Uncovering Morphoproteomic Relationships Using Probabilistic Graphical Models and Resource Description Framework Knowledgebases

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**Uncovering Morphoproteomic Relationships Using
Probabilistic Graphical Models and Resource Description
Framework Knowledgebases**

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DEDICATION

I would like to thank my wife, Olga, and my two wonderful sons, Alex and Max, for the support and love that they have given me. I would also like to thank my parents for the happy childhood and the values that they passed to me.

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ABSTRACT

The heterogeneity of individual patient responses to conventional drug therapies is one of the central problems in personalized medicine and has great impact on clinical outcomes. To address this problem a new field of morphoproteomics was recently introduced. Morphoproteomics is a new method aimed at comprehensive analysis of protein circuitries in diseased cells to design effective drug therapies for individual patient cases. However, due to the overwhelming amount of molecular information that needs to be processed, successful adoption of morphoproteomics will greatly depend on availability of a comprehensive computerized knowledgebase and intelligent retrieval technologies.

We have, therefore, initiated new research with the overall goal to develop informatics methods to support morphoproteomic studies. We integrate evidence and information extracted from Whole Slide Imaging (WSI) and Immunohistochemistry (IHC) as well as from a semantic “mashup” of publicly available knowledge sources to provide pathologists a comprehensive picture of morphoproteomic mechanisms. This dissertation introduces novel methods for improving IHC antibody/antigen test selection as well as uncovering morphoproteomic relationships using probabilistic graphical models and Resource Description Framework (RDF) graphs of biomedical knowledgebases. Our methods have great potential to bring a broad impact in to pathology and personalize medicine as well as to be extended to more general systems biology domain.

Chapter 1: Introduction

1.1 Challenges in Personalized Medicine

Traditionally the standards of medical care focused on studies of large patient populations. Clinical practices based on these standards seldom took into account the variability of individual patients' genetic make-up, which often resulted in significant levels of variability in terms of clinical outcomes. To address this problem a new model of *personalized medicine* was introduced with the overall goal of redirecting clinical practices toward more *individualized* care. The early attempts to customize patient care, however, were limited to incorporating family history, environmental conditions and circumstances into the diagnostic and therapeutic decision-making. With the advances in microarray technology, molecular profiling, genetic testing, molecular pathology and related fields, it became possible to define the genetic level of the individual patient's make-up to provide more refined diagnosis and treatment. There was, however, a number of new challenges that personalized medicine brought into the picture [1], with one of the most prominent being the *complexity of human cancer and drugs response to it*. A pathologist can no longer restrict his work to just identifying a type of malignancy through morphological examination of the tissue. The discoveries of new disease subclasses and phenotypes dictate the need to classify cancer into these more refined categories. Furthermore, pathology seems to be the medical specialty assigned to identify new drug targets, which will help oncologists design efficient therapies tailored to the specifics of individual patient cases. Therefore, pathology as a field of diagnostic medicine has been undergoing a rapid transformation in which molecular pathology is

gaining ultimate importance; but there are barriers as well. The rapid genome-wide analysis of singular nucleotide polymorphisms (SNPs), which can affect the individual patients responses to drugs, is still a challenge. Moreover, microarray-based expression profiling is not directly indicative of the location of expressed proteins, biomarkers of certain biological processes. For instance, nuclear expression of a protein can constitute its phosphorylation and activation of a signal transduction pathway involved in a tumorigenesis, whereas cytoplasmic expression of the same protein may not be significant. Such cellular translocation events can be detected by microscopic analysis of protein expression patterns on histopathological slides “highlighted” by immunohistochemistry (IHC). One of the first attempts to use this technique to uncover biochemical mechanisms of the individual patient cases and to find new unconventional drug targets was performed by Robert Brown, MD et al. [2, 3]. He and his collaborators used comprehensive assessment of the expression of antibody-linked proteins on histopathological slides to come up with hypotheses on why traditional therapies failed for some patients. They then proposed new drug targets and reported improved clinical outcomes [4, 5]. The new method called *morphoproteomics*.

1.2 Morphoproteomics and Its Challenges

Morphoproteomics is a new method aimed at comprehensive analysis of protein circuitries in diseased cells to design effective drug therapies in a *personalized medicine setting* [6]. It combines histopathology, immunohistochemistry, proteomics, molecular pathology and protein biochemistry to draw as complete a picture of involved cellular pathways as possible. Morphoproteomics helps to reveal possible inter- and intra- cellular

translocations of proteins, i.e., their expression in “unusual” cellular compartments and to create an overall schema of biochemical mechanisms in a given case. The generated schema can be used to identify unconventional drug targets and assess susceptibility to inhibitors and cytotoxic agents that can be combined in effective individualized combinatorial therapies. There have been a number of studies that demonstrated clinical utility of morphoproteomics [7-22]. The problem, however, lies in the fact that morphoproteomics analysis requires accessing a large number of biochemical mechanisms, something that many pathologists may find challenging. As previously demonstrated, the complexity of protein expression patterns creates a combinatorial explosion of data for pathologists to process [23, 24]. It is even more challenging to identify relevant biological pathways that support certain protein expression patterns. *In this dissertation we introduce a computerized knowledge base and retrieval methods to help pathologists extract relevant morphoproteomic information based on IHC protein expression patterns that would have great impact on their abilities to construct morphoproteomic schemas. This might be crucial in finding new drug targets for individual patient cases and therefore reduce the number of “genetic gaps” in personalized medicine setting.*

1.3 Review of Related Research

1.3.1 Theranostics

Several research groups focus on the use of IHC to improve pathology diagnostics and therapeutics, also known as *theranostics* [25]. There are also research groups that work on data integration problem and use Semantic Web technologies [26] to consolidate

online knowledge sources into semantic “mashup” that can be queried to produce useful knowledge for biomedical domain. These methods are summarized in Table 1.

Table 1 Related research

Research group	Imaging data	Genetic, genomic data	Proteomic data	Pathway data	Disease data	Ontology	Analysis
Robert Brown et al [6]	BFM with IHC slides, FISH	literature, expert knowledge	literature, expert knowledge	literature, expert knowledge	literature, expert knowledge	N/A	Manual, based on subjective impression of protein level expression and individual researchers' knowledge
Russell Higgins et al [27]	BFM IHC slides	expert knowledge literature	expert knowledge literature	expert knowledge literature	expert knowledge literature	N/A	Manual, based on subjective impression of protein level expression and individual researchers' knowledge
Michael Feldman [28]	BFM IHC with multi spectral CRI systems, Aperio WSI	expert knowledge literature	expert knowledge literature	expert knowledge literature	expert knowledge literature	N/A	Computer-based image processing algorithms. System for de-multiplexing stains.
Satya S. Sahoo et al [29]	N/A	Entrez Gene (EG) digital knowledge base	N/A	N/A	NCBI OMIM – database of genetic disorders	Gene Ontology (GO)	Sematic Web based framework provides storage of data as RDF, inference through OWL and ontology and querying through SPARQL
Satya S. Sahoo et al [30]	N/A	Entrez Gene (EG), HomoloGene	N/A	KEGG, Reactome, BioCyc	N/A	GO, Biopax	Sematic Web based framework provides storage of data as RDF, inference through OWL and ontologies , SPARQL
Charles Vaske et al [31]	N/A	Microarray Data	Microarray Data	NCI PID	N/A	BioPax	SPARQL, Factor Graphs modeling, multidimensional modeling using probabilistic inference
The proposed research	IHC WSI	EG, GEO	UniProtKB , HPRD	KEGG, Reactome, BioCyc, BioCarta, NCI PID	OMIM	GO, BioPAX, Uniprot, custom ontologies	Semi-automatic image processing, Sematic Web, RDF, OWL, rulebases, SPARQ, PGM

BFM – bright field microscopy, IHC – immunohistochemistry, FISH – fluorescence in situ hybridization, CRI – Cambridge Research and Instrumentation Inc, WSI – Whole Slide Imaging, NCBI – National Center for Biotechnology Information, RDF – Resource Description Framework, OWL – Web Ontology Language, SPARQL – Semantic Web query language, CV – computer vision, IM – image processing, NCI PID – National Cancer Institute Pathway Interaction database, PGM – Probabilistic Graphical Models, HPRD - Human Protein Reference Database, OMIM - NCBI Mendelian Inheritance in Man

Brown and his group at Department of Pathology in University of Texas Health Science Center at Houston pioneered new methods of *morphoproteomics* and *morphogenomics* [6] (first research group in Table 1). One of the main goals for these new methods is to guide targeted cancer therapies. By studying the morphology of protein expression (e.g. *nuclear* vs *plasmalemmal* vs *cytoplasmic* expression) from IHC

slides (morphoproteomics), the researchers try to explain underlying biological processes in a diseased tissue. The assumption behind their method is that a more thorough than “conventional” study of protein expression from IHC and FISH probes can point out what signal transduction pathways are activated, whether the drug worked or not, and finally, based on discovered facts, design effective cancer therapies (e.g. combinatorial therapies integrating *cytotoxic* and *differentiating agents* and *signal transduction inhibitors*). They use expert- and literature-derived knowledge as well as manual assessment of protein expression patterns by bright field microscopy (BFM) and semi-quantitative analyses of combined IHC scores to manually analyze biological processes for therapy design. They emphasize the fact that a response to the same therapy can be inconsistent across different patient cases. Because of that, more thorough analyses of companionate cells and processes need to be performed to attempt to resolve such inconsistencies.

Russell Higgins, Jennifer Blankenship and Marsha Kinney at Department of Pathology in University of Texas Health Science Center at San Antonio have published a work with an overall guidance for practicing pathologists for using IHC in diagnosis of Hodgkin and Non-Hodgkin lymphomas [27]. The authors suggest carefully designed panels to study and diagnose malignant hematopoietic tumors. They point out that an explosion in the number of antigens (proteins) in cluster designation system (CD) “*necessitates judicious use of carefully selected panels rather than a ‘shotgun approach’ to diagnosis.*” The researchers discuss expression patterns of many proteins associated with lymphomas and show how to determine cell lineage and to detect specific malignancies. Higgins et al use IHC to phenotype abnormal population detected by

conventional morphology on hematoxylin and eosin (H&E) stained slides, characterize an abnormal population detected by flow cytometry and to screen “suspected reactive” tissue for subtle abnormalities. The authors use conventional BFM with IHC to study protein expression patterns and expert knowledge for manual analysis of the biological processes. Higgins et al. also warned about the difficulties (pitfalls) in the interpretation of IHC data.

Michael Feldman at the University of Pennsylvania Health System and his collaborators at Cambridge Research & Instrumentation, Inc. (CRi), Rutgers University and Rensselaer Polytechnic Institute have been practicing a new method that they named *slide-based histocytometry* [28]. They developed a set of staining protocols and a range of computational algorithms to analyze and interpret protein expression patterns for therapeutic and diagnostic purposes. The developed methods include protein quantification algorithms, color de-multiplexing approaches, image segmentation and tumor classification software. The authors base their analysis on conventional, expert and literature-derived knowledge of biological processes in a manual manner.

