetic method would score them as spanning a large evolutionary distance. OTU-based and phylogenetic methods tend to be complementary, in that each will reveal different aspects of community structure. OTUs are highly sensitive to the specific means by which taxa are binned, for example, whereas phylogenetic measures are sensitive to the method of tree construction. Like the OTU-based diversity estimators discussed above, several standard metrics such as UniFrac [20] exist for quantifying phylogenetic diversity, and these can be treated as single-sample descriptors or as multiple-sample similarity measures.

It is critically important in any microbiome richness analysis to account for the contribution that technical noise will make to apparent diversity. As a simple example, consider that a single base pair error in a 100 bp sequence read will create a new OTU at the 99% similarity threshold. Apparent diversity can thus be dramatically modified by the choice of marker gene, the region within it that is sequenced, the biochemical marker extraction and amplification processes, and the read length and noise characteristics of the sequencing platform. Accounting for such errors computationally continues to be a fruitful area of research, particularly as 454-based technologies have transitioned to the Illumina platform, as current solutions can discard all but the highest-quality sequence regions [18]. A major confound in many early molecular richness analyses was the abundance of chimeric sequences, or reads in which two unique marker sequences (typically 16S regions) adhere during the amplification process, creating an apparently novel taxon. Although sequence chimeras can now be reliably removed computationally [13,19,35], this filtering process is still an essential early step in any microbiome analysis.

A final consideration in the computational analysis of community structure assays is the use of microarray-based methods for 16S (and other marker) quantification within a microbiome. Just as high-throughput RNA sequencing parallels gene expression microarrays, 16S rDNA sequencing parallels phylochips, microarrays constructed with probes complementary to a variety of 16S and other marker sequences [36]. The design and analysis of such arrays can be challenging, as 16S sequences (or any good genomic markers) will be highly similar, and the potential for extensive cross-hybridization must be taken into account both when determining what sequences to place on a chip and how to quantify their abundance after hybridization [37]. The continued usefulness of such arrays will be dictated by future trends in high-throughput sequencing costs and barcoding, but at present phylochips are beginning to be constructed to capture functional sequences in combination with measures of taxon abundances in high throughput, and they represent an interesting option for population-level microbiome assays.

4. Shotgun Sequencing and Metagenomics

While measures of community diversity have dominated historical analyses, modern high-throughput methods are being developed for a host of other "meta" assays from uncultured microbes. The term metagenomics is used with some frequency to describe the entire body of high-throughput studies now possible with microbial communities, although it also refers more specifically to whole-metagenome shotgun (WMS) sequencing of genomic DNA fragments from a community's metagenome [38,39]. Metatranscriptomics, a close relative, implies shotgun sequencing of reverse-transcribed RNA transcripts [40,41], metaproteomics [42,43] the quantification of protein or peptide levels, and metametabolomics (or less awkwardly community metabolomics) [44,45] the investigation of small-molecule metabolites. Of these assays, the latter three in particular are still in their infancy, but are carried out using roughly the same technologies as their culture-based counterparts, and the resulting data can typically be analyzed using comparable computational methods.

As of this writing, no complete meta-metabolomic studies from uncultured microbiomes have yet been published, although their potential usefulness in understanding e.g. the human gut microbiome and its role in energy harvest, obesity, and metabolic disorders is clear [44]. Metaproteomic and metatranscriptomic studies have primarily focused on environmental samples [46,47,48], but human stool metatranscriptomics [41,49] and medium-throughput human gut metaproteomics [42,43] have also been successfully executed and analyzed using bioinformatics similar to those for metagenomes (see below) [42]. Quantification of the human stool metatranscriptome and metaproteome in tandem with host biomolecular activities should yield fascinating insights into our relationship with our microbial majority.

DNA extraction and WMS sequencing from uncultured samples developed, like many sequencing technologies, concurrently with the Human Genome Project [2,50,51,52], and as with other community genomic assays, the earliest applications were to environmental microbes due to the ease of isolation and extraction [53,54]. WMS techniques are in some ways much the same now as they were then, modulo the need for complex Sanger clone library construction: isolate microbial cells of a target size range (e.g. viral, bacterial, or eukaryotic), lyse the cells (taking care not to lose DNA to native DNAses), isolate DNA, fragment it to a target length, and sequence the resulting fragments [55,56]. Since this procedure can be performed on essentially any heterogeneous population, does not suffer from the single-copy and evolutionary

assumptions of marker genes, and does not require (although can include) amplification, it can to some degree produce a less biased community profile than does 16S sequencing [57].

4.1 Metagenome Data Analysis

Unlike whole-genome shotgun (WGS) sequencing of individual organisms, in which the end product is typically a single fully assembled genome, metagenomes tend not to have a single "finish line" and have been successfully analyzed using a range of assembly techniques. The simplest is no assembly at all - the short reads produced as primary data can, after cleaning to reduce sequencing error [18], be treated as taxonomic markers or as gene fragments and analyzed directly. Since microbial genomes typically contain few intergenic sequences, most fragments will contain pieces of one or more genes; these can be used to quantify enzymatic or pathway abundances directly as described below [1,58,59,60]. Alternatively, metagenome-specific assembly algorithms have been proposed that reconstruct only the open reading frames from a population (its ORFeome), recruiting highly sequence-similar fragments on an as-needed basis to complete single gene sequences and avoiding assembly of larger contigs [61,62]. The most challenging option is to attempt full assemblies for complete genomes present in the community, which is rarely possible save in very simple communities or with extreme sequencing depth [53,54]. When successful, this has the obvious benefit of establishing synteny, structural variation, and opening up the range of tools developed for whole-genome analysis [63], and guided assemblies using read mapping (rather than *de novo* assembly) can be used when appropriate reference genomes are available. However, care must be taken in interpreting any such assemblies, since horizontal transfer and community complexity prevent unambiguous assemblies in essentially all realistic cases [64]. A more feasible middle ground is emerging around maximal assemblies that capture the largest unambiguous contigs in a community [65], allowing e.g. local operon structure to be studied without introducing artificial homogeneity into the data. In any of these cases - direct analysis of reads, ORF assembly, maximal unambiguous scaffolds, or whole genomes - subsequent analyses typically focus on the functional aspects of the resulting genes and pathways as detailed below.

A key bioinformatic tradeoff in analyzing metagenomic WMS sequences, regardless of their degree of assembly, is whether they should be analyzed by homology, *de novo*, or a combination thereof. An illustrative example is the task of determining which parts of each sequence read (or ORF/contig/etc.) encode one or more genes, i.e. gene finding or calling. By homology, each sequence can be BLASTed [66] against a large database of reference genomes, which will retrieve any similar known reading frames; the boundaries of these regions of similarity thus become the start and stop of the metagenomic open reading frames. This method is robust to sequencing and assembly errors, but it is sensitive to the contents of the reference database. Conversely, *de novo* methods have been developed to directly bin [67,68,69] and call genes within [61,62] metagenomic sequences using DNA features alone (GC content, codon usage, etc.). As with genome analysis for newly sequenced single organisms, most *de novo* methods rely on interpolated [70] or profile [71] Hidden Markov Models (HMMs) or on other machine learners that perform classification based on encoded sequence features [72,73]. This is a far more challenging task, making it sensitive to errors in the computational prediction process, but it enables a greater range of discovery and community characterization efforts by relying less on prior knowledge. Hybrid methods for e.g. taxonomic binning [69] have recently been developed that consume both sequence similarity and *de novo* sequence features as input, and for some tasks such systems might represent a sweet spot between computational complexity, availability of prior knowledge, and biological accuracy. This tradeoff between knowledge transfer by homology and *de novo* prediction from sequence is even more pronounced when characterizing predicted genes, as discussed below.

5. Computational Functional Metagenomics

Essentially any analysis of a microbial community is "functional" in the sense that it aims to determine the overall phenotypic consequences of the community's composition and biomolecular activity. For example, the Human Microbiome Project began to investigate what typical human microbial community members are doing [60], how they are affecting their human hosts [2], what impact they have on health or disease, and these help to suggest how pro- or antibiotics can be used to change community behavior for the better [74]. The approaches referred to as computational functional metagenomics, however, typically focus on the function (either biochemically or phenotypically) of individual genes and gene products within a community and fall into one of two categories. Top-down approaches screen a metagenome for a functional class of interest, e.g. a particular enzyme family, transporter or chelator, pathway, or biological activity, essentially asking the question, "Does this community carry out this function and, if so, in what way?" Bottom-up approaches attempt to reconstruct profiles, either descriptive or predictive, of overall functionality within a community, typically relying on pathway and/or metabolic reconstructions and asking the question, "What functions are carried out by this community?"

Either approach relies, first, on cataloging some or all of the gene products present in a community and assigning them molecular functions and/or biological roles in the typical sense of protein function predictions [53,54,59]. As with so many bioinformatic methods, the simplest techniques rely on BLAST [66]: a top-down investigation can BLAST representatives of gene families of interest into the community metagenome to determine their presence and abundance [63], and a bottom-up approach can BLAST reads or contigs from a metagenome into a large annotated reference database such as nr to perform knowledge transfer by homology [75,76,77]. Top-down approaches dovetail well with experimental screens for individual gene product function [6], and bottom-up approaches are more descriptive of the community as a whole [78].

As each metagenomic sample can contain millions of reads and databases such as nr in turn contain millions of sequences, computational efficiency is a critical consideration in either approach. On one hand, stricter nucleotide searches or direct read mapping to reference genomes [79,80] improve runtime and specificity at the cost of sensitivity; on the other, more flexible characterizations of sequence function such as HMMs [72,73] tend to simultaneously increase coverage, accuracy, and computational expense. Any of these sequence annotation methods can be run directly on short reads, on ORF assemblies, or on assembled contigs, and statistical methods have been proposed to more accurately estimate the frequencies of functions in the underlying community when they are under-sampled (requiring the estimation of unobserved values [81]) or over-sampled (correcting for loci with greater than 1× coverage [82]). In any of these cases, the end result

of such an analysis is an abundance profile for each metagenomic sample quantifying the frequency of gene products in the community; the profiles for several related communities can be assembled into a frequency matrix resembling a microarray dataset. Gene products (rows) in such a profile can be identified by functional descriptors such as Gene Ontology [83] or KEGG [84] terms, protein families such as Pfams [73] or TIGRfams [72], enzymatic [85], transport [86], or other structural classes [87], or most often as orthologous families such as HomoloGenes [88], COGs [89], NOGs [90], or KOs [84].

A logical next step, given such an abundance profile of orthologous families, is to assemble them into profiles of community metabolic and functional pathways. This requires an appropriate catalog of reference pathways such as KEGG [84], MetaCyc [91], or GO [83], although it should be noted that none of these is currently optimized for modeling communities rather than single organisms in monoculture [90]. The pathway inference process is similar to that performed when annotating an individual newly sequenced genome [92] and consists of three main steps: A) assigning each ortholog to one or more pathways, B) gap filling or interpolation of missing annotations, and C) determining the presence and/or abundance of each pathway. The first ortholog assignment step is necessary since many gene families participate in multiple pathways; phosphoenolpyruvate carboxykinase, for example, is used in the TCA cycle, glycolysis, and in various intercellular signaling mechanisms [93]. The abundance mass for each enzyme is distributed across its functions in one or more possible pathways; methods for doing this range from the simple assumption that it is equally active in all reference pathways (as currently done by KAAS [94] or MG-RAST [76]) to the elimination of unlikely pathways and the redistribution of associated mass in a maximum parsimony fashion [95]. Second, once all observed orthologs have been assigned to pathways (when possible), gaps or holes in the reference pathways can be filled, using the assumption that the enzymes necessary to operate a nearly complete pathway should be present somewhere in the community. Essentially three methods have been successfully employed for gap filling: searching for alternative pathway fragments to explain the discrepancy [96,97], purely mathematical smoothing to replace the missing enzymes' abundances with numerically plausible values [81], and targeted searches of the metagenome of interest for more distant homologues with which to fill the hole [98]. Since we are currently able to infer function for only a fraction of the genes in any given complete genome, let alone metagenome, any of these approaches should be deemed hypothetical at best; nevertheless, like any missing value imputation process, they can provide numerically stable guesses that are substantially better than random [99]. Finally, as described above for taxa, the resulting data can be used to summarize each reference pathway either qualitatively (i.e. with what likelihood is it present in the community?) or quantitatively (how abundant is it in the community?), and in its simplest form condenses the abundance matrix of orthologous families into an abundance (or presence/absence) matrix of pathways. Either the ortholog or pathway matrices can then be tested for differentially abundant features representing diagnostic biomarkers with potential explanatory power for the phenotype of interest, using statistical methods developed for identical tests in expression biomarker discovery [100] and genome-wide association studies [101].

However, our prior knowledge of (primarily) metabolic pathways can be leveraged to produce richer inferences from such pathway abundance information. Given sufficient information about the pathways in a community, it is relatively straightforward to predict what metabolic compounds have the potential to be produced. However, it is much more difficult to infer what metabolite pools and fluxes in the community will actually be under a specific set of environmental conditions [102,103]. Multi-organism flux balance analysis (FBA) is an emerging tool to enable such analyses [104], but given the extreme difficulty of constructing accurate models for even single organisms [105] or of determining model parameters in a multi-organism community [53], no successful reconstructions have yet been performed for complex microbiomes. The area holds tremendous promise, however, first with respect to metabolic engineering - it is not yet clear what successes might be achieved with respect to biofuel production or bioremediation using synthetically manipulated communities in place of individual organisms [106,107]. Second, in addition to metabolite profiling, multi-organism growth prediction allows the determination of mutualisms, parasitisms, and commensalisms among taxa in the community [108] [109,110], opening the door to basic biological discoveries regarding community dynamics [25,111,112] and to therapeutic probiotic treatments for dysbioses in the human microbiome [113,114].

6. Host Interactions and Interventions

A final but critical aspect of translational metagenomics lies in understanding not only a microbial community but also its environment - that is, its interaction with a human host. Our microbiota would be of interest to basic research alone if they were not heavily influenced by host immunity and, in turn, a major influence on host health and disease. The skin of humans hosts relatively few taxa (e.g. *Propionibacterium* [115]), the nasal cavity somewhat more (e.g. *Corynebacterium* [116]), the oral cavity (dominated by *Streptococcus*) several hundred taxa (with remarkable diversity even among saliva, tongue, teeth, and other substrates [117,118]) and the gut over 500 taxa with densities over 10^{11} cells/g [2,119]. Almost none of these communities are yet well-understood, although anecdotes abound. The skin microbiome is thought to be a key factor in antibiotic resistant *Staphylococcus aureus* infections [120,121]; nasal communities have interacted with the pneumococcus population to influence its epidemiological carriage patterns subsequent to vaccination programs [122]; and extreme dysbiosis in cystic fibrosis can be a precursor to pathogenic infection [123].

The gut, however, is currently the best-studied human microbiome [119,124,125]. It is a dynamic community changing over the course of days [126,127], over the longer time scales of infant development [112,128,129,130] and aging [131,132], in response to natural perturbations such as diet [59,133,134,135] and illness [114,136], and modified in as-yet-unknown ways by the modern prevalence of travel, chemical additives, and antibiotics [126]. Indeed, the human gut microbiome has proven difficult to study exactly because it is so intimately related to the physiology of its host; inasmuch as no two people share identical microbiota, most microbiomes are strikingly divergent between distinct host species, rendering results from model organisms difficult to interpret [137,138]. Nevertheless, studies in wild type vertebrates such as mice [139,140] and zebrafish [141,142] have found a number of similarities in their microbiotic function and host interactions. In particular, germ-free organisms have yielded insights into the microbiota's role in maturation of the host immune system and, surprisingly, even

anatomical development of the intestine [143,144]. Similarly, gnotobiotic systems in which an organism's natural microbiota are replaced with their human analog are a current growth area for closer study of the phenotypic consequences of controlled microbiotic perturbations [145].

One of the highest-profile demonstrations of this technique and of the microbiota's influence on human health has been in an ongoing study of the microbiome in obesity [146]. Early studies in wild-type mice [139] demonstrated gross taxonomic shifts in the composition and diversity of the microbiomes of obese individuals; follow-ups in gnotobiotic mice confirmed that this phenotype was transmissible via the microbiome [147]. These initial studies were taxonomically focused and found that, while high-level phyla were robustly perturbed in obesity (which incurs a reduction in *Bacteroidetes* and concomitant increase in *Firmicutes* [139]), few if any specific taxa seemed to be similarly correlated [138,140]. Subsequent functional metagenomics, first in mouse [148] and later a small human cohort [59], established that the functional consistency of these shifts operates more consistently, enriching the microbiome's capacity for energy harvest and disregulating fat storage and signaling within the host. While these observations represent major descriptive triumphs, further computational and experimental work must yet be performed to establish the underlying biomolecular mechanisms and whether they are correlative, causative, or may be targeted by interventions to actively treat obesity [59].

A similarly complex community for which we have a greater understanding of the functional mechanisms at play is the formation of biofilms in the oral cavity preceding caries (cavities) or periodontitis [149]. While we are still investigating the microbiota of the saliva [150] and of the oral soft tissues [151], colonization of the tooth enamel is somewhat better understood due to the removal of significant interaction with host tissue. Even more strikingly, this biofilm, or physically structured consortium of multiple microbial taxa, must reestablish itself from almost nothing each time we brush our teeth - a process that can be achieved within hours [152]. Streptococci in particular possess a number of surface adhesins and receptors that enable them to behave as early colonizers on bare tooth surface and to bind together a variety of subsequent microbes [153]. These fairly minimal bacteria are metabolically supported by *Veillonella* and *Actinomyces* species, and their aggregation leads to local nutritive and structural environments favorable to e.g. *Fusobacterium* and *Porphyromonas* [154]. Each of these steps is mediated by a combination of cell surface recognition molecules, extracellular physical interactions, metabolic codependencies, and explicit intercellular signaling, providing an excellent example of the complexity with which structured microbiomes can evolve. Indeed, the evolvability of such systems, both as a whole [155] and at the molecular level [156], is yet another aspect of the work remaining to computationally characterize microbiotic biomolecular and community function.

Finally, the microbiota clearly represent a key component of future personalized medicine. First, the number and diversity of phenotypes linked to the composition of the microbiota is immense: obesity, diabetes, allergies, autism, inflammatory bowel disease, fibromyalgia, cardiac function, various cancers, and depression have all been reported to correlate with microbiome function [157]. Even without causative or modulatory roles, there is tremendous potential in the ability to use the taxonomic or metagenomic composition of a subject's gut or oral flora (both easily sampled) as a diagnostic or prognostic biomarker for any or all of these conditions. Commercial personal genomics services such as 23andMe (Mountain View, CA) promise to decode your disease risk based on somatic DNA from a saliva sample; bioinformatic techniques have yet to be developed that will allow us to do the same using microbial DNA.

Second, the microbiota are amazingly plastic; they change metagenomically within hours and metatranscriptomically within minutes in response to perturbations ranging from broad-spectrum antibiotics to your breakfast bacon and eggs [41,126,127]. For any phenotype to which they are causally linked, this opens the possibility of pharmaceutical, prebiotic (nutrients promoting the growth of beneficial microbes [113,119]), or probiotic treatments. Indeed, Nobel Prize winner Ilya Mechnikov famously named *Lactobacillus bulgaricus*, a primary yogurt-producing bacterium, for its apparent contribution to the longevity of yogurt-consuming Bulgarians [158], and despite a degree of unfortunate popular hype, the potential health benefits of a variety of probiotic organisms are indeed supported by recent findings [125,159]. Unfortunately, we currently understand few of the mechanisms by which these interventions operate. Do the supplemented organisms outcompete specific pathogens, do they simply increase their own numbers, or do they shift the overall systems-level balance of many taxa within the community? Do they reduce the levels of detrimental metabolites in the host, or do they increase the levels of beneficial compounds? Do they change biomolecular activity being carried out in microbial cells, adjacent host epithelial or immune cells, or distal cells through host signaling mechanisms? Or, as in polygenic genetic disorders, does a combination of many factors result in health or disease status as an emergent phenotype?

The human microbiome has been referred to as a "forgotten organ" [160], and the truth of both words is striking. Our trillions of microbial passengers account for a proportion of our metabolism and signaling as least as great as that performed by more integral body parts, and after a century of molecular biology, we have only begun to realize their importance within the last few years. To close with a success story, the popular press [161] recently reported on the full recovery of a patient suffering from *Clostridium difficile*-associated diarrhea, which had led her to lose over 60 pounds in less than a year. *C. difficile* is often refractory to antibiotics, with spores able to repopulate from very low levels, and the patient's normal microbiota had been decimated by the infection and subsequent treatment. Finally, she received a simple fecal transplant from her husband, in which the host microbiome was replaced with that of a donor. Within days, not only had she begun a complete recovery, but a metagenomic survey of her microbiota showed that the new community was almost completely established and had restored normal taxonomic abundances [162]. While this is an extreme case, similar treatments have shown a success rate of some 90% historically [163], all of which occurred before modern genomic techniques allowed us to more closely examine the microbiota. Imagine performing any other organ transplant with such a high rate of success - while blindfolded! Like so many other discoveries of the genomic era, the study of the human microbiome has begun with amazing achievements, and it will require continued experimental and bioinformatic efforts to better understand the biology of these microbial communities and to see it translated into clinical practice.

7. Summary

The human microbiome consists of unicellular microbes - mainly bacterial, but also archaeal, viral, and eukaryotic -

that occupy nearly every surface of our bodies and have been linked to a wide range of phenotypes in health and disease. High-throughput assays have offered the first comprehensive culture-free techniques for surveying the members of these communities and their biomolecular activities at the transcript, protein, and metabolic levels. Most current technologies rely on DNA sequencing to examine either individual taxonomic markers in a microbial community, typically the 16S ribosomal subunit gene, or the composite metagenome of the entire community. Taxonomic analyses lend themselves to computational techniques rooted in microbial ecology, including diversity measures within (alpha) and between (beta) samples; these can be defined quantitatively (based on abundance) or qualitatively (based on presence/absence), and they may or may not take into account the phylogenetic relatedness of the taxa being investigated. Finally, in the absence of information regarding specific named species in a community, sequences are often clustered by similarity into Operational Taxonomic Units (OTUs) as the fundamental unit of analysis within a sample.

In contrast, whole-genome shotgun analyses begin with sequences sampled from the entire community metagenome. These can also be taxonomically binned, or they can be assembled, partially assembled into ORFeomes, or characterized directly at the read level. Characterization typically consists of function assignment similar to that performed for genes during annotation of a single organism's genome; once genes in the metagenome are defined, they can be mapped or BLASTed to reference sequence databases or analyzed intrinsically using e.g. codon frequencies or HMM profiles. Finally, the frequencies of enzymes and other gene products so determined can be assigned to pathways, allowing inference of the overall metabolic potential of the community and inference of diagnostic and potentially explanatory functional biomarkers. Ongoing studies are beginning to investigate the ways in which the microbiota can be directly engineered using pharmaceuticals, prebiotics, probiotics, or diet as a preventative or treatment for a wide range of disorders.

8. Exercises

Q1. You have a collection of 16S rRNA gene sequencing data, which consists of an Illumina run in which the 100 bp V6 hypervariable region has been amplified. The error rate of Illumina sequencing has been estimated as 1.3×10^{-3} per base pair [164], and you have 30 million Illumina reads. Will binning your reads into OTUs at 100% or 97% give you a more interpretable estimation of the number of OTUs present? Why?

Q2. You have collections of 16S rRNA gene reads from two environmental samples, A and B. You examine 50 reads each from sample A and sample B, which correspond to four taxa in A and two taxa in B. You examine 25 more reads from each library and detect two more taxa in A and one more in B. In total, two of these taxa are present in both communities A and B. Which sample has higher alpha diversity by counting taxonomic richness? What is the beta diversity between A and B using simple overlap of taxa? Using Bray-Curtis dissimilarity?

Q3. You examine 1,000 more sequences from samples A and B, detecting 10 additional taxa in A and 25 in B. Which sample has higher alpha diversity now, as measured by taxonomic richness? Why is this different from your previous answer? What statement can you make about the ecological evenness of communities A and B as a result?

Q4. What factors in the microbial environment might you expect to be reflected in metabolism, signaling, and biomolecular function between skin bacteria and oral bacteria? What impact would you expect this to have on the pathways carried in these community metagenomes, or on their alpha diversities?

Q5. It is estimated that 2–5% of the population has *Clostridium difficile* in their intestines. Why is this not usually a problem?

Q6. Consider the impact upon the human microbiome of two perturbations: social contact and brushing your teeth. What short-term and long-term impact do you expect on alpha diversity? Beta diversity?

Q7. Calculate richness, the inverse Simpson index, and the Shannon index for each sample described in the table below. Which has the highest alpha diversity? Why is the answer different according to which measurement you use?

OTU	*Sample 1*	*Sample 2*	*Sample 3*
A	20	20	30
B	20	20	30
C	1	20	30
D	1	20	0
E	1	0	1

Answers to the Exercises can be found in Text S1.

Supporting Information

Text S1 Answers to Exercises.
(DOCX)

Acknowledgments

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Further Reading

It is difficult to recommend comprehensive literature in an area that is changing so rapidly, but the bioinformatics of microbial community studies are currently best covered by the reviews in [22,56,165]. Computational tools for metagenomic analysis include [13,19,63,75,76,77,166]. An overview of microbial ecology from a phylogenetic perspective is provided in [167,168], and the use of the 16S subunit as a marker gene is reviewed in [12]. Likewise, experimental and computational functional metagenomics are discussed in [6,25,169]. The clinical relevance of the human microbiome is far-ranging and is comprehensively reviewed in [157].

Glossary

alpha diversity: within-sample taxonomic diversity

beta diversity: between-sample taxonomic diversity

binning: assignment of sequences to taxonomic units

biofilm: a physically (and often temporally) structured aggregate of microorganisms, often containing multiple taxa, and often adhered to each other and/or to a defined substrate

chimera: an artificial DNA sequence generated during amplification, consisting of a combination of two (or more) true underlying sequences

collector's curve: a plot in which the horizontal axis represents samples (often DNA sequences) and the vertical axis represents diversity (e.g. number of distinct taxa)

community structure: used most commonly to refer to the taxonomic composition of a microbial community; can also refer to the spatiotemporal distribution of taxa

diversity: a measure of the taxonomic distribution within a community, either in terms of distinct taxa or in terms of their evolutionary/phylogenetic distance

FBA: Flux Balance Analysis, a computational method for inferring the metabolic behavior of a system given prior knowledge of the enzymatic reactions of which it is capable

functional metagenomics: computational or experimental analysis of a microbial community with respect to the biochemical and other biomolecular activities encoded by its composite genome

gap filling: the process of imputing missing or inaccurate gene abundances in a set of pathways

germ-free: a host animal containing no microorganisms

gnotobiotic: a host animal containing a defined set of microorganisms, either synthetically implanted or transferred from another host; often used to refer to model organisms with humanized microbiota

holes: missing genes in a set of reference pathways; see gap filling

interpolation: see gap filling

marker: a gene or other DNA sequence that can be (ideally) unambiguously assigned to a particular taxon or function

metagenome: the total genomic DNA of all organisms within a community

metagenomics: the study of uncultured microbial communities, typically relying on high-throughput experimental data and bioinformatic techniques

metametabolome: the total metabolite pool (and possibly fluxes) of a community

metaproteome: the total proteome of all organisms within a community

metatranscriptome: the total transcribed RNA pool of all organisms within a community

microbiome: the total microbial community and biomolecules within a defined environment

microbiota: the total collection of microbial organisms within a community, typically used in reference to an animal host

microflora: an older term used synonymously with microbiota

ORFeome: the total collection of open reading frames within a metagenome

ortholog: in strict usage, a homologous gene in two species distinguished only by a speciation event; in practice, used to denote any gene sufficiently homologous as to represent strong evidence for conserved biological function

OTU: Operational Taxonomic Unit, a cluster of organisms similar at the sequence level beyond some threshhold (e.g. 95%) used in place of species, genus, etc.

phylochip: a microarray containing taxonomic (and sometimes functional) marker sequences

phylotype: see OTU

prebiotic: a food substance metabolized by the microbiota so as to directly or indirectly benefit the host

probiotic: a live microorganism consumed by the host with direct or indirect health benefits

rarefaction curve: see collector's curve

richness: see diversity

16S rRNA: the transcribed form of the 16S ribosomal subunit gene, the smaller RNA component of the prokaryotic ribosome, used as the most common taxonomic marker for microbial communities

WGS: Whole-Genome Shotgun, used to describe shotgun sequencing of individual organisms and, sometimes, microbial communities, although this is not completely accurate as no "whole-genome" is typically involved

WMS: Whole-Metagenome Shotgun sequencing, used in reference to undirected metagenomic sequencing to distinguish it from sequencing directed to specific taxonomic marker genes

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Education

Chapter 13: Mining Electronic Health Records in the Genomics Era

Joshua C. Denny*

Departments of Biomedical Informatics and Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, United States of America

Abstract: The combination of improved genomic analysis methods, decreasing genotyping costs, and increasing computing resources has led to an explosion of clinical genomic knowledge in the last decade. Similarly, healthcare systems are increasingly adopting robust electronic health record (EHR) systems that not only can improve health care, but also contain a vast repository of disease and treatment data that could be mined for genomic research. Indeed, institutions are creating EHR-linked DNA biobanks to enable genomic and pharmacogenomic research, using EHR data for phenotypic information. However, EHRs are designed primarily for clinical care, not research, so reuse of clinical EHR data for research purposes can be challenging. Difficulties in use of EHR data include: data availability, missing data, incorrect data, and vast quantities of unstructured narrative text data. Structured information includes billing codes, most laboratory reports, and other variables such as physiologic measurements and demographic information. Significant information, however, remains locked within EHR narrative text documents, including clinical notes and certain categories of test results, such as pathology and radiology reports. For relatively rare observations, combinations of simple free-text searches and billing codes may prove adequate when followed by manual chart review. However, to extract the large cohorts necessary for genome-wide association studies, natural language processing methods to process narrative text data may be needed. Combinations of structured and unstructured textual data can be mined to generate high-validity collections of cases and controls for a given condition. Once high-quality cases and controls are identified, EHR-derived cases can be used for genomic discovery and validation. Since EHR data includes a broad sampling of clinically-relevant phenotypic information, it may enable multiple genomic investigations upon a single set of genotyped individuals. This chapter reviews several examples of phenotype extraction and their application to genetic research, demonstrating a viable future for genomic discovery using EHR-linked data.

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1. Introduction and Motivation

Typical genetic research studies have used purpose-built cohorts or observational studies for genetic research. As of 2012, more than 1000 genome-wide association analyses have been performed, not to mention a vast quantity of candidate gene studies [1]. Many of these studies have investigated multiple disease and phenotypic traits within a single patient cohort, such as the Wellcome Trust [2] and Framingham research cohorts [3–5]. Typically, patient questionnaires and/or research staff are used to ascertain phenotypic traits for a patient. While these study designs may offer high validity and repeatability in their assessment of a given trait, these models are typically very costly and often represent only a cross-section of time. In addition, rare diseases may take a significant time to accrue in these datasets.

Another model that is gaining acceptance is genetic discovery based solely or partially from phenotype information derived solely from the electronic health record (EHR) [6]. In these models, a hospital collects DNA for research, and maintains a linkage between the DNA sample and the EHR data for that patient. The primary source of phenotypic information, therefore, is the EHR. Depending on the design of the biobank model, some EHR-linked biobanks have the ability to supplement EHR-accrued data with purpose-collected research data.

The EHR model for genetic research offers several key advantages, but also faces prominent challenges to successful implementation. A primary advantage is cost. EHRs contain a longitudinal record of robust clinical data that is produced as a byproduct of routine clinical care. Thus, it is a rich, real-world dataset that requires little additional funding to obtain. Both study designs share costs for obtaining and storing DNA.

Another advantage of EHR-linked DNA databanks is the potential to reuse genetic information to investigate a broad range of additional phenotypes beyond the original study. This is particularly true for dense genetic data such as generated through genome-wide association studies or large-scale sequencing data. For instance, a patient may be genotyped once as part of a study on diabetes, and then later participate in another analysis for cardiovascular disease.

Major efforts in EHR DNA biobanking are underway at a number of institutions. One of the major driving forces has been the National Human Genome Research Institute (NHGRI)-sponsored Electronic Medical Records and Genomics (eMERGE) network [7], which began in

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* E-mail: josh.denny@vanderbilt.edu

What to Learn in This Chapter

- Describe the types of information available in Electronic Health Records (EHRs), and the relative sensitivity and positive predictive value of each
- Describe the difference between unstructured and structured information in the EHR
- Describe methods for developing accurate phenotype algorithms that integrate structured and unstructured EHR information, and the roles played by billing codes, laboratory values, medication data, and natural language processing
- Describe recent uses of EHR-derived phenotypes to study genome-phenome relationships
- Describe the cost advantages unique to EHR-linked biobanks, and the ability to reuse genetic data for many studies
- Understand the role of EHRs to enable phenome-wide association studies of genetic variants

2007 and, as of 2012, consists of nine sites that are performing genome-wide association studies using phenotypic data derived from EHR. The National Institutes of Health (NIH)-sponsored Pharmacogenomics Research Network (PGRN) also include sites performing genetic research using EHR data as their source of phenotypic data. Another example is the Kaiser Permanente Research Program on Genes, Environment and Health, which has genotyped 100,000 members with linked EHR data [8].

2. Classes of Data Available in EHRs

EHRs are designed primarily to support clinical care, billing, and, increasingly, other functions such as quality improvement initiatives aimed at improving the health of a population. Thus, the types of data and their methods of storing this data are optimized to support these missions. The primary types of information available from EHRs are: billing data, laboratory results and vital signs, provider documentation, documentation from reports and tests, and medication records. Billing data and many laboratory results are available in most systems as structured "name-value pair" data. Clinical documentation, many test results (such as echocardiograms and radiology testing), and medication records are often found in narrative or semi-narrative text formats. Researchers creating "electronic phenotype algorithms" (discussed in Section 6.2) typically utilize multiple types of informatics (e.g., billing codes, laboratory results, medication data, and/or NLP) to achieve high accuracy when identifying cases and controls from the EHR.

Table 1 summarizes the types of data available in the EHR and their strengths and weaknesses.

2.1 Billing Data

Billing data typically consists of codes derived from the International Classification of Diseases (ICD) and Current Procedural Terminology (CPT). ICD is a hierarchical terminology of diseases, signs, symptoms, and procedure codes maintained by the World Health Organization (WHO). While the majority of the world uses ICD version 10, the United States (as of 2012) uses ICD version 9-CM; the current Center for Medicare and Medicaid Services guidelines mandate a transition to ICD-10-CM in the United States by October 1, 2014. Because of their widespread use as required components for billing, and due to their ubiquity within EHR systems, billing codes are frequently used for research purposes [9–14]. Prior research has demonstrated that such administrative data can have poor sensitivity and specificity [15,16]. Despite this, they remain an important part of more complex phenotype algorithms that achieve high performance [17–19].

CPT codes are created and maintained by the American Medical Association. They serve as the chief coding system providers use to bill for clinical services. Typically, CPTs are paired with ICD codes, the latter providing the reason (e.g., a disease or symptom) for a clinical encounter or procedure. This satisfies the requirements of insurers, who require certain allowable diagnoses and symptoms to pay for a given procedure. For example, insurance companies will pay for a brain magnetic resonance imaging (MRI) scan that is ordered for a number of complaints (such as known cancers or symptoms such as headache), but not for unrelated symptoms such as chest pain.

Within the context of establishing a particular diagnosis from EHR data, CPT codes tend to have high specificity but low sensitivity, while ICD9 codes have comparatively lower specificity but higher sensitivity. For instance, to establish the diagnosis of coronary artery disease, one could look for a CPT code for "coronary artery bypass surgery" or "percutaneous coronary angioplasty" disease, or for one of several ICD9 codes. If the CPT code is present, there is a high probability that the patient has corresponding diagnosis of coronary disease. However, many patients without these CPT codes also have coronary disease, but either have not received these interventions or received them at a different hospital. In contrast, a clinician may bill an ICD9 code for coronary disease based on clinical suspicion without a firm diagnosis. Figure 1 shows the results of a study that compared the use of natural language processing (NLP) and CPT codes to detect patients who have received colorectal cancer screening, via a colonoscopy within the last ten years, at one institution. In this study, only 61% (106 out of 174 total) of the documented completed colonoscopies were found via CPT codes [20]. The most common cause of false negatives was a colonoscopy completed at another hospital. CPT codes, however, had a very high precision (i.e., positive predictive value; see Box 1), with only one false positive.

2.2 Laboratory and Vital Signs

Laboratory data and vital signs form a longitudinal record of mostly structured data in the medical record. In addition to being stored as name-value pair data, these fields and values can be encoded using standard terminologies. The most common controlled vocabulary used to represent laboratory tests and vital signs is the Logical Observation Identifiers Names and Codes (LOINC®), which is a Consolidated Health Informatics standard for representation of laboratory and test names and is part of Health Language 7 (HL7) [21,22]. Despite the growing use of LOINC, many (perhaps most) hospital lab systems still use local dictionaries to encode laboratory results internally. Hospital laboratory systems or testing companies may change over time, resulting in different internal codes for the same test result. Thus, care is needed to implement selection logic based on laboratory results. Indeed, a 2009–2010 data standardization effort at Vanderbilt University Medical Center found that the concept of "weight" and "height" each had more than five internal representations. Weights and heights were also recorded by different systems using different field names and stored internally with different units (e.g., kilograms, grams, and pounds for weight;

Table 1. Strengths and weakness of data classes within EHRs.

	ICD codes	CPT codes	Laboratory Data	Medication records	Clinical Documentation
Availability in EHR systems	Near-universal	Near-universal	Near-universal	Variable	Variable
Recall	Medium	Poor	Medium	Inpatient: High Outpatient: Variable	Medium
Precision	Medium	High	High	Inpatient: High Outpatient: Variable	Medium-High
Fragmentation effect	Medium	High	Medium-High	Medium	Low-Medium
Query method	Structured	Structured	Mostly structured	Structured, text queries, and NLP	NLP, text queries, and rarely structured
Strengths	-Easy to query -Serves as a good first pass of disease status	-Easy to query -High precision	-Value depends on test -High data validity	Can have high validity	Best record of what providers thought
Weaknesses	-Disease codes often used for screening when disease not a ctually present -Accuracy hindered by billing realities and clinic workflow	-Most susceptible to missing data errors (e.g., performed at another hospital) -Procedure receipt influenced by patient and payer factors external to disease process	-May need to aggregate different variations of the same data elements -Normal ranges and units may change over time	-Often need to interface inpatient and outpatient records -Medication records from outside providers not present -Medications prescribed not necessary taken	-Difficult to process automatically -Interpretation accuracy depends on assessment method -May suffer from significant "cut and paste" -Not universally available in EHRs -May be self-contradictory
Summary	Essential first element for electronic phenotyping	Helpful addition if relevant	Helpful addition if relevant	Useful for confirmation and a marker of severity	Useful for confirming common diagnoses or for finding rare ones

doi:10.1371/journal.pcbi.1002823.t001

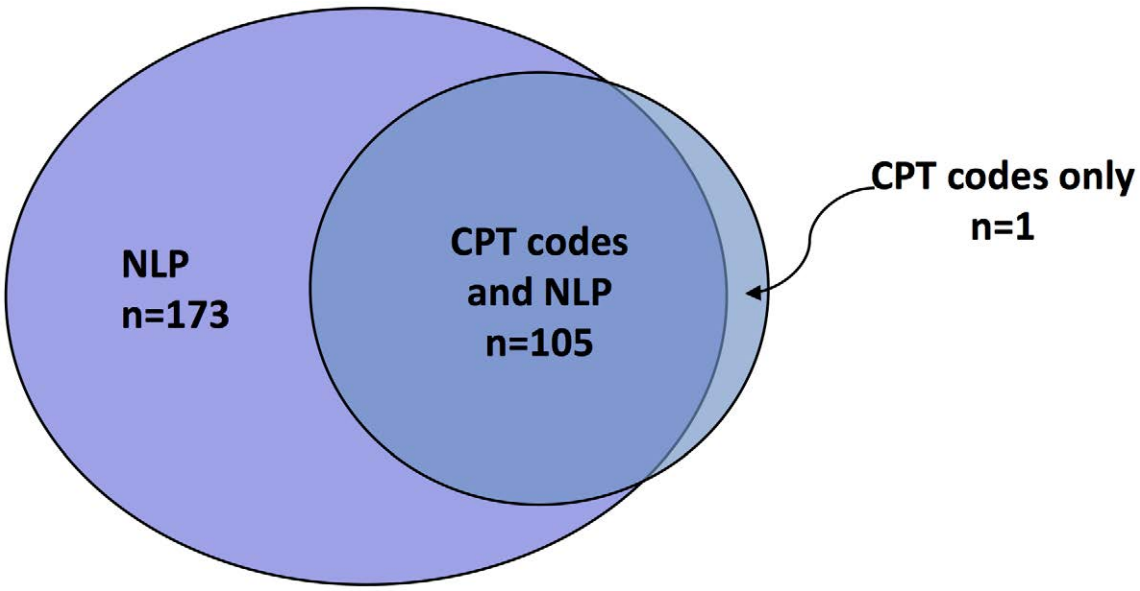


Figure 1. Comparison of natural language processing (NLP) and CPT codes to detect completed colonoscopies in 200 patients. In this study, more completed colonoscopies were found via NLP than with billing codes alone, and only one colonoscopy was found with billing codes that was not found with NLP. NLP examples were reviewed for accuracy.
doi:10.1371/journal.pcbi.1002823.g001

Box 1. Metrics Commonly Used to Evaluate Phenotype Selection Algorithms

$$Sensitivity(Recall) = \frac{True\ Positives}{Gold\ standard\ positives}$$

$$Specificity = \frac{True\ Negatives}{Gold\ standard\ negatives}$$

$$Positive\ Predictive\ Value(PPV, Precision) = \frac{True\ Positives}{True\ Positives + False\ Positives}$$

$$Negative\ Predictive\ Value(NPV) = \frac{True\ Negatives}{True\ Negatives + False\ Negatives}$$

$$F-measure = \frac{2 \times Recall \times Precision}{Recall + Precision}$$

centimeters, meters, inches, and feet for height).