Sahoo et al. were among the first to show that publicly available electronic biomedical knowledge sources such as Entrez Gene (EG) [32], Gene Ontology (GO) [33] and OMIM [34] databases can be linked in a unified framework based on RDF [29]. This framework can supply not only the information *directly stored* in the above mentioned sources, but also information about *inferred* relationships. For instance, to find a relationship between not just a particular gene and a particular disease but also between related genes and related disorders would require writing specialized software or manual effort. This is because these online resources were primarily designed for consultation by

humans. With EG, OMIM and GO imported into Sematic Web repository based on Oracle 10g, the authors were able to demonstrate that such queries can be handled automatically. For example, a query: “*Find all genes annotated with glycosyltransferase-related terms in GO and associated with disease congenital muscular dystrophy in OMIM*” will return all genes carrying molecular function *glycosyltransferase* as well as all “descendants” with specialized types of *glycosyltransferase*, such as *acetylglucosaminyltransferase*. This is possible due to inference capabilities of Web Ontology Language OWL [35] and query language SPARQL [36]. The research was not concerned with disease diagnosis or treatment. Its primary goal was to *explore* Sematic Web capabilities for data integration of publicly available biomedical sources to answer complex biomedical questions.

In another work [30] Sahoo et al. added information about cellular pathways. They created the Entrez Knowledge Model (EKoM) genetic semantic “mashup” and then incorporated knowledge from KEGG [37], Reactome [38] and BioCyc [39] pathways databases with the help of standardized Biopax ontology [40]. By using pathway information, researchers had demonstrated that even simple SPARQL queries could easily identify *hub* genes, i.e., genes that participate in a large number of pathways or interact with many other gene products.

In [31] Vaske et al. used breast cancer and glioblastoma multiform microarray data to infer gene involvement in signal transduction pathways. They modeled it by factor graphs and probabilistic inference using a proposed PARADIGM method. The main goal was to generate a matrix of integrated pathway activity (IPA) for each gene and assess involvement of each gene in a pathway using statistics. The involvement of a gene in a

pathway is assessed based on a deviation of a gene measurement from a null distribution, which they generate based on "within" permutation. They then ordered pathways by gene involvement and visualized using a novel CircleMaps tool. The authors claim that their factor graph based modeling can incorporate several functional aspects for each gene like DNA methylation, copy number, overexpression etc.

As can be seen from this review of related research, the first two groups are concerned with pathology theranostics issues and employ *tissue-based proteomics* methods (i.e., IHC). However, they use traditional BFM tools and expert knowledge coupled with the review of the literature to analyze IHC protein expression patterns and make sense out of it. The next group in Table 1 uses advanced WSI and multispectral microscopy imaging (CRi) technologies in addition to BFM. They also develop computer algorithms and design staining protocols for more quantitative analysis of IHC protein expression. But they still use expert knowledge to recreate a picture of biological processes. The Sahoo et al. group, on the other hand, utilizes computer knowledge-based systems to derive biological knowledge. Their frameworks, however, are not linked to any *imaging data* and are not designed to recreate the morphoproteomic picture of biological processes in a given tissue sample. Vaske et al. used microarray data to infer involvement of a gene in a pathway as well as curated data from NCI pathway database and BioPax ontology. However, data from microarray experiments lack locational information, i.e., it just shows expression (or methylation etc) levels but not where the particular gene product is expressed, which is an important diagnostic factor in pathology. For example, if a certain protein is expressed in the cytoplasm, it may be of no importance; if it is expressed in the nucleus, it may indicate phosphorylation and

activation of a "corresponding" pathway. Inconsistency of some high-throughput experiments and, therefore, inability to use it in a personalized medicine setting can be attributed to this lack of locational information.

1.3.2 Gene Regulatory Networks

Modeling of Gene Regulatory Networks (GRN) using computational means is a related research area that is also concerned with studying relationships among genes and gene products (protein, transcription factors). However, GRN modeling sets more “basic research” goals of *discovering or confirming biological networks* in which gene and their products interact rather than *identifying activated networks and mechanisms based on clinical tests*.

Here we briefly list traditional methods for modeling GRN that have been developed over the past decades.

Boolean Networks were first proposed by Kaufman [41] and have been since used to model GRNs [42]. In these networks each node represents a dichotomous variable, the two values of which code for *active* and *inactive* states of the underlying biological entity (e.g. gene). The edges of a Boolean Network correspond to Boolean operations AND, OR and NOT. The whole network of n nodes can be viewed as a binary feature vector going through 2^n -dimensional space of different states. Some equilibrium states can correspond to gene regulatory processes under certain conditions such as disease or immunoreactive response of an organism. The goal of learning Boolean Network is to find a Boolean function for each gene in such a way that it fits the observed data. There are a number of

algorithms for learning Boolean Network, including REVEAL by Liang et al. [43]. Boolean Networks are limited by the fact that each gene can be represented only by two states, the values of which are approximated by a discretization procedure. That raises a question as to what is the appropriate threshold to process continuous gene expression values. A new type of Probabilistic Boolean Networks (PBN) was proposed by Shmulevich [44] that overcomes the deterministic nature of the traditional Boolean Networks. PBNs model the stochastic nature of gene regulation by introducing the state transition mechanism that is dependent on a random variable. One example of usage of PBN to model GRNs is T lymphocytes receptor signaling in which PBN models immune response to different foreign entities [44].

Differential and Difference equations model dynamic aspects of GRN (i.e., changes in gene expression) [45]. Due to potential existence of multiple solutions for ordinary differential equations (ODE), some constraints are utilized to reduce complexity. Such constraints might be represented by a prior knowledge or other simplifications. It is believed that gene regulatory processes have a complex non-linear character. Therefore, even though “linearization” can make modeling simpler, linear models still are not sufficient to model large scale GRNs from gene expression data accurately [46]. Still, there are both linear and non-linear differential equations that can capture kinetics of GRNs, with the latter having limitations related to the computational difficulties.

Bayesian Networks (BNs), in which the structure of a network is represented by directed acyclic graph (DAG) and nodes are represented by random variables, can be used to capture stochastic nature of gene regulation. The expression values of each gene

are determined by the Bayes rule and local Markov property [47], which flexibly incorporates prior knowledge in the process of GRN inference to generate a correct network structure. There are several aspects of BNs that we will discuss in more detail in the Section 1.3.3 as well as in Chapter 3 as they represent the main underlying formalism that is used in this dissertation’s research methods.

There are a number of other GRN architecture approaches such as Petri Nets, Hybrid Models and Finite State Linear Models (FSLMs) which can all be viewed as extensions of graph models and have been successfully used to model, for example, metabolic networks or to incorporate discrete and continuous aspects of DNA expression. For a review of these models see Schlitt et al. [45].

1.3.3 Probabilistic Graphical Models

Probabilistic Graphical Models (PGMs) are a large family of representational and computational methods [47-54]. The common feature of all PGMs is that they all combine probability and graph theories to manage complexity of stochastic processes and provide means for inference. In fact, PGMs can be considered as an underlying fundamental formalism for many methods used in machine learning, statistics, physics and other fields. PGMs manage complexity through a decomposition of a large system into “digestible” parts, whose parameters can be successfully learned. These “parts” together represent one network that can then be used for inference. Depending on the type of decomposition there have been developed several constituent formalisms including Markov Random Fields (MRFs), Bayesian Networks (BNs) and recently

introduced Hybrid Random Fields (HRFs). Since our main research focus in this dissertation is a development of a methodology to manage complexity of IHC and morphoproteomic studies using BNs, we will briefly describe MRFs and HRFs and then discuss BNs in more detail.

The first usage of MRFs can be traced to the field of stochastic mechanics, to so-known Ising model [55]. MRFs represent a joint probability distribution of a stochastic process by an *undirected* graph, where each node is a random variable and each maximal *clique* (a sub-graph where all vertices are connected) is assigned a *potential function* φ_c . An argument of a potential function φ_c is a *state* of a clique C and output is a non-negative number. So, the overall joint probability distribution is computed as follows:

$$P(\mathbf{x}) = \frac{1}{Z} \prod_{\varphi_c} \varphi_c(\mathbf{x}_c) \quad \text{Eq. 1}$$

where $\mathbf{x}_c$ is a state of the clique C and Z is a normalization factor, which is the sum over all products of all possible configurations of $\mathbf{x}$. After some manipulations it can be shown that this distribution is actually a *Gibbs* distribution. The connection between *Gibbs* distribution and topological nature of MRF is better understood by the Hammersley-Clifford theorem. This theorem states that to say that a process can be modeled by Gibbs distribution is equivalent to saying that the process satisfies the Markov property and vice versa [56]. The Markov property reflects the statistical independence of a node in a network from all other nodes given the outcomes of that node's immediate neighbors. In context of MRFs it is called *Markov Blanket* and in context of BNs it is called *Directed Markov Property*. MRFs can be used to model processes where the direction of

connections among entities is not specified (e.g. correlation). It is relatively difficult to learn the structure of an MRF from experimental data.

Hybrid Random Fields [57] is a new approach that was designed to overcome some weak properties of BNs and MRFs. In particular, learning structure of a BN network can be very costly in a high-dimensional domain. Even though there are many heuristic methods for learning parameters and structures of BNs, these methods may not scale well because the number of possible DAGs grows exponentially with the number of nodes. Since heuristic methods search through the space of all possible DAGs, such methods may quickly become computationally intractable. In HRFs parameter learning is processed by *local* BNs, consisting of a set of only suitable variables. Therefore, the whole HRF is modeled as a union of such local BNs, each of which can be learned relatively efficiently. Both parameter and structure learning algorithms have been proposed by Freno et al. [56] for discrete distributions. Later, HRFs were extended to continuous variable domains.

BNs can be taken to be, perhaps, the most popular graphical methods to model conditional probability distributions. One of the first investigators to propose a rigorous mathematical formalism for BNs was Judea Pearl [52]. He introduced the notions of *d-separation*, *minimal I-Map* and *faithfulness condition* that are considered to be the main fundamental principles underlying all graphical models. In this dissertation Pearl formalism is exploited to derive causal relationships among proteins and signaling pathways (see Chapter 3). Namely, we extend *d-separation* principles into the domain of RDF graphs and apply it to generate a set of independence statements, which can then be

fed into structure learning algorithms. There are two main tasks associated with learning BNs: (i) parameter learning and (ii) structure learning.

Parameter learning in BNs is a task of assigning conditional probabilities to each node in a BN network, given that the BN's structure is provided. A probability is computed for all node values (discrete case) for every possible instantiation of parents' node values. For example in a binary variable BN network, if a node x_1 has two parents x_2 and x_3 , we need to learn eight conditional probabilities: $P(x_1=0|x_2=0, x_3=0)$, $P(x_1=0|x_2=0, x_3=1)$, $P(x_1=0|x_2=1, x_3=0)$, $P(x_1=0|x_2=1, x_3=1)$, $P(x_1=1|x_2=0, x_3=0)$, $P(x_1=1|x_2=0, x_3=1)$, $P(x_1=1|x_2=1, x_3=0)$, and $P(x_1=1|x_2=1, x_3=1)$. These conditional probabilities can be readily approximated using frequencies and computed from experimental data.

Structure learning is a more computationally challenging task because of the large number of possible DAGs that need to be considered. Two distinct approaches have been developed: (i) score-based structure learning and (ii) constrained-based structure learning [58].

In score-based structure learning, some kind of heuristic algorithm (e.g., *hill-climbing*) is used to iterate over DAG search space. Each DAG is assigned a score. The DAG with the best score is then selected as a solution. There are several types of scores that can be used including Minimum Description Length (MDL) [59] and BDe score [60, 61].

Constrained based structure learning of BNs exploits some kind of restrictions to generate a graph of a BN. SGS algorithm named after its authors Spirtes, Glymour and Scheines [53] is perhaps the most simple algorithm that tries to reconstruct a network by removing an edge in between two nodes if they are conditionally independent given sets of other nodes. Since, the number of such sets can be big, SGS algorithm can run very slow. The PC structure-learning algorithm optimizes search by pre-ordering the conditioned sets by their cardinality, from smallest to largest, so the complexity is bounded by the largest *degree* in the graph of a BN. The GS algorithm exploits sparseness of BN graphs to avoid processing a *fully connected* graph as is done in SGS and PC algorithms [58].