Structured laboratory results are often a very important component of phenotype algorithms, and can represent targets for genomic investigation [3,4,23]. An algorithm to identify type 2 diabetes (T2D) cases and controls, for instance, used laboratory values (e.g., hemoglobin A1c and glucose values) combined with billing codes and medication mentions [17]. Similarly, an algorithm to determine genomic determinants of normal cardiac conduction required normal electrolyte (potassium, calcium, and magnesium) values [16]. In these settings, investigation of the determinants of the values requires careful selection of the value to be investigated. For instance, an analysis of determinants of uric acid or red blood cell indices would exclude patients treated with certain antineoplastic agents (which can increase uric acid or suppression of erythrocyte production), and, similarly, an analysis of white blood cell indices also excludes patients with active infections and certain medications at the time of the laboratory measurement.

2.3 Provider Documentation

Clinical documentation represents perhaps the richest and most diverse source of phenotype information. Provider documentation is required for nearly all billing of tests and clinical visits, and is frequently found in EHR systems. To be useful for phenotyping efforts, clinical documentation must be in the form of electronically-available text that can be used for subsequent manual review, text searches, or NLP. They can be created via computer-based documentation (CBD) systems or dictated and transcribed. The most common form of computable text is in unstructured narrative text documents, although a number of developers have also created structured documentation tools [24]. Narrative text documents can be processed by text queries or by NLP systems, as discussed in the following section.

For some phenotypes, crucial documents may only be available as hand-written documents, and thus not amenable to text searching or NLP. Unavailability may result from clinics that are slow adopters, have very high patient volumes, or have specific workflows not well accommodated by the EHR system [25]. However, these hand-written documents may be available electronically as scanned copies. Recent efforts have shown that intelligent character recognition (ICR) software may be useful for processing scanned documents containing hand-written fields (Figure 2) [26,27]. This task can be challenging, however, and works best when the providers are completing pre-formatted forms.

2.4 Documentation from Reports and Tests

Provider-generated reports and test results include radiology and pathology reports and some procedure results such as echocardiograms. They are often in the form of narrative text results. Many of these contain a mixture of structured and unstructured results. Examples include an electrocardiogram report, which typically has structured interval durations and may contain a structured field indicating whether the test was abnormal or not. However, most electrocardiogram (ECG) reports also contain a narrative text "impression" representing the cardiologist's interpretation of the result (e.g., "consider anterolateral myocardial ischemia" or "Since last ECG, patient has developed atrial fibrillation") [28]. For ECGs, the structured content (e.g., the intervals measured on the ECG) are generated using automated algorithms and have varying accuracy [29].

2.5 Medication Records

Medication records serve an important role in accurate phenotype characterization. They can be used to increase the precision of case identification, and to help ensure that patients believed to be controls do not actually have the disease. Medications received by a patient serve as confirmation that the treating physician believed the disease was present to a sufficient degree that they prescribed a treating medication. It is particularly helpful to find presence or absence of medications highly specific or sensitive for the disease. For instance, a patient with diabetes will receive either oral or injectable hypoglycemic agents; these medications are both highly sensitive and specific for treating diabetes, and can also be used to help differentiate type I diabetes (treated almost exclusively with insulin) from T2D (which is typically a disease of insulin resistance and thus can be treated with a combination of oral and injectable hypoglycemic agents).

Medication records can be in varying forms within an electronic record. With the increased use of computerized provider order entry (CPOE) systems to manage hospital stays, inpatient medication records are often available in highly structured records that may be mapped to controlled vocabularies. In addition, many hospital systems are installing automated bar-code medication administration records by which hospital staff record each individual drug administration for each patient [30]. With this information, accurate drug exposures and their times can be

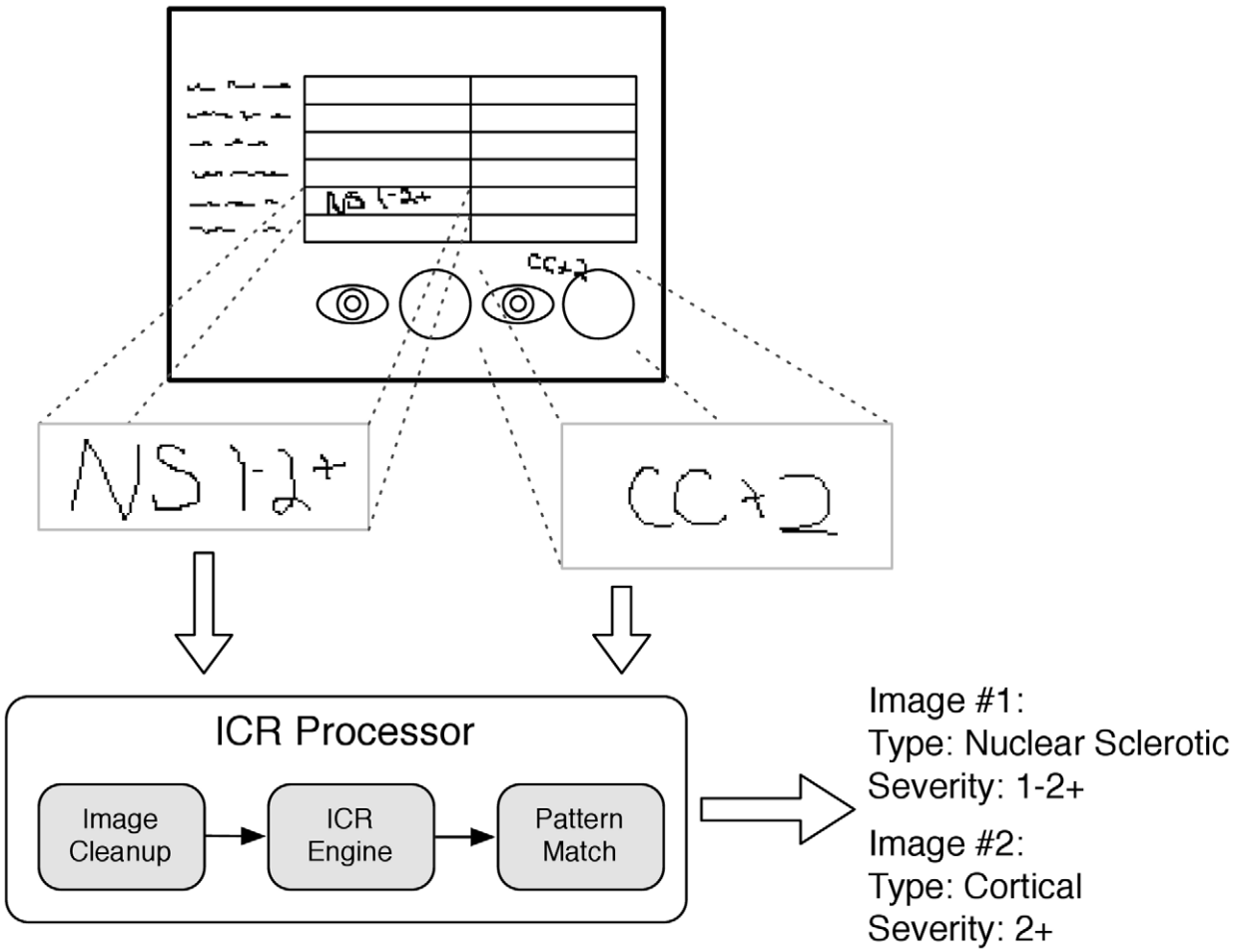


Figure 2. Use of Intelligent Character Recognition to codify handwriting. Figure courtesy of Luke Rasmussen, Northwestern University.
doi:10.1371/journal.pcbi.1002823.g002

constructed for each inpatient. Even without electronic medication administration records (such as bar-code systems), research has shown that CPOE-ordered medications are given with fairly high reliability [31].

Outpatient medication records are often recorded via narrative text entries within clinical documentation, patient problem lists, or communications with patients through telephone calls or patient portals. Many EHR systems have incorporated outpatient prescribing systems, which create structured medical records during generation of new prescriptions and refills. However, within many EHR systems, electronic prescribing tools are optional, not yet widely adopted, or have only been used within recent history. Thus, accurate construction of a patient's medication exposure history often requires NLP techniques. For specific algorithms, focused free-text searching for a set of medications can be efficient and effective [17]. This approach requires the researcher to generate the list of brand names, generics, combination medications, and abbreviations that would be used, but has the advantage that it can be easily accomplished using relational database queries. The downside is that this approach requires re-engineering for each medication or set of medications to be searched, and does not allow for the retrieval of other medication data, such as dose, frequency, and duration. A more general-purpose approach can be achieved with NLP, which is discussed in greater detail in Section 3 below.

3. Natural Language Processing to Support Clinical Knowledge Extraction

Although many documentation tools include structured and semi-structured elements, the vast majority of computer based documentation (CBD) remains in "natural language" narrative formats [24]. Thus, to be useful for data mining, narrative data must be processed through use of text-searching (e.g., keyword searching) or NLP systems. Keyword searching can effectively identify rare physical exam findings in text [32], and extension to use of regular expression pattern matching has been used to extract blood pressure readings [33]. NLP computer algorithms scan and parse unstructured "free-text" documents, applying syntactic and semantic rules to extract structured representations of the information content, such as concepts recognized from a controlled terminology [34–37]. Early NLP efforts to extract medical concepts from clinical text documents focused on coding in the Systematic Nomenclature of Pathology or the ICD for financial and billing purposes [38], while more recent efforts often use complete versions of the Unified Medical Language System (UMLS) [39–41], SNOMED-CT [16], and/or domain-specific vocabularies such as RxNorm for medication extraction [42]. NLP systems utilize varying approaches to "understanding text," including rule-based and statistical approaches using syntactic and/or semantic information. Natural language

processors can achieve classification rates similar to those of manual reviewers, and can be superior to keyword searches. A number of researchers have demonstrated the effectiveness of NLP for large-scale text-processing tasks. Melton and Hripcsak used MedLEE to recognize instances of adverse events in hospital discharge summaries [43]. Friedman and colleagues evaluated NLP for pharmacovigilance to discover adverse drug events from clinical records by using statistical methods that associate extracted UMLS disease concepts with extracted medication names [40]. These studies show the potential for NLP to aid in specific phenotype recognition.

Using either NLP systems or keyword searching, the primary task in identifying a particular phenotype is to filter out concepts (or keywords) within a corpus of documents that indicate statements other than the patient having the disease. Researchers may desire to specify particular document types (e.g., documents within a given domain, problem lists, etc.) or particular types of visits or specialists (e.g., requiring a visit with an ophthalmologist). Some common NLP tasks needed in phenotype classification include identifying family medical history context and negated terms (e.g., "no cardiac disease"), and removing drug allergies when searching for patients taking a certain medication. Recognition of sections within documents can be handled using structured section labels, specialized NLP systems such as SecTag [44], or more general-purpose NLP systems such as MedLEE [45] or HITEX [46]. A number of solutions have been proposed for negation detection; among the more widespread are adaptations of the NegEx algorithm developed by Chapman et al., which uses a series of negation phrases and boundary words to identify negated text [47]. NegEx or similar algorithms can be used as a standalone system or be integrated within a number of general-purpose NLP systems including MedLEE [48], the KnowledgeMap concept identifier [49], cTAKES [50], and the National Library of Medicine's MetaMap [51].

Medication information extraction is an important area for clinical applications that benefits from specialized NLP tools. Most general-purpose NLP systems will recognize medications by the medication ingredient mentioned in the text but may not identify the relevant medication metadata such as dose, frequency, and route. In addition, a general purpose NLP system using as its vocabulary the UMLS will likely recognize "atenolol" and "Tenormin" (a United States brand name for atenolol) as two different concepts, since each is represented by separate concepts in the UMLS. Medication-specific NLP systems focus on extracting such metadata for a medication. Sirohl and Peissig applied a commercial medication NLP system to derived structured medication information [52], which was later linked to laboratory data and used to explore the pharmacodynamics of statin efficacy (a cholesterol-lowering medication) [53]. Xu et al. developed a similar system at Vanderbilt called MedEx, which had recall and precision $\geq$0.90 for discharge summaries and clinic notes on Vanderbilt clinical documents [42]. Additionally, the 2009 i2b2 NLP challenge focused on medication extraction using de-identified discharge summaries from Partners Healthcare, and 20 teams competed to identify medications and their signatures. The best systems achieved F-measures $\geq$0.80 [54]. Much work remains to be done in this area, as extraction of both medication names and associated signature information can be challenging when considering the full breadth of clinical documentation formats available, including provider-staff and provider-patient communications, which often contain less formal and misspelled representations of prescribed medications.

For more information on NLP methods and applications, please see the article on text mining elsewhere in this collection (submitted).

4. EHR-Associated Biobanks: Enabling EHR-Based Genomic Science

DNA biobanks associated with EHR systems can be composed of either "all comers" or a focused collection, and pursue either a conventional consented "opt-in" or an "opt-out" approach. Currently, the majority of DNA biobanks have an opt-in approach that selects patients for particular research studies. Two population-based models in the eMERGE network are the Personalized Medicine Research Population (PMRP) project of the Marshfield Clinic (Marshfield, WI) [55] and Northwestern University's NUgene project (Chicago, IL). The PMRP project selected 20,000 individuals who receive care in the geographic region of the Marshfield Clinic. These patients have been consented, surveyed, and have given permission to the investigators for recontact in the future if additional information is needed. The NUgene project, which has enrolled nearly 10,000 people through 2012, uses a similar approach, obtaining patients' consent during outpatient clinic visits [56]. Another example of an EHR-associated biobank is the Kaiser-Permanente biobank, which has genotyped 100,000 individuals [57].

The alternative "opt-out" approach is evidenced by Vanderbilt University's BioVU, which associates DNA with de-identified EHR data [58]. In this model, patients have the opportunity to "opt out" of the DNA biobank by checking a box on the standard "Consent to Treatment" form signed as part of routine clinical care. A majority of patients (>90%) do not check this box, indicating assent to the use of their DNA in the biobank [58]. If the patient does not opt-out, blood that is scheduled to be discarded after routine laboratory testing is instead sent for DNA extraction, which is stored for potential future use. To ensure that no one knows with certainty if a subject's DNA is in BioVU, an additional small percentage of patients are randomly excluded.

The BioVU model requires that the DNA and associated EHR data be de-identified in order to assure that the model complies with the policies of non-human subjects research. The full-text of the EHR undergoes a process of de-identification with software programs that remove Health Insurance Portability and Accountability Act (HIPAA) identifiers from all clinical documentation in the medical record. At the time of this writing, text de-identification for BioVU is performed using the commercial product DE-ID [59] with additional pre- and post-processing steps. However, a number of other clinical text de-identification software packages have been studied, some of which are open source [60,61]. Multiple reviews by both the local institutional review board and the federal Office for Human Research Protections have affirmed this status as nonhuman subjects research according to 45 CFR 46 [58]. Nonetheless, all research conducted within BioVU and the associated de-identified EHR (called the "Synthetic Derivative") is overseen by the local Institutional Review Board. An opt-out model similar to BioVU is used by Partners Healthcare for the Crimson biobank, which can accrue patients who meet specific phenotype criteria as they have routine blood draws.

An advantage of the opt-out approach is rapid sample accrual. BioVU began col-

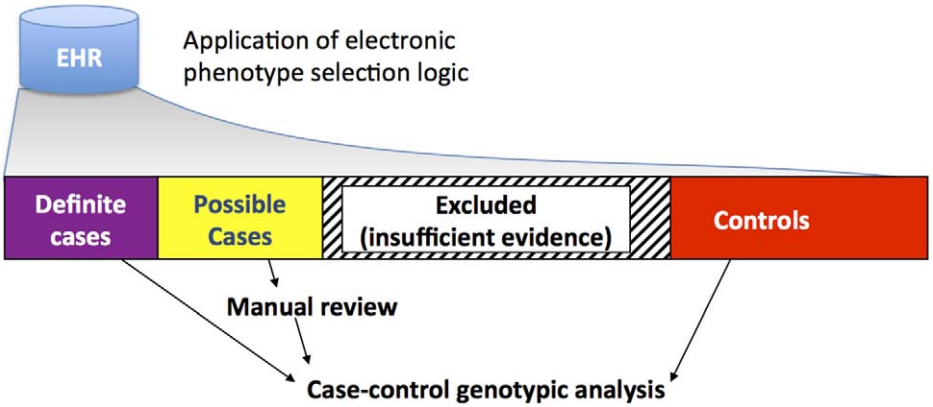


Figure 3. General figure for identifying cases and controls using EHR data. Application of electronic selection algorithms lead to division of a population of patients into four groups, the largest of which comprises patients who were excluded because they lack sufficient evidence to be either a case or control patient. Definite cases and controls cross some predefined threshold of positive predictive value (e.g., PPV≥95%), and thus do not require manual review. For very rare phenotypes or complicated case definitions, the category of "possible" cases may need to be reviewed manually to increase the sample size.
doi:10.1371/journal.pcbi.1002823.g003

lecting DNA samples in 2007, adding about 500 new samples weekly, and has over 150,000 subjects as of September 2012. Since it enrolls subjects prospectively, investigation of rare phenotypes may be possible with such systems. The major disadvantage of the opt-out approach is that it precludes recontact of the patients since their identity has been removed. However, the Synthetic Derivative is continually updated as new information is added to the EHR, such that the amount of phenotypic information for included patients grows over time.

5. Race and Ethnicity in EHR-Derived Biobanks

Given that much genetic information varies greatly within ancestral populations, accurate knowledge of genetic ancestry information is essential to allow for proper genetic study design and control of population stratification. Without it, one can see numerous spurious genetic associations due solely to race/ethnicity [62]. Single nucleotide polymorphisms (SNPs) common in one population may be rare in another. In large-scale GWA analyses, one can tolerate less accurate knowledge of ancestry *a priori*, since the large amount of genetic data allows one to calculate the genetic ancestry of the subject using catalogs of SNPs known to vary between races. Alternatively, one can also adjust for genetic ancestry using tools such as EIGENSTRAT [63]. However, in smaller candidate gene studies, it is important to know the ancestry beforehand.

Self-reported race/ethnicity data is often used in genetic studies. In contrast race/ethnicity as recorded within an EHR may be entered through a variety of sources. Most commonly, administrative staff record race/ethnicity via structured data collection tools in the EHR. Often, this field can be ignored (left as "unknown"), especially in busy clinical environments, such as emergency departments. "Unknown" percentages of patients can range between 9% and 23% of subjects [17,18]. Among those patients for whom data is entered, a study of genetic ancestry informative markers correlated well with EHR-reported race/ethnicities [64]. In addition, a study within the Veterans Administration (VA) hospital system noted that over 95% of all EHR-derived race/ethnicity agreed with self-reported race/ethnicity using nearly one million records [65]. Thus, despite concerns over EHR-derived ancestral information, such information, when present, appears similar to self-report ancestry information.

6. Phenotype-Driven Discovery in EHRs

6.1 Measure of Phenotype Selection Logic Performance

The evaluation of phenotype selection logic can use metrics similar to information retrieval tasks. Common metrics are sensitivity (or recall), specificity, positive predictive value (PPV, also known as precision), and negative predictive value (see Box 1). If a population is assessed for case and control status, then another useful metric is comparing the receiver operator characteristic (ROC) curves. ROC curves graph the sensitivity vs. false positive rate (or, 1-specificity) given a continuous measure of the outcome of the algorithm. By calculating the area under the ROC curve (AUC), one has a single measure of the overall performance of an algorithm that can be used to compare two algorithms or selection logics. Since the scale of the graph is 0 to 1 on both axes, the performance of a perfect algorithm is 1, and random chance is 0.5.

6.2 Creation of Phenotype Selection Logic

Initial work in phenotype detection has often focused on a single modality of EHR data. A number of studies have used billing data, some comparing directly to other genres of data, such as NLP. Li et al. compared the results of ICD-9 encoded diagnoses and NLP-processed discharge summaries for clinical trial eligibility queries, finding that use of NLP provided more valuable data sources for clinical trial pre-screening than ICD-9 codes [15]. Savova et al. has used cTAKES to discover peripheral arterial disease cases by looking for particular key words in radiology reports, and then aggregating the individual instances using "AND-OR-NOT" Boolean logic to classify cases into four categories: positive, negative, probable, and unknown [66].

Phenotype algorithms can be created multiple ways, depending of the rarity of the phenotype, the capabilities of the EHR system, and the desired sample size of the study. Generally, phenotype selection logics (algorithms) are composed of one or more of four elements: billing code data, other structured (coded) data such as laboratory values and demographic data, medication information, and NLP-derived data. Structured data can be retrieved effectively from most EHR systems. These data can be combined through simple Boolean logic

Table 2. Methods of finding cases and controls for genetic analysis of five common diseases.

Disease	Methods	Cases	Controls	Case PPV	Control PPV
Atrial fibrillation	NLP of ECG impressions ICD9 codes CPT codes	168	1695	98%	100%
Crohn's Disease	ICD9 codes Medications (text)	116	2643	100%	100%
Type 2 Diabetes	ICD9 codes Medications (text) Text searches (controls)	570	764	100%	100%
Multiple Sclerosis	ICD9 codes or text diagnosis	66	1857	87%*	100%
Rheumatoid Arthritis	ICD9 codes Medications (text) Text searches (exclusions)	170	701	97%	100%

*Given the small number of multiple sclerosis cases, all possible cases were manually validated to ensure high recall.
doi:10.1371/journal.pcbi.1002823.t002

[17] or through machine learning methods such as logistic regression [18], to achieve a predefined specificity or positive predictive value. A drawback to the use of machine learning data (such as logistic regression models) is that it may not be as portable to other EHR systems as more simple Boolean logic, depending on how the models are constructed. The application of many phenotype selection logics can be thought of partitioning individuals into four buckets – definite cases (with sufficiently high PPV), possible cases (which can be manually reviewed if needed), controls (which do not have the disease with acceptable PPV), and individuals excluded from the analysis due to either potentially overlapping diagnoses or insufficient evidence (Figure 3).

For many algorithms, sensitivity (or recall) is not necessarily evaluated, assuming there are an adequate number of cases. A possible concern in not evaluating recall (sensitivity) of a phenotype algorithm is that there may be a systematic bias in how patients were selected. For example, consider a hypothetical algorithm to find patients with T2D whose logic was to select all patients that had at least one billing code for T2D and also required that cases receive an oral hypoglycemic medication. This algorithm may be highly specific for finding patients with T2D (instead of type 1 diabetes), but would miss those patients who had progressed in disease severity such that oral hypoglycemic agents no longer worked and who now require insulin treatment. Thus, this phenotype algorithm could miss the more severe cases of T2D. However, for a practical application, such assessments of recall can be challenging given large samples sizes of rare diseases. Certain assumptions (e.g., that a patient should have at least one billing code for the disease) are reasonable and likely do not lead to significant bias.

For other algorithms, the temporal relationships of certain elements are very important. Consider an algorithm to determine whether a certain combination of medication adversely impacted a given lab, such as kidney function or glucose [67]. Such an algorithm would need to take into account the temporal sequence and time between the particular medications and laboratory tests. For example, glucose changes within minutes to hours of a single administration of insulin, but the development of glaucoma from corticosteroids (a known side effect) would not be expected to happen acutely following a single dose.

For very rare diseases or findings, one may desire to find every case, and thus the logic may simply be a union of keyword text queries and billing codes followed by manual review of all returned cases. Examples include the rare physical exam finding hippus (exaggerated pupillary oscillations occurring in the setting of altered mental status) [32], or potential drug adverse events (e.g., Stevens-Johnson syndrome), which are often very rare but severe.

Since EHRs represent longitudinal records of patient care, they are biased to recording those events that are recorded as part of medical care. Thus, they are particularly useful for investigating disease-based phenotypes, but potentially less efficacious for investigating non-disease phenotypes such as hair or eye color, left vs. right handedness, cognitive attributes, biochemical measures (beyond routine labs), etc. On the other hand, they may be particularly useful for analyzing disease progression over time.

7. Examples of Genetic Discovery Using EHRs

The growth of "EHR-driven genomic research" (EDGR) – that is, genomic research proceeding primarily from EHR data linked to DNA samples – is a recent phenomenon [6]. Preceding these most recent research initiatives, other studies laid the groundwork for use of EHR data to study genetic phenomena. Rzhetsky et al. used billing codes from the EHRs of 1.5 million patients to analyze disease co-occurrence in 161 conditions as a proxy for possible genetic overlap [68]. Chen et al. compared laboratory measurements and age with gene expression data to identify rates of change that correlated with genes known to be involved in aging [69]. A study at Geisinger Clinic evaluated SNPs in the 9p21 region that are known to be associated to cardiovascular disease and early myocardial infarction [70]. They found these SNPs were associated with heart disease and T2D using EHR-derived data. Several specific examples of EDGR are detailed below.

7.1 Replicating Known Genetic Associations for Five Diseases

An early replication study of known genetic associations with five diseases with known genetic associations was performed in BioVU. The study was designed to test the hypothesis that an EHR-linked DNA biobank could be used for genetic association analyses. The goal was to use only EHR data for phenotype information. The first 10,000 samples accrued in BioVU were genotyped at 21 SNPs that are known to be associated with these five diseases (atrial fibrillation, Crohn's disease, multiple sclerosis, rheumatoid arthritis,

Table 3. eMERGE network participants.

Institution	Biorepository Overview	Model	Size	EHR Summary	Phenotyping Methods
Group Health[1] (Seattle, WA)	**GHC Biobank** Alzheimer's Disease Patient Registry and Adult Changes in Thought Study	Disease specific Cohort	4000	Comprehensive vendor-based EHR since 2004	Structured data extraction, NLP
Marshfield Clinic Research Foundation[1] (Marshfield, WI)	**Personalized Medicine Research Project** Marshfield Clinic, an integrated regional health system	Population based	20,000	Comprehensive internally developed EHR since 1985	Structured data extraction, NLP, Intelligent Character Recognition
Mayo Clinic[1] (Rochester, MN)	**Disease cohort** Derived from vascular laboratory & exercise stress testing labs	Disease specific Cohorts	16,500	Comprehensive internally developed EHR since 1995	Structured data extraction, NLP
Northwestern University[1] (Chicago, IL)	**NUgene Project** Northwestern affiliated hospitals and outpatient clinics	Population based	>10,000	Comprehensive vendor based Inpatient and Outpatient (different systems) EHR since 2000	Structured data extraction, text searches, NLP
Vanderbilt University[1] (Nashville, TN)	**BioVU** Primarily drawn from outpatient routine laboratory samples	Population based	150,000	Comprehensive internally developed EHR since 2000	Structured data extraction, NLP
Geisinger Health System[2] (Pennsylvania)	**MyCode** Enrollment of health plan participants	Population based	>30,000	Comprehensive vendor-based EHR	Structured data extraction, NLP
Mount Sinai Medical Center[2] (New York, NY)	**Institute for Personalized Medicine Biobank** Outpatient enrollment	Population based	>30,000	Comprehensive vendor-based EHR since 2004	Structured data extraction, NLP
Cincinnati Children's Hospital[3] (Cincinnati, OH)	General and disease cohorts.	Population based	>3,000	Comprehensive vendor-based EHR	Structured data extraction, NLP
Children's Hospital of Philadelphia[3] (Philadelphia, PA)	General and disease cohorts.	Population based	>100,000	Comprehensive vendor-based EHR	Structured data extraction, NLP
Boston Children's[3] (Boston MA)	**Crimson** On-demand, de-identified phenotype-driven collection	Disease based	Virtual	Comprehensive internally developed EHR	Structured data extraction, NLP

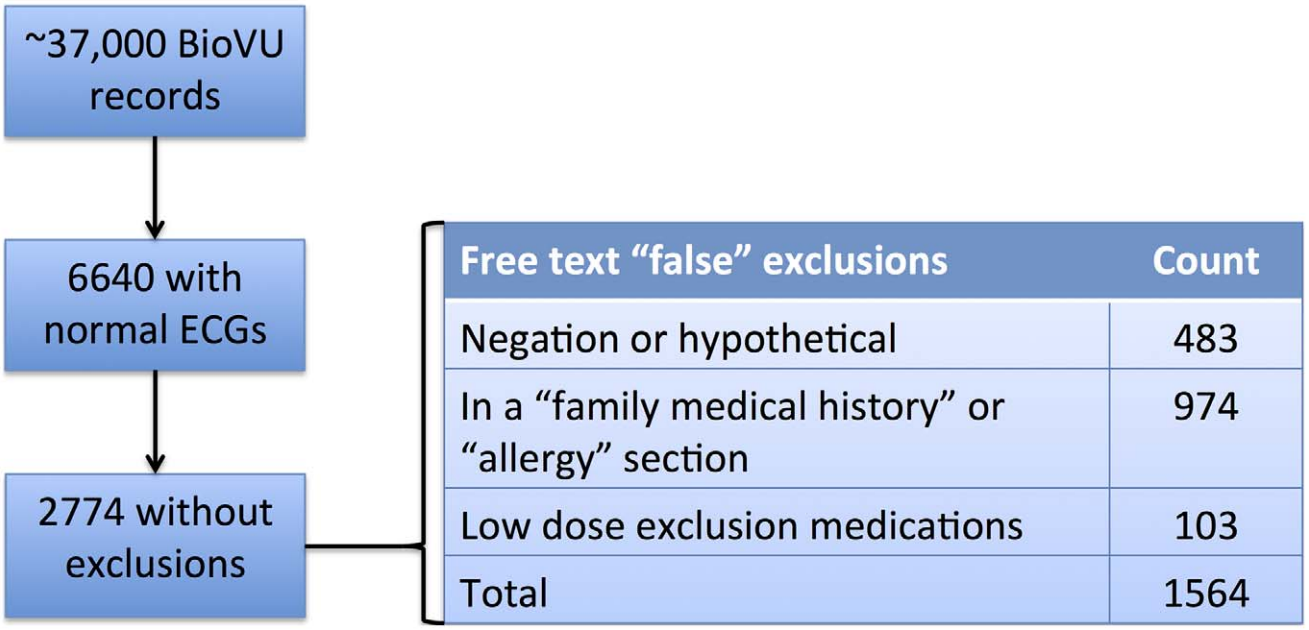
Sizes represent approximate sizes as of 2012; many sites are still actively recruiting. NLP = Natural Language Processing. Sites joined with [1]eMERGE-I in 2007, [2]eMERGE-II in 2011, or as [3]pediatric sites in 2012.
doi:10.1371/journal.pcbi.1002823.t003

and T2D). Reported odds ratios were 1.14–2.36 in at least two previous studies prior to the analysis. Automated phenotype identification algorithms were developed using NLP techniques (to identify key findings, medication names, and family history), billing code queries, and structured data elements (such as laboratory results) to identify cases (n = 70–698) and controls (n = 808–3818). Final algorithms achieved PPV of ≥97% for cases and 100% for controls on randomly selected cases and controls (Table 2) [17]. For each of the target diseases, the phenotype algorithms were developed iteratively, with a proposed selection logic applied to a set of EHR subjects, and random cases and controls evaluated for accuracy. The results of these reviews were used to refine the algorithms, which were then redeployed and reevaluated on a unique set of randomly selected records to provide final PPVs.

Used alone, ICD9 codes had PPVs of 56–89% compared to a gold standard represented by the final algorithm. Errors were due to coding errors (e.g., typos), misdiagnoses from non-specialists (e.g., a non-specialist diagnosed a patient as having rheumatoid arthritis followed by a rheumatologist who revised the diagnosis to psoriatic arthritis), and indeterminate diagnoses that later evolved into well-defined ones (e.g., a patient thought to have Crohn's disease was later determined to have ulcerative colitis, another type of inflammatory bowel disease). Each of the 21 tests of association yielded point estimates in the expected direction, and eight of the known associations achieved statistical significance [17].

7.2 Demonstrating Multiethnic Associations with Rheumatoid Arthritis

Using a logistic regression algorithm operating on billing data, NLP-derived features, medication records, and laboratory data, Liao et al. developed an algorithm to accurately identify rheumatoid arthritis patients [18]. Kurreeman et al. used this algorithm on EHR data to identify a population of 1,515 cases and 1,480 matched controls [71]. These researchers genotyped 29 SNPs that had been associated with RA in at least one prior study. Sixteen of these SNPs achieved statistical significance, and 26/29 had odds ratios in the same direction and with similar effect sizes. The authors also demonstrated that these portions of these risk alleles were associated with rheumatoid arthritis in



Free text "false" exclusions	Count
Negation or hypothetical	483
In a "family medical history" or "allergy" section	974
Low dose exclusion medications	103
Total	1564

Figure 4. Use of NLP to identify patients without heart disease for a genome-wide analysis of normal cardiac conduction. Using simple text searching, 1564 patients would have been eliminated unnecessarily due to negated terms, family medical history of heart disease, or low dose medication use that would not affect measurements on the electrocardiogram. Use of NLP improves recall of these cases without sacrificing positive predictive value. The final case cohort represented the patients used for GWAS in [71].
doi:10.1371/journal.pcbi.1002823.g004

East Asian, African, and Hispanic American populations.

7.3 eMERGE Network

The eMERGE network is composed of nine institutions as of 2012 (http://gwas.org; Table 3). Each site has a DNA biobank linked to robust, longitudinal EHR data. The initial goal of the eMERGE network was to investigate the feasibility of genome-wide association studies using EHR data as the primary source for phenotypic information. Each of these sites initially set out to investigate one or two primary phenotypes (Table 3). Network sites have currently created and evaluated electronic phenotype algorithms for 14 different primary and secondary phenotypes, with nearly 30 more planned. After defining phenotype algorithms, each site then performed genome-wide genotyping at one of two NIH-supported genotyping centers.

The primary goals of an algorithm are to perform with high precision (≥95%) and reasonable recall. Algorithms incorporate billing codes, laboratory and vital signs data, test and procedure results, and clinical documentation. NLP is used to both increase recall (find additional cases) and achieve greater precision (via improved specificity). These phenotype algorithms are available for download from PheKB (http://phekb.org).

Initial plans were for each site to analyze their own phenotypes independently. However, the network has realized the benefits of synergy. Central efforts across the network were involved in harmonization of the collective genetic data.

7.4 Early Genome-Wide Association Studies from the eMERGE Network

As of 2012, the eMERGE Network has published GWAS on atrioventricular conduction [72], red blood cell [23] and white blood cell [73] traits, primary hypothyroidism [74], and erythrocyte sedimentation rate [75], with others ongoing. The first two studies published by the network were using single-site GWAS studies; latter studies have realized the advantage of pooling data across multiple sites to increase the sample size available for a study. Importantly, several studies in eMERGE have explicitly evaluated the portability of the electronic phenotype algorithms by reviewing algorithms at multiple sites. Evaluation of the hypothyroidism algorithm at the five eMERGE-I sites, for instance, noted an overall weighted PPV of 92.4% and 98.5% for cases and controls, respectively [74]. Similar results have been found with T2D [76], cataracts [27], and rheumatoid arthritis [77] algorithms.

As a case study, the GWAS for atrioventricular conduction (as measured by the PR interval on the ECG), conducted entirely within samples drawn from one site, identified variants in *SCN10A*. *SCN10A* is a sodium channel expressed in autonomic nervous system tissue and is now known to be involved in cardiac regulation. The phenotype algorithm identified patients with normal ECGs who did not have evidence of prior heart disease, were not on medications that would interfere with cardiac conduction, and had normal electrolytes. The phenotype algorithm used NLP and billing code queries to search for the presence of prior heart disease and medication use [72]. Of note, the algorithm highlights the importance of using clinical note section tagging and negation to exclude only those patients with heart disease, as opposed to patients whose records contained negated heart disease concepts (e.g., "no myocardial infarction") or heart disease concepts in related individuals (e.g., "mother died of a heart attack"). Use of NLP improved recall of cases by 129% compared with simple text searching, while maintaining a positive predictive value of 97% (Figure 4) [78,72].

The study of RBC traits identified four variants associated with RBC traits. One of these, *SLC17A1*, had not been previously identified, and is involved in sodium-phosphate co-transport in the kidney. The latter study of RBC traits utilized patients genotyped at one site as cases and controls

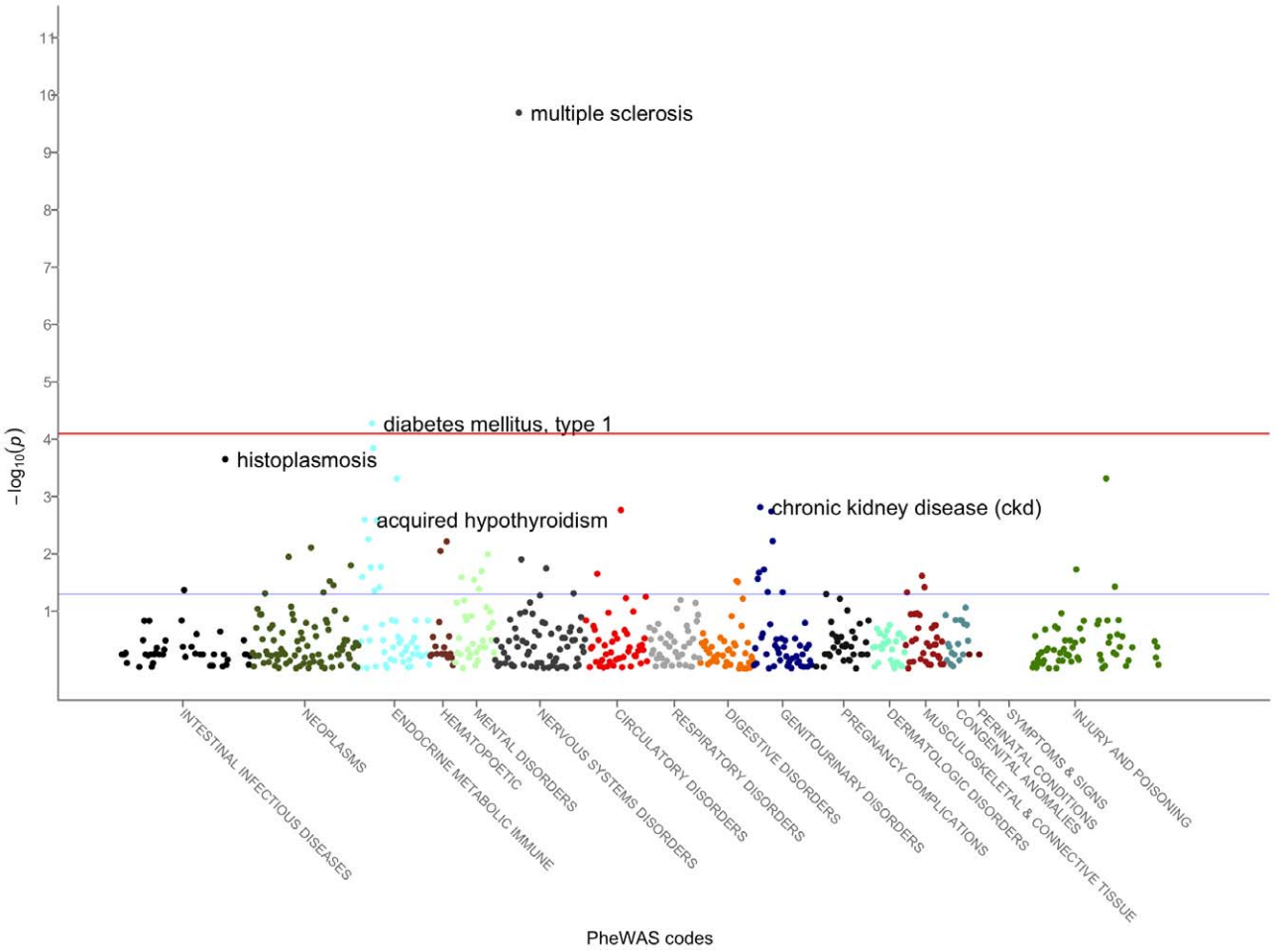


Figure 5. A PheWAS plot for rs3135388 in HLA-DRA. This region has known associations with multiple sclerosis. The red line indicates statistical significance at Bonferroni correction. The blue line represents $p<0.05$. This plot is generated from updated data from [78] and the updated PheWAS methods as described in [73].
doi:10.1371/journal.pcbi.1002823.g005

for their primary phenotype of peripheral arterial disease (PAD). Thus, this represents an *in silico* GWAS for a new finding that did not require new genotyping, but instead leveraged the available data within the EHR. The eMERGE study of primary hypothyroidism, similarly, identified a novel association with *FOXE1*, a thyroid transcription factor, without any new genotyping by using samples derived from five eMERGE sites.