1.3.4 PGM and Semantic Web Knowledgebases

There have been a number of research projects involving probabilistic graphical models and Semantic Web technologies. Perhaps the most obvious way is to use RDF graphs to encode for probabilistic information (see for instance [62]). In this case, RDF plays a role of the “storage” for a conditional probability distribution. Potential benefits may include data integration between heterogeneous systems that need to exchange, for instance, BNs for processing. Since there is not a standard persistent model for BN, this RDF-based method for storing BN can find utilization in design of software systems.

There have also been attempts to derive the structure of BNs from ontologies [63-65]. Since, the main goal in these investigations was to show a possibility of an automatic reconstruction of a BN from ontology, the resulting network represented a graph of

ontological concepts rather their instances. For example, in [64] the resulting BN shows probabilistic relation among such concepts as “Threat” and “Asset.”. In our case, we are most interested to establish relationships not just among proteins and pathways as concepts (or families of concepts) but particular “instances” of proteins such as AKT, NF-kappa-B and others. To our knowledge, there has not been any work done to develop a methodology that uses RDF graphs of instance data knowledgebases to derive structure of a graphical model that reflects relationships among protein and pathways.

1.4 The Objectives of This Research

In this work we set two goals: 1) to develop a computerized method to manage complexity of different IHC tests, and 2) to develop a framework that will allow generating morphoproteomic schemas based on a *Semantic Web driven knowledge base* and probabilistic graphical models. The central part of the system is a semantic “mashup” of a *more comprehensive set of biomedical databases* than was used by Sahoo et al. (see Table 1). Moreover, the biomedical problem that this research tackles is more challenging than the one from Sahoo’s group. *We are interested not just in finding direct relationships between stand alone biological entities, but more in generating a comprehensive schema of processes involved in tumorigenesis, given protein expression patterns from a particular patient case.* We will use the words *antibody, antigen and protein* interchangeably in this document.

Chapter 2: A Pathologist-In-The-Loop Antibody Test Selection Using Entropy Based Probabilistic Method

This chapter introduces the ATOS - Antigen Tests Optimized Selection method, a novel informatics tool to help pathologists streamline IHC studies.

2.1 Overview

An extensive corpus of fundamental medical knowledge has been gained through decades of the scientific examination of *tissues stained with the basic hematoxylin and eosin stains*. Countless studies have identified precise diagnostic entities based on fine visual and logical distinctions evident from *alterations of cellular morphology* [66]. Even more precise diagnostic entities are being discovered based on *genomic and proteomic signatures of the diseases and their subclasses*; hence, it has become increasingly important to utilize methods of diagnostic molecular pathology to provide precise diagnosis as well at the *genomic/proteomic level*, which can lead to improved treatment and therefore better clinical outcomes. However, there are several challenges associated with genomic/proteomic studies.

Figure 1 A typical pathology diagnostic workflow

Fig. 1 depicts a typical pathology diagnostic workflow involving extensive iterative processes. A diagnostic process usually starts with some *preliminary hypothesis* (Step 1) after receiving initial information and gathering initial evidence. If further morphological observations and clinical findings (Step 2) reject that hypothesis (Step 3), a new hypothesis is generated (Step 4). However, if the evidence supports the hypothesis (Step 3), the pathologist may or may not immediately report it as a diagnosis. Instead, he or she might be interested in knowing if the diagnosis can be refined to reveal possible *disease subclasses* and/or additional important *genomic and proteomic characteristics* (Step 5). If so, a refined hypothesis is generated (Step 7). New evidence is then collected (Step 8) and analyzed to see if the refined hypothesis can be accepted (Step 3). Steps 3, 5, 7, and 8 can be repeated in a loop several times until after all possible refinement attempts, which can provide precise evidence for treatment, are exhausted. This diagnostic loop is a technique that pathologists use to gradually drill down to the most precise diagnosis. The

technique can be especially important in solving difficult cases and/or to tailor treatment to *individual patient genomic makeup* in a *personalized medicine* setting. Among the most important types of evidence being collected during the diagnosis refinement step are the results of application of antibody-linked stains, a.k.a. immunohistochemistry (IHC). IHC tests have permitted the highly specific identification of a diverse variety of cellular proteins that play an essential role in the molecular pathology of a wide range of diseases. These diagnostic tools have also allowed the emergence of new fields of *morphoproteomics* and *morphogenomics* [6], which have great potential to provide more specific and accurate diagnoses and, consequently, a more reliable *estimation of prognosis*. As a consequence of this transformative technology, great demands have been imposed upon the integrative intellectual skills of even the most experienced and well-trained surgical pathologists. The mental retention and recall of the large number of facts generated during diagnosis refinement in the interpretation of specific protein patterns in biopsies from even a single organ system can be problematic. For example, just for one family of diseases, lymphomas, which can have more than 60 variations [67], there could be more than 80 different antigens [68, 69], that may need to be analyzed before an interpretation of the underlying biological processes of a given case can be obtained [27]. Currently, the cluster designation system includes more than 400 antigens [70] and the number of proteins functioning as biomarkers keeps increasing with continuous developments in molecular biology [71-73]. Since pathologists can consider more than just two states of protein expression (e.g. *weakly expressed*, *strongly expressed* etc.), the number of all possible combinations of antigen expression creates a *combinatorial explosion* that a practicing pathologist can face during the diagnostic process.

Typically, IHC is performed using *panels of antibodies* ranging from five to a dozen or more as judged appropriate for the tissue and tumor being examined. The composition of a panel is selected to both confirm and rule out diseases for further diagnostic consideration. The exact content of antibody test panels and the sequence in which tests are performed can sometimes vary significantly from pathologist to pathologist (*inter-pathologist variability*). Moreover, the same pathologist may order different tests for very similar cases on different days (*intra-pathologist variability*). Such variability results in high inconsistency rates in pathology disease diagnosis [74]. It is very easy for a pathologist to overlook the inclusion of a certain antigen into the panel and miss an opportunity to obtain a crucial clue about the disease in question. On the other hand, a pathologist might include unnecessary tests in the panels with discriminatory power that may not be required. Therefore, it appears to be inefficient to design panels of fixed size to examine predetermined disease groups rather than to permit a *dynamic selection of the antibodies* required to evaluate a more realistic set of diseases with which the pathologist may be confronted.

Given the central diagnostic importance of IHC and predicted rapid increase in its utilization, it is timely to question whether there may be more effective and efficient schema for the implementation of IHC than those that have been developed empirically over decades. It is becoming increasingly important for modern healthcare to deliver cost-effective service in general and reduce cost of pathology and laboratory medicine in particular [75]. Hence, a method that can save both in pathologists' time and cost of reagents can find widespread application in the *Digital Pathology* era [76, 77], when

pathology diagnosis is more and more reliant on the assistance of information technology and computational tools [28].

Previously, there have been attempts with certain degrees of success to develop computerized methods to assist pathologists in their decision-making. Early studies include *Pathfinder* expert system created by Heckerman et al. [78]. While this method helps to organize the diagnostic process using etiological and morphological features, it is not suitable for the new challenges and complexity posed by increasing usage of IHC in the pathology workflow. A number of other works had used probabilistic methods to improve decision-making in pathology [79, 80]. More recent studies, using a non-computational approach, on solving problems due to the complexity of IHC tests include works by Taylor [68, 69].

We have identified a few distinct features of the diagnostic workflow discussed earlier (Fig. 1) that, in our view, make it hard to directly apply most of the abovementioned traditional methods or human heuristic approaches. First of all, due to its iterative nature, the *disease diagnosis refinement process* (Fig. 1) cannot be simply viewed as a *classification problem* mainly because there is not a clearly defined *final classification* goal for such a type of process. A pathologist keeps diagnosis refinement (into disease class, subclass, grade, therapeutic target etc) as long as he or she can obtain new information. Second of all, in such a diagnostic workflow, there is not a *fixed feature space*. In this work, we use *feature*, *fact*, *factor*, and *test* interchangeably. Instead, the process is based on a *dynamic set of facts at hand*. Depending on the *diagnostic path*, the process may require a different set of tests to be conducted. Thirdly, pathologists often use different tests to reveal the same biological mechanisms (e.g., proliferation of B-cells

can be identified by a test for CD20 *or* CD23 antigen). Therefore, the IHC *panel design philosophy* is a complex *mental process* aimed at constructing a *comprehensive* but *limited* set of IHC tests that can generate valuable information at each diagnostic refinement step.

The pathology community needs a robust computational tool that can propose a *preliminary diagnostic path* and dynamically suggest a set of IHC tests to be performed. To answer this need, we have developed a novel method that helps to streamline IHC studies by dynamically suggesting an efficient set of IHC tests at each step of the pathology workflow. Our approach, while not entirely new computationally, is a novel application of information entropies and Bayesian probabilities in IHC that can significantly improve speed of IHC diagnostic workflow and reduce intra- and inter-pathologist variability. Our tool also helps to visualize possible diagnostic paths, which brings a great deal of clarity into the diagnostic process.

2.2 Methods

2.2.1 Dataset

The data in our work is represented by a matrix of antigen expression profiles (*MAEP*) across multiple diseases. In general, this type of matrix can be constructed from existing medical literature for specific diseases. Table 2 shows a subset of the matrix used in this study.

Table 2 Excerpt from a matrix of antigen expression scores across multiple diseases

Disease Inc Rate	Antigens (ant expr probability)			
	CD11c	CD15	CD19	CD20
MCL 0.51	- 0.95	- 0.95	+ 0.95	+ 0.95
CLL/SLL 5.17	+ 0.95	ND	+ 0.95	+ 0.95
FL 3.18	- 0.95	- 0.95	+ 0.95	+ 0.95
MALT 2.35	+ 0.75	- 0.95	+ 0.95	- 0.95
MZL nodal 0.66	- 0.95	- 0.95	+ 0.95	+ 0.95
MZL splenic 0.31	- 0.95	- 0.95	+ 0.95	+ 0.95
LPL/WM 0.62	ND	- 0.95	+ 0.95	+ 0.95
HCL 0.33	+ 0.95	- 0.95	ND	+ 0.95
PCM 5.86	- 0.95	- 0.95	- 0.95	+ 0.75
Burkitt 0.3	- 0.95	- 0.95	+ 0.95	+ 0.95
DLBCL GC 3.21	- 0.95	- 0.95	+ 0.95	+ 0.95
DLBCL Non-GC 3.93	- 0.95	- 0.95	+ 0.95	+ 0.95
ALCL ALK + 0.25	ND	- 0.95	ND	- 0.95
MF 0.52	ND	- 0.95	- 0.95	- 0.95
B-ALL/LBL 0.76	ND	- 0.95	+ 0.95	- 0.75
T-ALL/LBL 0.22	ND	- 0.95	ND	- 0.95
PTCL NOS 0.3	ND	- 0.95	- 0.95	- 0.95
AITL 0.05	ND	- 0.95	- 0.95	+ 0.95
CHL 2.59	ND	+ 0.95	ND	- 0.75
NLPHL 0.08	ND	- 0.95	ND	+ 0.95

Rows and columns of this matrix A represent correspondingly *disease immunotypic profiles* across multiple antigens and *antigen expression profiles* across multiple diseases. Each row starts with the name of a disease followed, after a vertical bar, by that disease's incidence rate. Disease incidence rates for each malignancy were obtained from the Cancer Statistics Review data provided by the Surveillance Epidemiology and End Results (SEER) website, maintained by the National Cancer Institute [81]. We have to mention here that to tune ATOS to *geographically local disease incidents rates*, the data from regional cancer registries can be used. An element $A_{i,j}$ of the matrix gives information about expression of the antigen j in disease i and probability of getting such expression value. In the tests reported here, the antigen expression scores and their

probabilities were derived using primarily studies by Higgins et al. [27] and the WHO disease classification [67] for several families of non-Hodgkin and Hodgkin lymphoma. For simplicity and clarity purposes, only two discrete levels for antigens expression were considered in this paper: expressed (“+”) and not expressed (“-”). *Antigen* is used interchangeably with *diagnostic factor* or just simply *factor* and *antigen test* is interchangeable with *antibody test*.