7.5 Phenome-Wide Association Studies (PheWAS)

Typical genetic analyses investigate many genetic loci against a single trait or disease. Such analyses cannot identify pleiotropic associations, and may miss important confounders in an analysis. Another approach, engender by the rich phenotype record included in the EHR, is to simultaneously investigate many phenotypes associated with a given genetic locus. A "phenome-wide association study" (PheWAS) is, in a sense, a "reverse GWAS." PheWAS investigations require large representative patient populations with definable phenotypic characteristics. Such studies only recently became feasible, facilitated by linkage of DNA biorepositories to EHR systems, which can provide a comprehensive, longitudinal record of disease.

The first PheWAS studies were performed on 6,005 patients genotyped for five SNPs with seven previously known disease associations [79]. This PheWAS used ICD9 codes linked to a code-translation table that mapped ICD9 codes to 776 disease phenotypes. In this study, PheWAS methods replicated four of seven previously known associations with $p<0.011$. Figure 5 shows one illustrative PheWAS plot of phenotype associations with an *HLA-DRA* SNP known to be associated with multiple sclerosis. Of note, this PheWAS not only demonstrates a strong association between this SNP and multiple sclerosis, but also highlights other possible associations, such as Type 1 diabetes and acquired hypothyroidism. Recent explorations into PheWAS methods using NLP have shown greater efficacy for detecting associations: with the same patients, NLP-based PheWAS replicated six of the seven known associations, generally with more significant p-values [80].

PheWAS methods may be particularly useful for highlighting pleiotropy and clinically associated diseases. For example, an early GWAS for T2D identified, among others, *FTO* loci as an associated variant [81]. A later GWAS demonstrated this risk association was mediated through the effect of *FTO* on increasing body mass index, and thus increasing risk of T2D within those individuals. Such effects may be identified through broad phenome scans made possible through PheWAS.

8. Conclusions and Future Directions

EHRs have long been seen as a vehicle to improve healthcare quality, cost, and safety. However, their growing adoption in the United States and elsewhere is demonstrating their capability as a broad tool for research. Enabling tools include enterprise data warehouses and software to process unstructured information, such as de-identification and NLP. When linked to biological data such as DNA or tissue biorepositories, EHRs can become a powerful tool for genomic analysis. One can imagine future repositories also storing intermittent plasma samples to allow for proteomic analyses.

A key advantage of EHR-based genetic studies is that they allow for the collection of phenotype information as a byproduct of routine healthcare. Moreover, this information collection grows over time and is continually refined as new information may confirm or refute a diagnosis for a given individual. Through the course of one's life, a number of information points concerning disease, response to treatment, and laboratory and test data are collected. Aggregation of this information can allow for generation of large sample sizes of patients with certain diseases or medication exposures. Moreover, once a subject receives dense genotyping for one EHR-based study, their genetic data can be reused for many other genotypic studies, allowing for relatively low-cost reuse of the genetic material (once a given phenotype can be found in the EHR).

Three major rate-limiting steps impede utilization of EHR data for genetic analysis. A major challenge is derivation of accurate collections of cases and controls for a given disease of interest, usually achieved through creation and validation of phenotype selection logics. These algorithms take significant time and effort to develop and often require adjustment and a skilled team to deploy at a secondary site. Another challenge is the availability of phenotypic information. Many patients may be observed at a given healthcare facility only for certain types of care (e.g., primary care or a certain subspecialist), leading to fragmented knowledge of a patient's medical history and medication exposures. Future growth of Health Information Exchanges could substantially improve these information gaps. Finally, DNA biobanks require significant institutional investment and ongoing financial, ethical, and logistical support to run effectively. Thus, they are not ubiquitous.

As genomics move beyond discovery into clinical practice, the future of personalized medicine is one in which our genetic information could be "simply a click of the mouse" away [82]. In this future, DNA-enabled EHR systems will assist in more accurate prescribing, risk stratification, and diagnosis. Genomic discovery in EHR systems provides a real-world test bed to validate and discover clinically meaningful genetic effects.

9. Exercises

1) Compare and contrast the basic types of data available in an Electronic Health Records (EHR) that are useful for mining genetic data. What are some of the strengths and drawbacks of each type of data?
2) Explain what a phenotype algorithm is and why it is necessary. For example, how can use of natural language processing improve upon use of billing codes alone?
3) Select a clinical disease and design a phenotype algorithm for it.
4) How might a phenotype algorithm be different for a very rare disease (e.g., prion diseases) vs. a more common one (e.g., Type 2 diabetes)? How would a phenotype algorithm be different for a physical exam finding (e.g., hippus or a particular type of heart murmur) vs. a disease?
5) Describe the differences between a DNA biobank linked to an EHR and one collected as part of a non-EHR research cohort. What are the advantages and disadvantages of a de-identified DNA biobank vs. an identified DNA biobank (either linked to an EHR or not).
6) It is often harder to create algorithms to find drug-response phenotypes (such as adverse drug events) than for a chronic disease. Give several reasons why this might be.

Answers to the Exercises can be found in Text S1.

Supporting Information

Text S1 Answers to Exercises.
(DOCX)

Further Reading

- Shortliffe EH, Cimino JJ, editors (2006) Biomedical informatics: computer applications in health care and biomedicine. 3rd edition. Springer. 1064 p.
  Chapters of particular relevance: Chapter 2 ("Biomedical data: their acquisition, storage, and use"), Chapter 8 ("Natural language and text processing in biomedicine"), Chapter 12 ("Electronic health record systems")
- Hristidis V, editor (2009) Information discovery on electronic health records. 1st edition. Chapman and Hall/CRC. 331 p. *Chapters of particular relevance: Chapter 2 ("Electronic health records"), Chapter 4 ("Data quality and integration issues in electronic health records"), 7 ("Data mining and knowledge discovery on EHRs").*
- Wilke RA, Xu H, Denny JC, Roden DM, Krauss RM, et al. (2011) The emerging role of electronic medical records in pharmacogenomics. Clin Pharmacol Ther 89: 379–386. doi:10.1038/clpt.2010.260.
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Glossary

- **Candidate gene study**: A study of specific genetic loci in which a phenotype-genotype association may exist (e.g., hypothesis-led genotype experiment)
- **Computer-based documentation (CBD)**: Any electronic note or report found within an EHR system. Typically, these can be dictated or typed directly into a ''note writer'' system (which may leverage ''templates'') available within the EHR. Notably, CBD excludes scanned documents.
- **Computerized Provider Order Entry (CPOE)**: A system for allowing a provider (typically a clinician or a nurse practitioner) to enter, electronically, an order for a patient. Typical examples include medication prescribing or test ordering. These systems allow for a precise electronic record of orders given and also can provide decision support to help improve care.
- **Electronic Health Record (EHR)**: Any comprehensive electronic medical record system storing all the data about a patient's encounters with a healthcare system, including medical diagnoses, physician notes, prescribing records. EHRs include CPOE and CBD systems (among others), and allow for easy information retrieval of clinical notes and results.
- **Genome-wide association study (GWAS)**: A broad scale study of a number of points selected along a genome without using a prior hypothesis. Typically, these studies analyze more than >500,000 loci on the genome.
- **Genotype**: The specific DNA sequence at a given location.
- **Natural language processing (NLP)**: Use of algorithms to created structured data from unstructured, narrative text documents. Examples include use of comprehensive NLP software solutions to find biomedical concepts in documents, as well as more focused applications of techniques to find extract features from notes, such as blood pressure readings.
- **Phenome-wide association study (PheWAS)**: A broad scale study of a number phenotypes selected along the genome without regard to a prior hypothesis as what phenotype(s) a given genetic locus may be associated.
- **Phenotype selection logic (or algorithm)**: A series of Boolean rules or machine learning algorithms incorporating such information as billing codes, laboratory values, medication records, and NLP designed to derive a case and control population. from EHR data.
- **Phenotype**: Any observable attribute of an individual.
- **Single nucleotide polymorphism (SNP)**: a single locus on the genome that shows variation in the human population.
- **Structured data**: Data that is already recorded in a system in a structured name-value pair format and can be easily queried via a database.
- **Unified Medical Language System (UMLS)**: A comprehensive metavocabulary maintained by the National Library of Medicine which combines >100 individual standardized vocabularies. The UMLS is composed of the Metathesaurus, the Specialist Lexicon, and the Semantic Network. The largest component of the UMLS is the Metathesaurus, which contains the term strings, concept groupings of terms, and concept interrelationships.
- **Unstructured data**: Data contained in narrative text documents such as the clinical notes generated by physicians and certain types of text reports, such as pathology results or procedures such as echocardiograms.

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Chapter 14: Cancer Genome Analysis

Miguel Vazquez, Victor de la Torre, Alfonso Valencia*

Structural Biology and BioComputing Programme, Spanish National Cancer Research Centre (CNIO), Madrid, Spain

Abstract: Although there is great promise in the benefits to be obtained by analyzing cancer genomes, numerous challenges hinder different stages of the process, from the problem of sample preparation and the validation of the experimental techniques, to the interpretation of the results. This chapter specifically focuses on the technical issues associated with the bioinformatics analysis of cancer genome data. The main issues addressed are the use of database and software resources, the use of analysis workflows and the presentation of clinically relevant action items. We attempt to aid new developers in the field by describing the different stages of analysis and discussing current approaches, as well as by providing practical advice on how to access and use resources, and how to implement recommendations. Real cases from cancer genome projects are used as examples.

This article is part of the "Translational Bioinformatics" collection for *PLOS Computational Biology*.

1. Introduction

Cancer is commonly defined as a "disease of the genes", a definition that emphasizes the importance of cataloguing and analyzing tumor-associated mutations. The recent advances in sequencing technology have underpinned the progress in several large-scale projects to systematically compile genomic information related to cancer. For example, the Cancer Genome Atlas (http://cancergenome.nih.gov/) and the projects overseen by the International Cancer Genome Consortium [1] (http://icgc.org/) have focused on identifying links between cancer and genomic variation. Unsurprisingly, the analysis of genomic mutations associated with cancer is also making its way into clinical applications [2–4].

Cancer may be favored by genetic predisposition, although it is thought to be primarily caused by mutations in specific tissues that accumulate over time. Genetic predisposition is represented by germline variants and indeed, many common germline variants have been associated with specific diseases, as well as with altered drug susceptibility and/or toxicity. The association of germline variants with clinical features and disease is mainly achieved through Genome Wide Association Studies (GWAS). GWAS use large cohorts of cases to analyze the relationship between the disease and thousands or millions of mutations across the entire genome, and they are the subject of a separate chapter in this issue.

The study of cancer genomes differs significantly from GWAS, as during the lifetime of the organism variants only accumulate in the tumor or the affected tissues, and they are not transmitted from generation to generation. These are known as somatic mutations. Mutations accumulate as the tumors progress through processes that are not completely understood and that depend on the evolution of the different cell types in the tumor, *i.e.*, clonal versus parallel evolution [5]. Regardless of which model is more relevant, the tumor genome includes mutations that facilitate tumorigenesis or are that essential for the generation of the tumor (known as tumor 'drivers'), and others that have accumulated during the growth of the tumor (known as 'passengers') [6]. Distinguishing 'driver' from 'passenger' mutations is crucial for the interpretation of cancer genomes [5].

Depending on the type of data and the aim of the analysis, cancer genome analysis may focus on the cancer type or on the patient. The first approach consists of examining a cohort of patients suffering from a particular type of cancer, and is used to identify biomarkers, characterize cancer subtypes with clinical or therapeutic implications, or to simply advance our understanding of the tumorigenic process. The second approach involves examining the genome of a particular cancer patient in the search for specific alterations that may be susceptible to tailored therapy. Although both approaches draw on common experimental and bioinformatics techniques, they analyze different types of information, have different goals and they require the presentation of the results in distinct ways.

The development of Next Generation Sequencing (NGS) has not only helped identify genetic variants but also, it represents an important aid in the study of epigenetics (DNAseq and ChipSeq of histone methylation marks), transcriptional regulation and splicing (RNAseq). The combined power of such genomic data provides a more complete definition of 'cancer genomes'.

To aid developers new to the field of cancer genomics, this chapter will discuss the particularities of cancer genome analysis, as well as the main scientific and technical challenges, and potential solutions.

2. Overview of Cancer Genome Analysis

The sequence of the steps in an idealized cancer genome analysis pipeline are presented in Figure 1. For each step listed, the biological disciplines involved, the bioinformatics techniques used and some of the most salient challenges that arise are listed.

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* E-mail: valencia@cnio.es

What to Learn in This Chapter

This chapter presents an overview of how cancer genomes can be analyzed, discussing some of the challenges involved and providing practical advice on how to address them. As the primary analysis of experimental data is described elsewhere (sequencing, alignment and variant calling), we will focus on the secondary analysis of the data, *i.e.*, the selection of candidate driver genes, functional interpretation and the presentation of the results. Emphasis is placed on how to build applications that meet the needs of researchers, academics and clinicians. The general features of such applications are laid out, along with advice on their design and implementation. This document should serve as a starter guide for bioinformaticians interested in the analysis of cancer genomes, although we also hope that more experienced bioinformaticians will find interesting solutions to some key technical issues.

2.1. Sequencing, Alignment and Variant Calling

After samples are sequenced, sequencing reads are aligned to a reference genome and all differences are identified through a process known as *variant calling*. The output of the variant calling is a list of genomic variations that is organized according to their genomic location (chromosome and position) and the variant allele. They may be accompanied by scores measuring the sequencing quality over that region or the prevalence of the variant allele in the samples. The workflow employed for this type of analysis is commonly known as a primary analysis (For more information on sequencing, alignment and variant calling, please refer to [7,8]).

This chapter describes the subsequent steps in the analysis of the variants detected at the genome level. This process is relatively well established and is the main subject of this chapter.

2.2. Consequence, Recurrence Analysis and Candidate Drivers

The list of somatic variants obtained from the primary analysis of DNA sequences is carefully examined to identify mutations that may alter the function of protein products. DNA mutations are translated into mutations in RNA transcripts, and from RNA into proteins, potentially altering their amino acid sequence. The impact of these amino acid alterations on protein function can range from largely irrelevant (if they do not affect any region of the protein involved in catalysis or binding, or if they do not significantly alter the structure and stability of the protein) to highly deleterious (for example if the amino acid changes result in the formation of a truncated protein lacking important functional regions). The severity of these alterations can be assessed

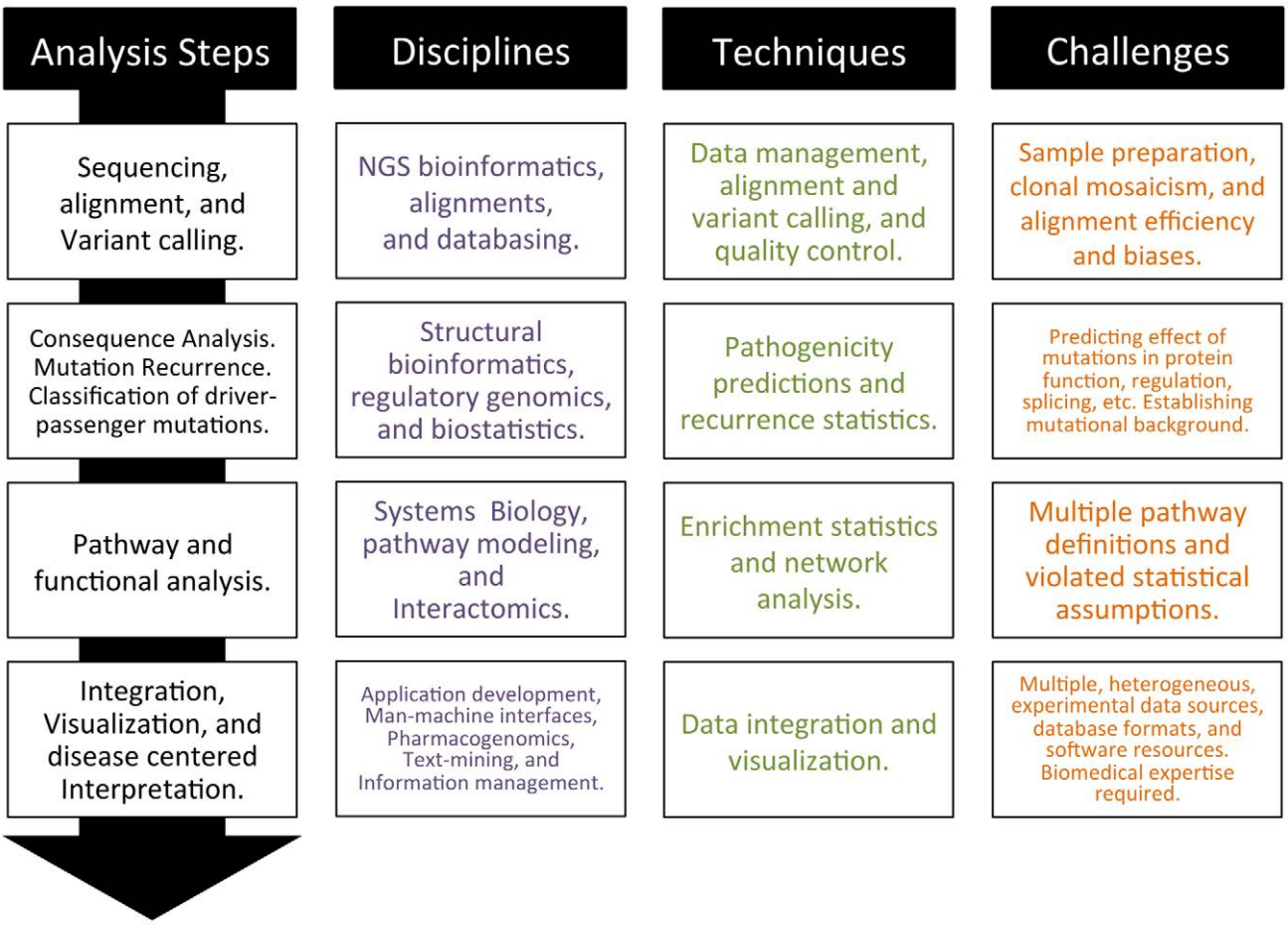

Figure 1. Idealized cancer analysis pipeline. The column on the left shows a list of sequential steps. The columns on the right show the bioinformatics and molecular biology disciplines involved at each step, the types of techniques employed and some of the current challenges faced.
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using specialized software tools known as *protein mutation pathogenicity predictors*.

Mutations are also examined to identify recurrence, which may point to key genes and mutational hotspots. The predicted consequences of the mutations and their recurrence are used to select potential driver mutations that may be directly involved in the tumorigenic process.

Note that not all mutations that have deleterious consequences for protein function are necessarily involved in cancer as the proteins affected may not play any fundamental role in tumorigenesis.

2.3. Pathways and Functional Analysis

Genes that are recurrently mutated in cancer tend to be easily identifiable, and obvious examples include TP53 and KRAS that are mutated in many cancer types. More often mutations are more widely distributed and the probability of finding the same gene mutated in several cases is low, making it more difficult to identify common functional features associated with a given cancer.

Pathway analysis offers a means to overcome this challenge by associating mutated genes with known signaling pathways, regulatory networks, clusters in protein interaction networks, protein complexes or general functional classes, such as those defined in the Gene Ontology database. A number of statistical methods have been developed to determine the significance of the associations between mutated genes and these functional classes. Pathways analysis has now become a fundamental component of cancer genome analysis and it is described in almost all cancer genome publications. In this sense, cancer is not only a 'disease of the genes' but also a 'disease of the pathways'.

2.4. Integration, Visualization and Interpretation

Information on the mutational status of genes can be better understood if it is integrated with information about gene expression and related to alterations in: the copy number of each gene (CNVs), a very common phenomenon in cancer; mutations in promoters and enhancers; variations in the affinity of transcription factors and DNA binding proteins; or dysregulation of epigenetic control.

The importance of the relationships between different genome data sources is illustrated by the case of chronic lymphocytic leukemia (CLL). The consequences of mutations in the SF3B1 splicing factor, detected by exon sequencing [9], were investigated in studies of DNA methylation [10] and RNA sequencing in the same patients (Ferreira et al. submitted). At the technical level, the analysis of heterogeneous genomic data adds further complications to analysis workflows, as the underlying biological bases are often not fully understood. Consequently, relatively few published studies have effectively combined more than a few combinations of such data [11–13]. These studies are usually supported by visualization tools to analyze the results within specialist applications tailored to fit the specific set of data generated.

Finally, in a personalized medicine application, the results must be related to information of clinical relevance, such as potentially related drugs and therapies.

2.5. Current Challenges

In general terms, three key challenges exist when analyzing cancer genomes: (1) the heterogeneity of the data to be analyzed, which ranges from genomic mutations in coding regions to alterations in gene expression or epigenetic marks; (2) the range of databases and software resources required to analyse and interpret the results; and (3) the comprehensive expertise required to understand the implications of such varied experimental data.

3. Critical Bioinformatics Tasks in Cancer Genome Analysis

An overview of the four main tasks that should be performed when analyzing the cancer genome is shown in Figure 2, along with the associated requirements. In the first instance, the mutations initially detected at the DNA level must be trimmed to include only somatic variations, removing the germline SNPs detected in healthy tissue of the same individuals or in the general population. The description of the different stages of analysis that we present begins with this list of somatic variants and their associated genomic locations.

3.1. Mapping between Coordinate Systems

Translating mutational information derived from genomic coordinates to other data types is an obvious first step. Although this may seem trivial, its importance should not be underestimated given that alterations in single nucleotides can have significant consequences.

The position of DNA mutations in transcripts and protein products must be obtained by translating their coordinates across various systems. For example, point mutations in coding regions can be mapped to different transcripts by finding the exon affected, the offset of the mutation inside that exon and the position of the exon inside the transcript. By removing the 5′ UTR region of the transcript sequence and dividing the rest into triplets, the affected codon can be identified, as well as the possible amino acid replacement. Ensembl BioMart provides all the information necessary to perform this type of mapping, while a number of other systems also provide this functionality (see Table 1).

One important technical consideration when mapping genomic variants is the version of the genome build. It is essential to use the correct build and many mapping tools support different versions of the genome build. Moreover, the data in Ensembl is thoroughly versioned, so that the BioMart interface can be used to gather all genomic information consistently for any particular build. Thus, entities (mutations, genes, transcripts or proteins) can be linked back to the appropriate version using the Ensembl web site archives.

3.2. Driver Mutations and Pathogenicity Prediction

In addition to false variants introduced by technical errors, some variants present in the samples may not contribute to cancer development. The terms 'driver' and 'passenger' were first used in 1964 in the context of viral infections that drive cancer [6]. However, they are now used to distinguish mutations that drive cancer onset and progression from those that play little or no role in such processes but that are propagated by their co-existence with driver mutations. The problem of distinguishing driver from passenger mutations remains unsolved as yet. Experimental assays of activity are one means of testing the tumorigenic potential of mutations [14], although such assays are difficult to perform to scale. Consequently, a number of complementary *in-silico* methods have been developed to identify driver mutations. Statistical approaches seek to identify traces of mutation selection during tumor formation by looking at the prevalence of mutations in particular genes in sample cohorts, or the ratios of synonymous versus non-synonymous mutations in particular candidate genes. However, such statistical approaches require large sample cohorts to achieve sufficient power. Alternatively, *in-silico* predictions of pathogenicity can be used to restrict the list of potential driver mutations to those that are likely to alter protein function [15].

Several tools that implement different versions of these general concepts can be

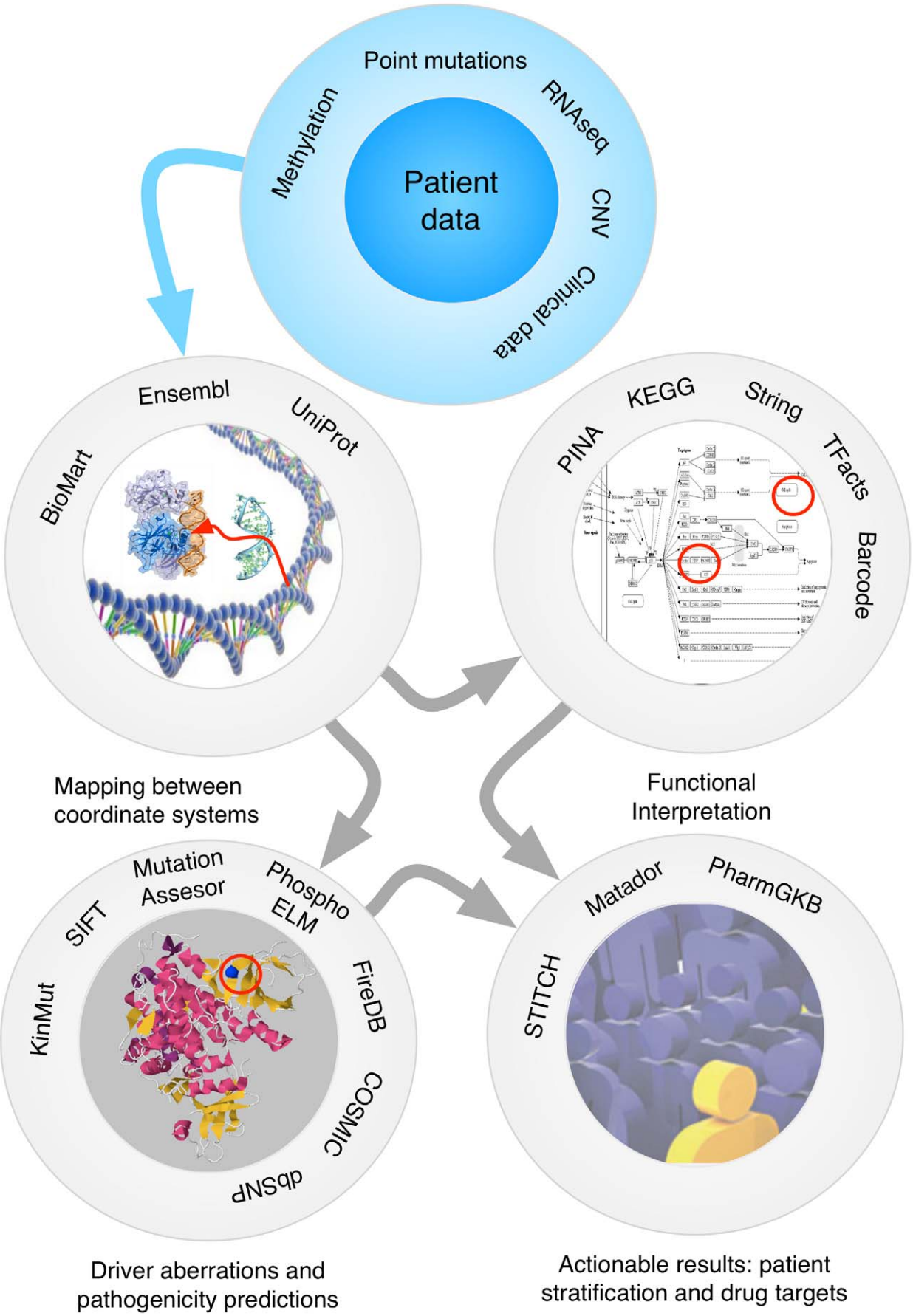
Point mutations
RNAseq
CNV
Clinical data
Methylation
Patient data
Ensembl
UniProt
BioMart
KEGG
String
TFacts
Barcode
PINA
Mapping between coordinate systems
Functional Interpretation
Mutation Assesor
Phospho ELM
FireDB
COSMIC
dbSNP
SIFT
KinMut
Matador
PharmGKB
STITCH
Driver aberrations and pathogenicity predictions
Actionable results: patient stratification and drug targets

Figure 2. Main tasks in an analysis pipeline. Starting with the patient information derived from NGS experiments, the variants are mapped between genes and proteins, evaluated for pathogenicity, considered systemically through functional analysis, and the resulting conclusions translated into actionable results.
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used to perform pathogenicity predictions for point mutations in coding regions (see Table 1). Prediction is far more complicated for genomic aberrations and mutations that affect non-coding regions of DNA, an area of basic research that is still in its early stages. However, the large collections of genomic information gathered by the ENCODE project [16] will doubtless play a key role in this research.

Despite their limited scope, mutations in coding regions are the most useful for cancer genome analysis. This is initially because it is still cheaper to sequence exomes than full genomes and also, because they are closer to actionable medical items, given that most drugs target proteins. Indeed, most clinical success stories based on cancer genome analysis have involved the analysis of point mutations in proteins [3].

In particular, we have focused on the need to analyze the consequences of mutations in alternative isoforms of each gene, in addition to those in the main isoforms. Despite the potential implications of alternative splicing, this problem remains largely overlooked by current applications. A common solution is to assign the genomic mutations to just one of the several potential isoforms, without considering their possible incidence of other splice isoforms, and in most cases without knowing which isoform is actually produced in that particular tissue. The availability of RNAseq data should solve this problem by demonstrating which isoforms are specifically expressed in the cell type of interest, in which case, additional software will be necessary to analyze the data generated by the new experiments.

3.3 Functional Interpretation

Some genes harbor a large number of mutations in cancer genomes, such as TP53 and KRAS, whose importance and relevance as cancer drivers have been well established. Frequently however, genomic data reveals the presence of mutated genes that are far less prevalent, and the significance of these genes must be considered in the context of the functional units they are part of. For example, SF3B1 was mutated in only 10 out of 105 samples of chronic lymphocytic leukemia (CLL) in the study conducted by the ICGC consortium [9], and in 14 out of 96 in the study performed in the Broad Institute [17]. While these numbers are statistically significant, many other components of the RNA splicing and transport machinery are also mutated in CLL. Even if these mutations occur at lower frequencies they further emphasize the importance of this gene [18].

Functional interpretation aims to identify large biological units that correlate better with the phenotype than individual mutated genes, and as such, it can produce a more general interpretation of the acquired genomic information. The involvement of genes in specific biological, metabolic and signaling pathways is the type of functional annotation most commonly considered and thus, functional analysis is often termed 'pathway analysis'. However, functional annotations may also include other types of biological associations such as cellular location, protein domain composition, and classes of cellular or biochemical terms, such as GO terms (Table 2 lists some useful databases along with the relevant functional annotations).

Over the last decade, multiple statistical approaches have been developed to identify functional annotations (also known as 'labels') that are significantly associated with lists of entities, collectively known as 'enrichment analysis'. Indeed, the current systems for functional interpretation have been derived from the systems previously developed to analyze expression arrays, and they have been adapted to analyze lists of cancer-related genes. As this step is critical to perform functional interpretations, special care must be taken when selecting methods to be incorporated into the analysis pipeline. Cases in which the characteristics of the data challenge the assumptions of the methods are particularly delicate. For instance, a hypergeometric test might be appropriate to analyze gene lists that are differentially expressed in gene expression arrays. However, when dealing with lists of mutated genes this approach does not account for factors such as the number of mutations per gene, the size of the genes, or the presence of genes in overlapping genomic clusters (where one mutation may simultaneously

Table 1. Selection of the software packages used in cancer genome analysis.

Software	Functionality	Availability
VEP	Mutation mapping	Local installation or web site
ANNOVAR	Mutation mapping	Local installation
VARIANT	Mutation mapping	Local installation, web site, and web service
Mutation Assessor, SIFT	For protein variants	Web site and web service
Condel	Consensus prediction	Web site and web service
wKinMut	Kinase specific	Web site and web service
Genecodis	Annotation enrichment for gene lists	Web site and web service
FatiGO, David	Annotation enrichment for gene lists	Web site
Cytoscape	Network visualization and analysis	Local installation. Can be embedded in browser applications
R	Statistics and plotting	Local installation
Taverna	Workflow enactment	Local installation
Galaxy	Workflow enactment	Browser application

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Table 2. Selection of databases commonly used in our workflows.

Database	Entities	Properties
Ensembl	Genes, proteins, transcripts, regulatory regions, variants	Genomic positions, relationships between them, identifiers in different formats, GO terms, PFAM domains
Entrez	Genes, articles	Articles for genes, abstracts of articles, links to full text
UniProt	Proteins	PDBs, known variants
KEGG, Reactome, Biocarta, Gene Ontology	Genes	Pathways, processes, function, cell location
TFacts	Genes	Transcription regulation
Barcode	Genes	Expression by tissue
PINA, HPRD, STRING	Proteins	Interactions
PharmaGKB	Drugs, proteins, variants	Drug targets, pharmacogenetics
STITCH, Matador	Drugs, proteins	Drug targets
Drug clinical trials	Investigational drugs	Diseases or conditions in they are being tested
GEO, ArrayExpress	Genes (microarray probes)	Expression values
ICGC, TCGA	Cancer Genomes	Point mutations, methylation, CNV, structural variants
dbSNP, 1000 genomes	Germline variations	Association with diseases or conditions
COSMIC	Somatic variations	Association with cancer types

doi:10.1371/journal.pcbi.1002824.t002

affect several genes). As none of these issues are accommodated by the standard approaches used for gene expression analysis, new developments are clearly required for cancer genome analysis.

To alleviate the rigidity introduced by the binary nature of set-based approaches, whereby genes are either on the list or they are not, some enrichment analysis approaches study the over-representation of annotations/labels using rank-based statistics. A common choice for rank-based approaches is to use some variation of the Kolmogorov-Smirnov non-parametric statistic, as employed in gene set enrichment analysis (GSEA) [19]. Another benefit of rank approaches is that the scores used can be designed to account for some of the features that are not well handled by set-based approaches. Accordingly, considerations of background mutation rates based on gene length, sequencing quality or heterogeneity in the initial tumor samples can be incorporated into the scoring scheme. However, rank statistics are still unable to handle other issues, such as mutations affecting clusters of genes that are functionally related (*e.g.*, proto-cadherins), which still challenge the assumption of independence made by most statistical approaches. Note that from a bioinformatics perspective, sets of entities are often conceptually simpler to work with than ranked lists when crossing information derived from different sources. Moreover, from an application perspective, information summarized in terms of sets of entities is often more actionable than ranks or scores.

A different type of analysis considers the relationships between entities based on their connections in protein interaction networks. This approach has been used to measure the proximity of groups of cancer-related genes and other groups of genes or functions, by labeling nodes with specific characteristics (such as roles in biological pathways or functional classes) [20].

Functional interpretation can therefore be facilitated by the use of a wide array of alternative analyses. Different approaches can potentially uncover hidden functional implications in genomic data, although the integration of these results remains a key challenge.

3.4. Applicable Results: Diagnosis, Patient Stratification and Drug Therapies

For clinical applications, the results of cancer genome analysis need to be translated into practical advice for clinicians, providing potential drug therapies, better tumor classification or early diagnostic markers. While bioinformatics systems can support these decisions, it will be up to expert users to present these findings in the context of the relevant medical and clinical information available at any given time. In the case of our institution's (CNIO) personalized cancer medicine approach, we use mouse xenografts (also known as 'avatar' models) to test the effects of drugs on tumors prior to considering their potential to treat patients [4]. In turn, the results of these xenograft studies are used as a feedback into the system for future analyses.

Drug-related information and the tools with which to analyze it is essential for the analysis of personalized data (some of the key databases linking known gene variants to diseases and drugs are listed in Table 2). Accessing this information and integrating chemical informatics methodologies into bioinformatics systems presents new challenges for bioinformaticians and system developers.

4. Resources for Genome Analysis in Cancer

4.1. Databases

Although complex, the data required for genome analysis can usually be represented in a tabular format. Tab separated values (TSV) files are the *de facto* standard when sharing database resources. For a developer, these files have several practical advantages over other standard formats popular in computer science (namely XML): they are easier to read, write and parse with scripts; they are relatively succinct; the format is straight-forward and the contents can be inferred from the first line of the file, which typically holds the names of the columns.

Some databases describe entities and their properties, such as: proteins and the drugs that target them; germline variations and the diseases with which they are associated; or genes along with the factors that regulate their transcription. Other databases are repositories of experimental data, such as the Gene Expression Omnibus and ArrayExpress, which contain data from microarray experiments on a wide range of

samples and under a variety of experimental conditions. For cancer genome studies, cancer-specific repositories will soon be the main reference, such as those developed by the ICGC and TCGA projects. Indeed, these repositories contain complete genotypes that offer a perfect opportunity to test new approaches with real data.

Bioinformaticians know that crossing information from different sources is not a trivial task, as different resources use a variety of identifiers. Even very similar entities can have different identifiers in two different databases (*e.g.*, genes in Entrez and Ensembl). Some resources borrow identifiers for their own data, along with HGNC gene symbols, while databases such as KEGG have their own identifiers for genes, and offer equivalence tables that map them to gene symbols or other common formats.

In addition to entities being referenced by different identifier formats, in distinct resources they may also adhere to slightly different definitions (*e.g.*, regarding what constitutes a gene). Furthermore, as mentioned above the differences between genome builds can substantially affect the mapping between coordinate systems, and they can also give rise to differences between entities.

In general, translating identifiers can be cumbersome and incompatibilities may exist between resources. For example, MutationAssessor, which predicts the pathogenicity of protein mutations [15], uses UniProt identifiers. Analysis systems using Ensembl data for coordinate mappings, such as our own, render mutations using Ensembl Protein IDs, and in some cases there are problems in translating identifiers, and even in assigning mutations to the wrong isoforms. To prevent these potential errors, MutationAssessor double checks that the original amino acid matches the sequence it is using and refuses to make a prediction otherwise. Although avoiding incorrect predictions is a valid strategy, in practice it substantially reduces the number of predictions that can be made.

Identifier translation is a very common task in Bioinformatics in general, and in cancer genome analysis in particular. In practice, we use the Ensembl BioMart web service to download identifier equivalence tables (in TSV format), which map different identifier formats between and across genes, proteins, array probes, *etc.* We build fast indexes over these equivalence tables and make them ubiquitously accessible to all our functionalities through simple API calls, web services, or command line statements. While potentially encumbered by semantic incompatibilities between entity definitions in multiple resources, a thoroughly versioned translation equivalence system is an invaluable asset for database integration.

4.2. Software Resources

In cancer analysis pipelines, several tasks must be performed that require supporting software. These range from simple database searches to cross-check lists of germline mutations with lists of known SNPs, to running complex computational methods to identify protein-protein interaction sub-networks affected by mutations. Some cancer analysis workflows opt to develop these functionalities in-house, while others delegate them to third party software with the implicit burdens of installation and configuration. Table 1 lists some software resources that are useful when implementing analysis workflows, and succinctly describes their functionality and availability.

The functionalities required in a genome analysis workflow can be divided into four classes, depending on how they are accessed (Table 3): via web services, local or browser based applications, command line tools, or application programming interfaces (APIs). It is not uncommon for resources to make their data and functionalities available in several ways, a trend that is already evident in databases like Ensembl, where the information can be examined using the web interface, downloaded via the BioMart web service, batch downloaded from an FTP server, or queried through the PERL API.

Bioinformaticians should strive to make their resources widely available to allow others to use them in the most convenient manner. In function of the workflow's characteristics, some accessibility modes (*e.g.*, web service, local application, or API) will be more convenient than others. For example, if a relatively systematic workflow has to be applied to a batch of datasets, then command-line tools are very convenient as they are easy to script. Because a cancer genome analysis pipeline may require several connected analytical steps, it is important to be able to script them to avoid manual operations, thereby guaranteeing the sustainability and reproducibility of the results. Conversely, if the user is concerned with the analysis of just one dataset but interpretation of the results requires more careful examination, visual interfaces such as browser-based applications may be the most convenient end-user interface, as these can link the results to knowledge databases to set the context.

5. Workflow Enactment Tools and Visual Interfaces

Given the complexity of cancer genome analysis, it is worth discussing how to design and execute (enact) workflows, which may become very elaborate. Workflows can be thought of as analysis recipes, whereby each analysis entails enacting that workflow using new data. Ideally a workflow should be comprehensive and cover the complete analysis process from the raw data to the final results. These workflows may involve processing different types of data and may require specific

Table 3. Types of third party software and their general characteristics.

Software type	Installation	User friendly	Scriptable	Reusable[1]
Browser app.	NO	YES	NO[2]	NO
Web server	NO	NO	YES	NO
Local app	YES	YES	NO[3]	NO
Command line	YES	NO	YES	YES[4]
API	YES	NO	YES	YES

[1]Reusable means that the code, in whole or in part, can be reused for some other purpose.
[2]May be scriptable using web scraping.
[3]May support some macro definitions and batch processing.
[4]If the source code is provided and is easy to pick apart.
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Further Reading

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adaptations for the analysis of certain types of experiments. Often, parts of the analysis will be repeated in a different context and thus, one of the objectives of workflow enactment tools is to reuse code efficiently. A number of systems have been designed to facilitate the construction of workflows (*e.g.*, Taverna [21] and Galaxy [22], which both offer visual interfaces to orchestrate workflows across a very wide range of available functionalities).

Although visual workflow enactment approaches have become reasonably popular, they still have several important limitations. Firstly, despite recent efforts, these approaches remain overly complex for non-bioinformaticians. Secondly, they are quite inflexible in terms of the presentation and exploration of the results, and thus, understanding the results requires the user to do additional work outside of the system. Finally, the expressiveness of these approaches is limited when compared with general purpose programming languages. Experienced developers will find them of limited utility, and prefer to have their functionalities accessible by APIs derived from general purpose programming languages.

The information presented to the user needs to closely match his/her needs, especially in more translational settings. Too much information may mask important conclusions, while too little may leave the user unsure as to the validity of their findings. This further emphasizes the need to customize workflows and the manner in which results are displayed, in order to best fit these aspects to the particularities of each user.

In a more academic setting, close collaboration between the researcher and the bioinformatician facilitates the development of custom interfaces that can better adapt to given datasets, and answer the very specific questions that may arise during data exploration. In our institution, we use a programmatic workflow enactment system that orchestrates a wide variety of tasks, ranging from coordinate mapping to enrichment analysis. This system is controlled via a browser application designed to rapidly produce custom reports using a template-based HTML report generation system. It is a system that was developed entirely in-house but that makes use of third party software, allowing us to address the requirements of our collaborators in a timely manner.

6. Summary

Cancer genome analysis involves the manipulation of large datasets and the application of complex methods. The heterogeneity of the data and the disparity of the software implementations represent an additional layer of complexity, which requires the use of systems that can be easily adapted and reconfigured. Additionally, interpretation of the results in terms of specific biological questions is more effective if done in close collaboration with experts in the field. This represents a specific challenge for software development in terms of interactivity and representation standards. Cancer genome analysis systems need to be capable of conveniently managing this complexity and of adapting to the specific characteristics of each analysis.

Finally, it is worth noting that bioinformatics systems will soon have to move beyond the current research environments and into clinical settings, a challenge that will involve more industrial development that can better cope with issues of sustainability, robustness and accreditation, while still incorporating the latest bioinformatics components that will continue to be generated in research laboratories. This constitutes a new and exciting frontier for bioinformatics software developers.

7. Exercise Questions

I. Name three general issues that bioinformaticians face when analyzing cancer genome data?

II. What are the four main tasks in cancer genome analysis in a clinical setting once the primary analysis has been performed?

III. Why is it important to use the correct genome build?

IV. What do we mean by driver mutation?

V. There are two key principles that help determine driver mutations *in-silico*. What are they?

VI. Give several reasons why point mutations in coding regions are so important.

VII. Name three issues that challenge the assumptions made by the standard pathway enrichment analysis tools when applied to genomic mutations.

VIII. Discuss the problems that arise with identifiers when integrating information across different databases.

IX. Why are command line tools generally more convenient than browser-based applications for processing a batch analyses?

X. How would an application aimed at researchers differ from one aimed at clinicians in terms of the information presented?

Answers to the Exercises can be found in Text S1.

Supporting Information

Text S1 Answers to Exercises
(DOCX)

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Chapter 15: Disease Gene Prioritization

Yana Bromberg*

Department of Biochemistry and Microbiology, School of Environmental and Biological Sciences, Rutgers University, New Brunswick, New Jersey, United States of America

Abstract: Disease-causing aberrations in the normal function of a gene define that gene as a disease gene. Proving a causal link between a gene and a disease experimentally is expensive and time-consuming. Comprehensive prioritization of candidate genes prior to experimental testing drastically reduces the associated costs. Computational gene prioritization is based on various pieces of correlative evidence that associate each gene with the given disease and suggest possible causal links. A fair amount of this evidence comes from high-throughput experimentation. Thus, well-developed methods are necessary to reliably deal with the quantity of information at hand. Existing gene prioritization techniques already significantly improve the outcomes of targeted experimental studies. Faster and more reliable techniques that account for novel data types are necessary for the development of new diagnostics, treatments, and cure for many diseases.

This article is part of the "Translational Bioinformatics" collection for *PLOS Computational Biology*.

1. Introduction

In 1904 Dr. James Herrick reported [1] the findings of "peculiar elongated and sickle shaped" red blood cells discovered by Dr. Ernest Irons in a hospital patient afflicted with shortness of breath, heart palpitations, and various other aches and pains. This was the first documented case of sickle cell disease in the United States. Forty years later, in 1949, sickle cell anemia became the first disease to be characterized on a molecular level [2,3]. Thus, implicitly, the first disease-associated gene, coding for beta-globin chain of hemoglobin A, was discovered.

It took another thirty years before in 1983 a study of the DNA of families afflicted with Huntington's disease has revealed its association with a gene on chromosome 4 called huntigtin (HTT) [4]. Huntington's became the first genetic disease mapped using polymorphism information (G8 DNA probe/genetic marker), closely followed by the same year discovery of phenylketonuria association with polymorphisms in a hepatic enzyme phenylalanine hydroxylase [5]. These advances provided a route for predicting the likelihood of disease development and even stirred some worries regarding the possibility of the rise of "medical eugenics" [6]. Interestingly, it took another ten years for HTT's sequence to be identified and for the precise nature of the Huntigton's-associated mutation to be determined [7].

The recent explosion in high-throughput experimental techniques has contributed significantly to the identification of disease-associated genes and mutations. For instance, the latest release of SwissVar [8], a variation centered view of the Swiss-Prot database of genes and proteins [9,10], reports nearly 20 thousand mutations in 35 hundred genes associated with over three thousand broad disease classes. Unfortunately, the improved efficiency in production of association data (*e.g.* genome-wide association studies, GWAS) has not been matched by its similarly improving accuracy. Thus, the sheer quantity of existing but yet unvalidated data resulted in information overflow. While association and linkage studies provide a lot of information, incorporation of other sources of evidence is necessary to narrow down the candidate search space. Computational methods - gene prioritization techniques, are therefore necessary to effectively translate the experimental data into legible disease-gene associations [11].

2. Background

The Merriam-Webster dictionary defines the word "disease" as a "a condition of the living animal or plant body or of one of its parts that impairs normal functioning and is typically manifested by distinguishing signs and symptoms." Thus, disease is defined *with respect to normal function* of said body or body part. Note, that this definition also describes the malfunction of individual cells or cell groups. In fact, many diseases can and should be defined on a cellular level. Understanding a disease, and potentially finding curative or preventive measures, requires answering three questions: (1) What is the affected function? (2) What functional activity levels are considered normal given the environmental contexts? (3) What is the direction and amount of change in this activity necessary to cause the observed phenotype?

Contrary to the view that historically prevailed in classical genetics it is rarely the case that one gene is responsible for one function. Rather, an assembly of genes constitutes a functional module or a molecular pathway. By definition, a molecular pathway leads to some specific end point in cellular functionality via a series of interactions between molecules in the cell. Alterations in any of the normally occurring processes, molecular interactions, and pathways lead to disease. For example, folate metabolism is an important molecular pathway, the disruptions in which have been associated with many disorders including colorectal cancer [12] and coronary heart disease [13]. Because this pathway involves 19 proteins interacting via numerous cycles and feedback loops [14], it is not surprising that there are a

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* E-mail: YanaB@rci.rutgers.edu

What to Learn in This Chapter

- Identification of specific disease genes is complicated by gene pleiotropy, polygenic nature of many diseases, varied influence of environmental factors, and overlying genome variation.
- Gene prioritization is the process of assigning likelihood of gene involvement in generating a disease phenotype. This approach narrows down, and arranges in the order of likelihood in disease involvement, the set of genes to be tested experimentally.
- The gene "priority" in disease is assigned by considering a set of relevant features such as gene expression and function, pathway involvement, and mutation effects.
- In general, disease genes tend to 1) interact with other disease genes, 2) harbor functionally deleterious mutations, 3) code for proteins localizing to the affected biological compartment (pathway, cellular space, or tissue), 4) have distinct sequence properties such as longer length and a higher number of exons, 5) have more orthologues and fewer paralogues.
- Data sources (directly experimental, extracted from knowledge-bases, or text-mining based) and mathematical/computational models used for gene prioritization vary widely.

number of different ways in which it can be broken. The changes in concentrations and/or activity levels of any of the pathway members directly affect the pathway end-products (*e.g.* pyrimidine and/or methylated DNA). The specifics of a given change define the severity and the type of the resulting disease; see Box 1 for discussion on disease types. Moreover, since the view of a single pathway as a discrete and independent entity (with no overlap with other pathways) is an oversimplification, it is increasingly evident that different diseases are also interdependent.

3. Interpreting What We Know

Identifying the genetic underpinnings of the observed disease is a major challenge in human genetics. Since disease results from the alteration of normal function, identifying disease genes requires defining molecular pathways whose disrupted functionality is necessary and sufficient to cause the observed disease. The pathway function changes due to the (1) changes in gene expression (*i.e.* quantity and concentration of product), (2) changes in structure of the gene-product (*e.g.* conformational change, binding site obstruction, loss of ligand affinity, etc.), (3) introduction of new pathway members (*e.g.* activation of previously silent genes), and (4) environmental disruptions (*e.g.* increased temperatures due to inflammation or decreased ligand concentrations due to malnutrition). While all members of the affected pathways can be construed as disease genes, the identification of a subset of the true causative culprits is difficult. Obscuring such identification are individual genome variation (*i.e.* the reference definition of "normal" is person-specific), multigenic nature and complex phenotypes of most diseases, varied influence of environmental factors, as well as experimental data heterogeneity and constraints.

Disease genes are most often identified using: (1) genome wide association or linkage analysis studies, (2) similarity or linkage to and co-regulation/co-expression/co-localization with known disease genes, and (3) participation in known disease-associated pathways or compartments. In bioinformatics, these are represented by multiple sources of evidence, both direct, *i.e.* evidence coming from own experimental work and from literature, and indirect, *i.e.* "guilt-by-association" data. The latter means that genes that are in any way related to already established disease-associated genes are promoted in the suspect list. Additionally, implied gene-disease links, such as functional deleteriousness of mutations affecting candidate genes, contributes to establishing associations. The manner in which each guilty association is derived varies from tool to tool and all of them deserve consideration. Very broadly, gene-disease associations are inferred from (Figure 1):

1. *Functional Evidence* – the suspect gene is a member of the same molecular pathways as other disease-genes; inferred from: direct molecular interactions, transcriptional co-(regulation/expression/localization), genetic linkage, sequence/structure similarity, and paralogy (in-species homology resulting from a gene duplication event)
2. *Cross-species Evidence* – the suspect gene has homologues implicated in generating similar phenotypes in other organisms
3. *Same-compartment Evidence* – the suspect gene is active in disease-associated pathways (*e.g.* ion channels), cellular compartments (*e.g.* cell membrane), and tissues (*e.g.* liver).
4. *Mutation Evidence* – suspect genes are affected by functionally deleterious

Box 1. Genetic similarities of different disease types.

Diseases can be very generally classified by their associated causes: *pathogenic* (caused by an infection), *environmentally determined* (caused by "inanimate" environmental stressors and deficiencies, such as physical trauma, nutrient deficiency, radiation exposure and sleep deprivation), and *genetically hereditary* or *spontaneous* (defined by germline mutations and spontaneous errors in DNA transcription, respectively). Moreover, certain genotypes are more susceptible to the effects of pathogens and environmental stress, contributing to a deadly interplay between disease causes. Regardless of the cause of disease, its manifestations are defined by the changes in the affected function. For example, cancer is the result of DNA damage occurring in a normal cell and leading toward a growth and survival advantage. The initial damage is generally limited to a fairly small number of mutations in key genes, such as proto-oncogenes and tumor suppressor genes [135]. The method of accumulation of these mutants is not very important. A viral infection may cause cancer by enhancing proto-oncogene function [136] or by inserting viral oncogenes into host cell genome. An inherited genetic variant may disrupt or silence a single allele of a mismatch-repair gene as in Lynch syndrome [137]. Spontaneous transcription errors and influence of environmental factors, *e.g.* continued exposure to high levels of ionizing radiation, may result in oncogene and tumor suppressor-gene mutations leading to the development of cancer [138]. Thus, the same broad types of disease can be caused by the disruption of the same mechanisms or pathways resulting from any of the three types of causes.

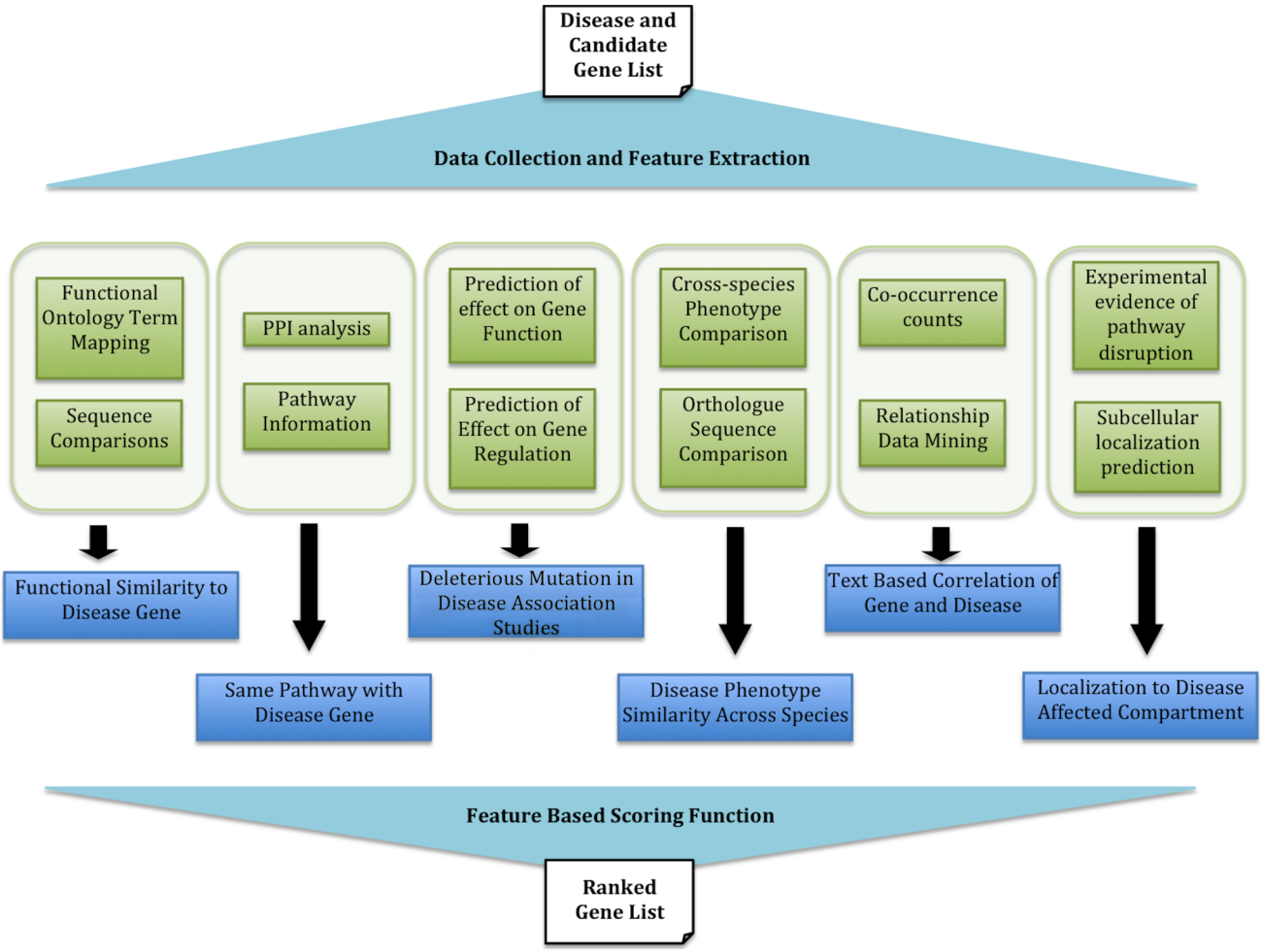

Figure 1. Overview of gene prioritization data flow. In order to prioritize disease-gene candidates various pieces of information about the disease and the candidate genetic interval are collected (green layer). These describe the biological relationships and concepts (blue layer) relating the disease to the possible causal genes. Note, the blue layer (representing the biological meaning) should ideally be blind to the content green layer (information collection); *i.e.* any resource that describes the needed concepts may be used by a gene prioritization method.
doi:10.1371/journal.pcbi.1002902.g001

mutations in genomes of diseased individuals

5. *Text Evidence* – there is ample co-occurrence of gene and disease terms in scientific texts. Note that textual co-occurrence represents some form of biological evidence, which does not yet lend itself to explicit documentation.

3.1 Functional Evidence

3.1.1 Molecular interactions. Gene prioritization tools, from the earliest field pioneers like G2D [15,16,17] to the more recent ENDEAVOUR [18,19] and GeneWanderer [20,21], among many others, have used gene-gene (protein-protein) interaction and/or pathway information to prioritize candidate genes. Biologically this makes sense, because if diseases result from pathway breakdown then disabling any of the pathway components can produce similar phenotypes; *i.e.* genes responsible for similar diseases often participate in the same interaction networks [22,23]. To illustrate this point, consider the interaction partners of the melanocortin 4 receptor (MC4R) in STRING [24,25] server generated Figure 2. Note, not all known interactions are shown – the inclusion parameter is STRING server likelihood >0.9.

MC4R is a hypothalamic receptor with a primary function of energy homeostasis and food intake regulation. Functionally deleterious polymorphisms in this receptor are known to be associated with severe obesity [26,27,28]. Here, MC1R, MC3R, and MC5R are membrane bound melanocortin (1,3,5) receptors that interact with MC4R via shared binding partners. Syndecan-3 (SDC3), agouti signaling protein precursor (ASIP), agouti related protein precursor (AgRP), pro-opiomelanocortin (POMC) and/or their processed derivatives directly bind MC4R for varied purposes of the MC4R signaling pathway. Finally, the reported interactions with Neuropeptide Y-precursor (NPY) and the growth hormone releasing protein (GHRL) are literature derived and may reflect indirect, but tight connectivity. By the token of "same pathway" evidence, *MC4R* interactors, whether agonists or antagonists, may be predicted to be linked to obesity. In fact, mutations that negatively affect normal POMC production or processing have been shown to be obesity-associated [29,30] and gene association studies have linked AgRP with anorexia and bulimia nervosa behavioral traits [31], representative of food intake abnormalities. Other pathway participants have also been marked and extensively studied for obesity association.

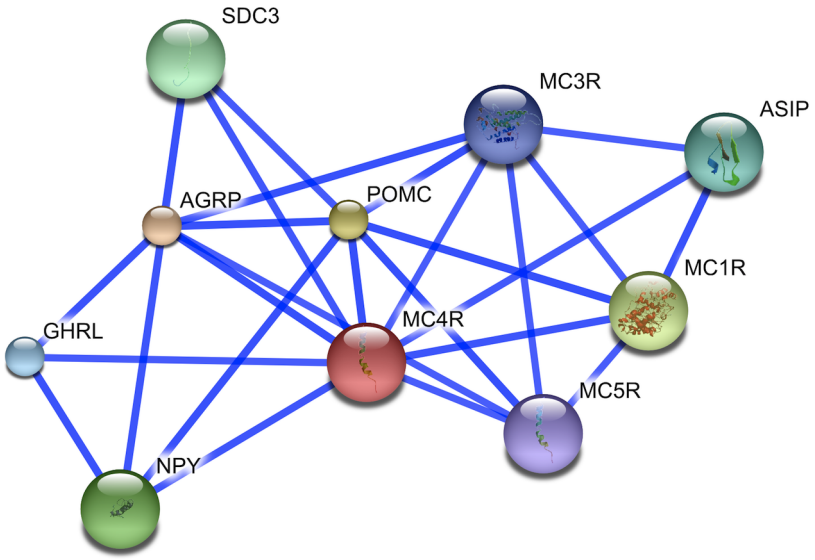

Figure 2. MC4R-centered protein-protein interaction network. The figure illustrates protein-protein interaction neighborhood of the human melanocortin 4 receptor (MC4R) as illustrated by the confidence view of the STRING 8.3 server. The nodes of the graph represent human proteins and the connections illustrate their known or predicted, direct and indirect interactions. The connection between any two protein-nodes is based on the available information mined from relevant databases and literature. The network includes all protein interactions that have >0.9 estimated probability.
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3.1.2 Regulatory and genetic linkage. Co-regulation of genes has traditionally been thought to point to their involvement in same molecular pathways [32] and, by that token, to similar disease phenotypes; *e.g.* [33,34]. For example, GPR30 a novel G-protein coupled estrogen receptor is co-expressed with the classical estrogen receptor ERβ [33]. The former (GPR30) has been linked to endometrial carcinoma [35] so it is no surprise that the latter (ERβ) is also associated with this type of cancer [33].

However, co-regulation doesn't *always* have to mean the same pathway – studies have shown that consistently co-expressed genes, while possibly genetically linked [36,37], may also reside in distinct pathways [38]. Additionally, co-expressed non-paralogous genes, independent of common pathway involvement, often cluster together in different species and fall into chromosomal regions with low recombination rates [39,40], suggesting genetic linkage [39,40]. These finding suggests that clusters of co-expressed genes are selectively advantageous [36]. Possibly, these clusters are groups of genes that despite the apparent functional heterogeneity may be jointly involved in orchestrating complicated cellular functionality [41]. Evolutionary pressure works on maintaining co-expression of these genes and on keeping recombination rates within the clusters low. Thus, the fine-tuned cooperation of alleles is not broken by recombination, but rather transmitted as one entity to the next generation. De-regulation of these clusters is therefore likely to be deleterious to the organism and develop into disease.

Genes co-expressed with or genetically linked to other disease genes are also likely to be disease-associated. However, while genetic linkage and co-regulation are valuable markers of disease association, they also pose a specificity problem; *i.e.* a given disease-associated gene may be co-regulated with or linked to another disease-associated gene, where the two diseases are not identical. Genetic linkage similarly poses a problem for GWAS where it is difficult to distinguish between "driver" mutations, the actual causes of disease, and "passenger" mutations, co-occurring with the disease-mutations due to genetic linkage.

3.1.3 Similar sequence/structure/function. Reduced or absent phenotypic effect in response to gene knockout/inactivation is a common occurrence [42,43], largely explained by functional compensation, *i.e.* partial interchangeability of paralogous genes. In humans, genes with at least one paralogue, approximated by 90% sequence identity, are about three times less likely to be associated with disease as compared to genes with more remote homologs [44]. However, in the cases where paralogous functional compensation is insufficient to restore normal function, inactivation of any of the paralogues leads to same or similar disease. Prioritization tools thus often use functional similarity as an input feature. For example, one GeneOntology (GO, [45]) defined MC4R function, is "melanocyte-stimulating hormone receptor activity" (GO:0004980). There are two other human gene products sharing this function: MSHR (MC1R, 52% sequence identity) and MC3R (61%). Predictors relying on functional similarity to annotate disease association would inevitably link both of these with obesity. These findings are confirmed by the recent studies for MC3R [46], but the jury still remains out for MC1R involvement.

Quantifying functional similarity is of utmost importance for the above approach. Using ontology-defined functions (*e.g.* GeneOntology) this problem reduces to finding a distance between two ontology nodes/subtrees (*e.g.* [47,48,49,50]). For un-annotated genes, however, sequence and structure homology is often used to transfer functional annotations from studied genes and proteins [51,52]. Since functionally similar genes are likely to produce similar disease phenotypes, sequence/structure similarities are good indicators of similar disease involvement. Additionally, disease genes are often associated with specific gene and protein features such as higher exon number and longer gene length, protein length, presence of signal peptides, higher distance to a neighboring gene and 3′ UTR length, and lower sequence divergence from their orthologues [53,54]. Moreover, disordered proteins are often implicated in cancer [55].

3.2 Cross-species Evidence

Animal models exist for a broad range of human diseases in a number of well-studied laboratory organisms, *i.e.* mouse, zebrafish, fruit fly, etc. However, straightforward cross-species comparisons of orthologues and their associated phenotypic traits are also very useful. A high number of orthologues (consistent presence in multiple species) generally highlights essential genes that are prone to disease involvement. Orthologues generally participate in similar molecular pathways although different levels of function are necessary for different organisms (*e.g.* human MC4R is more functional then its polar bear orthologue [56]). Thus, cross-species tissue-specific phenotypic differentiation due to slightly varied sequences may be useful for gene prioritization. For example, the human MC4R and almost

all of its close orthologues (*e.g.* in mouse, rat, pig, and cow) contain a conserved valine residue in the 95th position of the amino acid sequence. In the polar bear orthologue, however, this position is frequently occupied by an isoleucine residue [56]. When considering MC4R involvement in generating an obesity phenotype, it is useful to note that polar bears have a need for increased body fat content for thermal insulation, water buoyancy, and energy storage requirements [56] as compared to humans and to other organisms that share a conserved V95. Thus, one can imagine that the V95I mutation, while deleterious to the function of the receptor, is a polar bear specific adaptation to its environment, and may have a similar (increased body fat) effect in humans. In fact, V95I does inactivate the human receptor [57,58] and associates with obesity.

Comparing human and animal phenotypes is not always straightforward. Washington *et al* [59] have shown that phenotype ontologies facilitate genotype-phenotype comparisons across species. Disease phenotypes recorded in their ontology (OBD, ontology based database) can be compared to the similarly built cross-species phenotype ontologies using a set of proposed similarity metrics. Finding related phenotypes across species suggests orthologous human candidate genes. For instance, phenotypic similarities of eye abnormalities recorded in human and fly suggest that *PAX6*, a human orthologue of the phenotype-associated fly gene *ey*, is a possible disease-gene candidate. Further investigation shows that mutations in *PAX6* may result in aniridia (absence of iris), corneal opacity (aniridia-related keratopathy), cataract (lens clouding), glaucoma, and long-term retinal degeneration (Figure 3) [59].

A correlation of gene co-expression across species is also useful for annotating disease genes [60,61]. Genes that are part of the same functional module are generally co-expressed. Also, there is evidence for co-expression of visibly functionally unrelated genes [37,62,63]. The explanation of these co-expression clusters having an evolutionary advantage only holds true for otherwise unjustified conservation of these clusters throughout different species; *i.e.* cross-species comparison of protein co-expression may be used for validation of disease-gene co-expression inference. Using this assumption, Ala *et al* [61] had narrowed down the initial list of 1,762 genes in the loci mapped via genetic linkage to 850 OMIM (Online Mendelian Inheritance in Man) [64] phenotypes to twenty times fewer (81) possible disease-causing genes. For example, in their analysis a cluster of functionally unrelated genes co-expressed in human and mouse contained a *bona fide* disease-gene KCNIP4 (partial epilepsy with pericentral spikes).

3.3 Compartment Evidence

Changes in gene expression in disease-affected tissues are associated with many complex diseases [65]. Tissue specificity is also important for choosing correct protein-protein interaction networks, as some proteins interact in some tissues, but rarely in others [66]. Disease-associated cellular pathways (*e.g.* ion channels or endocytic membrane transport) and compartments (*e.g.* membrane or nucleus) implicate pathway/compartment-specific gene-products in disease as well. For example, autosomal recessive generalized myotonia (Becker's disease) (GM) and autosomal dominant myotoniacongenita (Thomsen's disease, MC) are characterized by skeletal muscle stiffness [67]. This phenotype is the result of muscle membrane hyperexcitability and, in conjunction with observed alterations in muscle chloride and sodium currents, points to possible involvement of deficiencies of the muscle chloride channel. In fact, studies point to the mutations in the transmembrane region of CLC-1, the muscle chloride channel coding gene, as the culprit [67]. Another example is that of the multiple storage diseases, such as Tay-Sachs, Gaucher, Niemann-Pick and Pompe disease, which are caused by the impairment of the degradation pathways of the intracellular vesicular transport. In fact, many of the genes implicated in these diseases encode for proteins localized to endosomes (*e.g. NPC1* in Neimann-Pick [68]) or lysosomes (*e.g. GBA* [69] in Gaucher, *GAA* in Pompe [70] and *HEXA* in Tay Sachs [71]).

3.4 Mutant Evidence

By definition, every genetic disease is associated with some sort of mutation that alters normal functionality. In fact, primary selection of candidates for further analysis is often largely based on observations of polymorphisms in diseased individuals, which are absent in healthy controls (*e.g.* GWAS). However, not all observed polymorphisms are associated with deleterious effects. Note, that on average gain and loss of function mutations are considered to alter normal functionality equally deleteriously. Most of the observed variation does not at all manifest phenotypically, some is weakly deleterious with respect to normal function, and less still is weakly beneficial. In nature strongly beneficial mutations are very rare; they spread rapidly in the population and cannot be considered disease-associated. On the other hand, strongly deleterious or inactivating mutations are often incompatible with life. A small percentage of mutations of this type, affecting genes whose function is not life-essential, are often associated with monogenic Mendelian disorders. Strongly dele-

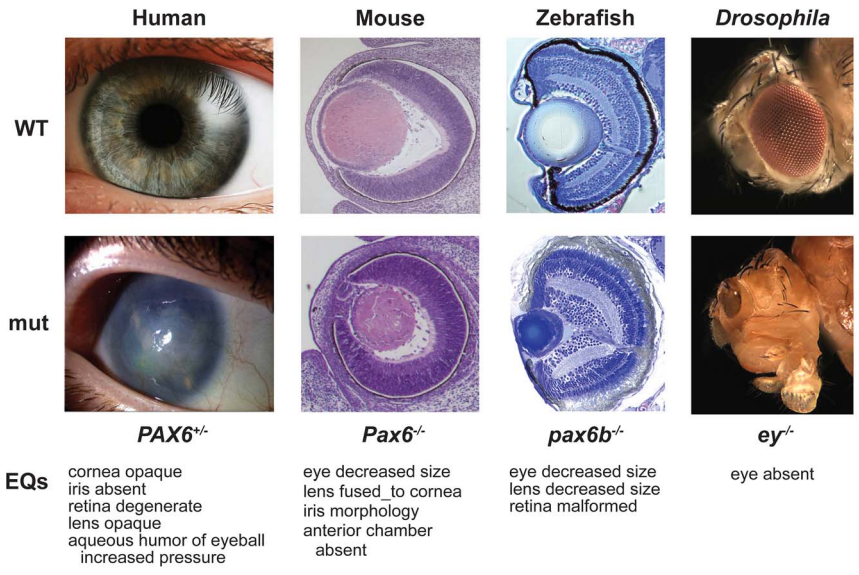

Figure 3. Correlating cross-species phenotypes. Phenotypes of wild-type (top) and PAX6 ortholog mutations (bottom) in human, mouse, zebrafish, and fly can be described with the EQ method suggested by Washington et al [59]. Once phenotypic descriptions are standardized across species, genotypic variations can be assessed as well.
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terious mutations in the genes whose function may somehow be compensated (*e.g.* via paralogue activity) are associated with complex disorders, where the level of compensation affects the observed phenotype. Complex disorders may also accumulate weakly deleterious mutations to generate a strongly negative phenotype. Intuitively it is clear that a selected candidate gene, carrying a deleterious mutation in an affected individual is more likely to be disease-associated than one which contains functionally neutral mutants or no variation at all.

3.4.1 Structural variation. Structural variation (SV) is the least studied of all types of mutations. It has long been assumed that less than 10% of human genetic variation is in the form of genome structural variants (insertions and deletions, inversions, translocations, aneuploidy, and copy number variations - CNVs). However, because each of the structural variants is large (kb-Mb scale), the total number of base pairs affected by SVs may actually be comparable to the number of base pairs affected by the much more common SNPs (single nucleotide polymorphisms). Moreover, high throughput detection of structural variants is notoriously difficult and is only now becoming possible with better sequencing techniques and CNV arrays. Thus, more SVs may be discovered in the near future. We do not currently know what proportion of genetic disease is caused by SVs, but we suspect that it is high.

Due to the above mentioned constraints on SV identification, there are only ~180 thousand structural variants reported in one of the most complete mutation collections – the Database of Genomic Variants, DGV [72]. Gross changes to genome sequence are very likely to be disease associated, but also frequently gene non-specific. For instance, Down's syndrome, trisomy 21, is an example of a whole extra chromosome gain and *cri du chat* syndrome results from the deletion of the short arm of chromosome 5 [73]. All of the genes found in these regions of the genome are, by default, associated with the observed disease but neither can be considered primarily causal. When the damage is less extensive the genes involved may be further evaluated for causation. For instance, several epilepsy-associated genes are known, but functionally-significant mutations in these account for only a small fraction of observed disease cases. One study [74] reports that CNV mutants found in epileptic individuals but not in the general population account for nearly nine percent of all cases. Among these are CNVs resulting from deletions in AUTS2 and CNTNAP2 genes. Both of these genes have been implicated in other neurological disorders [75,76] reaffirming the possible disease link. Inversions, translocations and large deletions and insertions have all been implicated in different forms of disease. Even very small indels, resulting in an open-reading frame shift (frameshift mutations), are often sufficient to cause disease. For instance, one of the causes of Tay-Sachs is a deletion of a single cytosine nucleotide in the coding sequence of a lysosomal enzyme beta-hexosaminidase [71].

In most cases of diseases that are associated with SVs the prioritization of disease-causing genes is reduced to finding those that are directly affected by the mutation. Lots of work has been done in this direction, including development of the CNVinetta package [77] for mining and visualizing CNVs, GASV approach for identifying structural variation boundaries more precisely [78], and software created by Ritz *et al* for searching for structural variants in strobe sequencing data [79]. SV identification is still a new field, but the advances in methodologies will have a great impact on our understanding and study of many of the known diseases.

3.4.2 Nucleotide polymorphisms. The other ~90% of human variation exists in the form of SNPs (single nucleotide polymorphisms) and MNPs (multi-nucleotide polymorphisms; consecutive nucleotide substitutions, usually of length two or three). A single human genome is expected to contain roughly 10–15 million SNPs per person [80]. As many as 93% of all human genes contain at least one SNP and 98% of all genes are in the vicinity (~5 kb) of a SNP [81]. The latest release of NCBI dbSNP database [82] (build 137) contains nearly 43 million validated human SNPs, 17.5 million of which have been experimentally mapped to functionally distinct regions of the genome (*i.e.* mRNA UTR, intron, or coding regions). Non-coding region SNPs (~17.2 million) are trivially more prevalent than coding SNPs (~432 thousand) as non-coding DNA makes up the vast majority of the genome. Coding SNPs, however, are over-represented in disease associations; *e.g.* OMIM contains 2430 non-coding SNPs (0.0001% of all) and 5327 coding ones (0.01% of all – 100-fold enrichment). Due to the redundancy of the genetic code, coding SNPs can be further subdivided into synonymous (no effect on protein sequence) and non-synonymous (single amino acid substitution) SNPs. Simple statistics of the genetic code suggest that synonymous SNPs should account for 24% of all coding-region SNPs. dbSNP data suggests an even larger percentage of synonymity – ~188 thousand (44%), which is possibly due to evolutionary pressure eliminating functionally deleterious non-synonymous SNPs. MNPs are rare as compared to SNPs, but are over-represented amongst the protein altering variants, almost always changing the affected amino acid, or two neighboring ones, or introducing a nonsense mutation (stop-codon) [83].

Identifying and annotating functional effects of SNPs and MNPs is important in the context of gene prioritization because genes selected for further disease-association studies are more likely to contain a deleterious mutation or be under the control of one (*e.g.* mutations affecting transcription factor or microRNA binding sites). In recent years a number of methods were created for identifying mutations as functionally deleterious. PromoLign [84], PupaSNP finder [85], and RAVEN [86] look for SNPs affecting transcription, SNPper [87] finds and annotates SNP locations, conservation, and possible functionalities so that they can be visually assessed, and SNPselector [88] and FASTSNP [89] assess various SNP features such as whether it alters the binding site of a transcription factor, affects the promoter/regulatory region, damages the 3′ UTR sequence that may affect post-transcriptional regulation, or eliminates a necessary splice site. Coding synonymous SNPs have recently been shown to have the same chance of being involved in a disease mechanism as non-coding SNPs [90]. This effect may be due to codon usage bias or to changes in splicing or miRNA binding sites [91]. However, few (if any) computational methods are able make predictions with regard to their functional effects.

Non-synonymous SNPs are somewhat more studied. Early termination of the protein is very often associated with disease so genes with nonsense mutants are automatically moved up in the list of possible suspects. Missense SNPs and MNPs, which alter the protein sequence without destroying it, may or may not be disease associated. In fact, most methods estimate that only 25–30% of the nsSNPs negatively affect protein function [92]. Databases like OMIM [93], and more explicitly, SNPdbe [94], SNPeffect [95], PolyDoms [96], Mutation@A Glance [97] and DMDM [98] map SNPs to known structural/functional effects and diseases. Computational tools that make predictions about functional and disease-associated effects of SNPs include SNAP [99,100], SIFT [101,102], PolyPhen [103,104],

Z score	Relevancy Score	Disease Name	Synonyms	PubMed Hits
Gene: PIK3CA				
13.2	7231 (74,106,118,291)	breast cancer	Breast Cancer; Cancer of the Breast; Cancer of Breast; Malignant Breast Tumor; Malignant Neoplasm of the Breast; Malignant Tumor of the Breast; Malignant Neoplasm of Breast; Malignant Breast Neoplasm…	128 (74,106,118,291)
12	6550 (68,90,101,395)	colorectal cancer	Cancer, Colorectal; Colorectal Cancer; Colorectal Cancers	172 (68,90,101,395)
Gene BRCA1				
13.2	7231 (74,106,118,291)	breast cancer	Breast Cancer; Cancer of the Breast; Cancer of Breast; Malignant Breast Tumor; Malignant Neoplasm of the Breast; Malignant Tumor of the Breast; Malignant Neoplasm of Breast; Malignant Breast Neoplasm…	128 (74,106,118,291)
12	6550 (68,90,101,395)	colorectal cancer	Cancer, Colorectal; Colorectal Cancer; Colorectal Cancers	172 (68,90,101,395)
Gene: MC4R				
9.8	1953 (21,28,30,53)	severe obesity	Severe obesity; Morbid obesity; Obesity, Morbid	44 (21,28,30,53)
5.1	1058 (9,17,25,58)	hyperphagia	Overeating; overeating; Gluttony; HYPERPHAGIA; polyphagia; Excessive eating;Polyphagia; Hyperalimentation	48 (9,17,25,58)
Gene CLC1				
3.2	85 (1,1,1,5)	myotonic dystrophy	Dystrophia myotonica; DYSTROPHY, MYOTONIC; Dystrophia Myotonica; Myotonia atrophica; Myotonic Dystrophy; STEINERT DISEASE; Myotonic Dystrophies; Myotonia Dystrophica…	2 (1,1,1,5)

Figure 4. PolySearch gene-disease associations. PolySearch uses PubMed lookup results to prioritize diseases associated with a given gene. Here, screen shots of the top two results (where available; sorted by relevancy score metric) from PolySearch are shown. According to these, BRCA1 and PIK3CA are associated with breast cancer, while MC4R and CLC1 are not. These results quantitatively confirm intuitive inferences made from simple PubMed searches.
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PHD-SNP [105], SNPs3D [106], and many others. Most of these methods are binary in essence – that is they point to a deficiency without suggesting specifics of the disease or molecular mechanisms of functional failure. Nevertheless, they are very useful in conjunction with other data described above. The recent trend in mutation analysis has seen the development of tools, like SNPNexus [107] and SNPEffectPredictor [108] that are no longer limited by DNA type and predict effects for both non-coding and coding region SNPs.