2.2.2 Antigen tree induction algorithm

Our method extends Shannon's information entropies [82] and Bayesian probabilities to iteratively build an *efficient antigen selection tree* (EAS-Tree) using a matrix of *antigen expression profiles* (Section 2.2.1). EAS-Tree can then be used by pathologists to streamline the antibody testing process. The main difference between a traditional decision tree (DT) approach [83], and our EAS-Tree is that a DT is usually used to confirm a single path of antigen expressions while an EAS-Tree is used to cover a comprehensive set of diseases and then rule out irrelevant ones. Moreover, a DT utilizing the *entropy minimization* concept likely will result in a skewed and deep tree. Such a tree likely will require a large number of antibody tests to provide conclusive recommendations to the pathologists. Unlike the DT approach, the EAS-Tree seeks a *balanced* tree structure, which suggests to pathologists a small number of antibody tests using an *entropy maximization* approach. We believe that our method strives to closely mimic the reasoning process of pathologists. To explain the ideas behind this, let us use the following *MAEP* corresponding to differential diagnoses of which the listed five diseases have equal possibilities for diagnosis (i.e. equal incidence rates) (Table 3)

Table 3 An example MAEP: “+” expressed, “-” not expressed

	Antigen A	Antigen B
Disease 1	+	+
Disease 2	-	+
Disease 3	-	-
Disease 4	-	-
Disease 5	-	-

From this example, *Antigen A* is expressed in only *Disease 1*. Intuitively, a pathologist would want to test for an expression of *Antigen A* because if the test comes out positive the diagnostic process would quickly end (with *Disease 1* as diagnosis in his/her mind). However, if we assume that no other factors can cause an expression of *Antigen A*, the probability of getting positive expression of *Antigen A* is 1/5, while the probability of getting negative expression results is 4/5. It means that no matter what the test outcome of *Antigen A*, it will suggest the following: (1) 20% chance to confirm *Disease 1* (“+” expression) or (2) 80% chance to rule out only *Disease 1* leaving four diseases (“-“ expressions) to be checked. From the same example if we choose another antigen, *Antigen B*, which is expressed in two out of five diseases, the test for that antigen in the most likely case would rule out two diseases. As mentioned previously, an efficient diagnostic process should start with a comprehensive list with diseases and quickly rule out as many irrelevant ones as possible. For this reason, the choice of testing *Antigen B* is over *Antigen A*. We select an antigen Ag_j with maximal entropy [84], which can be computed by the following formula:

$$H(Ag_j) = - \sum_{l=1}^{M_j} P_{Ag_j}(l) \log_2 P_{Ag_j}(l) \quad \text{Eq. 2}$$

where $P_{Ag_j}(l)$ is a fraction of diseases in $MAEP$ for which antigen Ag_j has l -th expression level.

The tree induction algorithm builds an EAS-Tree by splitting the current $MAEP$ into partitions, one for every level of expression of a certain antigen. The antigen is then represented by a node with child nodes for each expression level. At each step of the tree induction, the algorithm picks an antigen that splits diseases in current $MAEP_j^l$ *as evenly as possible*. To represent the matrix of antigen expression profiles, we use $MAEP$ with super/sub scripts to identify parts of the matrix that were generated during a split by the antigen tree induction step. For example, $MAEP_j^l$ is a sub-matrix that is resulted from splitting the matrix by j -th antigen Ag_j and consisting of diseases in which Ag_j has expression value l . Fig. 2 lists the pseudo code for the selection of antigen and the procedure of node splitting for EAS-Tree building.

Procedure EntropySplit(*MAEP*, *Parent*)**Input:***MAEP* - current matrix of antigen expressions*Parent* - parent node in EAS-Tree*A* = {*Ag*₁, *Ag*₂, ..., *Ag*_{*n*}} – set of *n* antigens in current *MAEP**D* = {*D*₁, *D*₂, ..., *D*_{*m*}} – set of *m* diseases in current *MAEP***Output:** EAS-Tree

```
1.  WHILE (A <> ∅ AND Size(D) > 1) {
2.    jmax = argmaxj Entropy(Agj), ∀ Agj ∈ A
3.    assign Agjmax to Parent node
4.    form  $MAEP_{j_{max}}^+ = (D_{j_{max}}^+, A^{new})$ ,
         $MAEP_{j_{max}}^- = (D_{j_{max}}^-, A^{new})$ , and
         $MAEP_{j_{max}}^m = (D_{j_{max}}^m, A^{new})$ ,
        where  $D_{j_{max}}^+ \subset D$ , for which Agjmax is expressed,
         $D_{j_{max}}^- \subset D$ , for which Agjmax is not expressed,
         $D_{j_{max}}^m \subset D$ , for which expression of
        Agjmax is missing and
         $A^{new} = A - Ag_{j_{max}}$ 
5.    add EAS-Tree child nodes Child+ and Child- to Parent
6.    call EntropySplit( $MAEP_{j_{max}}^+ \cup MAEP_{j_{max}}^m$ , Child+)
7.    call EntropySplit( $MAEP_{j_{max}}^- \cup MAEP_{j_{max}}^m$ , Child-)
8.  }
```

Figure 2 Pseudo code of ATOS's EntropySplit() procedure

Every call of this procedure finds an antigen in *MAEP* with the maximum entropy (Fig. 2, line 2), splits current *MAEP* using that antigen (Fig. 2, line 4) and creates new child nodes in EAS-Tree (Fig. 2, line 5). The antigen with maximum entropy splits current *MAEP* into *l* partitions, one per expression level of the splitting antigen (*l*=2 in this dissertation). For every resulting *MAEP* partition, the *EntropySplit()* procedure calls itself recursively (Fig. 2, lines 6 and 7) and the process repeats. The algorithm stops when there are no available antigens or there is only one disease profile left in the database (Fig. 2, line 1).

Since antigen expression information for some diseases might not be available, *MAEP* may have missing values. In our implementation of ATOS we do not impute missing expression values. After splitting *MAEP* into partitions, diseases for which expression value of the splitting antigen is not available are grouped together in $MAEP_{j_{max}}^m$ (Fig. 2, line 4) and passed to the next recursive calls of *EntropySplit()* (Fig. 2, lines 6 and 7).

Figure 3 An example of splitting of MAEP

In Fig. 3 (a) the initial *MAEP* is shown with 17 diseases and is used to explain the logic of the tree induction process in a recursive way. During the first call, the algorithm selects antigen Ag_3 as having the largest entropy value of:

$$(Ag_3) = \left(\frac{8}{17}\right) \log_2 \left(\frac{17}{8}\right) + \left(\frac{9}{17}\right) \log_2 \left(\frac{17}{9}\right) = 0.997$$

Indeed, the expression values of Ag_3 almost evenly splits $MAEP$ into two partitions $MAEP_3^+$ and $MAEP_3^-$, one having diseases with "+" expression values of Ag_3 and the other one with "-" expression values. This is illustrated by the leftmost column in Fig. 3(a) where disease rows are grouped by expression values to form partitions. Ag_3 becomes the root of the antigen tree (Fig. 4). For each resulting partition, the tree induction algorithm recursively calls itself by passing $MAEP_3^+$ and $MAEP_3^-$. At the next level of this recursion, the selected antigens are Ag_{11} and Ag_7 for partitions $MAEP_3^+$ and $MAEP_3^-$ respectively (Fig. 3(a)). Ag_{11} and Ag_7 are added to the tree as child nodes for Ag_3 (Fig. 2-5). At this step there are four new partitions $MAEP_{11}^+$ and $MAEP_{11}^-$ (Fig. 3(b)), $MAEP_7^+$ and $MAEP_7^-$ (Fig. 3(c)). For each of these partitions, the induction procedure calls itself recursively again and then adds new antigen nodes Ag_4 and Ag_2 to their parent node Ag_{11} and new nodes Ag_{10} and Ag_5 to Ag_7 (Fig. 4).

When the algorithm looks for an antigen that best splits a current partition, it chooses from all antigens with the exception of those that appear in the path to the root antigen node. For example, when the algorithm processes partitions generated by Ag_4 , it can choose any available antigen except Ag_4 , Ag_{11} , or Ag_3 , since any of these antigens will have zero entropy for partitions $MAEP_4^+$ and $MAEP_4^-$ (see Fig. 3(a)). However, some antigens may appear in different branches of the antigen tree (see double circled nodes on Fig. 4).

Figure 4 An efficient antigen selection tree (EAS-Tree) based on MAEP from Fig. 3

To reflect a more realistic picture, instead of simply counting occurrences of expression levels over diseases for each antigen in *MAEP*, there are other important parameters to be considered for entropy calculations. During disease diagnosis pathologists usually take into account disease incidence rates. The rationale behind this is that some diseases are prevalent while others are not. A specific antigen expression level is less likely to manifest the presence of a disease with a lower incidence rate than a disease with a high incidence rate. To address this fact we modify entropy computation and include disease incidence statistics in the following way. First of all, the “raw” disease incidence rates, as discussed in Section 2.2.1, have to be normalized. Since the differential diagnosis assumes that the pathology case in question should be diagnosed only with a disease from *MAEP*, we need to make sure that the incidence rates will sum to unity, i.e., $\sum_{i=1}^N P_{in}(D_i) = 1$, where $P_{in}(D_i)$ is a normalized incidence rate for disease D_i . We compute $P_{in}(D_i)$ using the following formula: $P_{in}(D_i) = P_{in}^{raw}(D_i) / \sum_{k=1}^N P_{in}^{raw}(D_k)$. Now the normalized incidence rates can be used as probabilities to

compute joint probability $P^*(l_{Ag_j}, D_i)$ for a certain expression value l for j -th antigen and i -th disease:

$$P^*(l_{Ag_j}, D_i) = P(l_{Ag_j}|D_i)P_{in}(D_i) \quad \text{Eq. 3}$$

By summing up all joint probabilities $P^*(l_{Ag_j}, D_i)$ for each level and then normalizing the sums, we obtain marginal probabilities for each expression level l of j -th antigen and use them to compute information entropies with the formula:

$$H(Ag_j) = - \sum_{l=1}^M P^*(l_{Ag_j}, D_i) \log_2 P^*(l_{Ag_j}, D_i) \quad \text{Eq. 4}$$

where the probability takes into account disease incidence rates and the probabilities for antigen expression levels of certain diseases as listed in Table 2.

2.2.3 Using EAST to guide immunohistochemical studies

After an EAS-Tree is built, a pathologist can interactively use it as a guidance to conduct actual antibody tests. A pathologist-in-the-loop interface provides a graphical interface for pathologists to select antibody tests and an indicator about the effectiveness of the selection. The outcomes of these tests will then be fed back to the system. If a test for a certain antigen has already been completed, this test will be excluded for future testing. Since ideally each test under any outcome should rule out as many disease diagnoses as possible, the whole testing process should result in an efficient testing workflow. In other words, the testing process using this method, on average, should result in faster turnaround.