3.5 Text Evidence

The body of science that addresses gene-disease associations has been growing in leaps and bounds since the mapping of a hemoglobin mutation to sickle cell anemia. Some researchers have been proactive in making their data computationally available from databases like dbSNP, GAD [109], COSMIC [110], *etc.* Others have contributed by depositing knowledge obtained through reading and manual curation into the likes of PMD [111], GeneRIF [112] and UniProt [9]. However, huge amounts of data, which could potentially improve the performance of any gene prioritization method, remains hidden in plain site in natural language text of scientific publications. Consider, for example, a scientist who is interested in prioritizing breast cancer genes. A casual search in PubMed for the term combination *breast cancer* generates over two hundred thousand matches. Limiting the field to *genetics of breast cancer* reduces the count to slightly fewer than fifty thousand. The past thirty days have brought about 46 new papers. Thus, someone interested in getting all the genetic information out of the PubMed collection would need to dedicate his or her life to reading. Fortunately, scientific text mining tools have recently come of age [113,114,115]. The new tools will allow for intelligent identification of possible gene-gene and disease-gene correlations [116,117,118]. For example, the Information Hyperlinked Over Proteins, IHOP method [119] links gene/protein names in scientific texts via associated phenotypes and interaction information. For automated link extraction, however, the existing gene prioritization techniques rely mostly on term co-occurrence statistics (*e.g.* PosMed [120] and GeneDistiller [121]) and gene-function annotations (*e.g.* ENDEAVOR [122] and PolySearch [123]), which can then be related to diseases as described above.

For a significantly oversimplified example of this type of processing consider searching PubMed for the terms *breast cancer* and *BRCA1*. The initial search returns 50 articles, as compared to 21 for *breast cancer* with *BRCA2*, 6 with *PIK3CA*, 1 with *TOX3*, and 0 for *MC4R* or *CLC1* associations. While the number of publications reflects many extraneous factors such as the popularity and "research age" of the protein, it is also very much reflective of the possibility of gene-disease association. Thus, BRCA1 and BRCA2 would be the most likely candidates for cancer association, followed by PIK3CA and TOX3. MC4R and CLC1 would not make the cut. Note that PubMed now defaults to a smart search engine, which identifies all aliases of the gene and the disease while cutting out more promiscuous matches; *i.e.* turning off the translation of terms would result in significantly more less accurate matches. Using specialized tools like PolySearch (or IHOP) to perform the same queries produces more refined and quantifiable results (Figure 4).

4. The Inputs and Outputs

Existing disease-gene prioritization methods vary based on the types of inputs that they use to produce their varied outputs. Functionality of prioritization methods is defined by previously known information about the disease and by candidate search space [124], which may be either submitted by the user or automatically selected by the tool. Disease information is generally limited to lists of known disease-associated genes, affected tissues and pathways and relevant keywords. The candidate search space does not have to be input at all (*i.e.* the entire genome) or be defined by the suspect (for varied experimental reasons) genomic region. The prioritization accuracy, in large part, depends on the accuracy and specificity of the inputs. Thus, providing a list of very broad keywords may reduce the performance specificity, while incorrect candidate search space automatically decreases sensitivity. Prioritization methods generally output ranked/ordered lists of genes, oftentimes associated with p-values, classifier scores, etc.

Overall, input and output requirements and formats are a very important part of establishing a tool's relevance for its users. As with other bioinformatics methods, the ease use and the steepness of learning curve for a given gene prioritization method often define the user base at least as strictly as does its performance.

Box 2. Illustrating basic functionality of a standard (on-line fully-interconnected feed-forward sigmoid-function back-propagating) neural network.

In Figure 5A example network there are three fully interconnected layers of neurons (input, hidden, and output layers); *i.e.* each neuron in one layer is connected to every neuron in the next layer. The three input neurons encode biologically relevant pieces of data relating a given gene G to a given disease D. For each G and D, *i_neuron1* is the fraction of articles (out of 1000) containing in-text co-occurrences of G and D and *i_neuron2* represents the presence/absence of a sequence-similar gene G′ associated with D (*i_neuron3* = G/G′ sequence identity). The hidden (inference) layer consists of two neurons *h_neuron1* and *h_neuron2* with activation thresholds θ_1 and θ_2, respectively. The single output, *o_neuron* (threshold θ_O) represents the involvement of G in causing D: 0 = no involvement, 1 = direct causation. The starting weights of the network ($w_{i1\text{-}h1}$, $w_{i1\text{-}h2}$…$w_{h2\text{-}o}$) are arbitrarily assigned random values between 0 and 1. Intuitively, the function of the network is to convert input neuron values into output neuron values via a network of weights and hidden neurons. Mathematically, the network is described as follows:

The value (d_x) of neuron x is the sum of inputs into x from the previous layer of neurons ($Y_{i=1\to n}$ in general; in our example: $I_{1\to 3}$, $H_{1\to 2}$). Each of the n inputs is a product of value of neuron Y_i and weight of connection between Y_i and x ($w_{Yi\to x}$).

$$d_x = \sum_{i=1}^{n} Y_i w_{Y_{i\to x}}$$

The value of the output (z_x) of a neuron x based on its d_x and its threshold θ_x is:

$$z_x = f(d_x + \theta_x)$$

In our case, the function (f) is a sigmoid, where a is a real number constant (optimized for any given network, but generally initially chosen to be between 0.5 and 2).

$$f(x) = \frac{1}{1+e^{-ax}}$$

Thus, to compute the output of every neuron in the network we need to use the formula:

$$z_x = \frac{1}{1+e^{-a(d_x+\theta_x)}}$$

Note, that to compute the output of the *o_neuron* (z_O; the prediction made by the network) we first have to compute the outputs of all *h_neurons* ($z_{Hi=1\to n}$).

In a supervised learning paradigm, experimentally established pairs of inputs and outputs are given to the network during training (Figure 5C). After each input, the network output (z_O) is compared to the observed result (R). If the network makes a classification error its weights are adjusted to reflect that error. Establishing the best way to update weights and thresholds in response to error is of the major challenges of neural networks. Many techniques use some form of the delta rule – a gradient descent-based optimization algorithm that makes changes to function variables proportionate to the negative of the approximate gradient of the function at the given point. [It's OK if you didn't understand that sentence – the basic idea is to change the weights and thresholds in the direction opposite of the direction of the error]. In our example, we use the delta rule with back-propagation. This means that to compute the error of the hidden layer, the threshold of the output layer (θ_O) and the weights connecting the hidden layer to the output layer ($w_{h1\to O}$, $w_{h2\to O}$) need to be changed first.

The steps are as follows:

1. Compute the error (e_O) of z_O as compared to result R. Note, that the difference between the expected and the observed values defines the gradient (g) at the output neuron.

$$e_O = z_O(1-z_O)(R-z_O)$$

2. Compute the change in the threshold of the output layer ($\Delta\theta_O$), using a variable λ, the learning rate constant - a real number, often initialized to 0.1–0.2 and optimized for each network)

$$\Delta\theta_O = \lambda e_O$$

3. Compute the change in the weights connecting the hidden layer to the output, $w_{Hi \rightarrow O}$.

$$\Delta W_{H_i \rightarrow O} = \Delta \theta_O H_i$$

4. Compute the gradient (g_i) at hidden neurons

$$g_i = e_O w_{H_i \rightarrow O}$$

Note, from here all steps are the same as above

5. Compute the error at z_{Hi}

$$e_{H_i} = z_{H_i}(1 - z_{H_i}) g_i$$

6. Compute the change in θ_{Hi}

$$\Delta \theta_{H_i} = \lambda e_{H_i}$$

7. Compute the change in $w_{Ij \rightarrow Hi}$

$$\Delta W_{I_j \rightarrow H_i} = \Delta \theta_{H_i} I_j$$

In on-line updating mode of our example, weights and thresholds are altered after each set of input transmissions. Once the network has "seen" the full set of input/output pairs (one epoch/iteration), training continues re-using the same set until the performance is satisfactory. Note that neural networks are sensitive to dataset imbalance. *I.e.* it is preferable to "balance" the training data, such that the number of instances of each class is presented a roughly equal number of times.

In testing, updating of the weights no longer takes place; *i.e.* the z_O for any given set of inputs is constant over time. See Exercise 8 for an experience with testing. Note, there are many variations on the type and parameters of network learning (propagation mode and direction, weight update rules, thresholds for stopping, etc.) Please consult the necessary literature for more information, *e.g.* [134].

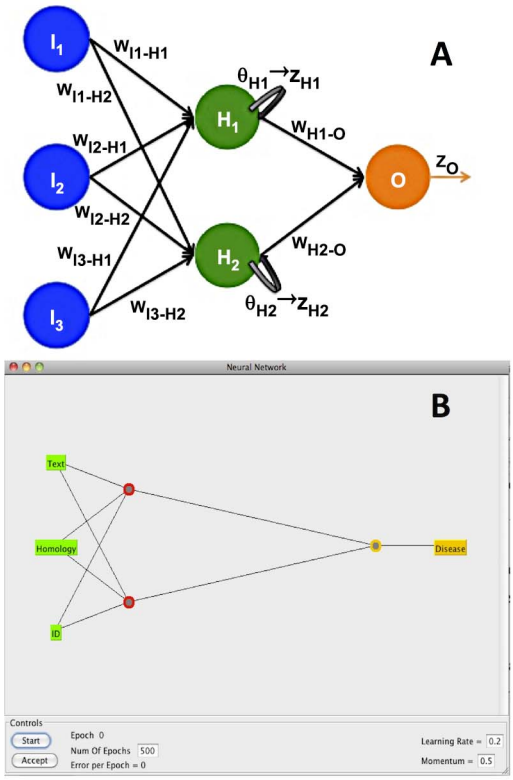
5. The Processing

Gene prioritization methods use different algorithms to make sense of all the data they extract, including mathematical/statistical models/methods (*e.g.* GeneProspector [125]), fuzzy logic (*e.g.* ToppGene [126,127]), and artificial learning devices (*e.g.* PROSPECTR [54]), among others. Some methods use combinations of the above. Objectively, there is no one methodology that is better than the others for all data inputs. For more details on computational methods used in the various approaches please refer to relevant tool publications and method-specific computer science/mathematics literature, *e.g.* [128,129,130,131,132,133,134].

To illustrate the general concepts of relying on the various computational techniques for gene prioritization we will consider the use of an artificial neural network (ANN). Keep in mind that while methods and their requirements differ, the notion of identifying patterns in the data that may be indicative disease-gene involvement remains the same throughout. In simplest terms, a neural network is essentially a mathematical model that defines a function $f\colon X \rightarrow Y$, where a distribution over X (the inputs to the network) is mapped to a distribution over Y (the outputs/classifications). The word "network" in the name "artificial neural network" refers to the set of connections between the "neurons" (Figure 5). The functionality of the network is defined by the transmission of signal from activated neurons in one layer to the neurons in another layer via established (and weighed) connections. Besides the choice and number of inputs and outputs, the parameters defining a given ANN are (1) interconnection patterns, (2) the process by which the weights of connections are selected/updated (learning function), and (3) the activation thresholds (functions) of any one given neuron. "Training" a network means optimizing these parameters using an existing set of inputs (and, possibly, outputs). Ultimately, a trained network could then relatively accurately recognize learned patterns in previously unseen data. For more details regarding the possible types and parameters of neural networks see [132,134]. For an illustration of network application see Box 2 and Figure 5.

6. Summary

The development of high throughput technologies has augmented our abilities to identify genetic deficiencies and inconsistencies that lead to the development of diseases. However, a large portion of information in the heaps of data that these methods produce is incomprehensible to the naked eye. Moreover, inferences that could potentially be made from combining



C

Text	Homology	ID	Disease	PREDICTION Actual	Binary
0.01	0	0	0	-0.015	0
0.01	1	0.3	0	-0.048	0
0.07	0	0	0	-0.016	0
0.02	1	0.1	0	-0.037	0
0.03	1	0.15	0	-0.043	0
0.01	1	0.09	0	-0.035	0
0.01	1	0.9	0	0.003	0
0.08	1	0.7	0	-0.045	0
0.12	1	0.95	1	0.971	1
0.4	0	0	0	-0.057	0
0.5	0	0	0	-0.056	0
0.6	1	0.09	1	1.006	1
0.5	1	0.09	1	0.969	1
0.45	1	0.21	1	0.985	1
0.63	1	0.22	1	0.98	1
0.43	1	0.9	1	0.996	1
0.53	1	0.76	1	0.967	1
0.45	1	0.97	1	0.977	1

D

TEST

0.01	1	0.55	1	-0.073	0
0.07	0	0	1	-0.016	0
0.55	1	0.95	1	0.951	1
0.1	1	0.3	0	-0.091	0
0.2	1	0.3	1	-0.148	0
0.01	0	0	0	-0.015	0
0.02	1	0.45	0	-0.067	0

Figure 5. Predicting gene-disease involvement using artificial neural networks (ANNs). In a supervised learning paradigm, the neural networks are trained using experimental data correlating inputs (descriptive features relating genes to diseases) to outputs (likelihood of gene-disease involvement). The training and testing procedures for the generalized network (Panel A) are described in text. In our example, the WEKA [129,130,131,139] ANN (Panel B; a = 0.5, λ = 0.2) is trained using the training set (Panel C) repeated 500 times (epochs). The network "memorizes" (Predictions in Panel C) the patterns in the training set and is capable of making accurate predictions for four out of seven instances it has not seen before (test set, Panel D). It is important to note here that the erroneously assigned instances (yellow highlight) in the test set are, for the most part, unlike the training. The first one has very little literature correlation (0.01), while sequence similarity to another disease-involved gene is fairly high 0.55). The second maps an unlikely candidate gene (very low literature, no homology) to disease, and the third has barely enough literature mapping and borderline homology. Representation of neither of these instances was consistently present in the training set. This example highlights the importance of training using a representative training set, while testing on a set that is not equivalent to training.
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different studies and existing research results are beyond reach for anyone of human (not cyborg) descent. Gene prioritization methods (Table 1) have been developed to make sense of this data by extracting and combining the various pieces necessary to link genes to diseases. These methods rely on experimental work such as disease gene linkage analysis and genome wide studies to establish the search space of candidate genes that may possibly be involved in generating the observed phenotype. Further, they utilize mathematical and computational models of disease to filter the original set of genes based on gene and protein sequence, structure, function, interaction, expression, and tissue and cellular localization information. Data repositories that contain the necessary information are diverse in both content and format and require deep knowledge of the stored information to be properly interpreted. Moreover, the models utilizing the various sources assign different weights to the information they extract based on perceived quality and importance of each piece of data available in the context of the entire set of descriptors – a function unlikely to be reproduced in manual data interpretation. Thus, computational gene prioritization techniques serve as interpreters of both of newly retrieved data and of information contained in previous studies. They also are the bridge that connects seemingly unrelated inferences creating an easily comprehensible outlook on an important problem of disease gene annotation.

7. Exercises

1. Search the GAD (http://geneticassociationdb.nih.gov/) database for all genes reported to be associated with diabetes. Refine this set to find only the positively associated genes. How many are there? Why was the total data set reduced? Count the number of unique diabetes associated genes or explain why this is not feasible. How many SNPs associate these genes with diabetes? Is it realistically possible to experimentally evaluate individual effects of each SNP in this set?
2. Using STRING (http://string-db.org/), find ***all*** genes (hint: use limit of 50) interacting with insulin (confidence >0.99). *Note, this confidence limit is extremely high – computational techniques would normally deal with lower limits and thus larger data sets.* What is the insulin gene name used by STRING? How many interaction partners does your query return? Switch to STRING evidence view. Pick three genes connected to insulin via text mining, but without "insulin" in their full name, and find one reference for each in PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) suggesting that these genes are involved with diabetes. Report Gene IDs (*e.g.* MC4R), PubMed IDs and publication citations. Use PolySearch

Table 1. The available data sources and gene prioritization tools.

Data Type	Data Content	Possible Sources	Tools
Experiment, observation	Linkage, association, pedigree, relevant texts and other data	User provided	CAESAR [140], CANDID [141], ENDEAVOR [122], G2D [15,16,17], Gentrepid [142], GeneDistiller [121], PGMapper [143], PRINCE [144], Prioritizer [145], SUSPECTS [146], ToppGene [126,127]
Sequence, structure, meta-data	Sequence conservation, exon number, coding region length, known structural domains and sequence motifs, chromosomal location, protein localization, and other gene-centered information and predictions	SCOP [147], PFam [148,149], ProSite [150], UniProt, Entrez Gene [151], ENSEMBL [152], InterPro [153], LocDB [154], GeneCards [155], PredictProtein [156]	CAESAR, CANDID, ENDEAVOR, G2D, Gentrepid, GeneDistiller, GeneProspector [125], MedSim [157], MimMiner [158], PGMapper, PhenoPred [159], Prioritizer, PROSPECTR [54], SNPs3D [106], SUSPECTS, ToppGene
Pathway, protein-protein interaction, genetic linkage, expression	Disease-gene associations, pathways and gene-gene/protein-protein interactions/ interaction predictions, and gene expression data	KEGG [160,161], STRING, Reactome [162,163], DIP [164], BioGRID [165], GEO [166,167], ArrayExpress [168], ReLiance [169]	CAESAR, CANDID, DiseaseNet [170], ENDEAVOR, G2D, Gentrepid, GeneDistiller, GeneWanderer [20], MaxLink [171], MedSim, PGMapper, PhenoPred, PRINCE, Prioritizer, SNPs3D, SUSPECTS, ToppGene
Non-human data	Information about related genes and phenotypes in other species	OrthoDisease [172], OrthoMCL [173], MGD [174], Pathbase [175]	CAESAR, CANDID, ENDEAVOR, GeneDistiller, GeneProspector, GeneWanderer, MedSim, Prioritizer, PROSPECTR, SNPs3D, SUSPECTS, ToppGene
Ontologies	Gene, disease, phenotype, and anatomic ontologies	GO, DO [176], MPO [177,178], HPO [179], eVOC [180]	CAESAR, ENDEAVOR, G2D, GeneDistiller, MedSim, PhenoPred, Prioritizer, SNPs3D, ToppGene
Mutation associations and effects	Information about existing mutations, their functional and structural effects and their association with diseases, predictions of functional or structural effects for the mutations in the gene in question	dbSNP, PMD [111], GAD, DMDM, SNAP, PolyDoms, SNPdbe, SNPselector, RAVEN, SNPeffect, PHD-SNP, Mutation@A Glance, PromoLign, SIFT, PolyPhen, PupaSNP finder, FASTSNP	CAESAR, CANDID, GeneProspector, GeneWanderer, PROSPECTR, SNPs3D, SUSPECTS
Literature	Mixed information of all types extracted from literature references (*e.g.* disease-gene correlation and non-ontology based gene-function assignment)	PubMed, PubMed Central, HGMD [181], GeneRIF, OMIM	CAESAR, CANDID, DiseaseNet, ENDEAVOR, G2D, Gentrepid, GeneDistiller, GeneProspector, GeneWanderer, MedSim, MimMiner, PGMapper, PolySearch [123], PRINCE, Prioritizer, PROSPECTR, SNPs3D, SUSPECTS, ToppGene

There is a wide range of data sources that can be used to infer the above-described pieces of evidence. The existing tools try to take advantage of many (if not all) of them. This table summarizes the collections and methodologies that make current state of the art in gene prioritization possible. Note, not all resources mentioned here are utilized by all gene prioritization tools nor are all data sources available listed. Moreover, some resources may be classified as more than one data-type. Many of the resources reported here are available electronically through the gene prioritization portal [124].

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(http://wishart.biology.ualberta.ca/polysearch) **gene** to **disease** mapping with your gene IDs to do the same. Does your experience confirm that the functional ''molecular interaction'' evidence works? Why?

3. In AmiGO (GO term browser, http://www.geneontology.org), find the human insulin record (hint: use the insulin ID obtained above). What is the Swiss-Prot ID for insulin? Go to the term view. How many GO term associations does insulin have? Reduce the view to ''molecular function'' terms. How many terms are left? Create a tree view of these terms (hint: use the ''Perform an action'' dropdown). Which of the terms is the most exact in defining the likely molecular function of insulin (lowest term in a tree hierarchy)? Display gene products in ''GO:0005158: insulin receptor binding'', reduce the set to human proteins, and look at the inferred tree. How many gene products are in this term? Pick a set of three gene products (report IDs) and use them to search PolySearch for diabetes associations. In question 3 we used the ''common pathway'' evidence to show the relationship of genes to diabetes. What type of predictive evidence is used here?
4. Search the Mammalian Phenotype Ontology for keyword ''diabetes'' and select increased susceptibility (MPO, http://www.informatics.jax.org/searches/MP_form.shtml). How many genotypes are returned? Display the genotypes and click on the $Aire^{tm1Mand}/Aire^{+}$ genotype for further exploration. What is the affected gene? Click on gene title (Gene link in Nomenclature section) to display further information. What is an orthologue? What is the human orthologue of your mouse gene? Look up this gene in OMIM (http://www.ncbi.nlm.nih.gov/omim) for association with diabetes. Copy/paste the *citation* from OMIM, describing the gene relationship to diabetes in humans. Do your

results confirm the "cross-species" evidence?

5. Search GeneCards (http://www.genecards.org, utilize advanced search) for genes expressing in the pancreas (hint: pancreatic tissue is often affected in diabetes). How many are there? Explore the GeneCard for CCKBR for diabetes association. Do you find that this gene confirms the "disease compartment" evidence? What database, referenced in GeneCards, contains the CCKBR-diabetes association? Now look at the GeneCard of PLEKHG4. Is there evidence for this gene being associated with diabetes (whether in the GeneCards record or otherwise)? Explain your ideas in detail, paying special attention to the "disease compartment" line of evidence.
6. Search UniProt (http://www.uniprot.org) for all reviewed [reviewed:yes] human [organism:"Homo sapiens [9606]"] protein entries that contain natural variants with reference to diabetes [annotation:(type:natural_variations diabetes)]. Use advanced search with specific limits (*i.e.* sequence annotation, natural_variations, term diabetes). How many proteins fit this description? Locate the entry for insulin (identifier from question 3) and find the total number of known coding variants of this sequence. How many are annotated as associated with any form of diabetes? (hint: read the general annotation section for correspondence of abbreviations to types of diabetes). Run SNAP (http://www.rostlab.org/services/snap/) to predict functional effects of all variants. (hint: use comma separated batch submit). How many are predicted to be functionally non-neutral? Do SNAP predictions of functional effect correlate with annotated disease associations? Does this result confirm the "mutant implication" for nsSNPs?
7. Search PolySearch for all genes associated with diabetes. How many results are returned? Look at the PubMed articles that associate "hemoglobin" with diabetes (follow the link from PolySearch). How many are there? Do you find this number large enough to convince you of hemoglobin-diabetes association and why? From reading article titles/extracted sentences, can you identify a biological reason for connecting hemoglobin to diabetes? If one looks especially convincing, cite that article (hint: its OK to not find one). For the first three articles, can you identify a biological reason for connecting hemoglobin to diabetes? Go back to the list of diabetes related genes and look at TCF7L2 articles. Are the biological reasons for matching TCF7L2 to diabetes clearly defined? Cite the most convincing article. Why do you think TCF7L2 is ranked lower in association than hemoglobin? Is there significant evidence for calcium channel (CACNA1E) involvement in diabetes? Consider the PubMed citations. Do you agree with PolySearch classification of this gene-disease association? Does your experience with PolySearch confirm the "text evidence" function of gene prioritization methods?
8. WEKA exercises (choose one).

   Download and install WEKA (http://www.cs.waikato.ac.nz/~ml/weka/). Using a text-editor (or Microsoft Excel) create comma delimited values (CSV) files identical to the ones described in Figure 5C–D (*i.e.* copy over the training and testing files and replace spaces with commas). Save the files and open the training file in WEKA's Explorer GUI. Open the training file in WEKA's Explorer GUI. You should have four columns of data (Text, Homology, ID, Disease) corresponding to four attributes of each data instance.

   8.1. Defined Questions: Run the MultiLayer Perceptron with parameters (momentum = 0.5, learning = 0.2, trained using the training set, Figure 5C, repeated 500 times/epochs). Test with the test set (Figure 5D) and output predictions for each test entry (make a screenshot). Assuming that everything predicted below 0 is 0, and everything above is 1. What is your performance (number of true/false positives/negatives, positive/negative accuracy/coverage, overall accuracy)? Try using the Decision Stump classifier with default parameters (take screenshot of output). If everything below 0.5 is 0, and everything above is 1, what is your performance? Is it better or worse than the neural net?

   8.2. Open ended: Experiment with different tools available from WEKA's Classify section setting the testing set to your test-file's location. First, run the MultiLayer Perceptron with parameters as described in Figure 5, then try to alter the parameters (momentum term, learning rate, and number of epochs). Try using Linear Regression, Decision Table, or Decision Stump classifiers with default parameters. Is your performance on the test set better or worse? Close the WEKA Explorer, reformat your train/test files in the text editor to replace Disease column values by Booleans (True/False) values, and re-open the training file. Use BayesianNet and RandomForest classifiers to test on the testing file. Does you performance improve? Note, that without further understanding of each of the tools, it is nearly impossible to determine which method is applicable to your data.

Answers to the Exercises can be found in Text S1.

Supporting Information

Text S1 Answers to Exercises.
(DOCX)

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Glossary

- **Annotation** – any additional information about a genetic sequence. Annotation types are extremely varied, including functional, structural, regulatory, location-related, organism-specific, experimentally derived, predicted, etc.
- **CNV**, copy number variation – an alteration of the genome, which results in an individual having a non-standard number of copies of one or more DNA sections.
- **Gene prioritization** – the process of arranging possible disease causing genes in order of their likelihood in disease involvement.
- **GWAS**, genome wide association studies – the examination of all genes in the genome to correlate their variation to phenotypic trait variation across individuals in a given population.
- **Genetic linkage** – tendency of certain genetic regions on the same chromosome to be inherited together more often than expected due to limited recombination between them.
- **Genetic marker** – a DNA sequence variant with a known location that can be used to identify specific subsets of individuals (cells, species, individual organisms, etc.).
- **Homologue** – a gene derived from a common ancestor with the reference gene. Generally, gene A is a homologue of gene B if both are derived from a common ancestor.
- **Linkage disequilibrium** – tendency of certain genetic regions (not necessarily on the same chromosome) to be inherited together more often that expected from considering their population frequencies. In reference to gene prioritization, this phenomenon may complicate establishment of causal genes due to their consistent inheritance in complex with non-causal genetic regions.
- **Orthologues** – homologous genes separated by a speciation event. Generally, gene A is an orthologue of gene B if A and B are homologous, but reside in different species. Orthologues often perform the same general function in different organisms.
- **Paralogues** – homologous genes separated by a duplication event (often followed by copy differentiation). Generally, gene A is a paralogue of gene B if A and B are homologous and reside in the same species. A and B can be functionally identical or, on contraire, very different, but are often only slightly dissimilar.
- **Pleiotropy** – the influence of a single gene on a number of phenotypic traits.

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Chapter 16: Text Mining for Translational Bioinformatics

K. Bretonnel Cohen*, Lawrence E. Hunter

Computational Bioscience Program, University of Colorado School of Medicine, Aurora, Colorado, United States of America

Abstract: Text mining for translational bioinformatics is a new field with tremendous research potential. It is a subfield of biomedical natural language processing that concerns itself directly with the problem of relating basic biomedical research to clinical practice, and vice versa. Applications of text mining fall both into the category of T1 translational research—translating basic science results into new interventions—and T2 translational research, or translational research for public health. Potential use cases include better phenotyping of research subjects, and pharmacogenomic research. A variety of methods for evaluating text mining applications exist, including corpora, structured test suites, and post hoc judging. Two basic principles of linguistic structure are relevant for building text mining applications. One is that linguistic structure consists of multiple levels. The other is that every level of linguistic structure is characterized by ambiguity. There are two basic approaches to text mining: rule-based, also known as knowledge-based; and machine-learning-based, also known as statistical. Many systems are hybrids of the two approaches. Shared tasks have had a strong effect on the direction of the field. Like all translational bioinformatics software, text mining software for translational bioinformatics can be considered health-critical and should be subject to the strictest standards of quality assurance and software testing.

This article is part of the "Translational Bioinformatics" collection for *PLOS Computational Biology*.

1. Introduction

Text mining for translational bioinformatics is a new field with enormous research potential. It is a subfield of biomedical natural language processing (BioNLP) that concerns itself directly with the problem of relating basic biomedical research to clinical practice, and vice versa.

1.1 Use Cases

The foundational question in text mining for translational bioinformatics is what the use cases are. It is not immediately obvious how the questions that text mining for translational bioinformatics should try to answer are different from the questions that are approached in BioNLP in general. The answer lies at least in part in the nature of the specific kinds of information that text mining should try to gather, and in the uses to which that information is intended to be put. However, these probably only scratch the surface of the domain of text mining for translational bioinformatics, and the latter has yet to be clearly defined.

One step in the direction of a definition for use cases for text mining for translational bioinformatics is to determine classes of information found in clinical text that would be useful for basic biological scientists, and classes of information found in the basic science literature that would be of use to clinicians. This in itself would be a step away from the usual task definitions of BioNLP, which tend to focus either on finding biological information for biologists, or on finding clinical information for clinicians. However, it is likely that there is no single set of data that would fit the needs of biological scientists on the one hand or clinicians on the other, and that information needs will have to be defined on a bespoke basis for any given translational bioinformatics task.

One potential application is better phenotyping. Experimental experience indicates that strict phenotyping of patients improves the ability to find disease genes. When phenotyping is too broad, the genetic association may be obscured by variability in the patient population. An example of the advantage of strict phenotyping comes from the work of [1,2]. They worked with patients with diagnoses of pulmonary fibrosis. However, having a diagnosis of pulmonary fibrosis in the medical record was not, in itself, a strict enough definition of the phenotype for their work [1]. They defined strict criteria for study inclusion and ensured that patients met the criteria through a number of methods, including manual review of the medical record. With their sharpened definition of the phenotype, they were able to identify 102 genes that were up-regulated and 89 genes that were down-regulated in the study group. This included Plunc (palate, lung and nasal epithelium associated), a gene not previously associated with pulmonary fibrosis. Automation of the step of manually reviewing medical records would potentially allow for the inclusion or exclusion of much larger populations of patients in similar studies.

Another use for text mining in translational bioinformatics is aiding in the preparation of Cochrane reviews and other meta-analyses of experimental studies. Again, text mining could be used to identify cohorts that should be included in the meta-analysis, as well as to determine P-values and other indicators of significance levels.

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* E-mail: kevin.cohen@gmail.com

What to Learn in This Chapter

Text mining is an established field, but its application to translational bioinformatics is quite new and it presents myriad research opportunities. It is made difficult by the fact that natural (human) language, unlike computer language, is characterized at all levels by rampant ambiguity and variability. Important sub-tasks include gene name recognition, or finding mentions of gene names in text; gene normalization, or mapping mentions of genes in text to standard database identifiers; phenotype recognition, or finding mentions of phenotypes in text; and phenotype normalization, or mapping mentions of phenotypes to concepts in ontologies. Text mining for translational bioinformatics can necessitate dealing with two widely varying genres of text—published journal articles, and prose fields in electronic medical records. Research into the latter has been impeded for years by lack of public availability of data sets, but this has very recently changed and the field is poised for rapid advances. Like all translational bioinformatics software, text mining software for translational bioinformatics can be considered health-critical and should be subject to the strictest standards of quality assurance and software testing.

Most of the applications discussed here fall into the category of *T1 translational research*—translating basic science results into new interventions (http://grants.nih.gov/grants/guide/notice-files/NOT-AG-08-003.html). There are also applications in translational research for public health, also known as *T2 translational research* (op. cit.). This is true both in the case of mining information for public health experts and for the general public. For public health experts, there is a growing body of work on various factors affecting disease monitoring in electronic medical records, such as work by Chapman and colleagues on biosurveillance and disease and syndrome outbreak detection (e.g., [3,4], among others). For the general public, simplifying technical texts can be helpful. [5] describes work in this area.

1.1.1 The pharmacogenomics perspective. One area of research that has made some steps towards defining a use case for text mining is pharmacogenomics. An example of this is the PharmGKB database. Essential elements of their definition of pharmacogenomics text mining include finding relationships between genotypes, phenotypes, and drugs. As in the case of other applications that we will examine in this chapter, mining this information requires as a first step the ability to find mentions of the semantic types of interest when they are mentioned in text. These will be of increasing utility if they can be mapped to concepts in a controlled vocabulary. Each semantic type presents unique challenges. For example, finding information about genotypes requires finding mentions of genes (see Section 4.3 below), finding mentions of mutations and alleles, and mapping these to each other; finding mentions of drugs, which is more difficult than it is often assumed to be [6]; and finding mentions of phenotypes. The latter is especially difficult, since so many things can fit within the definition of "phenotype." A phenotype is the entirety of observable characteristics of an organism [7]. The wide range and rapidly changing technologies for measuring observable features of patient phenotypes require the text mining user to be very specific about what observables they want to capture. For example, phenotypes can include any behavior, ranging from duration of mating dances in flies to alcohol-seeking in humans. They can also include any measurable physical characteristic, ranging from very "macro" characteristics such as hair color to very granular ones such as specific values for any of the myriad laboratory assays used in modern clinical medicine.

There is some evidence from the PharmGKB and the Comparative Toxicogenomics Database experiences that text mining can scale up processing in terms of the number of diseases studied and the number of gene-disease, drug-disease, and drug-gene associations discovered [8]. Furthermore, experiments with the PharmGKB database suggest that pharmacogenomics is currently more powerful than genomics for finding such associations and has reached the point of being ready for translation of research results to clinical practice [9].

1.1.2 The i2b2 perspective. *Informatics for Integrating Biology and the Bedside* (i2b2) is a National Center for Biomedical Computing devoted to translational bioinformatics. It has included text mining within its scope of research areas. Towards this end, it has sponsored a number of shared tasks (see Section 5 below) on the subject of text mining. These give us some insight into i2b2's definition of use cases for text mining for translational bioinformatics. i2b2's focus has been on extracting information from free text in clinical records. Towards this end, i2b2 has sponsored shared tasks on deidentification of clinical documents, determining smoking status, detecting obesity and its comorbidities, medical problems, treatments, and tests. Note that there are no genomic components to this data.

1.2 Text Mining, Natural Language Processing, and Computational Linguistics

Text mining, natural language processing, and computational linguistics are often used more or less interchangeably, and indeed one can find papers on text mining and natural language processing at the annual meeting of the Association for Computational Linguistics, and papers from any of these categories at meetings for any of the other categories. However, technically speaking, some differences exist between them. Computational linguistics strictly defined deals with building computationally testable models of human linguistic behavior. Natural language processing has to do with building a wide range of applications that take natural language as their input. Text mining is more narrow than natural language processing, and deals with the construction of applications that provide a solution to a specific information need. For example, a syntactic analyzer would be an example of a natural language processing application; a text mining application might use that syntactic analyzer as part of the process for filling the very specific information need of finding information about protein-protein interactions. This chapter will include information about both natural language processing and text mining [10–12].

1.3 Evaluation Techniques and Evaluation Metrics in Text Mining

A variety of methods for evaluating text mining applications exist. They typically apply the same small family of metrics as figures of merit.

1.3.1 Corpora. One paradigm of evaluation in text mining is based on the assumption that all evaluation should take place on naturally occurring texts. These texts are annotated with data or metadata about what constitutes the right answers for some task. For example, if the intended application to be tested is designed to locate mentions of gene names in free text, then the occurrence of every gene name in the text would be marked. The mark-up is known as *annotation*. (Note that this is a very different use of the word "annotation" from its use in the model organism database construction community.) The resulting set of

annotated documents is known as a *corpus* (plural *corpora*). Given a corpus, an application is judged by its ability to replicate the set of annotations in the corpus. Some types of corpora are best built by linguists, e.g., those involving syntactic analysis, but there is abundant evidence that biomedical scientists can build good corpora if they follow best practices in corpus design (see e.g., [13]).

1.3.2 Structured test suites. Structured test suites are built on the principles of software testing. They contain groups of inputs that are classified according to aspects of the input. For example, a test suite for applications that recognize gene names might contain sentences with gene names that end with numbers, that do not end with numbers, that consist of common English words, or that are identical to the names of diseases. Unlike a standard corpus, test suites may contain data that is manufactured for the purposes of the test suite. For example, a test suite for recognizing Gene Ontology terms [14] contains the term *cell migration*, but also the manufactured variant *migration of cells*. (Note that being manufactured does not imply being unrealistic.) Structured test suites have the major advantage of making it much more straightforward to evaluate both the strengths and the weaknesses of an application. For example, application of a structured test suite to an application for recognizing Gene Ontology terms made it clear that the application was incapable of recognizing terms that contain the word *in*. This was immediately obvious because the test suite contained sets of terms that contain function words, including a set of terms that all contain the word *in*. To duplicate this insight with a corpus would require assembling all errors, then hoping that the fact that no terms containing the word *in* were recognized jumped out at the analyst. In general, structured test suites should not be reflective of performance as measured by the standard metrics using a corpus, since the distribution of types of inputs in the test suite does not reflect the distribution of those types of inputs in naturally occurring data. However, it has been shown that structured test suites can be used to predict values of metrics for specific equivalence classes of data (inputs that should all be expected to test the same condition and produce the same result) [15]. We return to the use of test suites in Section 6.

1.3.3 Post hoc judging. Sometimes preparation of corpora is impractical. For example, there may be too many inputs that need to be annotated. In these cases, post hoc judging is sometimes applied. That is, a program produces outputs, and then a human judges whether or not they are correct. This is especially commonly used when a large number of systems are being evaluated. In this case, the outputs of the systems can be pooled, and the most common outputs (i.e., the ones produced by the most systems) are selected for judging.

1.3.4 Metrics. A small family of related metrics is usually used to evaluate text mining systems. *Accuracy*, or the number of correct answers divided by the total number of answers, is rarely used.

Precision. Precision is defined as the number of correct system outputs ("true positives," or TP) divided by the total number of system outputs (the count of TP plus the "false positives" (FP) —erroneous system outputs). It is often compared loosely to specificity, but is actually more analogous to positive predictive value.

$$\text{Precision} = \frac{TP}{TP+FP}$$

Recall. Recall is defined as the number of true positives divided by the total number of potential system outputs, i.e. true positives plus "false negatives" (FN) —things that should have been output by the system, but were not. This will differ from task type to task type. For example, in information retrieval (Section 4.1), it is the number of documents judged relevant divided by the total number of actual relevant documents. In named entity recognition of genes (Section 4.3), it is defined as the total number of correct gene names output by the system divided by the total number of gene names in the corpus.

$$\text{Recall} = \frac{TP}{TP+FN}$$

Balanced F-measure. The balanced F-measure attempts to reduce precision and recall to a single measure. It is calculated as the harmonic mean of precision and recall. It includes a parameter β that is usually set to one, giving precision and recall equal weight. Setting β greater than one weights precision more heavily. Setting β less than one weights recall more heavily.

$$\text{F} = \frac{(\beta^2+1)PR}{\beta^2 P+R}$$

2. Linguistic Fundamentals

Building applications for text mining for translational bioinformatics is made easier by some understanding of the nature of linguistic structure. Two basic principles are relevant. One is that linguistic structure consists of multiple layers. The other is that every layer of linguistic structure is characterized by ambiguity.

All linguistic analyses in text mining are *descriptive* in nature. That is, they seek only to describe the nature of human linguistic productions, much as one might attempt to describe the multi-dimensional structure of a protein. Linguistic analyses are *not* prescriptive—that is, they do not attempt to prescribe or enforce standards for language use.

2.1 Layers of Linguistic Structure

The layers of linguistic structure vary somewhat between written and spoken language (although many are shared). We focus here on the layers that are relevant to written language, focusing particularly on scientific journal articles and on clinical documents.

2.1.1 Document structure. The first layer of the structure of written documents that is relevant to text mining for translational bioinformatics is the structure of individual documents. In the case of journal articles, this consists first of all of the division of the document into discrete sections, typically in what is known as the IMRD model—an abstract, introduction, methods section, results section, discussion, and bibliography. Acknowledgments may be present, as well.

The ability to segment a document into these sections is important because different sections often require different processing techniques and because different sections should be focused on for different types of information. For example, methods sections are frequent sources of false positives for various semantic classes, which led researchers to ignore them in much early research. However, they are also fruitful sections for finding information about experimental methods, and as it has become clear that mining information about experimental methods is important to biologists [16], it has become clear that methods must be developed for dealing with methods sections. Abstracts have been shown to have different structural and content characteristics from article bodies [17]; most research to date has focused on abstracts, and it is clear that new approaches will be required to fully exploit the information in article bodies.

Segmenting and labeling document sections can be simple when documents are provided in XML and a DTD is available. However, this is often not the

case; for instance, many documents are available for processing only in HTML format. In this situation, two topics exist: finding the boundaries of the sections, and labelling the sections. The latter is made more complicated by the fact that a surprising range of phrases are used to label the different sections of a scientific document. For example, the methods section may be called *Methods, Methods and Materials, Materials and Methods, Experimental Procedures, Patients and Methods, Study Design*, etc. Similar issues exist for structured abstracts; in the case of unstructured abstracts, it has been demonstrated that they can be segmented into sections using a generative technique [18].

Clinical documents present a far more complex set of challenges than even scientific journal articles. For one thing, there is a much wider range of clinical document types—admission notes, discharge summaries, radiology reports, pathology reports, office visit notes, etc. Hospitals frequently differ from each other in the types of documents that they use, as do individual physicians' practices. Furthermore, even within a given hospital, different physicians may structure the same document type differently. For example, just in the case of emergency room visit reports, one of the authors built a classification system that determined, for a given document, what specialty it would belong to (e.g., cardiology or pediatrics) if it had been generated by a specialist. He found that not only did each hospital require a different classification system, but different doctors within the same emergency room required different classifiers. [19] describes an iterative procedure for building a segmenter for a range of clinical document types.

Once the document has been segmented into sections, paragraphs must be identified. Here the segmentation task is typically easy, but ordering may present a problem. For example, it may not be clear where figure and table captions should be placed.

2.1.2 Sentences. Once the document has been segmented into paragraphs, the paragraphs must be further segmented into sentences. Sentence segmentation is a surprisingly difficult task. Even for newswire text, it is difficult enough to constitute a substantial homework problem. For biomedical text, it is considerably more difficult. Two main difficulties arise. One is the fact that the function of periods is ambiguous—that is, a period may serve more than one function in a written text, such as marking the end of an abbreviation (*Dr.*), marking the individual letters of an abbreviation (*p.r.n.*), indicating the rational parts of real numbers (*3.14*), and so on. A period may even serve two functions, as for example when *etc.* is at the end of a sentence, in which case the period marks both the end of the abbreviation and the end of the sentence. Furthermore, some of the expected cues to sentence boundaries are absent in biomedical text. For example, in texts about molecular biology, it is possible for a sentence to begin with a lower-case letter when a mutant form of a gene is being mentioned. Various approaches have been taken to the sentence segmentation task. The KeX/PROPER system [20] uses a rule-based approach. The LingPipe system provides a popular machine-learning-based approach through its LingPipe API. Its model is built on PubMed/MEDLINE documents and works well for journal articles, but it is not likely to work well for clinical text (although this has not been evaluated). In clinical documents, it is often difficult to define any notion of "sentence" at all.

2.1.3 Tokens. Written sentences are built up of tokens. Tokens include words, but also punctuation marks, in cases where those punctuation marks should be separated from words that they are attached to. The process of segmenting a sentence into tokens is known as *tokenization*. For example, consider the simple case of periods. When a period marks the end of a sentence, it should be separated from the word that it is attached to. *regulation.* will not be found in any biomedical dictionary, but *regulation* will. However, in many other instances, such as when it is part of an abbreviation or a number, it should not be separated. The case of hyphens is even more difficult. Hyphens may have several functions in biomedical text. If they indicate the absence of a symptom (e.g., *-fever*), they should probably be separated, since they have their own meaning, indicating the absence of the symptom. On the other hand, they should remain in place when separating parts of a word, such as *up-regulate*.

The status of tokenization in building pipelines of text mining applications is complicated. It may be the case that a component early in the pipeline requires tokenized text, while a component later in the pipeline requires untokenized text. Also, many applications have a built-in tokenizer, and conflicts between different tokenization strategies may cause conflicts in later analytical strategies.

2.1.4 Stems and lemmata. For some applications, it is advantageous to reduce words to stems or lemmata. Stems are normalized forms of words that reduce all inflected forms to the same string. They are not necessarily actual words themselves—for example, the stem of *city* and *cities* is *citi*, which is not a word in the English language. Their utility comes in applications that benefit from this kind of normalization without needing to know exactly which words are the roots—primarily machine-learning-based applications.

The term *lemma* (plural *lemmata*) is overloaded. It can mean the root word that represents a set of related words. For example, the lemma of the set {*phosphorylate, phosphorylates, phosphorylated, phosphorylating*} is *phosphorylate*. Note that in this case, we have an actual word. *Lemma* can also mean the set of words that can instantiate a particular root word form; on this meaning, the lemma of *phosphorylate* is {*phosphorylate, phosphorylates, phosphorylated, phosphorylating*}. Lemmas have a clear advantage of stems for some applications. However, while it is always possible to determine the stem of a word (typically using a rule-based approach, such as the Porter stemmer [21], it is not always possible to determine the lemma of a word automatically. The BioLemmatizer [22] is a recently released tool that shows high performance on the lemmatization task.

2.1.5 Part of speech. It is often useful to know the part of speech, technically known as *lexical category*, of the tokens in a sentence. However, the notion of part of speech is very different in linguistic analysis than in the elementary school conception, and text mining systems typically make use of about eighty parts of speech, rather than the eight or so that are taught in school. We go from eight to eighty primarily by subdividing parts of speech further than the traditional categories, but also by adding new ones, such as parts of speech of sentence-medial and sentence-final punctuation. Parts of speech are typically assigned to tokens by applications called *part of speech taggers*. Part of speech tagging is made difficult by the fact that many words are ambiguous as to their part of speech. For example, in medical text, the word *cold* can be an adjective or it can be a reference to a medical condition. A word can have several parts of speech, e.g., *still*. A variety of part of speech taggers that are specialized for biomedical text exist, including MedPOST [23], LingPipe, and the GENIA tagger [24].

2.1.6 Syntactic structure. The *syntactic structure* of a sentence is the way in which the phrases of the sentence relate to each other. For example, in the article title *Visualization of bacterial glycocalyx with a scanning electron microscope* (PMID 9520897), the phrase *with a scanning electron microscope* is associated with *visualization*, not with *bacterial glycocalyx*. Automatic syntactic analysis is made difficult by the existence of massive ambiguity. For example, while one possible interpretation of that title is that the visualization is done with a scanning electron microscope, another possible interpretation is that the bacterial glycocalyx has a scanning electron microscope. (Consider the analogous famous example *I saw the man with the binoculars*, where one possible interpretation is that I used the bionoculars to visualize the man, whereas another possible interpretation is that I saw a man and that man had some binoculars.) It is very easy for humans to determine which interpretation of the article title is correct. However, it is very difficult for computers to make this determination. There are *many* varieties of syntactic ambiguity, and it is likely that any nontrivial sentence contains at least one.

Syntactic analysis is known as *parsing*. The traditional approach to automated syntactic analysis attempts to discover the phrasal structure of a sentence, as described above. A new approach called *dependency parsing* focuses instead on relationships between individual words. It is thought to better reflect the semantics of a sentence, and is currently popular in BioNLP.

Along with determining the phrasal or dependency structure of a sentence, some parsers also make limited attempts to label the syntactic functions, such as *subject* and *object*, of parts of a sentence.

2.2 The Nature of Linguistic Rules

When we think of linguistic rules, we are most likely to think of the rules that we learn in school that impose arbitrary norms on language usage, such as *Say "you and I", not "you and me"*, or *a preposition is a bad thing with which to end a sentence*. These are known as *prescriptive rules*. Text mining never deals with prescriptive rules. Rather, it always deals with *descriptive rules*. Descriptive rules *describe* the parts of the language and the ways in which they can combine, without any implied judgement as to whether they are "good" or "bad." For example, a linguistic rule might specify that certain classes of verbs can be converted to nouns by adding *-tion* to their end, or that when a passive form of a verb is used, the subject can be omitted.

3. The Two Families of Approaches: Rule-Based and Learning-Based

There are two basic approaches to text mining: rule-based, also known as knowledge-based, and machine-learning-based, also known as statistical.

Rule-based approaches to text mining are based on the application of rules, typically manually constructed, to linguistic inputs. For example, a rule-based approach to syntactic analysis might postulate that given a string like *phosphorylation of MAPK by MAPKK*, the phrase that follows the word *by* is the doer of the phosphorylation, and the phrase that follows the word *of* is the undergoer of the phosphorylation. Or, a rule-based approach might specify that in the pattern *A X noun* the *X* is an adjective, while in the pattern *The adjective X verb* the X is a noun, allowing us to differentiate between the word *cold* as an adjective in the former case and as a medical condition in the latter case. Rule-based solutions can be constructed for all levels of linguistic analysis.

Machine-learning-based approaches to text mining are based on an initial step of feeding the system a set of data that is labelled with the correct answers, be they parts of speech for tokens or the locations of gene names in text. The job of the system is then to figure out cues that indicate which of the ambiguous analyses should be applied. For instance, a system for document classification may learn that if a document contains the word *murine*, then it is likely to be of interest to researchers who are interested in mice. Many different algorithms for machine learning exist, but the key to a successful system is the set of features that are used to perform the classification. For example, a part of speech tagger may use the apparent parts of speech of the two preceding words as a feature for deciding the part of speech of a third word.

It is often claimed that machine learning systems can be built more quickly than rule-based systems due to the time that it takes to build rules manually. However, building feature extractors is time-consuming, and building the labelled "training" data with the right answers is much more so. There is no empirical support for the claim that learning-based systems can be built more quickly than rule-based systems. Furthermore, it is frequently the case that putative learning-based systems actually apply rules in pre- or post-processing steps, making them hybrid systems.

4. Text Mining Tasks

In Section 2.1, we discussed elements of linguistic analysis. These analytical tasks are carried out in support of some higher-level text mining tasks. Many types of text mining tasks exist. We will discuss only the most common ones here, but a partial list includes:

- Information retrieval
- Document classification
- Named entity recognition
- Named entity normalization
- Relation or information extraction
- Question-answering
- Summarization

4.1 Information Retrieval

Information retrieval is the task of, given an information need and a set of documents, finding the documents that are relevant to filling that information need. PubMed/MEDLINE is an example of a biomedical information retrieval system for scientific journal articles; Google is an information retrieval system for web pages. Early information retrieval assumed that all documents were classified with some code and typically required the assistance of a librarian to determine the appropriate code of interest. *Keyword*-based retrieval, in which the user enters a set of words that a relevant text would be expected to contain and the content of the texts in the set of documents are searched for those words, was a revolution made possible by the introduction of computers and electronic forms of documents in the hospital or research environment. The naive approach to keyword-based retrieval simply checks for the presence or absence of the words in the query, known as boolean search. Modern approaches use relatively simple mathematical techniques to determine (a) the relative importance of words in the query in deciding whether or not a document is relevant—the assumption here is that not all words are equally important—and (b) how well a given word reflects the actual relevance of a given document to the query. For example, we can determine, given a count of how often the words *hypoperfusion* and *kidney* occur in the set of documents as a whole, that if we are looking for documents about kidney hypoperfusion, we should give more weight to the rarer of the two words; given a count of how often the words *kidney*

and *hypoperfusion* occur in two documents, we can determine which of the two documents is most relevant to the query.

4.2 Document Classification

Document classification is the task of classifying a document as a member of one or more categories. In a typical document classification workflow, one is supplied with a stream of documents, and each one requires classification. This differs from the information retrieval situation, in which information needs are typically ad hoc. For example, curators of a model organism database may require journal articles to be classified as to whether or not they are relevant for further examination. Other classification tasks motivated by curation have been classifying journal articles as to whether or not they are about embryogenesis. Document classification typically uses very simple feature sets, such as the presence or absence of the words from the training data. When this is the only feature, it is known as a "bag of words" representation. However, it has also been found useful to use more abstract, conceptual features. For example, [25] found the presence or absence of mentions of mouse strains to be a useful feature, regardless of the identity of the particular strain.

4.3 Named Entity Recognition

Named entity recognition is the task of finding mentions of specific semantic classes in a text. In general language processing, the most heavily studied semantic classes have been persons, places, and organizations—thus, the term "named entity." In genomic BioNLP, the most heavily studied semantic class has been gene and protein names. However, other semantic classes have been studied as well, including cell lines and cell types. In clinical NLP, the range of semantic classes is wider, encompassing a large number of types included in the Unified Medical Language System [26]. The UMLS includes a "Metathesaurus" which combines a large number of clinically and biologically relevant controlled vocabularies, comprising many semantic classes. In the clinical domain, there is an "industry standard" tool for named entity recognition, called MetaMap [27,28]. Biological named entity recognition remains a subject of current research. Machine learning methods predominate. Feature sets generally include typographical features of a token—e.g., having mixed-case letters or not, containing a hyphen or not, ending with a numeral or not, etc. —as well as features of the surrounding tokens.

Early results in named entity recognition were consistent with the hypothesis that this task could not be achieved by simply starting with a "dictionary" of gene names and looking for those gene names in text. At least three problems were immediately evident with this approach—the fact that new gene names are coined constantly, the fact that a number of gene names are homographs of common English words, and the fact that many genes have names or synonyms that are unhelpful, such as *putative oxidoreductase* (Entrez Gene ID 6393330). However, recent evidence has suggested that dictionary-based approaches can achieve moderate success if the dictionary and the data to be processed are subjected to extensive preprocessing [29] or post-hoc filtering, e.g., by the success or failure of a subsequent gene normalization step (see Section 4.4 [30]).

4.4 Named Entity Normalization

Named entity normalization is the process of taking a mention of a named entity in free text and returning a specific database identifier that it refers to. In the biological domain, this has been studied most extensively in the case of genes and proteins, and the corresponding task is known as *gene normalization*. In the clinical domain, it has been approached simultaneously with named entity recognition, again using the MetaMap application (see Section 4.3). There are two major problems in gene normalization. The first is that many species have genes with the same name. For example, the BRCA1 gene is found in an enormous number of animals. Thus, finding the appropriate gene identifier requires knowing the species under discussion, which is a research problem in itself. The other problem is that a single species may have multiple genes with the same name. For example, humans have five genes named *TRP-1*. Gene normalization is often approached as a problem in *word sense disambiguation*, the task of deciding which dictionary entry a given text string refers to (e.g., the *cold* example referred to above). A popular approach to this utilizes knowledge about the gene and the context in which the gene is mentioned. For example, the SUMMARY fields of the candidate genes might be used as a source of words that indicate what we know about the gene. Then, if we see the words *cation* and *channel* in the text surrounding the gene name, we should expect that we have an instance of the *TRP1* with Entrez Gene ID 7220, while if we see the word *proline*, we should suspect that we have an instance of the *TRP1* with Entrez Gene ID 189930. Approaches might vary with respect to what they use as the knowledge source (e.g., Entrez Gene SUMMARY fields, Entrez Gene PRODUCT fields, the contents of publications linked to the Entrez Gene entry), and what they consider the context of the gene mention, e.g., the sentence, the surrounding sentences, the entire abstract, etc.

4.5 Relation or Information Extraction

Information extraction, or more recently *relation extraction*, is the process of mining very specific types of facts from text. Information extraction systems are by definition restricted to a very specific type of information. For example, a typical genomic information extraction system might extract assertions about protein-protein interactions, or a clinical information extraction system might mine assertions about relationships between diseases and their treatments. Most systems target binary relations, such as the ones just described. However, more ambitious systems have extracted relationships with as many as four participants. One system [31] targeted protein transport relations, with a four-way relationship that included the transporting protein, the transported protein, the beginning location of the transported protein, and the destination.

Rule-based approaches use typical sentence patterns. These may consist of text literals or may involve syntactic analyses [32]. Learning-based approaches have classically used bag-of-words representations (see Section 4.2), but more recent approaches have had success using features taken from syntactic analysis, particularly dependency parsing [33].

4.6 Question-Answering

Question-answering is the task of taking a question and a source of information as input and returning an answer. Early approaches to question-answering assumed that the source of information was a database, but modern approaches assume that the answer exists in some PubMed/MEDLINE document or (for non-biomedical applications) in some web page. Question-answering differs from information retrieval in that the goal is to return a specific answer, not a document containing the answer. It differs from information extraction in that it is meant to allow for ad hoc queries, while information extraction focuses on very specific information needs. Question-an-

Table 1. Some knowledge sources for biomedical natural language processing.

Informatics for Integrating Biology and the Bedside (i2b2 - https://www.i2b2.org/)	National Center for Biomedical Computing with focus on translational research that facilitates and proves data sets for clinical natural language processing research
Gene Ontology (https://www.geneontology.org)	Controlled vocabulary with relationships including partonymy and inheritance, designed for describing gene functions, broadly construed
Entrez Gene (https://www.ncbi.nlm.nih.gov/gene)	Source for gene names, symbols, and synonyms; also the source for GeneRIFs and SUMMARY fields
PubMed/MEDLINE (https://www.ncbi.nlm.nih.gov/pubmed)	The National Library of Medicine's database of abstracts of biomedical publications (MEDLINE) and search interface for accessing them (PubMed)
Unified Medical Language System (https://www.nlm.nih.gov/research/umls/)	Large lexical and conceptual resource, including the UMLS Metathesaurus, which aggregates a large number of biomedical and some genomic vocabularies
SWISSPROT (https://www.uniprot.org/)	Database of information about proteins with literature references, useful as a gold standard
PharmGKB (https://www.pharmgkb.org/)	Database of relationships between a number of clinical, genomic, and other entities with literature references, useful as a gold standard
Comparative Toxicogenomics Database (https://ctdbase.org/)	Database of relationships between genes, diseases, and chemicals, with literature references, useful as a gold standard

Various terminological resources, data sources, and gold-standard databases for biomedical natural language processing.
doi:10.1371/journal.pcbi.1003044.t001

swering typically involves determining the type of answer that is expected (a time? a location? a person?), formulating a query that will return documents containing the answer, and then finding the answer within the documents that are returned.

Various types of questions have varying degrees of difficulty. The best results are achieved for so-called "factoid" questions, such as *where are lipid rafts located?*, while "why" questions are very difficult. In the biomedical domain, definition questions have been extensively studied [34–36]. The medical domain presents some unique challenges. For example, questions beginning with *when* might require times as their answer (e.g., *when does blastocyst formation occur in humans?*, but also may require very different sorts of answers, e.g.,*when should antibiotics be given for a sore throat?* [37]. A shared task in 2005 involved a variety of types of genomic questions adhering to specific templates (and thus overlapping with information extraction), such as *what is the biological impact of a mutation in the gene X?*.

4.7 Summarization

Summarization is the task of taking a document or set of documents as input and returning a shorter text that conveys the information in the longer text(s). There is a great need for this capability in the biomedical domain—a search in PubMed/MEDLINE for the gene p53 returns 56,464 publications as of the date of writing.

In the medical domain, summarization has been applied to clinical notes, journal articles, and a variety of other input types. For example, one system, MITRE'S MiTAP, does multi-document summarization of epidemiological reports, newswire feeds, email, online news, television news, and radio news to detect disease outbreaks.

In the genomics domain, there have been three major areas of summarization research. One has been the automatic generation of GeneRIFs. GeneRIFs are short text snippets, less than 255 characters in length, associated with specific Entrez Gene entries. Typically they are manually cut-and-pasted from article abstracts. Lu et al. developed a method for finding them automatically using a variant of the Edmundsonian paradigm, a classic approach to single-document summarization [38,39]. In the Edmundsonian paradigm, sentences in a document are given points according to a relatively simple set of features, including position in the document, presence of "cue words" (words that indicate that a document is a good summary sentence), and absence of "stigma words" (words that indicate that a sentence is not likely to be a good summary sentence).

Another summarization problem is finding the best sentence for asserting a protein-protein interaction. This task was made popular by the BioCreative shared task. The idea is to boil down a set of articles to the single sentence that best gives evidence that the interaction occurs. Again, simple features work well, such as looking for references to figures or tables [40].

Finally, a small body of work on the generation of SUMMARY fields has been seen. More sophisticated measures have been applied here, such as the PageRank algorithm [41].

5. Shared Tasks

The natural language processing community has a long history of evaluating applications through the shared task paradigm. Similar to CASP, a *shared task* involves agreeing on a task definition, a data set, and a scoring mechanism. In biomedical text mining, shared tasks have had a strong effect on the direction of the field. There have been both clinically oriented and genomically oriented shared tasks.

In the clinical domain, the 2007 NLP Challenge [42] involved assigning ICD9-CM codes to radiology reports of chest x-rays and renal procedures. Also in the clinical domain, i2b2 has sponsored a number of shared tasks, described in Section 1.1.2. (At the time of writing, the National Institute of Standards and Technology is preparing a shared task involving electronic medical records under the aegis of the annual Text Retrieval Conference. The task definition is not yet defined.)

In the genomics domain, the predominant shared tasks have been the BioCreative shared tasks and a five-year series of tasks in a special genomics track of the Text Retrieval Conference [43]. Some of the tasks were directly relevant to translational bioinformatics. The tasks varied from year to year and included information retrieval (Section 4.1), production of GeneRIFs (Section 4.7), document classification (Section 4.2), and question-answering (Section 4.6). A topic that was frequently investigated by participants was the contribution of controlled vocabularies to performance on text mining tasks. Results were equivocal; it was found that

they could occasionally increase performance, but only when used intelligently, e.g., with appropriate preprocessing or filtering of items in the terminologies—blind use of vocabulary resources does not improve performance.

The BioCreative series of shared tasks has been oriented more towards model organism database curation than towards translational bioinformatics, but some of the subtasks that were involved are of utility in translational bioinformatics. BioCreative tasks have included gene name recognition in text (Section 4.3), mining information about relationships between genes and their functions (Section 4.5), mining information about protein-protein interactions (Section 4.5), information retrieval (Section 4.1), and relating mentions of genes in text to database entries in Entrez Gene and SWISSPROT (Section 4.4).

6. Software Engineering for Text Mining

Like all translational bioinformatics software, text mining software for translational bioinformatics can be considered health-critical and should be subject to the strictest standards of quality assurance and software testing. General software testing is covered in such standard books as [44]. The special requirements of software testing for natural language processing applications are not covered in the standard books on software testing, but a small but growing body of literature discusses the special issues that arise here. There are two basic paradigms for evaluating text mining applications. The standard paradigm involves running large corpora through the application and determining the F-measure achieved. However, this approach is not satisfactory for quality assurance and software testing. It is good for achieving overall estimates of performance, but does a poor job of indicating what the application is good at and what it is bad at. For this task, structured test suites and application of the general principles of software testing are much more appropriate. Structured test suites are discussed in Section 1.3.2. It is helpful to consult with a descriptive linguist when designing test suites for assessing an application's ability to handle linguistic phenomena. [15] and [14] describe basic principles for constructing test suites for linguistic phenomena by applying the techniques of software testing and of descriptive linguistics. The former includes a methodology for the automatic generation of test suites of arbitrary size and complexity. [45] presents a quantitative examination of the effectiveness of corpora versus structured test suites for software testing, and demonstrates that structured test suites achieve better code coverage (percentage of code that is executed during the test phase—bugs cannot be discovered in code that is not executed) than corpora, and also offer a significant advantage in terms of time and efficiency. They found that a structured test suite that achieved higher code coverage than a 3.9 million word corpus could be run in about 11 seconds, while it took about four and a half hours to process the corpus. [46] discusses the application of the software engineering concept of the "fault model," informed by insights from linguistics, to discovering a serious error in their ontology linking tool.

User interface assessment requires special techniques not found in other areas of software testing for natural language processing. User interface testing has been most heavily studied in the case of literature search interfaces. Here the work of [47,48] is most useful, and can serve as a tutorial on interface evaluation.

7. Exercises

1. Obtain a copy of a patient record collection from the i2b2 National Center for Biomedical Computing (see e.g., [49]). Download the MetaMap application or API and run it over a set of ten discharge summaries. Use Google to find the current links for the i2b2 data sets and for downloading MetaMap. Note that using the MetaMap application will require writing code to extract results from the MetaMap output file, while using the API will require writing your own application. Which outputs might you consider to identify phenotypes that could be relevant for your research interests?
2. Obtain a collection of 1,000 PubMed abstracts by querying with the terms *gene* and *mutation* and downloading the 1,000 most recent. Run the EMU mutation extractor (http://bioinf.umbc.edu/EMU/ftp) or a similar tool on them. What genotypes can you identify in the output?
3. A researcher has a collection of 10,000 documents. She wants to retrieve all documents relevant to pulmonary hypertension. The collection contains 250 documents that are relevant to pulmonary hypertension. An information retrieval program written by a colleague returns 100 documents. 80 of these are actually relevant to pulmonary hypertension. What is the precision, recall, and F-measure for this system?
4. Explain the difference between *descriptive linguistic rules* and *prescriptive linguistic rules*. Be sure to say which type text mining is concerned with.

Answers to the Exercises can be found in Text S1.

Further Reading

[50] is a book-length treatment of biomedical natural language processing, oriented towards readers with some background in NLP. [51] takes the perspective of model organism database curation as the primary motivating task for text mining. It includes a review of the ten most important papers and resources for BioNLP. [52] is an excellent introduction to text mining in general. [10] is the standard textbook on natural language processing.

There are a number of seminal papers on biomedical text mining besides those already cited in the text. These include [20,53–57].

Table 1 lists a variety of terminological resources, data sources, and gold-standard databases for biomedical natural language processing.

Supporting Information

Text S1 Answers to Exercises.
(DOCX)

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Chapter 17: Bioimage Informatics for Systems Pharmacology

Fuhai Li, Zheng Yin, Guangxu Jin, Hong Zhao, Stephen T. C. Wong*

NCI Center for Modeling Cancer Development, Department of Systems Medicine and Bioengineering, The Methodist Hospital Research Institute, Weil Medical College of Cornell University, Houston, Texas, United States of America

Abstract: Recent advances in automated high-resolution fluorescence microscopy and robotic handling have made the systematic and cost effective study of diverse morphological changes within a large population of cells possible under a variety of perturbations, e.g., drugs, compounds, metal catalysts, RNA interference (RNAi). Cell population-based studies deviate from conventional microscopy studies on a few cells, and could provide stronger statistical power for drawing experimental observations and conclusions. However, it is challenging to manually extract and quantify phenotypic changes from the large amounts of complex image data generated. Thus, bioimage informatics approaches are needed to rapidly and objectively quantify and analyze the image data. This paper provides an overview of the bioimage informatics challenges and approaches in image-based studies for drug and target discovery. The concepts and capabilities of image-based screening are first illustrated by a few practical examples investigating different kinds of phenotypic changes caused by drugs, compounds, or RNAi. The bioimage analysis approaches, including object detection, segmentation, and tracking, are then described. Subsequently, the quantitative features, phenotype identification, and multidimensional profile analysis for profiling the effects of drugs and targets are summarized. Moreover, a number of publicly available software packages for bioimage informatics are listed for further reference. It is expected that this review will help readers, including those without bioimage informatics expertise, understand the capabilities, approaches, and tools of bioimage informatics and apply them to advance their own studies.

This article is part of the "Translational Bioinformatics" collection for *PLOS Computational Biology*.

1. Introduction

The old adage that a picture is worth a thousand words certainly applies to the identification of phenotypic variations in biomedical studies. Bright field microscopy, by detecting light transmitted through thin and transparent specimens, has been widely used to investigate cell size, shape, and movement. The recent development of fluorescent proteins, e.g., green fluorescent protein and its derivatives [1], enabled the investigation of the phenotypic changes of subcellular protein structures, e.g., chromosomes and microtubules, revolutionizing optical imaging in biomedical studies. Fluorescent proteins are bound to specific proteins that are uniformly located in relevant cellular structures, e.g., chromosomes, and emit longer wavelength light, e.g., green light, after exposure to shorter wavelength light, e.g., blue light. Thus, the spatial morphology and temporal dynamic activities of subcellular protein structures can be imaged with a fluorescence microscope - an optical microscope that can specifically detect emitted fluorescence of a specific wavelength [2]. In current image-based studies, five-dimensional (5D) image data of thousands of cells (cell populations) can be acquired: spatial (3D), time lapse (1D), and multiple fluorescent probes (1D).

With advances to automated high-resolution microscopy, fluorescent labeling, and robotic handling, image-based studies have become popular in drug and target discovery. These image-based studies are often referred to as the High Content Analysis (HCA) [3], which focuses on extracting and analyzing quantitative phenotypic data automatically from large amounts of cell images with approaches in image analysis, computation vision and machine learning [3,4]. Applications of HCA for screening drugs and targets are referred to as High Content Screening (HCS), which focuses on identifying compounds or genes that cause desired phenotypic changes [5–7]. The image data contain rich information content for understanding biological processes and drug effects, indicate diverse and heterogeneous behaviors of individual cells, and provide stronger statistical power in drawing experimental observations and conclusions, compared to conventional microscopy studies on a few cells. However, extracting and mining the phenotypic changes from the large scale, complex image data is daunting. It is not feasible to manually analyze these data. Hence, bioimage informatics approaches were needed to automatically and objectively analyze large scale image data, extract and quantify the phenotypic changes to profile the effects of drugs and targets.

Bioimage informatics in image-based studies usually consists of multiple analysis modules [3,8,9], as shown in Figure 1. Each of the analysis tasks is challenging, and different approaches are often required for the analysis of different types of images. To facilitate image-based screening studies, a number of bioimage informatics software packages have been developed and are publicly available [9]. This chapter provides an overview of the bioimage informatics approaches in im-

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* E-mail: stwong@tmhs.org

What to Learn in This Chapter

- What automated approaches are necessary for analysis of phenotypic changes, especially for drug and target discovery?
- What quantitative features and machine learning approaches are commonly used for quantifying phenotypic changes?
- What resources are available for bioimage informatics studies?

age-based studies for drug and target discovery to help readers, including those without bioimage informatics expertise, understand the capabilities, approaches, and tools of bioimage informatics and apply them to advance their own studies. The remainder of this chapter is organized as follows. Section 2 introduces a number of practical screening applications for discovery of potential drugs and targets. Section 3 describes the challenges and approaches for quantitative image analysis, e.g., object detection, segmentation, and tracking. Section 4 introduces techniques for quantification of segmented objectives, including feature extraction, phenotype classification, and clustering. Section 5 reviews a number of prevalent approaches for profiling drug effects based on the quantitative phenotypic data. Section 6 lists major, publicly available software packages of bioimage informatics analysis, and finally, a brief summary is provided in Section 7.

2. Example Image-based Studies for Drug and Target Discovery

There are a variety of image-based studies for discovery of drugs, targets, and mechanisms of biological processes. A good starting point for learning about bioimage informatics approaches is to study practical image-based studies, and a number of examples are summarized below.

2.1 Multicolor Cell Imaging-based Studies for Drug and Target Discovery

Fixed cell images with multiple fluorescent markers have been widely used for drug and target screening in scientific research. For example, the effects of hundreds of compounds were profiled for phenotypic changes using multicolor cell images in [10–12]. Hundreds of quantitative features were extracted to indicate the phenotypic changes caused by these compounds, and then computational approaches were proposed to identify the effective compounds, categorize them, characterize their dose-dependent response, and suggest novel targets and mechanisms for these compounds [10–12]. Moreover, phenotypic heterogeneity was investigated by using a subpopulation based approach to characterize drug effects in [13], and distinguish cell populations with distinct drug sensitivities in [14]. Also in [15,16], the phenotypic changes of proteins inside individual Drosophila Kc167 cells treated with RNAi libraries were investigated by using high resolution fluorescent microscopy, and bioimage informatics analysis was applied to quantify these images to identify genes regulating the phenotypic changes of interest. Figure 2 shows an image of *Drosophila* Kc167 cells, which were treated with RNAi and stained to visualize the nuclear DNA (red), F-actin (green), and α-tubulin (blue). Freely available software packages, such as CellProfiler [17], Fiji

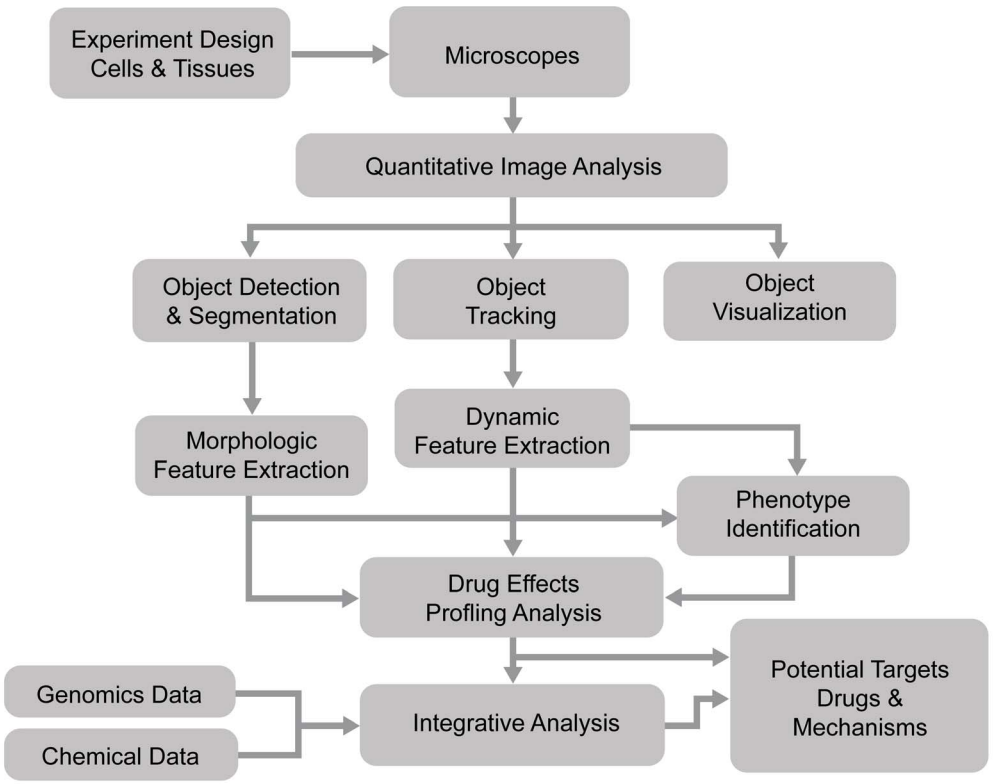

Figure 1. The flowchart of bioimage informatics for drug and target discovery.
doi:10.1371/journal.pcbi.1003043.g001

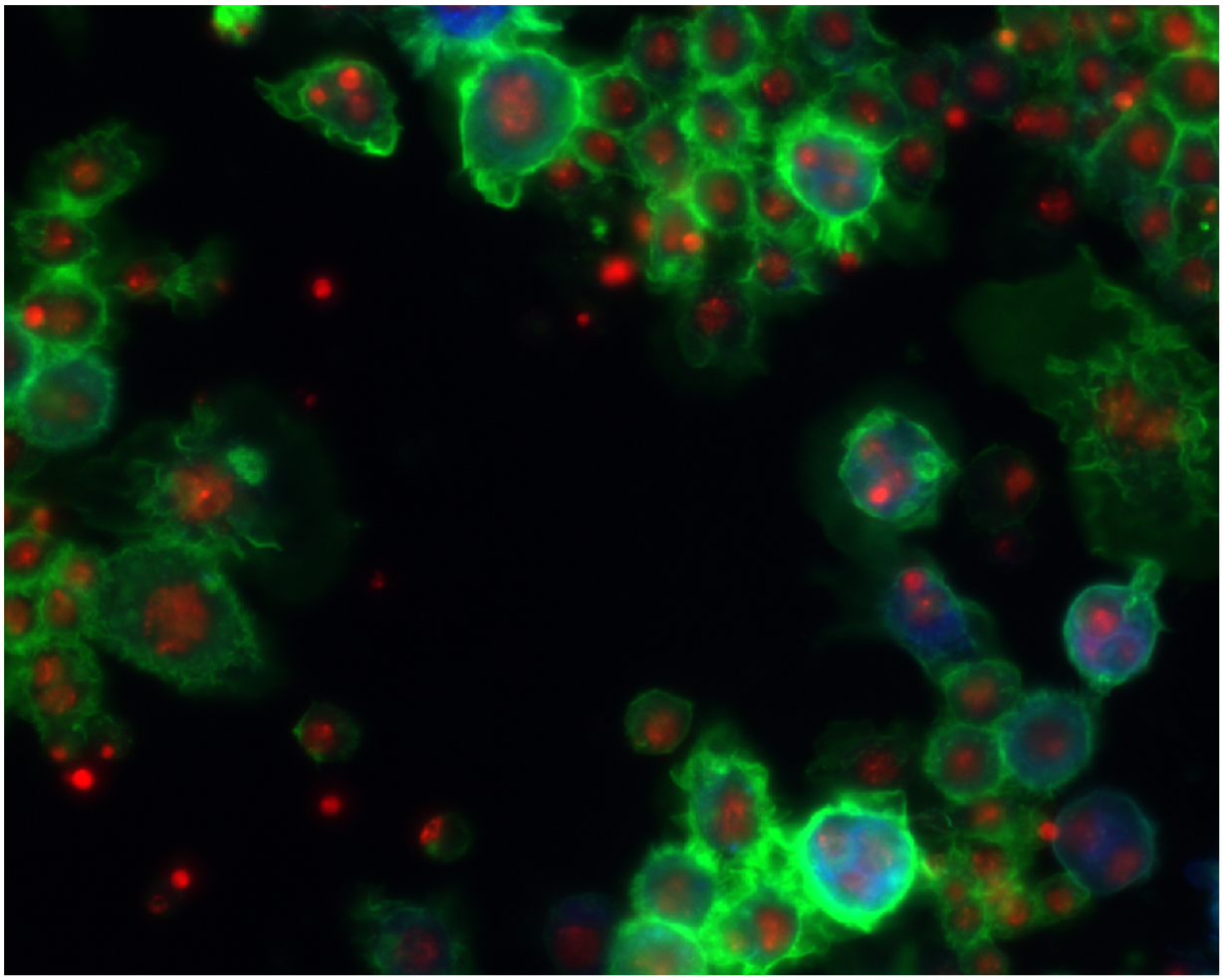

Figure 2. A representative image of *Drosophila* Kc167 cells treated with RNAi. The red, green, and blue colors are the DNA, F-actin, and α-tubulin channels.
doi:10.1371/journal.pcbi.1003043.g002

[18], Icy [19], GCELLIQ [20], and PhenoRipper [21] can be used for the multicolor cell image analysis.