Another way, which resembles the traditional approach (see for instance [27]) is to aggregate nodes of the EAS-Tree into test panels. One of the ways of doing that is to aggregate adjacent levels and construct an antigen *panel* tree (see Fig. 5). If the number of distinct expression levels l is constant for all antigens in the database (as in our example), the number of antigens in a panel for a given number of aggregated antigen tree levels K equals to $\sum_{l=0}^{K-1} l^i$. In the general case, the number of the antigens in panels might be different for each node in the antigen panel tree depending on values of l . It has to be noted that while this method does not fully eliminate the redundancy of the expert based IHC classification schemas [27], it can result in substantial decrease in the number of unnecessary tests and significantly reduce inter- and intra- pathologist variability by making the antibody test selection process uniform.

Figure 5 An example of antigen panel tree. The numbers in square brackets identify antigens. The numbers with signs in parentheses indicate outcomes of the testing for corresponding antigens

2.3 Results and Discussion

2.3.1 Method evaluation

A typical model of the conventional, recommended IHC classification schema for lymphoid tumors was constructed in accordance with text and tables presented in an expert review article [27] as well as WHO guidelines [67]. This model employs two to

three distinct panels performed in series as determined in conjunction with morphologic features within the tumor. To compare our method with this model, a *MAEP* for hematopoietic tumors was compiled from the same sources (see Section 2.2). This comprises 20 lymphoma/leukemia cases and 37 antibodies, which represent a relatively broad range of neoplasms and a sizable selection of antibodies. Our first assessment of the quality of the resulting EAS-Tree is to validate nodes from the first few levels with recommendations from the published literature or guidelines.

Figure 6 ATOS user interface for diagnostic tree building using automatic entropy-based probabilistic algorithm

As shown in Fig. 6, the three antigen nodes from the first two levels of the tree are CD20, CD79a and BCL-6 antigens, which are B-Cell markers and suggested to be analyzed first by guidelines in [27]. This tree would be valuable for pathologists to determine if the case is B-Cell or T-Cell based lymphoma/leukemia. Since the tree is balanced, the method finds the shortest path to the smallest subset of disease diagnosis candidates. In the worst-case scenario, a pathologist would need to test eight antibodies out of total 37, and in the best test scenario only six.

2.3.2 *Comparison of the conventional model (WHO, experts' guidelines) and entropy based ATOS*

No diagnostic discrepancies were identified using either the conventional model or our method; however, in general, our method's pathways were distinctly shorter for the majority of disease diagnoses. On average, our method requires slightly less than half of the positive markers and less than a third of the negative markers to reach a definitive diagnosis. This brevity is inherent in the algorithm, which is designed to produce the *shallowest* diagnostic tree. The method stops when the diagnostic branch contains only a single entity. However, if desired, the algorithm could be readily extended to identify any specified number of additional positive or negative confirmatory antigen tests. Table 2 shows a comparison of number of antibody (AB) tests required to reach a diagnostic conclusion using our method (ATOS) and conventional (Conv.) methods [27], [67]. A two-sample T-test, conducted with the assumption of unequal variances, was significant for the difference between mean number of tests in conventional and ATOS panels. For

positive, negative and total tests the p-values were correspondingly 2.619e-04, 1.214e-10, and 3.394e-11.

Table 4 Comparison of immunotypic findings for 14 representative lymphomas/leukemias between human expert model (*Conv.*) and the entropy-based computer algorithm (*ATOS*) (“+”: positive, “-”: negative outcomes)

Disease	Conv. ABs +	Conv. ABs -	ATOS ABs +	ATOS ABs -
ALCL	7	13	4	2
MF	4	16	1	5
MCL	5	10	3	3
CLL	5	10	4	2
FL	5	10	2	4
MALT	3	13	3	4
HCL	7	9	4	1
MZL nodal	5	10	3	3
B-ALL/LBL	7	11	2	4
T-ALL/LBL	8	13	3	3
DLBCL GC	8	10	5	1
DLBCL NGC	9	9	4	3
CHL	7	8	3	3
PCM	2	13	3	3
MEAN	5.86	11.07	3.14	2.93

Table 5 Abbreviations of diseases listed in Table 4

Abbreviation	Disease
ALCL	Anaplastic large cell lymphoma
MF	Mycosis <u>fungoides</u>
MCL	Mantle cell lymphoma
CLL	Chronic lymphocytic leukemia
FL	Follicular lymphoma
MALT	Mucosa-associated lymphoid tissue lymphoma
HCL	Hairy cell leukemia
MZL	Marginal zone lymphoma
B-ALL/LBL	Acute B lymphoblastic leukemia
T-ALL/LBL	Acute T lymphoblastic leukemia
DLBCL	Diffuse large B-cell lymphoma
CHL	Classic Hodgkin's lymphoma
PCM	Plasma cell myeloma

2.3.3 Comparison of manual antibody test selection using ATOS GUI and entropy based ATOS

For the next series of experiments we have developed a simulator program that uses the *MAEP* and ATOS GUI and allows a pathologist to build an antigen selection tree manually. The goal was to capture the way each pathologist approaches the task of reducing the number of suspected diagnoses at every step. The program includes a Graphical User Interface (GUI), which makes the process user friendly and helps to retain the logical order of the diagnostic process rather than rely on memory. This interface is shown in Fig. 7.

Figure 7 ATOS GUI in simulator program during manual antibody test selection

Moreover, the program provides linkages for the antigen descriptions and their genomic/ proteomic functions derived from well-known online knowledge bases such as UniProtKB/Swiss-Prot [85] and Gene Wiki [86].

Five experienced pathologists participated in the experiments, each with overall experience in practicing anatomic pathology ranging from 7 to 30 years. They were asked to build an antigen selection tree manually. The requirements for an experiment session were to select an antibody test for a given set of remaining (suspected) diseases at each step. The pathologists were not told to use any specific selection criteria; they had to use their own methods. Similar to our entropy-based algorithm, once an antibody test has been used, it could not be used again down in the corresponding decision branch. However, it could be used in a different branch of the tree.

To perform comparative analysis of the outcomes of manual test selection using the simulator program with experts [27], WHO IHC guidelines [67], and our entropy-based probabilistic algorithm, we plotted a bar chart in Fig. 8 that shows the number of tests required to diagnose each disease (for manual test selection averages across pathologists are shown). Just by using the ATOS-GUI (the simulator program) and the knowledge base of genomic/proteomic functions, the pathologists could significantly improve the antibody test selection process in terms of reducing the overall number of IHC tests required to reach a diagnostic conclusion. On average, the simulator program helps to reduce the number of tests up to twofold, based on comparisons to the expert IHC classification schema and WHO guidelines. Furthermore, by using our entropy-based probabilistic algorithm, we obtain an additional 35% to 40% decrease in the number of antibody tests.

Figure 8 Comparison of conventional test selection (WHO, Higgins), manual test selection using ATOS GUI in simulator program and automatic entropy-based probabilistic method (ATOS-method)

2.3.4 Inter-pathologist variability

To measure an inter-pathology variability, we calculated an average *difference between numbers of antibody tests required to diagnose the same disease by different pathologists* who participated in our research. We then constructed histograms showing the frequency for each such *difference* (Fig. 9). For example, from the top panel of Fig. 9, we can see that there were three diseases for which pathologists used the same number of tests, five diseases when the number of tests differed on average by 1, one disease when the number of tests differed by 3, 6 diseases when the number of tests differed by 4 tests

and so on. The bars appearing on the left side of the histogram correspond to lower variability, while the bars on the right side correspond to the higher variability. We repeated the experiment and plotted another histogram, which is shown on the bottom panel of Fig. 9. It can be seen from the chart that the difference in the number of tests in some cases can reach 10 tests.

Figure 9 Inter-pathologist variability (two experiments)

The overall level of inter-pathologist variability is significant, showing that the current antibody selection process is not well standardized. Even though the pathologists were aware of the general guidelines for using IHC for malignancies of lymphoid tissue (e.g., WHO [67]), each of them used his or her own *heuristics* to assemble the diagnostic tree. The use of such tacit knowledge by pathologists during decision-making has been previously discussed in the literature [87, 88]. Due to consistent tree building by our algorithm based on *MAEP* obtained by literature, variability using our method is zero in

all runs to emphasize the benefit of utilizing our entropy-based probabilistic algorithm in light of maintaining low inter-pathologist variability.

2.3.5 *Intra-pathologist variability*

Contrary to our expectation that intra-pathologist variability would be smaller than inter-pathologist variability, interestingly we did not observe that. The overall level of intra-pathologist variability had not been found significantly different from inter-pathologist variability, based on the analysis of the corresponding histograms (Figures 9 and 10). Studies on other types of cancers have reported similar findings [74]. After further analyses of the EAS-Trees, we concluded that the differences in diagnostic paths generated by the same pathologist can be attributed to the fact that some antigens carry similar functions. The pathologist could have picked a different antigen in place of the one that was no longer available.

Figure 10 Intra-pathologist variability

2.3.6 Experimental clinical study

To preliminary assess clinical utility of ATOS, we have conducted a small retrospective study. Twenty cases of hematopoietic tumors were selected. The diagnoses of these cases were performed in the past by five different pathologists that used traditional methods to choose IHC tests. The cases were then processed again by three expert hematopathologists in context of this study. They analyzed hematoxylin and eosin (H&E) slides and came up with differential diagnoses, which then were fed into ATOS. For each case, the number of tests suggested by ATOS that lead to original diagnosis was noted. We then set to answer the following two questions: (1) Is the number of ATOS tests that lead to original diagnosis *statistically* lower than the number of tests in traditional antibody panels? (2) Can ATOS panel be used by a pathologist to arrive at the proper diagnosis?

To answer the first question we conducted statistical tests using SAS and R tools. Since each case was diagnosed again by three pathologists, a four-sample data setup was established: one traditional and three ATOS. Statistically the question was to compare means of three ATOS samples with the traditional one. Bartlett's, Flinger-Killeen's and Levene's null hypothesis tests showed that the data failed to satisfy *homoscedasticity* requirement with corresponding p-values 6.474e-07, 4.133e-04, and 2.000e-04. The *normality* assumption violation was demonstrated by Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises and Anderson-Darling tests (p-values: 0.0001, <0.0100, <0.0050, <0.0050). We therefore decided to use nonparametric tests. The Kruskal-Wallis test was significant (p-value 6.678e-06), which showed overall high level of difference in mean number of tests across all samples. Pairwise differences between each ATOS

sample and traditional one were measured by Behrens-Fisher and Steel tests. The results were significant with p-values for Behrens-Fisher test for all pairs being less than $5.84e-06$ and for Steel test being less than $3.08e-04$. We have, therefore, come to the conclusion that *ATOS suggests statistically significantly lower number of tests than the number of tests in traditional IHC panels in real clinical cases.*

To answer the second question the ATOS panels of several cases from the study were manually analyzed. This instance of an ATOS algorithm is based on immunohistochemical data derived from a limited literature review [27, 67] and *appears to accurately derive the proper diagnosis.* Given the clinical setting and H&E findings, the analysis demonstrated the use of redundant or non-contributory stains during original processing of the cases by conventional panels. Several cases illustrated the type of diagnostic situations in which ATOS might be of value in the evaluation of lymphomas. For instance, it would: (1) exclude examination for widely positive antigens; (2) eliminate the implementation of multiple B-cell antibodies simultaneously; (3) avoid the use of stains which are listed as +/- for multiple tumors in the differential; (4) avoid the use of antibodies to evaluate large cell lymphomas when no large cells are present; (5) exclude the application of epithelial-associated antibodies for tumors with a purely lymphoid appearance. Of course, these would be presented as guidelines, not absolute rules for all cases.

The conducted experiments have shown that the pathology diagnostic process can greatly benefit from our new improved methodology for streamlining antibody test selection in IHC studies. One of the most important advantages of our computerized approach is that it is more *quantitative* than *qualitative*. For instance, substitution of

antibody tests may lead to significantly different results because of the subtle differences in expression patterns of corresponding antigens. For example, different B-cell surface expressed proteins can produce slightly different staining in the same disease. A pathologist may not recall and account for that at the time of diagnosis, which can, therefore, complicate the diagnostic process and increase turnaround time. Another problem is related to the fact that many antibodies have been poorly clinically validated [89] and are often selected based on *sensitivity* only. Due to the low *specificity* some of the tests do not have sufficient discriminative power and may lead to highly variable results. These issues are accurately handled in our algorithm by the antigen expression probabilities across diseases, which is a rather *quantitative task* that may not be easily managed by a human. The same applies to the disease incidence rates. It is not a simple task to account accurately for disease incidence rates during diagnosis. Our method provides a solution to these problems, while being flexible enough to allow a pathologist to have full control of the decision process through pathologist-in-the-loop interface.

Our method is very flexible in the sense that it just *eliminates* diseases that do not support revealed facts. The overall goal of the method is not to suggest a single diagnosis, but to *streamline* pathology decision-making. For example, in a traditional rule-based expert system, the inference process produces “useful” results only when information about all “key” antecedent terms is entered into the system. If the antecedent part of a rule is only partially satisfied, the rule may not fire and it is hard to extract any benefit from the system in this case. Our method, on the other hand, finds the shortest diagnostic paths using only *available facts*. It “optimizes” the process by finding the “most efficient” factors at each step (the ones that will lead to ruling out larger number of possible

diagnoses). The more such facts are available, the more diseases can be ruled out and the leaf nodes of a corresponding EAS-Tree will have fewer possible diagnostic solutions. A pathologist is free to remove from or add diseases/antigens into consideration and the algorithm will dynamically rebuild the EAS-Tree. This gives the pathologist control over the decision process. Moreover, being involved in an interactive decision-making provides the pathologist with better understanding of why one or another disease should be *ruled out* or *ruled in* and with what confidence level. ATOS is not one-answer fit system with predefined rules and answers. Based on the differential diagnosis provided by a pathologist, it builds diagnostic trees that reflect the individual practice patterns of that pathologist. Furthermore, the Bayesian probabilistic model used in our method naturally incorporates disease incidence rates as prior probabilities. ATOS can be easily adjusted to account for specific rates of disease occurrences across *different demographical and geographical categories*.

We share the view that personalized medicine involves the integration of diagnostics and therapeutics into what has become known as *theranostics* [25]. As such, a more granular sub-classification of diseases based on an increasingly complex proteomic knowledge would be expected to contribute significantly to this process. Granted, our current state of practice of personalized medicine in pathology largely seems to be dependent upon the identification of the expression or activation of proteins that may be unique to an individual's particular cellular pathways rather than representative of a population. Therefore, our algorithm could not be expected to provide much assistance. However, as diagnostics evolves *to include pathway-based molecular biological evidence*

(see for instance [90]), new patterns of protein expression may need to be evaluated in which computational support in the manner of this algorithm may be useful.

2.4 Conclusion

We have developed ATOS - a novel informatics tool to help pathologists streamline antibody selection process. Using the ATOS-GUI alone, pathologists can significantly speed up antibody selection process. Furthermore, our entropy maximization probabilistic algorithm brings up to 40% additional decrease in the number of antibody tests required to reach a diagnostic conclusion. Moreover, it significantly reduces inter- and intra-pathologist variability, which makes the process more consistent and predictable. A comparative analysis of our method with the World Health Organization classification guidelines and conventional approaches showed that the proposed tool brings approximately a threefold reduction in number of antibody tests required to reach a diagnostic conclusion. Therefore, ATOS has great potential to streamline antibody test selection and significantly decrease costs associated with it.

Chapter 3: Uncovering Morphoproteomic Relationships Using Probabilistic Graphical Models and RDF Graphs

In this chapter we introduce a novel method for discovering morphoproteomic relationships using graphical models and public knowledge sources about protein, signal transduction pathways and drugs.

3.1 Overview

The heterogeneity of individual patient responses to conventional drug therapies is one of the central problems in personalized medicine and has great impact on clinical outcomes. To address this problem a new field of *morphoproteomics* was recently introduced [6]. Morphoproteomics is a new method aimed at comprehensive analysis of protein circuitries in diseased cells to design effective drug therapies for individual patient cases. The analysis is done based on the visual expression of antibody-linked (IHC) proteins on histopathological slides.

It has already been a few decades since pathologists began to use IHC to identify antigens of aberrantly expressed proteins - biomarkers of certain diseases. However, this process is often complicated by an inexact nature of protein expression patterns and the overwhelming number of their different combinations that a pathologist has to analyze before coming to a diagnostic conclusion. On the other hand, the vast amounts of information related to cancer genetics, epigenetics, proteomics and other "omics" have been collected and are available in electronic databases. In this dissertation we propose a methodology to automatically "project" protein expression pattern information from the

histopathological slide into an "omics" knowledgebase in such a way that a pathologist could get a detailed "picture" of the abnormalities in the specimen. It includes not only morphological information of the *microanatomy of the tissue*, but also information about what proteins are expressed, whether they should be expressed or not, what signaling pathways are triggered and so on. In other words, it enables a pathologist to incorporate molecular knowledge of biochemical pathways into the *theranostic* process. That could lead to better understanding of high-level physiological processes and allow more precise treatment schemes. We call this approach a "Systems Pathology" paradigm.

The feasibility of this task has been improved by the recent advances in whole slide scanning technology [76, 77, 91]. Several major vendors began to offer scanners that can generate images from whole histopathological slides taken at very high optical magnification [92]. It has become feasible to perform computerized image processing of whole slides in a clinical setting, instead of occasional use of cropped digital images for education or research purposes only. However, there was a need to develop efficient methods to “make sense” of different protein expression patterns. It would be invaluable to use knowledge from high quality knowledgebases to construct morphoproteomic diagrams in an automatic or semi-automatic manner. The computer generated morphoproteomic schemas would have great potential to help identifying inhibitors and cytotoxic agents that can be combined in effective individualized combinatorial therapies. This work introduces such a method that employs probabilistic graphical models for uncovering of relationships between proteins and signal transduction pathways.

3.2 Methods

3.2.1 Theoretical Foundation

To infer causal relationships among proteins and pathways, we extend the theory of causation and information relevance, the first comprehensive treatment of which was given by Pearl and Verma [52], followed later by works of Spirtes [53], Verma and Pearl [93, 94]. The researchers showed that under certain conditions graphical models could be used to represent dependencies and independencies embedded in probability distributions. In particular, Pearl and Verma introduced notions of *graphoids*.

According to Pearl [95], for probability distribution F over a set of discrete variables $\{a, b, c, \dots\}$, a *graphoid* is a relation of the form $I(a, b|c)$ that satisfies the following independence conditions:

$$\text{Symmetry: } I(X, Y|Z) \Leftrightarrow I(Y, X|Z)$$

$$\text{Decomposition: } I(X, Y \cup W|Z) \Rightarrow I(X, Y|Z) \wedge I(X, W|Z)$$

$$\text{Weak Union: } I(X, Y \cup W|Z) \Rightarrow I(X, Y|Z \cup W)$$

$$\text{Contraction: } I(X, Y|Z) \& I(X, W|Z \cup Y) \Rightarrow I(X, Y \cup W|Z)$$

$$\text{Intersection: } I(X, Y|Z \cup W) \& I(X, W|Z \cup Y) \Rightarrow I(X, Y \cup W|Z),$$

where X , Y , W and Z are disjoint subsets of a finite set $U = \{a, b, \dots\}$ of discrete variables over which F is defined. The *Intersection* condition is considered only for strictly positive distributions F , distributions for which every combinations of events has some chance of being true (for more details see [52]). The graphoid $I(x, y|z)$ stands for “ x is independent

of y given z ” where $x \in X$, $y \in Y$ and $z \in Z$. Pearl and Paz [95] introduced the following *Completeness Conjecture*:

$$\exists P: P(x, y|z) = P(x|z) \text{ iff } I(x, y|z) \quad \text{Eq. 5}$$

The Completeness Conjecture implies that a set of graphoids is sufficient to specify independencies in probability distributions.

There was, however, an issue of learning and encoding independencies from experimental data. For example, given a set of n measurements (observations) consisting of m binary variables, there could be over $m!/(2(m-2)!)$ combinations of variables that need to be specified. For each such combination, we need to compute frequencies of variable values in the set of n measurements. For instance, given three variables a , b , and c , and assuming symmetry property $P(x, y|z) = P(y, x|z)$ (first axiom on p. 84 in [52]), there will be three distinct conditional probability distributions: $P(a, b|c)$, $P(a, c|b)$, $P(b, c|a)$. For each such distribution we need to compute and store eight different conditional probabilities, e.g., $P(a, b|c)$: $P(a=0, b=0|c=0)$, $P(a=0, b=1|c=0)$, $P(a=1, b=0|c=0)$, $P(a=1, b=1|c=0)$, $P(a=0, b=0|c=1)$, $P(a=0, b=1|c=1)$, $P(a=1, b=0|c=1)$, $P(a=1, b=1|c=1)$, resulting in a total of 24 probabilities for all three distributions. Adding one more variable d , will increase the number of different conditional probability distributions to 18: $P(a, b|c)$, $P(a, c|b)$, $P(b, c|a)$, $P(a, b|d)$, $P(a, d|b)$, $P(b, d|a)$, $P(a, d|c)$, $P(a, c|d)$, $P(d, c|a)$, $P(d, b|c)$, $P(d, c|b)$, $P(b, c|d)$, $P(a, b|c, d)$, $P(a, c|b, d)$, $P(a, d|c, b)$, $P(b, c|a, d)$, $P(b, d|a, c)$, $P(c, d|a, b)$ requiring computation and storage of 144 (8 for each of 18 distributions) conditional probabilities. Further increases to the number of variables will lead to a *combinatorial explosion*, which makes the task of computing and storing conditional probabilities intractable.

Using graphs makes learning and encoding of conditional probabilities computationally feasible. Pearl was among the first investigators to demonstrate that a probability distribution can be *decomposed* into a set of *local dependencies* using graphical models [52]. He showed that probability of an event could be treated as dependent only on the outcomes of its immediate causes. In the graph terminology it would translate to the statement that the probability of an event associated with a graph node is dependent only on the outcomes of the node's immediate neighbors, the so-called *Markov Blanket* (MB) nodes for undirected graphs and the *Directed Markov Blanket* nodes for *Directed Acyclic Graphs*. A *directed* graph, $G=(N, E)$, with a set of nodes N and a set of edges E is a graph where all edges E are directed:

$$\forall e_k = (n_i, n_j) \in E, i < j, n_i, n_j \in N, k, i, j = 1, 2 \dots: e_k = \begin{cases} n_i \rightarrow n_j \\ \text{or} \\ n_i \leftarrow n_j \end{cases} \quad \text{Eq. 6}$$

where arrows $\rightarrow$ and $\leftarrow$ denote direction of an edge. Graphs for which Eq. 6 does not hold are considered to be *undirected*. A *directed acyclic* graph (DAG) is a *directed* graph that does not have any *cycles*. A *cycle* in a directed graph is a path $Path = (n_1, n_2, \dots, n_m)$, $n_i \in N$, $i = 1 \dots m$ such that $n_1 = n_m$ and $\forall i=1 \dots m-1: n_i \rightarrow n_{i+1}$. Decomposition of a probability distribution P into local dependencies using graphs and MB property requires computation of a significantly smaller subset of all conditional probabilities $P(\cdot/\cdot)$. Therefore, extraction of conditional independencies from these probabilities using Eq. 5 becomes computationally feasible even for large values of m . The independence relations themselves are also naturally represented by graphs. For instance, the statement “ x is independent of y given z ” is equivalent to saying that “node z separates node x from node

y ” in the graph language. That is, for every set of nodes x, y, z of a DAG G and the set of all paths $S_{Path} = \{Path(x, y)\}$ from node x to node y we have:

$$I(x, y|z) \equiv \forall Path(x, y), Path(x, y) \in S_{Path}: z \in Path(x, y) \quad \text{Eq. 7}$$

This means there is no edge between nodes x and y and *all* paths between these nodes should pass through the node z .