2.2 Live-cell Imaging-based Studies for Cell Cycle and Migration Regulator Discovery

Two hallmarks of cancer cells are uncontrolled cell proliferation and migration. These are also good phenotypes for screening drugs and targets that regulate cell cycle progression and cell migration in time-lapse images. For example, out of 22,000 human genes, about 600 were identified as related to mitosis by using live cell (time-lapse) imaging and RNAi treatment in the MitoCheck project (www.mitocheck.org) [22,23]. The project is now being expanded to study how these identified genes work together to regulate cell mitosis, in which mistakes can lead to cancer, in the MitoSys (systems biology of mitosis) project (http://www.mitosys.org/). Also, live cell imaging of Hela cells was used to discover drugs and compounds that regulate cell mitosis in [24,25]. Moreover, the time-lapse images of live cells were used to study the dynamic behaviors of stem cells in [26,27] and predict cell fates of neural progenitor cells using their dynamic behaviors in [28]. Figure 3 shows a single frame of live HeLa cell images and the images of four cell cycle phases: interphase, prophase, metaphase, and anaphase [25]. The publicly available software packages for time-lapse image analysis include, for example, the plugins of CellProfiler [17], Fiji [18], BioimageXD [29], Icy [19], CellCognition [23], DCELLIQ [30], and TLM-Tracker [31].

2.3 Neuron Imaging-based Studies for Neurodegenerative Disease Drug and Target Discovery

Neuronal morphology is illustrative of neuronal function and can be instructive toward the dysfunctions seen in neurodegenerative diseases, such as Alzheimer's and Parkinson's disease [32,33]. For example, the 3D neuron synaptic morphological and structural changes were investigated by using super-resolution microscopy, e.g., STED microscopy, to study brain functions and disorders under different stimulations [34–36]. Also other advanced optical techniques were proposed in [37,38] to image and reconstruct the 3D structure of live neurons. Figure 4 shows an example of 2D neuron image used in [39]. In [40], neuronal degeneration was mimicked by treating mice with different dosages of Aβ peptide, which may cause the loss of neuritis, and drugs that rescue the loss of neurites were identified as candidates for AD therapy. Figure 5 shows an example of neurites and nuclei images acquired in [40]. To quantitatively analyze neuron images, a number of publicly available software packages have been developed, for example, NeurphologyJ [41], NeuronJ [42], NeuriteTracer (Fiji plugin) [43], NeuriteIQ [44], NeuronMetrics [45], NeuronStudio

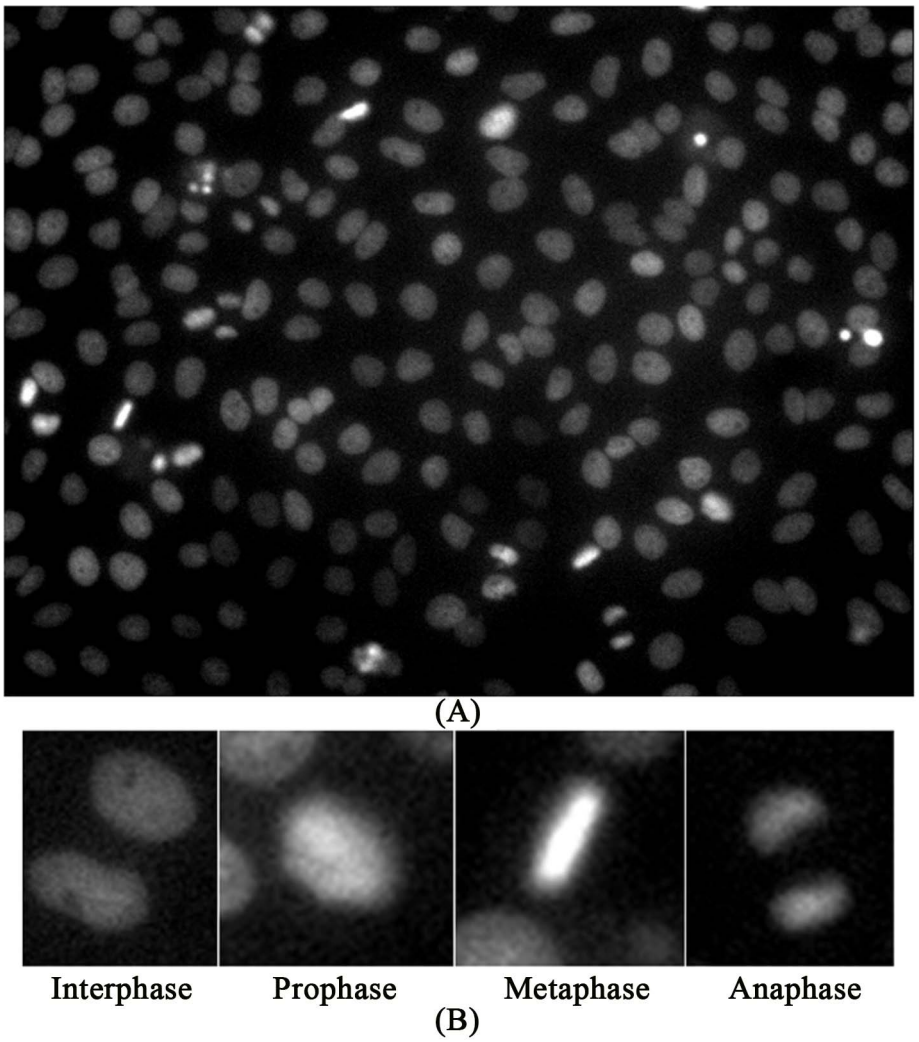

Figure 3. Examples of HeLa cell nuclei and cell cycle phase images. (A) A frame of HeLa cell nuclei time-lapse image sequence; (B) Example images of four cell cycle phases.
doi:10.1371/journal.pcbi.1003043.g003

[46,47], NeuronJ [42], NeuronIQ [39,48], and Vaa3D [49,50]. A review of software packages for neuron image analysis was also reported in [51].

2.4 *Caenorhabditis elegans* Imaging-based Studies for Drug and Target Discovery

Caenorhabditis elegans (C. elegans) is a common animal model for drug and target discovery. Consisting of only hundreds of cells, it is an excellent model to study cellular development and organization. For example, the invariant embryonic development of *C. elegans* was recorded by time-lapse imaging, and the embryonic lineages of each cell were then reconstructed by cell tracking to study the functions of genes underpinning the development process [52–54]. Moreover, an atlas of *C. elegans*, which quantified the nuclear locations and statistics on their spatial patterns in development, was built based on the confocal image stacks via the software, CellExplorer [55,56]. In addition, CellProfiler provides an image analysis pipeline for delineating bodies, and quantifying the expression changes of specific proteins, e.g., clec-60 and pharynx, of individual *C. elegans* under different treatments [57].

These examples have demonstrated diverse cellular phenotypes in different image-based studies. To quantify and analyze the complex phenotypic changes of cells and sub-cellular components from large scale image data, bioimage informatics approaches are needed.

3. Quantitative Bioimage Analysis

After image acquisition, phenotypic changes need to be quantified for characterizing functions of drugs and targets. Due to the large amounts of images generated, it is not feasible to quantify the images manually. Therefore, automated image analysis is essential for the quantification of phenotypic changes. In general, the challenges of quantitative image analysis include object detection, segmentation, tracking, and visualization. The word 'object' in this context means the object captured in the bioimages, e.g., the nucleus and cell. The following sections will introduce techniques used to address these challenges.

3.1 Object Detection

Object detection is to detect the locations of individual objects. It is important, especially when the objects cluster together, to facilitate the segmentation task by providing the position and initial boundary information of individual objects. Based on the shape of objects, two

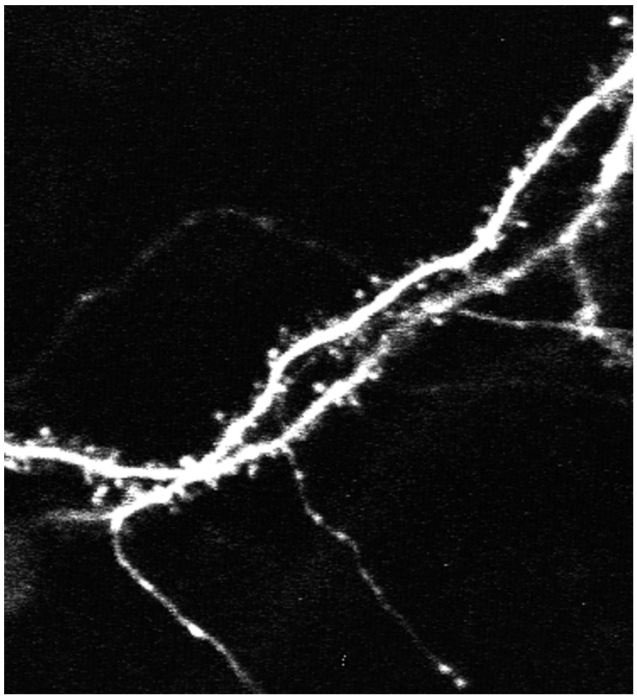

Figure 4. A representative 2D neuron images. The bright spots near the backbones of neurons are the dendritic spines.
doi:10.1371/journal.pcbi.1003043.g004

categories of object detection techniques are developed: blob structure detection, e.g., particles and cell nuclei, and tube structure detection, e.g., neurons, blood vessels.

The shape information of blob objects can be used to detect the centers of objects using distance transformation [58]. The concavity of two touching objects would cause two local maxima in the distance image, such that thresholding or seeded watershed can be employed to the distance image to detect and separate the touching blob objects [59]. The intensity information is also often used for blob detection. Blob objects usually have relatively high intensity in the center, and relatively low intensity in the peripheral regions. For example, the Laplacian-of-Gaussian (LOG) filter is effective [60–63] to detect blob objects based on the intensity information. After LOG filtering, local maximum response points often correspond to centers of blob objects, as shown in Figure 6. Moreover, the intensity gradient information is also used for blob detection. For example, in [64] the intensity gradient vectors were smoothed by using the gradient vector flow approach [65] so that the smoothed gradient vectors continuously point to the object centers. Consequently, the blob object centers can be detected by following the gradient vectors [64]. In addition, the boundary points of blob objects with high gradient amplitude can be used to detect their centers, based on the idea of Hough Transform [66]. For example, in [67] an iterative radial voting method was developed to detect such object centers based on the boundary points. In brief, the detected boundary points vote the blob center with oriented kernels iteratively, and the orientation and size of the kernels are updated based on the voting results. Finally, the maximum response points in the voting image are selected as the centers of objects. The advantage of this method is that it can detect the centers of objects with noise appearance [67]. The distance transform and the intensity gradient information also can be combined for the object detection [68]. For other blob objects with complex appearances, the machine learning approaches based on local features [69,70] can also be used for object detection [71,72], as in the Fiji (trainable segmentation plugin) [18] and Ilastik [73].

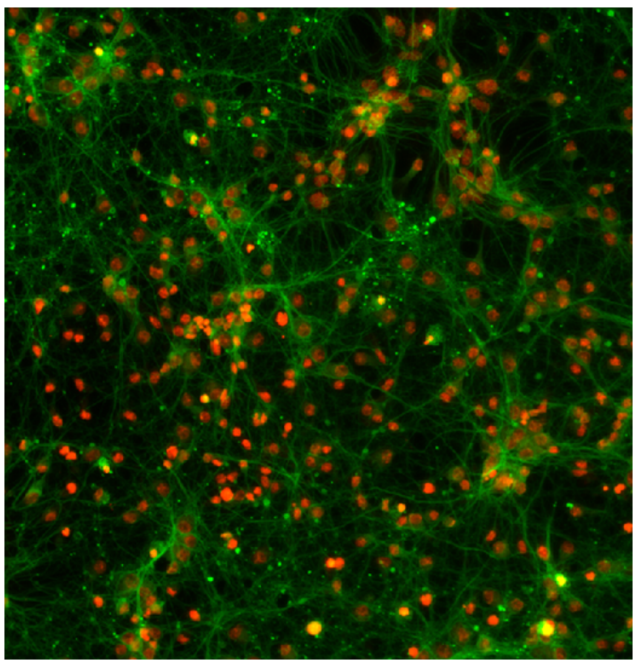

Figure 5. A representative image of neurites. Red indicates nuclei and green represents neurites.
doi:10.1371/journal.pcbi.1003043.g005

Tubular structure detection is based on the premise that the intensity remains constant in the direction along the tube, and varies dramatically in the direction perpendicular to the tube. To find the local direction of tube center lines, the eigenvector corresponding to the minimum and negative eigenvalue of Hessian matrix was proposed in [44,74]. Center line points can be characterized by their local geometric attributes, i.e., the first derivative is close to zero and the magnitude of second derivatives is large in a direction perpendicular to tube center line [42,44,74]. After the center line point

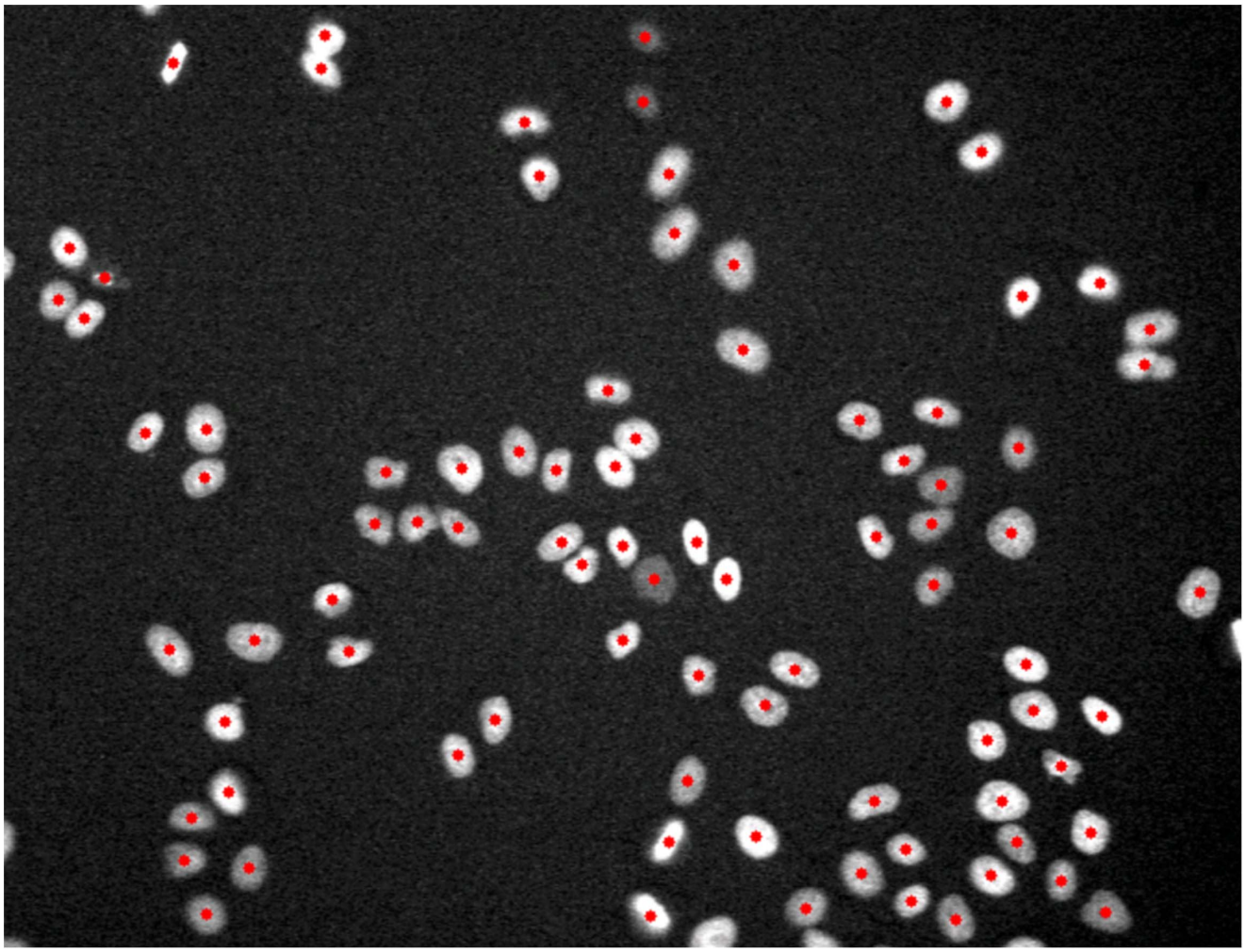

Figure 6. An example of blob-structure (HeLa cell nuclei) detection. The red spots indicate the detected centers of objects.
doi:10.1371/journal.pcbi.1003043.g006

detection, a linking process is needed to connect these center line points into continuous center lines based on their direction and distance. For example, in NeuronJ, Dijkstra's shortest-path was used based on the Gaussian derivative features to detect the neuron's centerline between two given points on the neuron [42]. Figure 7 provides an example of neurite images, and Figure 8 shows the corresponding centerline detection results [44] based on the local Gaussian derivative features. In addition to the approaches based on Gaussian derivatives, there are other tubular structure detection approaches. For example, four sets of kernels (edge detectors) were designed to detect the neuron edges and centerlines [75], and super-ellipsoid modeling was designed to fit the local geometry of blood vessels [76].

Moreover, machine learning-based tubular structure detection is a widely used method. For example, blood vessel detection in retinal images is a representative tubular structure detection task with the supervised learning approaches [77,78]. In these methods, the local features, e.g., intensity and wavelet features, of an image patch containing a given pixel are calculated, and then a classifier is trained using these local features based on a set of training points [77,78]. A good survey of blood vessel (tube structure) detection approaches in retinal images was reported in [79]. For more approaches and details of tubular structure detection, readers should refer to the aforementioned neuron image analysis software packages.

In summary, blobs and tubes are the dominating structures in bioimages. The detection results provide the position and initial boundary information for the quantification and segmentation processes. In other words, the segmentation process tries to delineate boundaries of objects starting from the detected centers or centerlines of objects. Without the guidance of detection results, object segmentation would be more challenging.

3.2 Object Segmentation

The goal of object segmentation is to delineate boundaries of individual objects of interest in images. Segmentation is the basis for quantifying phenotypic changes. Although a number of image segmentation methods have been reported, this remains an open challenge due to the complexity of morphological appearances of objects. This section introduces a number of widely used segmentation methods.

Threshold segmentation [80] is the simplest method: $T(I)=\begin{cases}1; t_2>I(x,y)>t_1\\ 0; otherwise\end{cases}$, where $I(x,y)$ is the image, and t_1 and t_2 are the intensity thresholds. As an extension of the thresholding method, Fuzzy-C-

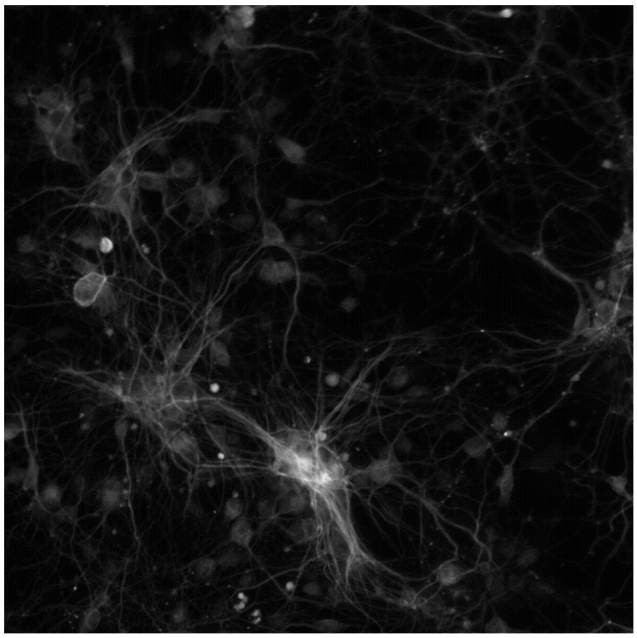

Figure 7. A representative neurite image for centerline detection.
doi:10.1371/journal.pcbi.1003043.g007

Means [81] can be used to separate images into more regions based on intensity information. These methods could divide the image into objects and background, but fail to separate the object clumps (i.e., multiple objects touching together). Watershed segmentation and its derivatives are widely used segmentation methods. They build object boundaries between objects on the pixels with local maximum intensity, which act like dams to avoid flooding from different basins (object regions) [82]. To avoid the over-segmentation problem of the watershed approach, the marker-controlled watershed (or seeded watershed) approach, in which the floods are from the 'marker' or 'seed' points (the object detection results), was proposed [68,83–85]. Figure 9 illustrates the segmentation result of HeLa cell nuclei using the seeded watershed method based on the cell detection results.

Active contour models are another set of widely used segmentation methods [86–90]. Generally, there are two kinds of active contour models: boundary-driven and region-competition models. In the boundary-driven model, the contours' (boundaries of objects) evolution is determined by the local gradient. In other words, the boundary fronts move toward the outside (or inside) quickly in the regions with low intensity variation (gradient), and slowly in the regions with high gradient (where the boundaries are). When great intensity variation appears inside cells, or the boundary is weak, this method often fails [91]. Instead of using gradient information, the region-competition model makes use of the intensity similarity

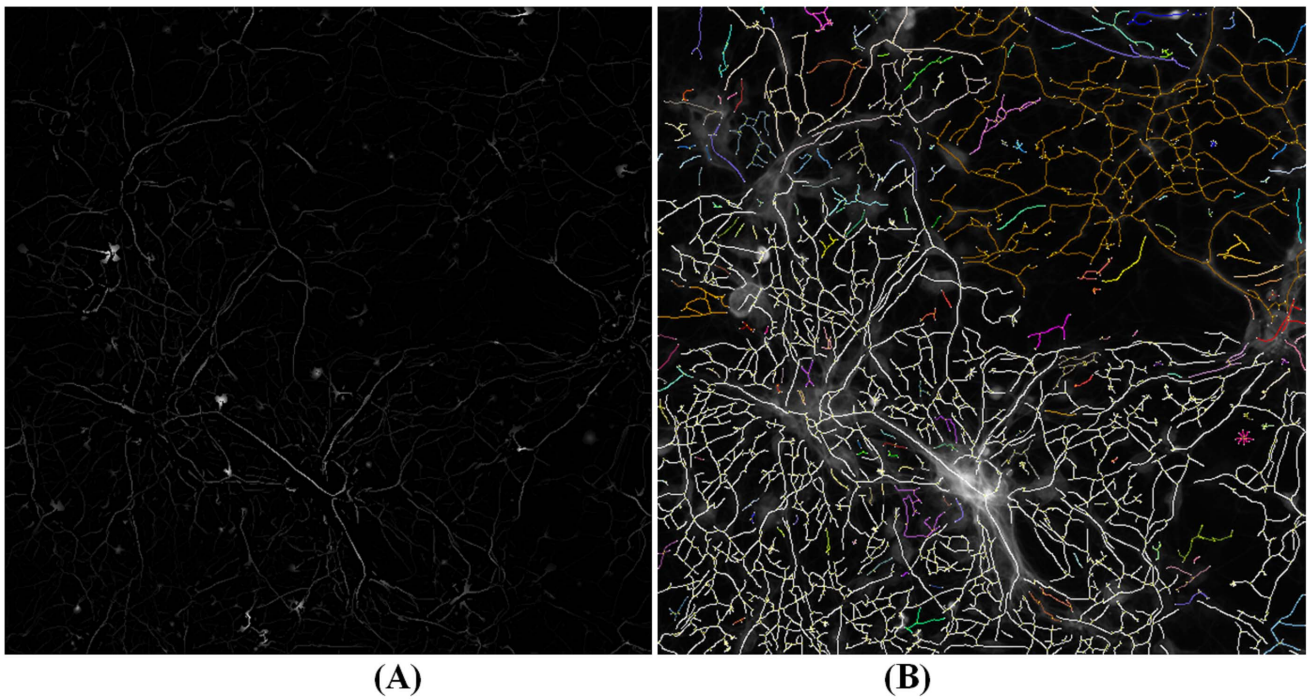

Figure 8. An example of neurite centerline detection. (A) The centerline confidence image obtained by using the local Gaussian derivative features. Higher intensity indicates higher confidence of pixels on the centerlines. (B) The neurite centerline detection result image. Different colors indicate the disconnected branches.
doi:10.1371/journal.pcbi.1003043.g008

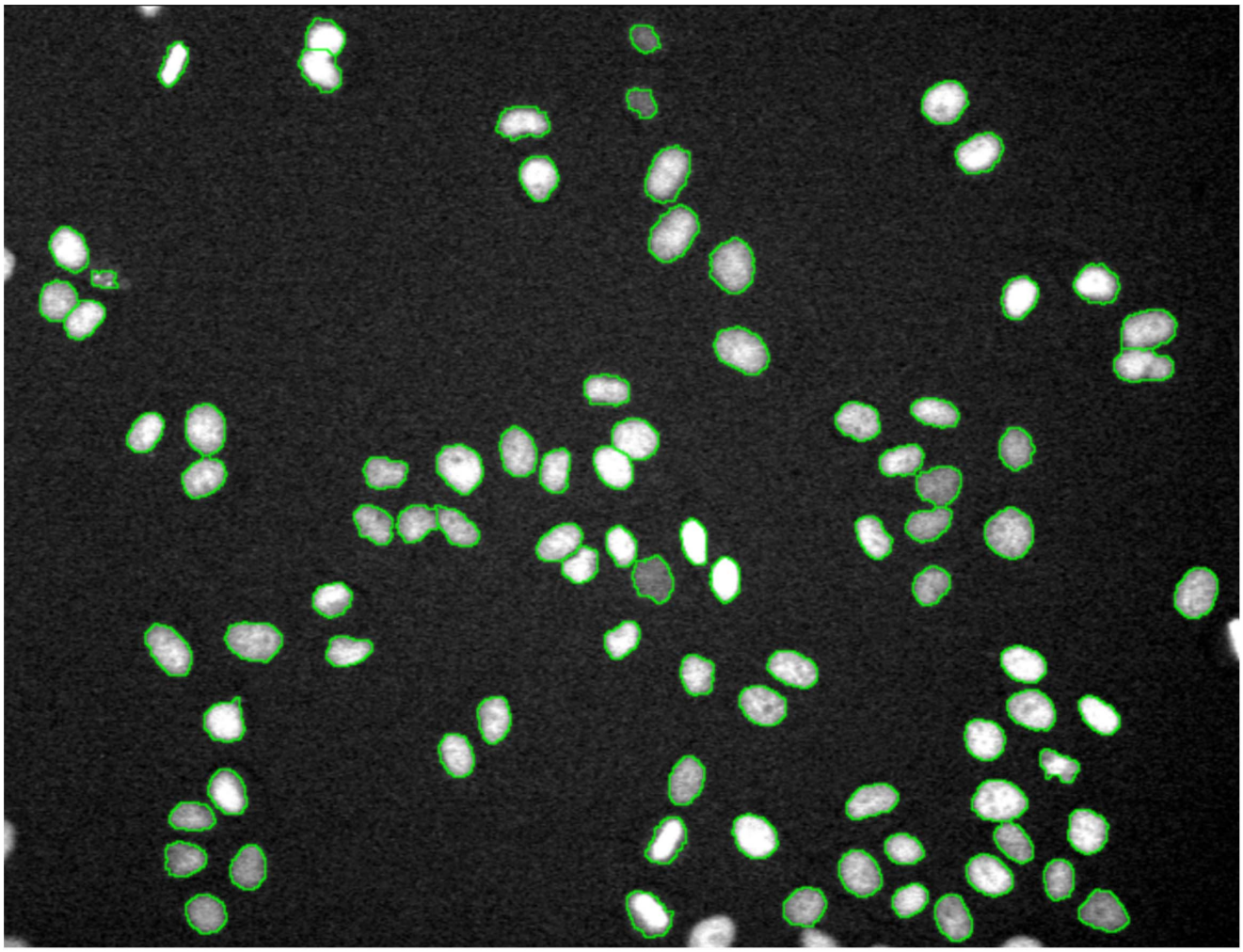

Figure 9. An example of HeLa nuclei segmentation using the seeded watershed algorithm. The green contours are the boundaries of nuclei.
doi:10.1371/journal.pcbi.1003043.g009

information to separate the image into regions with similar intensity. Region competition-based active contour models could solve the weak boundary problem; however, they require that the intensity of touching objects is separable [87]. To implement these active contour models, level set representation is widely used [92]. Level set is an *n+1* dimensional function that can easily represent any *n* dimensional shape without parameters. The inside regions of objects are indicated by using positive levels, and outside regions are represented using negative levels. For this implementation, the initial boundary (zero level) is required, and the signed distance function is often used to initialize the level set function [92,93]. To evolve the level set functions (grow the boundaries of objects), the following two equations are classical models. The first equation is often called geodesic active contour (GAC) [86], and the second one is often named the Chan and Vese active contour (CV) [87].

$$\frac{d}{dt}\psi = \alpha(\nabla g \cdot \nabla\psi) + g(\kappa + c)|\nabla\psi|$$

(GAC level set evolution equation),

$$\frac{d}{dt}\psi = \delta_\varepsilon(\psi)\left[\mu\cdot\kappa - v - \lambda_1(I-c_1)^2 + \lambda_2(I-c_2)^2\right]$$

(CV level set evolution equation),

where ψ denotes the level-set function, and g indicates the gradient function, ∇ is the gradient operator, c, c_1, and c_2 are constant variables. $\delta_\varepsilon(x) = \frac{1}{\pi}\frac{\varepsilon}{\varepsilon^2+x^2}$ is an approximation of the Dirac function to indicate the boundary bands), which is the derivative function of Heaviside function denoting inside/outside regions of objects: $H(x) = \frac{1}{2}\left[1 + \frac{2}{\pi}\arg\tan\left(\frac{x}{\varepsilon}\right)\right]$, and the curvature term, $\kappa = div\left(\frac{\nabla\psi}{|\nabla\psi|}\right) = \frac{\psi_{xx}\psi_y^2 - 2\psi_x\psi_{xy}\psi_y + \psi_x^2\psi_{yy}}{\left(\psi_x^2+\psi_y^2\right)^{3/2}}$ indicates the local smoothness of boundaries, and '*div*' is the divergence operation. Figure 10 demonstrates the segmentation result using GAC level set approach. An additional segmentation method, Voronoi segmentation [94], first defines the centers of objects and then constructs the boundaries between two objects on the pixels, from which the distances are the same to the two centers. In CellProfiler, the Voronoi segmentation method was extended by considering the local intensity variations in the distance metric to achieve better segmentation results [95]. This method is fast and generates level set comparable

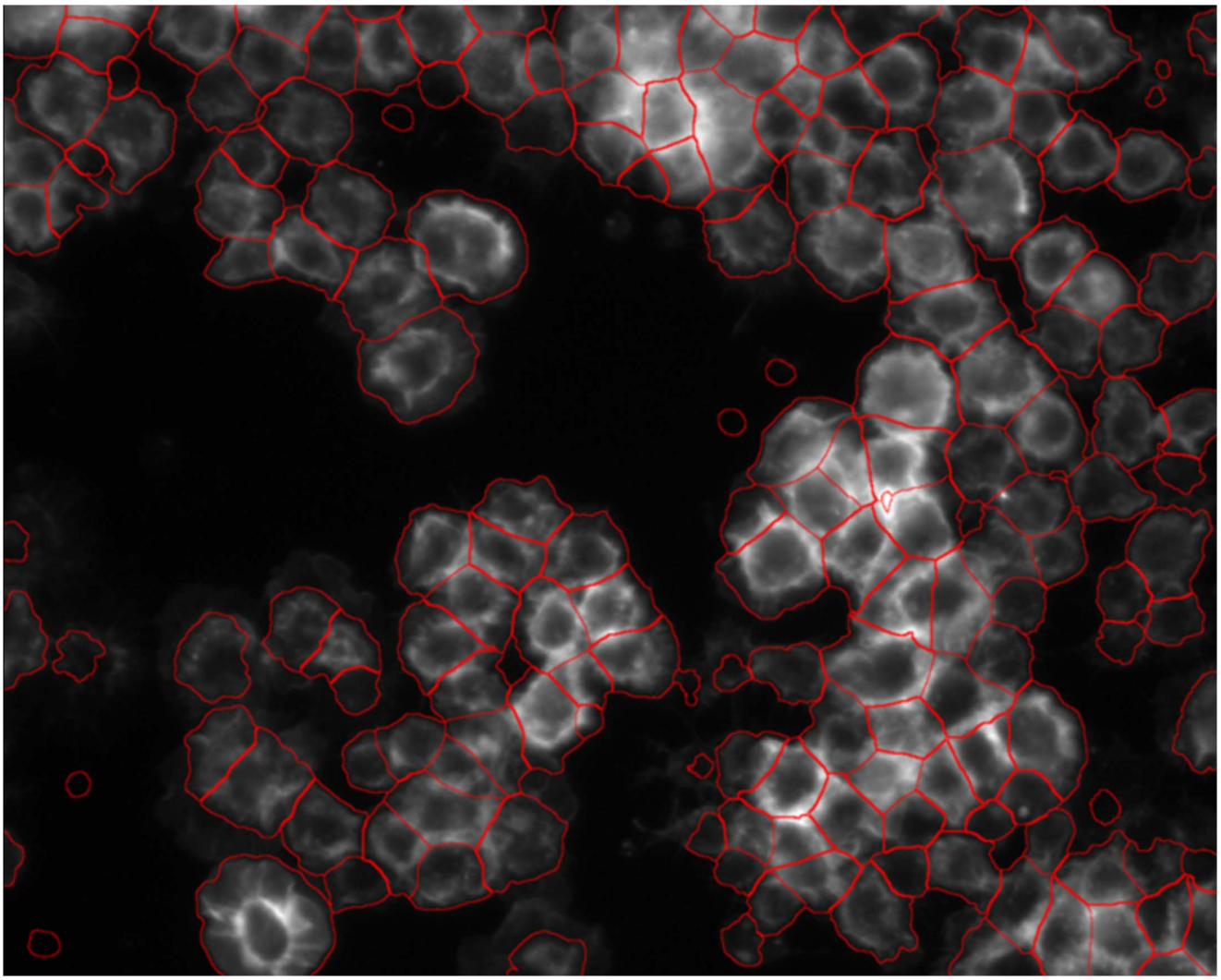

Figure 10. An example of segmentation of *Drosophila* cell images using the level set approach.
doi:10.1371/journal.pcbi.1003043.g010

results. Graph cut segmentation method views the image as a graph, in which each pixel is a vertex and adjacent pixels are connected [63,96,97]. It 'cuts' the graph into several small graphs from the regions where adjacent pixels have the most different properties, e.g., intensity.

Different from the aforementioned segmentation approaches, local feature and machine learning-based segmentation approaches are implemented, for example, in Fiji (trainable segmentation plugin) [18] and Ilastik [73]. Users can interactively select the training sample pixels/voxels or small image patches conveniently, and then classifiers are automatically trained based on the features of the training pixels or voxels (or patches) to predict the classes, e.g., cells or background, of the pixels or voxels (or patches) in a new image. The image patches could be a circle or square neighbor regions of a given point, and also could be regions (superpixel) obtained by the clustering analysis. For example, Simple Linear Iterative Clustering (SLIC) made use of the intensity and coordinate information of pixels to separate the image into uniformly sized and biologically meaningful regions [98,99], and then the machine learning approaches were used to identify the regions of interest, e.g., boundary superpixels, for object segmentation [99].

3.3 Object Tracking

To study the dynamic behaviors and phenotypic changes of objects over time (e.g., cell cycle progression and migration), object tracking using time lapse image sequences is necessary. Figure 11 shows a Hela cell's division process in four frames at different time points, and Figures 12 and 13 show the examples of cell migration trajectories and cell lineages reconstructed from the time-lapse images of Hela cells [30]. Object tracking is a challenging task due to the complex dynamic behaviors of objects over time. In general, cell tracking approaches can be classified into three categories: model evolution-based tracking, spatial-temporal volume segmentation-based tracking, and segmentation-based tracking.

In the model evolution based tracking approaches, cells or nuclei are initially detected and segmented in the first frame, and then their boundaries and positions evolve frame by frame. Some tracking techniques in this category are mean-shift [100] and parametric active contours [88,101]. However, neither mean-shift nor parametric active contours can cope well with cell division and nuclei clusters. Though the level set method enables topological change, e.g., cell division, it also allows the fusion of overlapping cells. Extending these methods to cope with

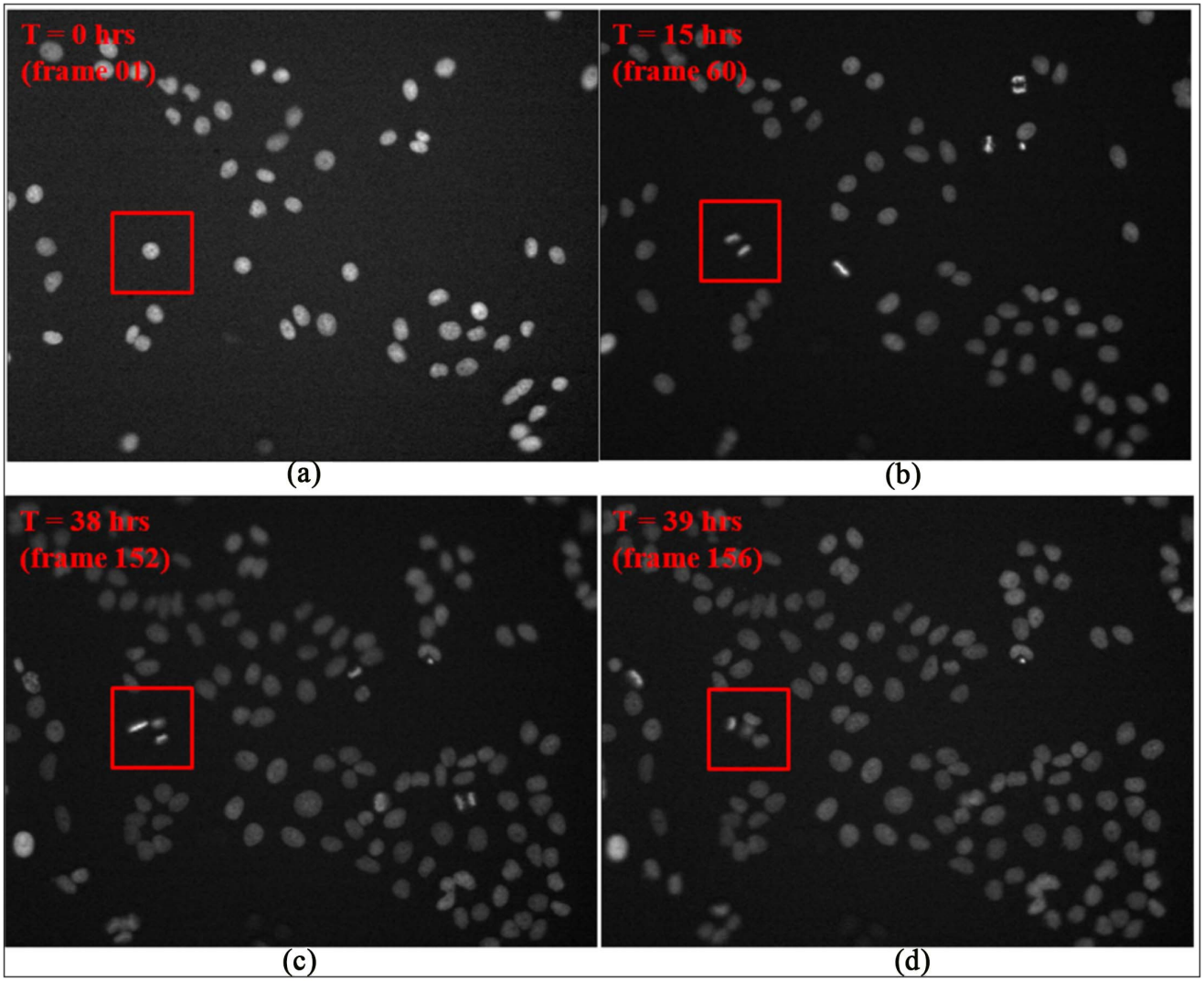

Figure 11. Time-lapse images indicating cell cycle progression. The cell in the red square in the first frame (A) divided into two cells in frame 60 (B). The descendent cells divided again in frame 152 and 156 respectively as shown in the red squares in (C) and (D).
doi:10.1371/journal.pcbi.1003043.g011

these tracking challenges is nontrivial and increases computation time [90,102–104]. For example, the coupled geometric active contours model was proposed to prevent object fusion by representing each object with an independent level set in [105], and this was further extended to the 3D cell tracking in [90]. The other approach explicitly blocking the cell merging is to introduce the topology constraints, i.e., labeling objects regions with different numbers or colors. For example, the region labeling map was employed in [27,106] to deal with the cell merging, and planar graph–vertex coloring was employed to separate the neighboring contours. From that four separate level set functions could easily deal with cell merging [107] based on the four-color theorem [108,109]. For the spatial-temporal volume segmentation based tracking, 2D image sequences were viewed as 3D volume data (2D spatial+temporal), and the shape and size constrained level set segmentation approaches were applied to segment the traces of objects, and reconstruct the cell lineage in [110–112].