The structure of a graph that encodes independencies of P can be constructed either manually by domain experts or learned from data. In any case, the resulting probabilistic network along with the set of conditional probabilities can be used to *make inferences*. Two types of networks were originally introduced: *Random Markov Field* (RMF) and *Bayesian Network* (BN) [52]. RMFs use *undirected graphs* to encode dependencies, whereas BNs use DAGs to do the same. Since DAGs encode *directional* probabilistic dependencies, they exhibit more expressive power and can be used to *infer causal relationships* when structure of a network is learned from experimental data. There are two main categories of learning BN structure from data. *Score-based structure learning* approaches use conditional probabilities for computing scores to assess the “quality” of heuristically generated network structures and to select the best. It is a more *quantitative* method because it involves manipulation of numerical parameters of the probability distribution P . *Constraint-based structure learning* uses independence relations directly to infer the structure of the network.

In this work we extend PGM formalisms of inferring casual relationships from observed data to learn complex relationships among proteins and pathways *using RDF graphs* of publicly available biological knowledgebases. Representing information from

the knowledgebases as RDF graphs allows us to induce conditional independence statements (graphoids) $I(\cdot, \cdot | \cdot)$ that reflect biological dependencies. The graphoids are then fed into constrained-based structure learning algorithms to learn casual relationships. Fig. 11 illustrates the difference between our and traditional approaches of inferring causation. In a probabilistic setting the graphoids are extracted from probability distributions (gray workflow arrows in Fig. 11), while in our method we use RDF graphs to do so (black workflow arrows in Fig. 11). In Section 3.3.4 we examine the benefits of using knowledgebases instead of experimental data that can be of great importance to the biomedical research in light of uncovering biological mechanisms.

Figure 11 Workflow for inferring casual relationships

In Section 3.2.2 we show how to induce sets of graphoids by translating the definition of conditional independence to be applicable to RDF graphs.

3.2.2 Conditional Independence in RDF Graphs

The theory of causation revolves around the notion of *information relevance*, which in traditional probabilistic systems is expressed through the mechanism of conditional probabilities [52] (gray workflow arrows in Fig. 11). To understand that, we note that in majority of cases humans use *causation* as an “artificial” construct that helps them to reason [96]. If for instance the outcome of event y does not bring any *new* information about the outcome of event x , given that we know the outcome of event z , we say that event y is *irrelevant* to event x , since event z already captures all information that can influence on x , in other words $P(x,y|z)=P(x|z)$. In such a case we can say that y is *not a direct cause of x* or x is *independent of y given z* : $I(x,y|z)$. With knowledgebases we do not have any probabilistic information that can be used to derive independencies $I(x,y|z)$ through *Completeness Conjecture* (Eq. 5). However, as will be shown in this section, we can “extract” conditional independencies $I(\cdot, \cdot | \cdot)$ from the knowledgebases *directly* if we represent them as RDF graphs.

Since the elementary unit of information in RDF format is a *subject-predicate-object* triple, where each *object* can in turn be the *subject* of another triple (and vice versa), a knowledgebase in RDF format forms a graph. We call it an RDF graph, with nodes representing RDF *subjects* and *objects* and edges representing RDF *predicates*. Unfortunately, the RDF graph of a knowledgebase cannot *directly* represent causal relationships for at least two reasons: (a) not all edges (RDF predicates) can be considered as reflecting causal relationships, and (b) there are many nodes that have no relevance to the domain in question but rather are introduced to maintain organizational structure of the knowledgebase.

In this work, we propose using RDF graphs of knowledgebases only to extract conditional independencies or graphoids $I(\cdot, \cdot | \cdot)$. The graphoids can then be used to generate probabilistic graphical models (e.g. Bayesian Networks) with causal links among entities of interest (Fig. 12).

Figure 12 The proposed schema of inferring causal links

Before defining conditional independencies in RDF graphs, this chapter will formally introduce basic concepts. In this work we will use simplified definitions for RDF knowledgebase and ontology.

An ontology $O = \{C, R\}$ is a set of concepts C along with a set of relations R over those concepts. In this research, concepts C include such biological entities such as *genes*, *proteins*, *protein complexes*, *biochemical reactions*, *signal transduction pathways* and others. Examples of relations R among concepts C are “COFACTOR” and “PATHWAY-COMPONENTS”. This research is concerned with a special type of relations R called *object properties* R_O , which is a subset of all relations R .

An RDF knowledgebase $KB = \{T, S_{RS}, S_O\}$ is a set of RDF triples T , RDF resources S_{RS} and a set of ontologies S_O . The set of all RDF resources $S_{RS} = \{RS_O, L\}$ consists of a set of *object resources* RS_O and a set of *literals* L . An object resource $rs_O \in RS_O$ is an instance of a concept from the corresponding ontology $O \in S_O$. For example, for a concept *protein* from ontology O there can be an RDF *object resource* *AKT1*. Literals L include string constants and numbers. Each RDF resource $rs \in S_{RS}$ and relation $r \in R$ from a set of ontologies S_O can appear in KB multiple times in so-called RDF triples. An RDF triple $t \in T$ is a relation of the form $t = (rs^S, r^P, rs^O)$, where rs^S , r^P and rs^O are called *subject*, *predicate* and *object* respectively. Now we can give a formal definition for an *RDF graph*.

Definition (RDF graph): Given an RDF knowledgebase $KB = \{T, S_{RS}, S_O\}$ and isomorphic mapping functions $fn()$ and $fe()$, an *RDF induced graph* or simply an *RDF graph* is a graph $G_{RDF} = \{N_{RDF}, E_{RDF}\}$ with set of nodes, N_{RDF} , and set edges, E_{RDF} , for which the following holds true:

$$\begin{aligned}
& \forall t = (rs^S, r^P, rs^O) \in T: \\
& \exists n_i \in N_{RDF} : n_i = fn(rs^S) \wedge \\
& \exists n_j \in N_{RDF} : n_j = fn(rs^O) \wedge \\
& \exists e(n_i, n_j) \in E_{RDF} : e = fe(r^P)
\end{aligned} \tag{Eq. 8}$$

In context of morphoproteomics we will work with RDF knowledgebases of *proteins*, *protein complexes*, and *pathways*. Every protein *pr*, protein complex *prc* and pathway *pw* is modeled by an *object resource* $rs_O \in RS_O$ in RDF knowledgebase KB and a corresponding node $n_i \in N_{RDF}$ in RDF graph G_{RDF} . A protein complex *prc* consists of

proteins pr , i.e., $prc = \{pr_i\}$, $i = 1 \dots m_{PR}$, and every pathway pw consists of proteins, protein complexes, and other pathways (which we call sub-pathways): $pw = \{pr_i, prc_j, pw_k\}$, $i = 1 \dots m_{PR}$, $j = 1 \dots m_{PRC}$, $k = 1 \dots m_{PW}$.

Next, let us define a state function $a()$. The state function $a()$ is defined for proteins pr , protein complexes prc and pathways pw and returns a state of these biological entities. This research considers only two states for every pr , prc , and pw : 1 or *active* and 0 or *inactive*. The biological meaning of *active* state of a protein pr is a condition where the protein pr is *expressed*, i.e., *present in quantities sufficient to carry out its functions*. Below are formal definitions for active states for proteins pr , protein complexes prc and pathways pw :

$$a(pr) = 1 \text{ if } q(pr) \geq T_f \quad \text{Eq. 9}$$

where functions $q()$ returns quantity of protein pr and T_f is the minimum amount of protein pr needed for it to carry out its functions. Then for every protein complex prc we have:

$$a(prc) = 1 \text{ if } \forall pr_i, pr_i \in prc: a(pr_i) = 1 \quad \text{Eq. 10}$$

and for all pathways pw we have:

$$\begin{aligned} a(pw) = 1 \text{ if } & \forall pr_i, pr_i \in pw: a(pr_i) = 1 \wedge \\ & \forall prc_j, prc_j \in pw: a(prc_j) = 1 \wedge \\ & \forall pw_k, pw_k \in pw: a(pw_k) = 1 \end{aligned} \quad \text{Eq. 11}$$

We define conditional independence of nodes in an RDF graph G of a knowledgebase KB according to some biological ontology O similarly to conditional independence defined by Pearl for DAGs [52]. First, we divide all object properties R_O of ontology O

into two groups: (i) *directional* and (ii) *non-directional RDF predicates*, according to some biological interpretation. Like directed edges in Pearl’s DAGS, *directional* RDF predicates reflect possible causal relationship between nodes. In our setting with *proteins*, *protein complexes*, and *signal transduction pathways*, we define a causal relationship as the one that signifies an *activation of one biological entity due to the active state of another* (possibly through mechanisms of *phosphorylation* or *ubiquitination*).

Definition (directional predicate): Given an RDF graph $G_{RDF} = \{N_{RDF}, E_{RDF}\}$ induced from an RDF knowledgebase $KB = \{T, S_{RS}, S_O\}$ with a set of *object properties* $R_O = \{r_O\}$ for each ontology $O \in S_O$, an isomorphic mapping function $fe()$, and state function $a()$, r_O is a *directional* predicate if the following holds:

$$\forall e(n_i, n_j) = f(r_O), e \in E_{RDF}, r_O \in R_O, n_i, n_j \in N_{RDF}, i \neq j: \quad \text{Eq. 12}$$

$$\begin{cases} \text{if } a(n_i) = 1 \text{ then } a(n_j) = 1 \\ \text{or} \\ \text{if } a(n_j) = 1 \text{ then } a(n_i) = 1 \end{cases}$$

All other object properties R_O of ontology O for which Eq. 3.2.2.5 does not hold are considered to be *unidirectional* predicates. Examples of directional predicates are object properties “CONTROLLER” and “CONTROLLED” of the BioPax 2 ontology [97], which can be viewed as reflecting causal relationships. Examples of *non-directional* predicates are “PATHWAY-COMPONENTS” and “PHYSICAL-ENTITY.” While these object properties cannot be considered as representing causation, they do indicate other types of relations between entities in KB (e.g., correlation of protein expressions, whole-part relationships).

All nodes in RDF graphs are classified as *colliders* and *non-colliders*. A directional predicate has a *head* and a *tail*. The *tail* is connected to an RDF node, whose corresponding biological entity's active state causes an RDF node connected to a *head* of the directional predicate to also switch to the active state. If three RDF nodes are connected by directional predicates and the middle RDF node is connected to the *heads* of both directional predicates, this middle node is called a *collider*.