For detection and segmentation-based tracking, objects are first detected and segmented, and then these objects are associated between two consecutive frames, based on their morphology, position, and motion [30,113–115]. The tracking approaches are usually done fast, but their accuracy is closely related to detection and segmentation results, similarity measurements, and association strategies. The cell center position, shape, intensity, migration distance, and spatial context information were used as similarity measurements in [113,115]. For the association approaches, the overlap region and distance based method was employed in [114], in which objects in the current frame were associated with the nearest objects in the next frame. Then the false matches, e.g., many-to-one or one-to-many, were further corrected through the post processing. Different from the individual object association above, all segmented objects were simultaneously associated by using the integer programming optimization in [113,116]: $\mathbf{x}^* = \max_{\mathbf{x}\in\{0,1\}^N} \mathbf{S}\mathbf{x}$, *s.t.* $\mathbf{A}\mathbf{x} \le 1$, where $\mathbf{A}\mathbf{x} \le 1$ restricts that one object can be associated to one object at most, $\mathbf{A}$ is an $(m+n)\times N$ matrix, and the first m rows correspond to m objects in frame t, and the last n rows denote objects in frame $t+1$. N is the

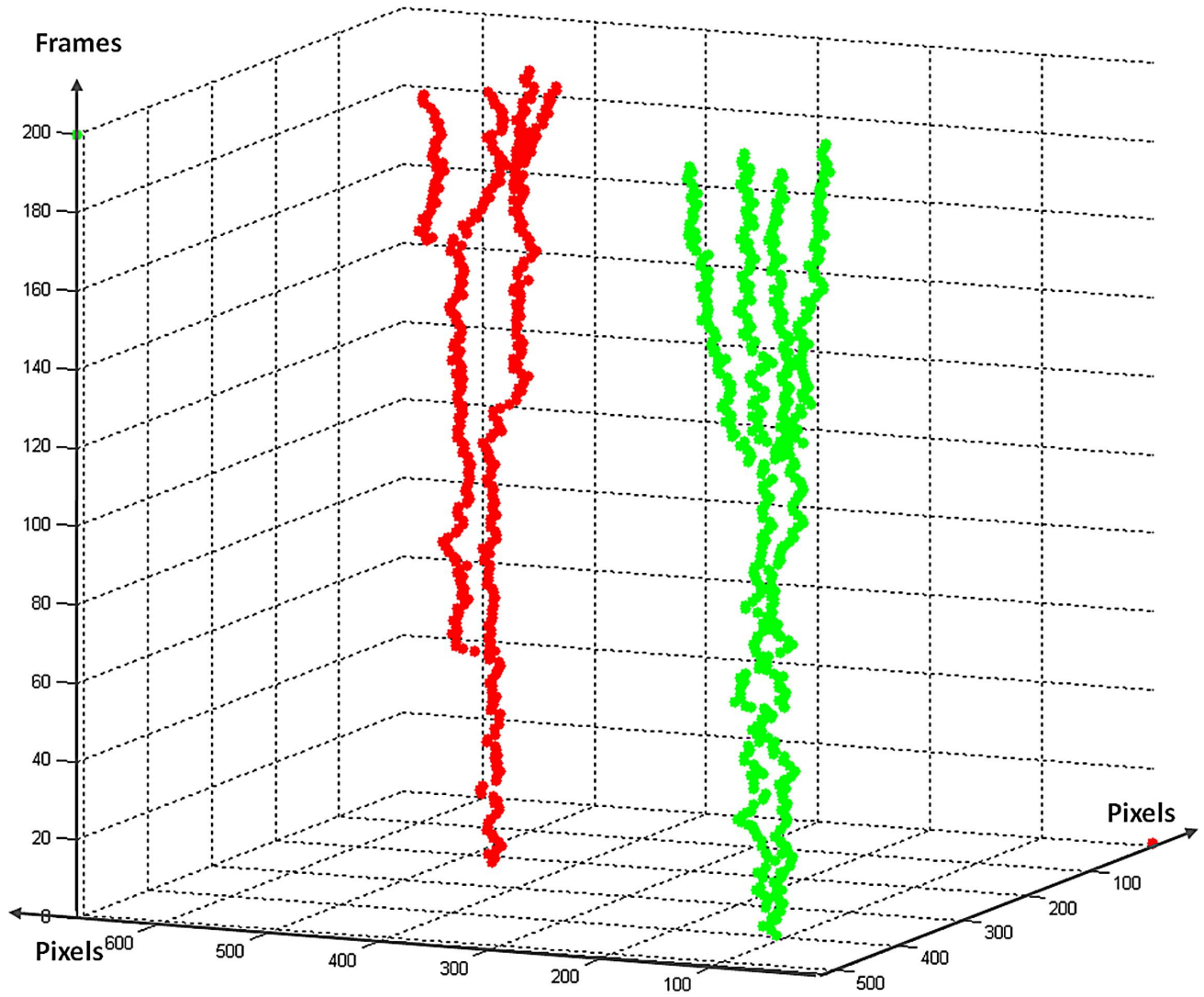

Figure 12. Examples of cell migration trajectories. Different colors represent different trajectories.
doi:10.1371/journal.pcbi.1003043.g012

number of all possible associations among objects in frame t and frame $t+1$. $\mathbf{S}$ is a $1\times N$ similarity matrix, and $\mathbf{S}(j) = S(\mathbf{c}_{kt+1}|\mathbf{c}_{it})$. For the unmatched cells, e.g., the new born or new entered cells, a linking process is usually needed to link them to the parent cells or as a new trajectory. This optimal matching strategy was also used to link the object trajectory segments in [27] to link the broken or newly appearing trajectories.

As an alternative to frame-by-frame association strategies, Bayesian filters, e.g., Particle filter and Interacting Multiple Model (IMM) filters [117,118], are also used for object tracking. The goal of these filters is to recursively estimate a model of object migration in an image sequence. Generally, in the Bayesian methods, a state vector, $\mathbf{x}_t$, is defined to indicate the characters of objects, e.g., position, velocity, and intensity. Then, two models are defined based on the state vector. The first is the state evolution model, $\mathbf{x}_t = f_t(\mathbf{x}_{t-1})+\varepsilon_t$, where f_t is the state evolution function at time point, t, and ε_t is a noise, e.g., Gaussian noise, which describes the evolution of the state. The other is the observation model, $\mathbf{z}_t = h_t(\mathbf{x}_{t-1})+\eta_t$, where h_t is the map function, and η_t is the noise, which maps the state vector into observations that are measurable in the image. Based on the two models and Bayes' rule, the posterior density of the object state is estimated as follows: $p(\mathbf{x}_t|\mathbf{z}_{1:t}) \propto p(\mathbf{z}_t|\mathbf{x}_t)\, p(\mathbf{x}_t|\mathbf{z}_{1:t-1})$, and $p(\mathbf{x}_t|\mathbf{z}_{1:t-1}) = \int p(\mathbf{x}_t|\mathbf{x}_{t-1}) p(\mathbf{x}_{t-1}|\mathbf{z}_{t-1}) d\mathbf{x}_{t-1}$ where the $p(\mathbf{z}_t|\mathbf{x}_t)$ is defined based on the observation model, and the $p(\mathbf{x}_t|\mathbf{x}_{t-1})$ is defined based on the state evolution model. The basic principle of particle filter is to approximate the posterior density by a set of samples (particles) being stochastically drawn, and it had been employed for object tracking in fluorescent images in [119–121]. In some biological studies, the motion dynamics of objects are complex. Therefore, one motion model might not be able to describe object motion dynamics well. The IMM filter is employed to incorporate multiple motion models, and the motion model of objects can be transitioned from one to another in the next frame with certain probabilities. For example, the IMM filter with three motion models, i.e., random walk, first-order, and second-order linear extrapolation, was used for 3D object tracking in [118], and for 2D cell tracking in [27].

3.4 Image Visualization

Most of the aforementioned software packages provide functions to visualize 2D images and the analysis results. However,

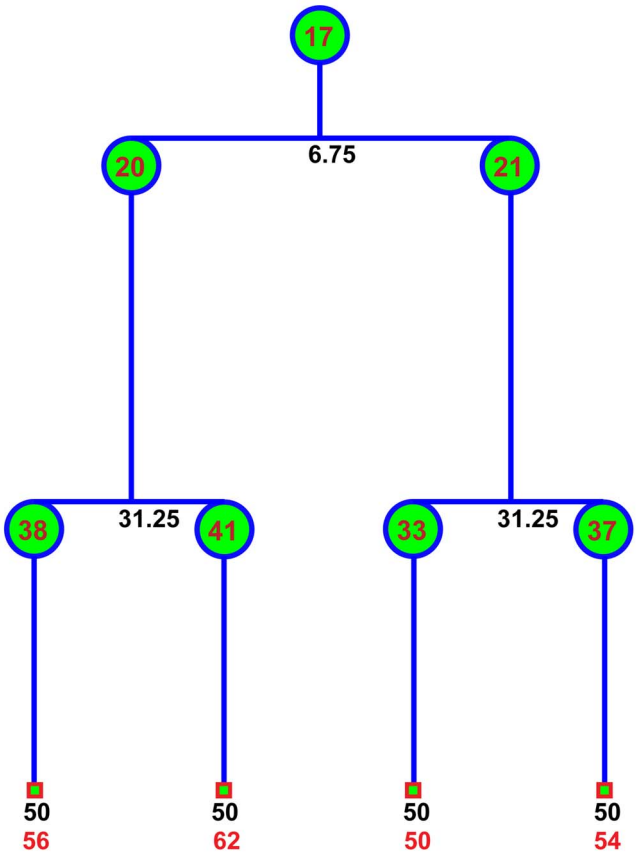

Figure 13. Examples of cell lineages constructed by the tracking algorithm. The black numbers are the time of cell division (hours). The bottom red numbers indicate the number of traces, and the numbers inside circles are the labels of cells in that frame.
doi:10.1371/journal.pcbi.1003043.g013

for higher dimensional images, e.g., 3D, 4D (including time), and 5D (including multiple color channels), visualization is challenging. Fiji [18], Icy [19], and BioimageXD [29], for example, are the widely used bioimage analysis and visualization software packages for higher dimensional images. In addition, NeuronStudio [46,47] is a software package tailored for neuron image analysis and visualization. Farsight [122] and vaa3D [123] are also developed for analysis and visualization of 3D, 4D, and 5D microscopy images. For developing customized visualization tools, the Visualization Toolkit (VTK) is a favorite choice (http://www.vtk.org/) as it is open source and developed specifically for 3D visualization. ParaView (http://www.paraview.org/) and ITK-SNAP (http://www.itksnap.org/) are the popular Insight Toolkit (ITK) (http://www.itk.org/) and VTK based 3D image analysis and visualization software packages.

This section has introduced a number of major methods for object detection, segmentation, tracking, and visualization in bioimage analysis. These analyses are essential and provide a basis for the following quantification of morphological changes.

4. Numerical Features and Morphological Phenotypes

4.1 Numerical Features

To quantitatively measure the phenotypic changes of segmented objects, a set of descriptive numerical features are needed. For example, four categories of quantitative features, measuring morphological appearances of segmented objects, are widely used in imaging informatics studies for object classification and identification, i.e., wavelets features [124,125], geometry features [126], Zernike moment features [127], and Haralick texture features [128]. In brief, Discrete Wavelet Transformation (DWT) features characterize images in both scale and frequency domains. Two important DWT feature sets are the Gabor wavelet [129] and the Cohen–Daubechies–Feauveau wavelet (CDF9/7) [130] features. Geometry features describe the shape and texture features of the individual cells, e.g., the maximum value, mean value, and standard deviation of the intensity, the lengths of the longest axis, the shortest axis, and their ratio, the area of the cell, the perimeter, the compactness of the cell (*compactness = perimeter^2/4π*area*), the area of the minimum convex image, and the roughness (*area of cell/area of convex shape*). The calculation of Zernike moments features was introduced in [131]. First, the center of mass of the cell image was calculated, then the average radius for each cell was computed, and the pixel p(x, y) of the cell image was mapped to a unit circle to obtain the projected pixel as p(x′, y′). Then Zernike moment features were calculated based on the projected image I(x′, y′). The Haralick texture features are extracted from the gray-level spatial-dependence matrices, including the angular second moment, contrast, correlation, sum of the squares, inverse difference moment, sum of the average, sum of the variance, sum of entropy, entropy, difference of the variance, difference of entropy, information measures of correlation, and maximal correlation coefficient [132]. More descriptions and calculation programs about these Subcellular Location Features (SLF) and SLF-based machine learning approaches for image classification can be found at: http://murphylab.web.cmu.edu/services/SLF/features.html.

4.2 Phenotype Identification

Although these numerical features are informative to describe the phenotypic changes, it can be difficult to understand these changes in terms of visual and understandable phenotypic changes. For example, the increase or decrease of cell size can be understood; however, it is not clear what the physical meaning of the increase or decrease is for certain wavelet features. Therefore, transforming the numerical features into biologically meaningful features (phenotypes) is important. This section introduces a number of widely used phenotype identification approaches.

4.2.1. Cell cycle phase identification. In cell cycle studies, drug and target effects are indicated by the dwelling time of cell cycle phases, e.g., interphase, prophase, metaphase and anaphase. Additional cell cycle phases, e.g., Prometa-, Ana 1-, Ana 2-, and Telophases, were also investigated in [133] and [23,134]. After object segmentation and tracking, cell motion traces can be extracted, as shown in Figure 14, and then the automated cell cycle phase identification is needed to calculate the dwelling time of individual cells on different phases.

Cell cycle phase identification can be viewed as a pattern classification problem. The aforementioned numerical features, and a number of classifiers can be used to identify the corresponding phases of individual segmented cells, e.g., support vector machine (SVM) [115,133,135], K-nearest neighbors (KNN), and naïve Bayesian classifiers [114]. However, the classification accuracy is often poor for cell cycle phases appearing for a short time, e.g., prophase and metaphase, due to the unbalance of sample size compared to interphase, and the segmentation bias. Fortunately, the cell cycle phase transition rules, e.g., from interphase to prophase, and from prophase to metaphase, can be used to reduce identification errors. Thus, a set of cell cycle phase identification approaches based on the cell tracking results were proposed to achieve high identification accuracy. This problem is often formulized as follows, and as shown in Figure 15. Let $\mathbf{x}=(x_1, x_2, \ldots, x_T)$ denote a cell image sequence of length T. Each cell image is represented by a numerical feature vector $\varphi(x_i)\in R^d$ (using the aforementioned numerical features). Let $\mathbf{y}=(y_1, y_2, \ldots, y_T)$ represent the corresponding cell cycle phase sequence that needs to be predicted. Based on the cell cycle progression rules, for example, the variation of nuclei size and intensity were used as an index to identify the mitosis phases of cells in [25], and Hidden Markov Modeling (HMM) was used to identify the cell cycle phases in CellCognition [23]. In brief, the transition possibility from one phase to the other was learned from the training data of cell cycle progressions, which could improve the accuracy of cell cycle phase identification. As an extension of HMM, Temporally Constrained Combinatorial Clustering (TC3), which is an unsupervised learning approach for cell cycle phase identification, was designed and combined with Gaussian Mixture Model (GMM) and HMM to achieve robust and accurate cell cycle identification results in [134]. Also, in [133] Finite State Machine (FSM) was employed to check the phase transition consistency and make corrections to the error cell cycle phases predicted by using SVM classifier [115]. Moreover, the cell cycle phases could be identified during the segmentation and linking process in the spatiotemporal volumetric segmentation-based tracking methods [110–112].

4.2.2 User defined phenotype, identification, and classification. In certain image-based studies, cells may not have an intrinsic phenotype, e.g., cell cycle phases, but may exhibit unpredicted and novel phenotypes caused by experimental perturbations, e.g., drugs or RNAi treatments. These phenotypes are often defined by well-trained biologists to characterize drug and target effects [16]. Figure 16 shows images of *Drosophila* cells with three defined phenotypes: Normal, Ruffling and Spiky [136].

In large scale screening studies, however, it is subjective and time-consuming for biologists to uncover novel phenotypes from millions of cells. Thus, automated discovery of novel phenotypes is important. For example, an automated phenotype discovery method was proposed in [20]. In brief, a GMM was constructed first for the existing phenotypes. Then the quantitative cellular data from new cellular images were combined with samples generated from the GMM, and the cluster number of the combined data was estimated using gap statistics [137]. Then, clustering analysis was performed on the combined data set, in which some of the cells from the new cellular images were merged into the existing phenotypes, and the clusters that could not be merged by any existing phenotype classes were considered as new phenotype candidates. After the phenotypes are defined, classifiers can be built conveniently based on the training data and the numerical features for classifying cells into one of the predefined phenotypes. However, it is tedious to manually collect enough training samples of the rare and unusual phenotypes. To solve this challenge, an iterative machine learning based approach was proposed in [138]. First, a tentative rule (classifier) was determined based on a few samples of a given phenotype, and then the classifier presented users a set of cells that were classified into the phenotype based on the tentative rule. Users would then manually correct the classification errors, and the corrections are used to refine the rule. This method could collect plenty of training samples after several rounds of error correction and rule refinement [138].

This section introduced numerical feature extraction, phenotype identification, and classification. These analyses provide quantitative phenotypic change data for identifying candidate targets and drug hits that cause desirable phenotypic changes. The following section will describe approaches to analyze the quantitative phenotypic profile data for drug and target identification.

5. Multidimensional Profiling Analysis

The aim of profiling analysis is to characterize the functions of drugs and targets, divide them into groups with similar phenotypic changes, and identify the candidates causing desired phenotypic changes. To help analyze and organize these multidimensional phenotypic profile data, some publicly available software packages have been designed, for example, CellProfiler Analyst (http://www.cellprofiler.org/) and PhenoRipper (http://www.phenoripper.org). In addition, KNIME (http://www.knime.org/) is a publicly available pipeline and workflow system to help organize different data flows. It also provides connections to bioimage analysis software packages, e.g., Fiji [18] and CellProfiler [9], and enables users to conveniently build specific data analysis pipelines in KNIME. This section describes some prevalent approaches in analyzing quantitative phenotypic profile data.

5.1 Clustering Analysis

Clustering analysis is to divide experimental perturbations, e.g., drugs, RNAis, into groups that have similar phenotypic changes. As clustering analysis approaches, e.g., Hierarchical Clustering [139] and Consensus Clustering [140], are well established, their technical details will not be discussed here. In addition to the aforementioned software, Cluster 3.0 (http://www.falw.vu/~huik/cluster.htm) and Java TreeView (http://jtreeview.sourceforge.net/) are two additional easy-to-use clustering analysis software packages available in public domain.

5.2 SVM-based Multivariate Profiling Analysis

SVM classifier was employed for analyzing the multivariate drug profiles in [141]. To measure the phenotypic change caused by drug treatments, the cell populations harvested from the drug-treated wells were compared with cells collected from the control wells (no drug treatment). The difference between the control and drug treatment was indicated by two factors that are the outputs of the SVM classifier. One is the accuracy of classification, which indicates the magnitude of the drug effect. The other is the normal vector (d-profile) of the hyperplane separating the two cell populations, which indicates the phenotypic changes caused by the drug. Figure 17 illustrates the idea; the yellow arrow is the d-profile indicating the direction of drug effects in the

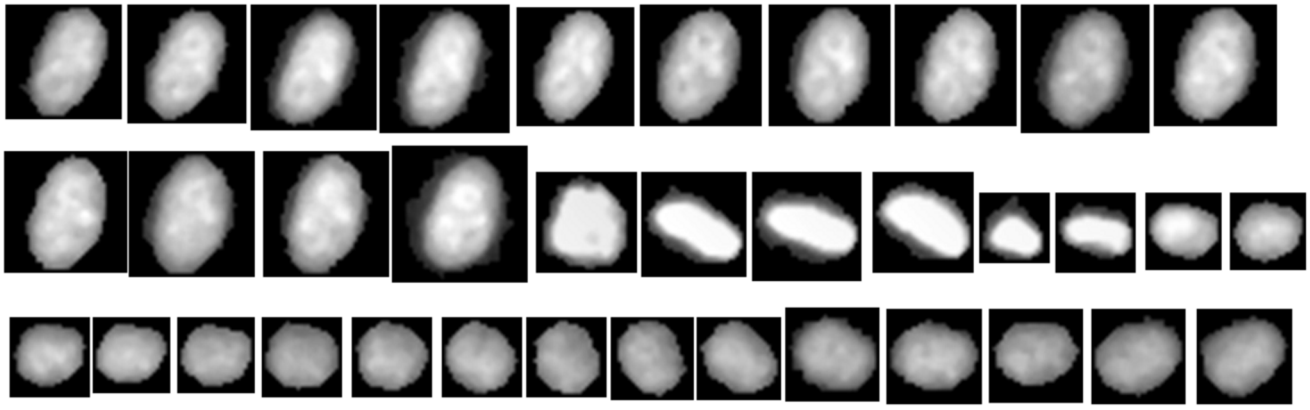

Figure 14. A segment of cell cycle procession sequence. Four cell cycle phases, interphase, prophase, metaphase, and anaphase, appear in order.
doi:10.1371/journal.pcbi.1003043.g014

phenotypic feature space. Drugs with similar d-profiles were found to have the same functional targets, and thus it could be used to predict functions of new drugs or compounds.

5.3 Factor-based Multidimensional Profiling Analysis

In the set of numerical features, some are highly correlated within groups but poorly correlated with features in other groups. One possible explanation is that the features in one group measure a common biological process, such as increase or decrease of nuclei size. The challenge using these numerical features directly is that biological meanings of certain phenotypic features are often vague. It is thus difficult to explain the phenotypic changes represented by these numerical features as aforementioned. To remove the redundant features and make the biological meanings of numerical features explicitly clear, factor analysis was employed in [12]. The basic principle of factor analysis is to determine the independent common 'traits' (factors). Mathematically it is formulated by the following equation.

$$\begin{bmatrix} x_{11},x_{12},\ldots,x_{1n} \\ x_{21},x_{22},\ldots,x_{2n} \\ \ldots \\ x_{m1},x_{m2},\ldots,x_{mn} \end{bmatrix} = X_{mn}$$

$$= \boldsymbol{\mu}_{mn} + L_{mk}F_{kn} + \varepsilon_{mn}$$

where $\boldsymbol{\mu}_{mn}$ is the mean value of each row, F_{kn} denotes the k factor, and the L_{mk} is the loading matrix, which is the coordinates of the n samples in the new k-dimensional space. In other words, k factors are independent and are the underlying biological processes that regulate the phenotypic changes. For example, six factors representing nuclei size, DNA replication, chromosome condensation, nuclei morphology, Edu texture, and nuclei ellipticity, were obtained through factor analysis in [12].

5.4 Subpopulation-based Heterogeneity Profiling Analysis

In image-based screening studies, heterogeneous phenotypes often appeared within a cell population, as shown in Figures 2 and 16, which indicated that individual cells responded to perturbations differently [142]. However, the heterogeneity information was ignored in most screening studies. To better make use of the heterogeneous phenotypic responses, a subpopulation based approach was proposed to study the phenotypic heterogeneity for characterizing drug effects in [13], and distinguishing cell populations with distinct drug sensitivities in [14]. The basic principle of the subpopulation based

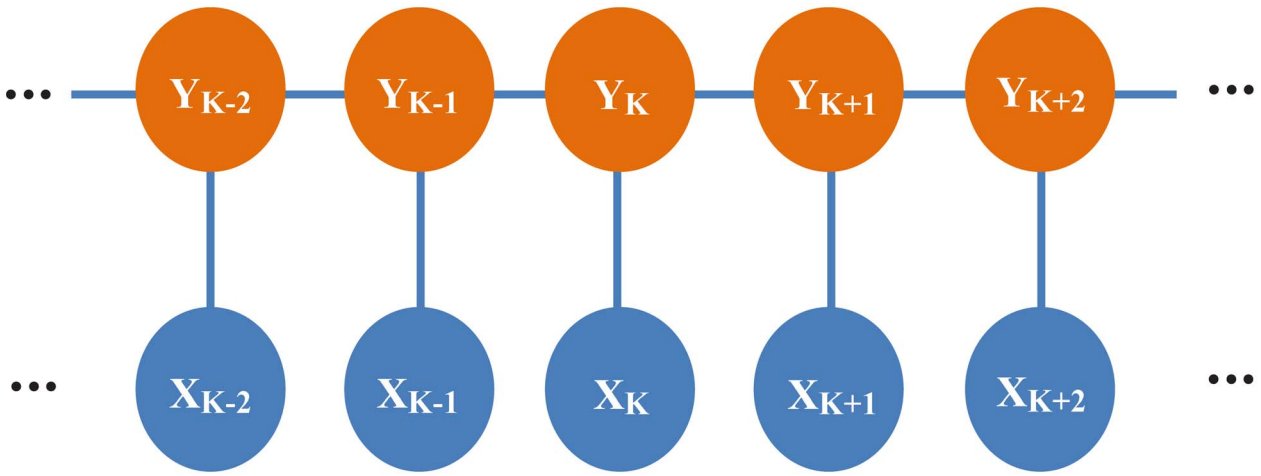

Figure 15. The graphical representation of cell cycle phase identification.
doi:10.1371/journal.pcbi.1003043.g015

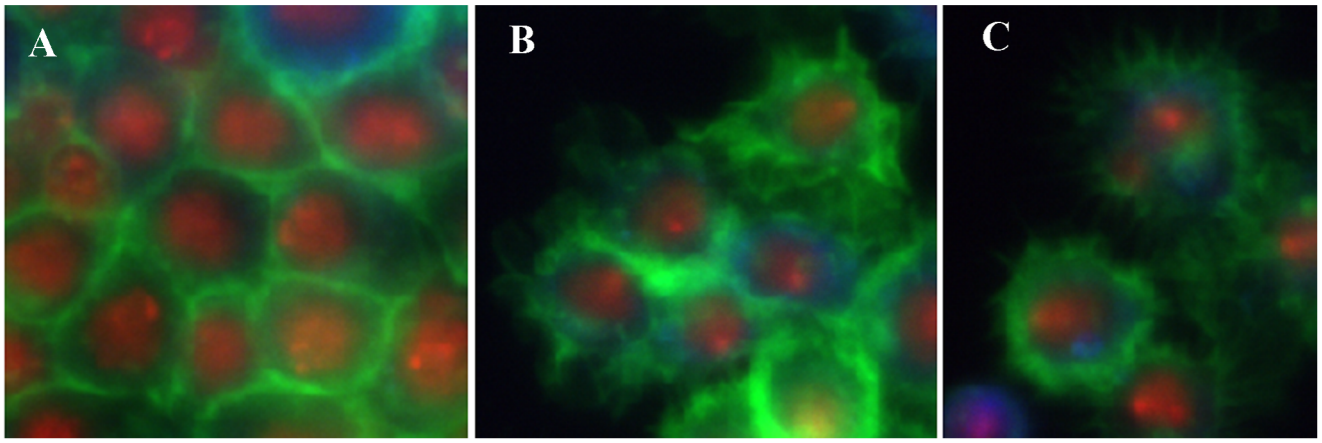

Figure 16. A representative image of *Drosophila* cells with three phenotypes: (A) Normal, (B) Ruffling and (C) Spiky phenotypes.
doi:10.1371/journal.pcbi.1003043.g016

method is to characterize the phenotypic heterogeneity with a mixture of phenotypically distinct subpopulations. This idea was implemented by fitting a GMM in the numerical space, and each model component of the GMM represents a distinct subpopulation. To profile the effects of perturbations, cells collected from perturbation conditions were first classified into

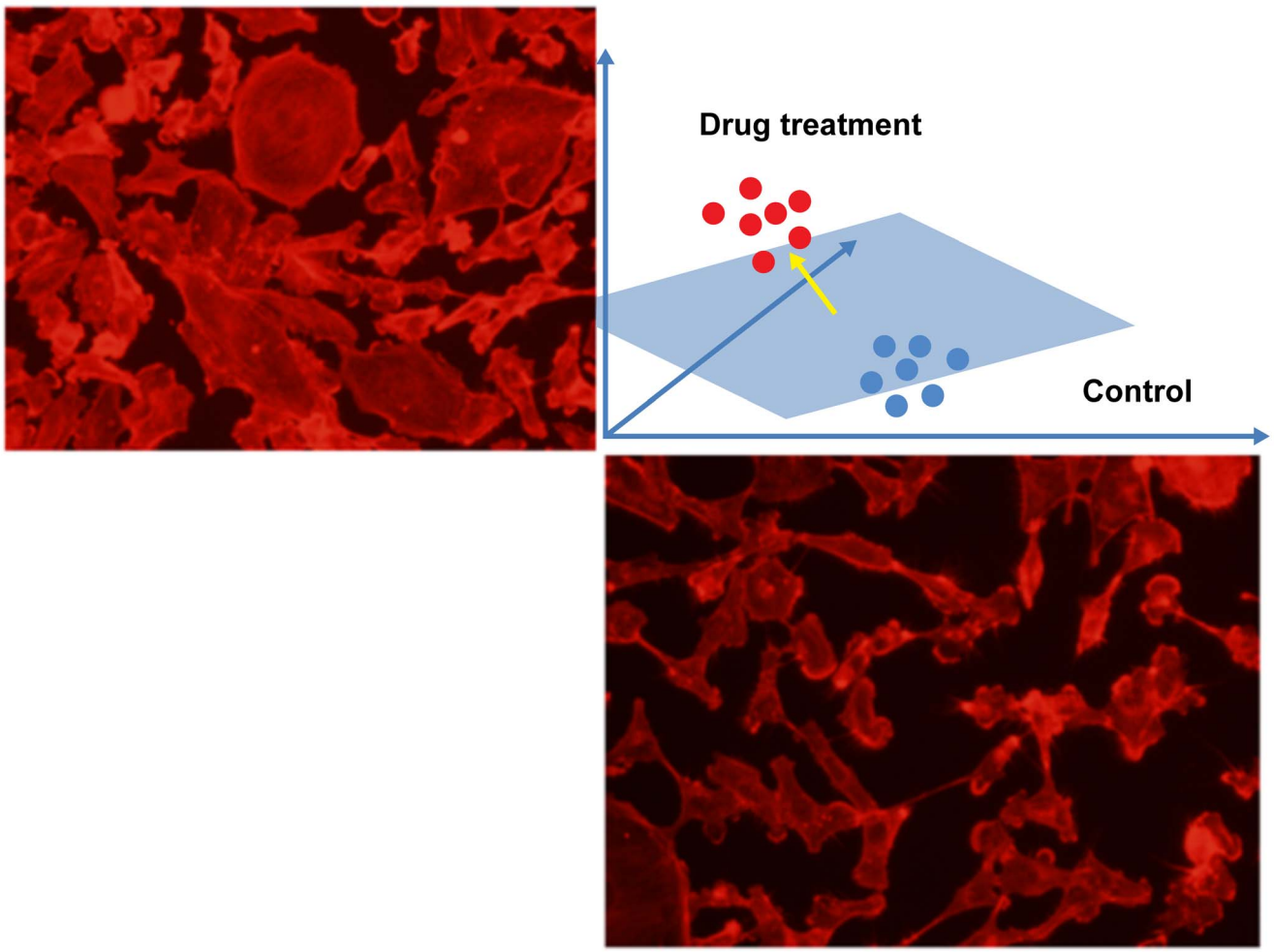

Figure 17. An illustration of drug profiling using the normal vector of hyperplane of SVM. The red and blue spots indicate the spatial distribution of cells in the numeric feature space. The yellow arrow represents the normal vector of the hyperplane (the blue plane). The top left and bottom right (MB231 cell) images are from drug treated and control conditions respectively.
doi:10.1371/journal.pcbi.1003043.g017

Table 1. List of publicly available bioimage informatics software packages.

Name	Link	Basic Functions
ImageJ	http://rsb.info.nih.gov/ij/	General image analysis with rich plugins
Fiji (A distribution of ImageJ)	http://fiji.sc/	Bioimage analysis with rich plugins
CellProfiler	http://www.cellprofiler.org/	Bioimage analysis with rich analysis pipelines
CellProfiler Analyst	http://www.cellprofiler.org/	Screening data analysis with machine learning approaches
Icy	http://icy.bioimageanalysis.org/index.php	Bioimage analysis
BioimageXD	http://www.bioimagexd.net/	3D Bioimage analysis and Visualization
PhenoRipper	http://www.phenoripper.org	Bioimage analysis for rapid exploration and interpretation of bioimage data in drug screening
FarSight	http://www.farsight-toolkit.org/wiki/Main_Page	Dynamic Biological Microenvironments from 4D/5D Microscopy Data
Vaa3D	http://penglab.janelia.org/proj/v3d/V3D/About_V3D.html	Bioimage visualization and analysis
Cell Analyzer	http://penglab.janelia.org/proj/cellexplorer/cellexplorer/What_is_Cell_Explorer.html	*C. elegans* image analysis
AceTree and StarryNite	http://starrynite.sourceforge.net/	*C. elegans*' embryo cell tracking and lineage reconstruction
Ilastik	http://www.ilastik.org/	Image classification and segmentation
Image Quantitators (ZFIQ, DCELLIQ, GCELLIQ, NeuriteIQ, NeuronIQ)	http://www.methodisthealth.com/bbpsoftware	A set of image analysis software packages for cell tracking in time-lapse images, and RNAi cell, neuron, neurite and Zebrafish image analysis
CellCognition	http://cellcognition.org/software/cecoganalyzer	Cell tracking in time-lapse image analysis
TLMTracker	http://www.tlmtracker.tu-bs.de/index.php/Main_Page	Cell tracking in time-lapse image analysis
NeuronJ	http://www.imagescience.org/meijering/software/neuronj/	Neurite Tracing and Quantification
NeurphologyJ	http://life.nctu.edu.tw/~microtubule/neurphologyJ.html	Neuron image analysis
NeuronStudio	http://research.mssm.edu/cnic/tools-ns.html	Neuron image analysis
CellOrganizer	http://cellorganizer.org/	Synthetically model and simulate fluorescent microscopic cell images
SimuCell	http://www.simucell.org	Synthetically model and simulate fluorescent microscopic cell images
PatternUnmixer	http://murphylab.web.cmu.edu/software/PatternUnmixer2.0/	Model fundamental sub-cellular patterns
μManager	http://valelab.ucsf.edu/~MM/MMwiki/	Control of automated microscopes
ScanImage	http://openwiki.janelia.org/wiki/display/ephus/ScanImage%2C+Ephus%2C+and+other+DAQ+software	Control of automated microscopes
OME	http://www.openmicroscopy.org/site	Image Database Software
Bisque	http://www.bioimage.ucsb.edu/bisque	Image Database Software
OMERO.searcher	http://murphylab.web.cmu.edu/software/searcher/	Content-based bioimage search
KNIME	http://www.knime.org/example-workflows	Workflow system for data analytics, reporting and integration

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one of the subpopulations, and then the portions of cells belonging to each subpopulation were calculated as features to further characterize the effects of perturbations. For more details, please refer to [13,14].

6. Publicly Available Bioimage Informatics Software Packages

A number of commercial bioimage informatics software tools e.g., GE-InCellAnalyzer [143], Cellomics [144], Cellumen [145], MetaXpress [146], BD Pathway [147] have been developed and are widely used in pharmaceutical companies, and academic institutions. In addition to the commercially available software packages, there are a number of publicly available bioimage informatics software packages [9], which provide even more powerful functions with cutting-edge algorithms and screening-specific analysis pipelines. For the convenience of finding these popular software packages, they are listed in Table 1. It is difficult to summarize all of their capabilities and functions because many of them are designed for flexible bioimage analysis with a set of diverse plugins and function modules, e.g., Fiji, CellProfiler, Icy, and BioimageXD. The software selection for specific applications is also non-trivial, and the best way might be to check their websites and online documents. In addition to the bioimage informatics software packages, there are other software packages, including the microscope control software for image acquisition (μManager and ScanImage) and image database software (OME, Bisque and OMERO.searcher). Also, certain cellular image simulation software packages, e.g., CellOrganizer and SimuCell, provide useful insights into the organizations of proteins of interest within individual cells. These software packages represent the prevalent directions of bioimage informatics research, thus their websites and features are worth checking.

7. Summary

With the advances of fluorescent microscopy and robotic handling, image-

based screening has been widely used for drug and target discovery by systematically investigating morphological changes within cell populations. The bioimage informatics approaches to automatically detect, quantify, and profile the phenotypic changes caused by various perturbations, e.g., drug compounds and RNAi, are essential to the success of these image-based screening studies. In this chapter, an overview of the current bioimage informatics approaches for systematic drug discovery was provided. A number of practical examples were first described to illustrate the concepts and capabilities of image-based screening for drug and target discovery. Then, the prevalent bioimage informatics techniques, e.g., object detection, segmentation, tracking and visualization, were discussed. Subsequently, the widely used numerical features, phenotypes identification, classification, and profiling analysis were introduced to characterize the effects of drugs and targets. Finally, the major publicly available bioimage informatics software packages were listed for future reference. We hope that this review provided sufficient information and insights for readers to apply the approaches and techniques of bioimage informatics to advance their research projects.

8. Exercises

Q1. Understand the principle of using green fluorescent protein (GFP) to label the chromosome of HeLa cells.

Q2. Download a cellular image processing software package, then download some cell images, and use them as examples to perform the cell detection, segmentation, and feature extraction, and provide the analysis results.

Q3. Download a time-lapse image analysis software package, then download some time-lapse images, and use them as examples to perform cell tracking, and cell cycle phase classification, and provide the analysis results.

Q4. Download a neuron image analysis software package, then download some neuron images, and use them as examples to perform dendrite and spine detection, and provide the analysis results.

Q5. Implement the watershed and level set segmentation methods by using ITK functions (http://www.itk.org/) and test them on some cell images.

Answers to the Exercises can be found in Text S1.

Supporting Information

Text S1 Answers to Exercises.
(DOCX)

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Further Reading

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Glossary

- **Cellular phenotype:** A cellular phenotype refers to a distinct morphological appearance or behavior of cells as observed under fluorescent, phase contrast, or bright field microscopy.
- **Green fluorescent protein (GFP):** GFP is used as a protein reporter by attaching to specific proteins, and exhibiting bright green fluorescence when exposed to light in the blue to ultraviolet range.
- **Fluorescence microscope:** A fluorescence microscope is an optical microscope that uses higher intensity light source to excite a fluorescent species in a sample of interest.
- **High content analysis (HCA):** HCA focuses on extracting and analyzing quantitative phenotypic data automatically from large amounts of cell images with automated image analysis, computer vision and machine learning approaches.
- **High content screening (HCS):** Applications of HCA for screening drugs and targets are referred to as HCS that aims to identify compounds or genes that cause desired phenotypic changes.
- **RNA interference (RNAi):** RNAi is a biological process, in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules.
- **Automated image analysis:** Automated image analysis aims to quantitatively analyze images automatically by computer programs with minimal human interventions.
- **Object detection:** Object detection is to automatically detect locations of objects of interest in images.
- **Blob structure detection:** Blob structure detection is to detect positions of objects of interest that have circle, sphere like structures, e.g., nuclei and particles.
- **Tube structure detection:** Tube structure is to detect centerlines of objects that have long tube like structures, e.g., neuron dendrite and blood vessel.
- **Object segmentation:** Object segmentation is to automatically delineate boundaries of objects of interest in images.
- **Object tracking:** Object tracking is to identify the motion traces of objects of interest in time-lapse images.
- **Feature extraction:** Feature extraction is to quantify the morphological appearances of segmented objects by calculating a set of numerical features.
- **Phenotype classification:** Phenotype classification is to assign each segmented object into a sub-group that has distinct phenotypes from other sub-groups.
- **Cell cycle phase identification:** Cell cycle phase identification is to automatically identify the corresponding cell cycle phase that a given cell is in according to its morphological appearances.

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