A set of RDF nodes $(n_1, n_2, \dots, n_m)$, $n_i \in N_{\text{RDF}}$, $i = 1 \dots m$ forms an *RDF path* $Path_{\text{RDF}} = (n_1, n_2, \dots, n_m)$, $n_i \in N$, $i = 1 \dots m$ if $\forall i = 1 \dots m-1$: $n_i \rightarrow n_{i+1}$ or $n_i \text{---} n_{i+1}$ or $n_i \leftarrow n_{i+1}$, where $\rightarrow$ and $\leftarrow$ denotes *directional* predicates and --- denotes *unidirectional* predicates. An RDF path $Path_{\text{RDF}}$ is called a *non-collider path* if none of its nodes is a collider, and an RDF path $Path_{\text{RDF}}$ is called a *collider path* if it contains a least one collider node. An *active path* between nodes A and B is the one that:

- I. For *non-collider path*, there are no nodes between A and B that are members of S;
- II. For each *collider* node in a *collider path*, the *collider* itself or any of its descendants is a member of S.

Figure 13 An RDF path without colliders

Figure 14 An RDF path with a collider node B_2

Fig. 13 shows a path in RDF graph (from now on an RDF path) that does not have any colliders. If neither of the nodes B_2 , B_3 or B_4 is a member of the set S , then the path is considered to be *active*, which means nodes B_1 and B_5 are *not* independent given the set S . Conversely, if some of the nodes B_2 , B_3 or B_4 are members of the set S , then the path is considered to be *inactive*, which means nodes B_1 and B_5 are independent given set S : $I(B_1, B_5 | S)$.

Fig. 14 presents an RDF path between nodes B_1 and B_4 that has a collider node B_2 , which is sometimes referred in the literature as forming a *v-structure* or a *common-effect structure* [47]. If the node B_2 or its descendant node B_5 (or any other possible descendant of the node B_2) is a member of the set S , then the path from the node B_1 to the node B_4 is *active*, which means nodes B_1 and B_4 are *not* independent given the set S . Conversely, if both node B_2 and its descendant node B_5 (and all other descendants of the node B_2) are not members of the set S , then the path is considered to be *inactive*, which means nodes B_1 and B_4 are independent given the set S : $I(B_1, B_4 | S)$.

We can now define conditional independence of nodes in RDF graphs as follows:

Conjecture 1 (Conditional independence in RDF graphs): Node a is independent of node b given a set of nodes S if and only if there exists no *active path* between nodes a and b .

Let us now examine a domain specific interpretation of the *Conjecture 1* as it applies to the proteins and signal transduction pathways to see if it can be accepted without a formal proof. We need to take a closer look at how *directional* and *non-directional* predicate edges affect conditional independence in both types of RDF paths *with* and *without* colliders.

There could be several *node-edge-node-edge-node* patterns, which we call *edge junctions*, in an RDF path *without colliders*. This chapter will review some of these patterns from Fig. 13. All others have either equivalent explanation or can be considered as their combinations. For each pattern, we must ask if the outer nodes are independent given the middle node.

Non-collider path. Pattern 1. In the RDF path from node B_1 to node B_3 , the middle node B_2 has adjacent edges that are directional predicates (according to ontology O) with their heads pointing away from the node B_2 . This is known as a *common cause structure* [47]. The protein/pathway B_1 is active when protein/pathway B_2 is active because the latter is a direct cause of the former. The protein/pathway node B_3 is also in active state because of node B_2 , which is its direct cause as well. We can therefore conclude that an expression of B_3 *indirectly* indicates an expression of B_1 if we do not consider the state of node B_2 . In other words, we have $\neg I(B_1, B_3 | \emptyset)$, which is equivalent to saying that the path from B_1 to B_3 is *active* because separating set S is empty ($\emptyset$ indicates an empty set) and B_2 is not in S . However, if we know the expression status of B_2 , the knowledge of the status of B_3

becomes irrelevant to the expression of B_1 . In other words, we have that $I(B_1, B_3 | B_2)$ which is the same as to say that path B_1 is B_3 is *inactive* because node B_2 is in the separating set S .

Non-collider path. Pattern 2. The independence rules for *Pattern 1* will hold for the RDF path from pathway/protein node B_3 to node B_1 because of the symmetry.

Non-collider path. Pattern 3. The RDF path from node B_2 to node B_4 has all directed predicates with a *head-to-tail* edge junction. This is an example of a *causal trail* pattern. In this case, expression of protein/pathway node B_4 serves as a *direct evidence* of expression of B_2 , if we have no information of the active status of the protein/pathway B_3 . Therefore B_2 and B_4 are not *marginally* independent, i.e., $\neg I(B_2, B_4 | \emptyset)$, and the path is *active*. As soon as we have knowledge of the expression state of B_3 , we will have that B_2 and B_4 are independent given B_3 , i.e. $I(B_2, B_4 | B_3)$ and that the path is *inactive*, because now B_3 serves as evidence of expression of B_2 and there is no need to know the active status of protein/pathway B_4 .

Non-collider path. Pattern 4. If we reverse direction of the path from *Pattern 3* and consider the path from protein/pathway node B_4 to node B_2 , we will get a so-called *evidential trail* pattern. The node B_4 will be dependent on the node B_2 through a *direct cause* relationship, given that we do not have information about B_3 . In other words, the expression $\neg I(B_4, B_2 | \emptyset)$ will hold. Once knowledge of the active status of B_3 is available, nodes B_4 and B_2 will be considered to be independent given B_3 : $I(B_4, B_2 | B_3)$.

Non-collider path. Pattern 5. The RDF path from the protein/pathway node B_3 to node B_5 has two types of predicates: directional and non-directional. Non-directional predicates,

as mentioned above, represent dependencies between biological entities that do not have *causal underpinning*. However, they still indicate other types of relationships between connected entities. Let us consider a case when expression of the protein/pathway node B_4 is *correlated* with the expression of protein/pathway B_5 . That means that even if the causal relationship between B_4 and B_5 is unknown, information about the status of the node B_5 is *relevant* to our understanding of what happens to the node B_4 . We can therefore say that expression of the protein/pathway node B_5 is an *indirect evidence* of the expression status of the node B_3 , if we do not have any information about active state of B_4 . We can then state that $\neg I(B_3, B_5 | \emptyset)$ is true and consider the path to be *active*. With the knowledge of the active status of B_4 , we will have that B_3 and B_5 are independent given B_4 , i.e. $I(B_3, B_5 | B_4)$, and the path will become *inactive*. If nodes B_4 and B_5 have *whole-part* relationship, i.e., either protein/pathway B_5 is a part of B_4 or vice versa; node B_5 can still be considered an *indirect evidence* for the node B_3 leading to the same conditional independence properties of the path as in the case of expression *correlation*. It can be readily shown that other types of non-directional connections will result in the same outcome as for *correlation* and *whole-part* relationships.

All other non-collider paths in RDF graphs of protein/pathways *KB* can either be composed of *Patterns 1-5* or be proven in a similar way to possess the same property as *Patterns 1-5*. For instance if we reverse the direction of predicate between nodes B_4 and B_5 in *Pattern 5*, the node B_5 becomes an *indirect cause* of the node B_3 . We can therefore conclude that for all *non-collider* paths, the node separation criterion (also known as vertex separation in graphs [52]) defines conditional dependence, which makes *Part 1 of Conjecture 1* valid.

Let us now look at an RDF path *with a collider* as shown on Fig. 14 where an example of such type of RDF path from protein/pathway node B_1 to node B_4 has a collider node B_2 . The protein/pathway associated with the node B_2 can be *casually* influenced by both node B_1 and node B_3 . For instance if the node B_2 represents an apoptotic pathway and the B_1 and B_3 nodes are apoptotic or anti-apoptotic agents, the active status of B_2 can be a result of either active status B_1 or/and B_3 . Normally, the expression of agents B_1 and B_3 might not to be dependent (*marginal independence*). However, giving the active status of B_2 enables us to draw some conclusions about the status of B_1 depending on the status of B_3 . For example if B_2 is activated, and we know that B_3 is expressed too, there are fewer chances that B_1 is *expressed* than in the case with B_3 *not expressed*. Since the expression of both protein/pathway B_1 and B_3 nodes can cause expression of B_2 , knowing that B_3 is expressed makes expression of B_1 less likely since we have already “*explained away*” the expression of B_2 . This case of conditional independence has been previously discussed in the literature [96]. Here, we simply demonstrate that it can be applied to RDF graphs of protein/pathway *KB* as a *conditional independence extraction technique* rather than a *depiction of conditional independencies of probability distributions by DAGs* [47]. We have to add that the existence of a non-directional link between the B_3 and B_4 nodes does not change the conditional independence of expression of B_1 and B_4 proteins/pathways given the status of B_2 . As we have already mentioned, *non-directional predicates* do indicate the dependence of expression of one protein/pathway on another (just not casual). Therefore, the status of B_4 will “propagate” to B_3 and influence the expression of B_1 the same way as expression B_3 does. In a similar way, since the expression of the B_5 protein/pathway is a *direct evidence*

of the expression of B_2 ; hence, we can “*substitute*” node B_2 with the node B_5 (or any its *direct* or *indirect evidences*) and state our conditional dependency of B_1 and B_3 (or B_4) given B_5 and not B_2 . It can be concluded now that the *Part 2 of Conjecture 1* is valid.

We have now *informally* shown that the *Conjecture 1* can be used to extract conditional independencies from RDF graphs of protein/pathway *KBs*. Fig. 15 shows an updated diagram from Fig. 11 indicating the functional place of *Conjecture 1*.

Figure 15 Updated workflow for inferring casual relationships

We formalize the fact that we can use *Conjecture 1* to extract independencies as follows:

Conjecture 2 (Extraction of independencies from RDF graphs): If an independence model M (set of graphoids $I(\cdot, \cdot | \cdot)$) is embedded in an RDF graph G , it can be extracted by iterative application of *Conjecture 1*.

3.2.3 Semantic “Mashup” Knowledgebase of Genetic, Proteomic and Drug Data

To mine for *cause and effect* relationships among proteins and pathways using ideas presented in Sections 3.2.1 and 3.2.2 we use a Semantic Web [98] “mashup” knowledge base (SWMKB) that unifies a number of online biomedical databases. The overall schema of the proposed semantic “mashup” is presented in Fig. 16.

Figure 16 Semantic Web “Mashup” Knowledgebase (SWMKB)

We investigated data integration issues related to the creation of SWMKB, which serves as the main source of genomic, proteomic and drug related data for building morphoproteomic diagrams. Oracle 11g [99] Semantic Web framework was chosen as an underlying platform for the knowledge base. SWMKB is a part of our SPIRIT (Systems Pathology InfoRmatics Toolbox) framework, which unites our IHC antibody test selection tool and computational morphoproteomic module with a common user interface. The instance data from EntrezGene [32] (~330,000 RDF triples), Reactome [38] (~700,000 RDF triples), HPRD [100] (~40,000 RDF triples), [85] UniprotKB (3,300,000 RDF triples), and NCI Nature Pathways [101] (~240,000 RDF triples) have been imported into the Oracle's RDF store totaling to more than 4,600,000 RDF triples of information. We then constructed and ran an initial set of SPARQL queries to assess performance of the “mashup” knowledge base. The speed of the simple queries was in the order of tens of a second, but the execution time of some queries containing a SPARQL UNION statement were in the order of a few minutes and more. We have observed that more work on refining the structure of the semantic “mashup” has to be done to satisfy the performance requirements of the complex morphoproteomic queries.

We have developed a method that tackles the problem of integrating heterogeneous data from publicly available “omics” data sources such as Uniprot, EntrezGene, BioCarta, Reactome. The data that are not available in Semantic Web compatible format are converted into RDF representation using N-ary relation modeling and custom ontologies.

The SWMKB consists of several KB-components imported from online repositories. The knowledge about protein functions is represented by the functional annotations from Uniprot and Protein Information Resource (PIR) [102]. The “low-level” pathway data are

imported from Reactome. This information helps to answer questions about “low-level” protein interactions at the level of biochemical reactions. We draw “high-level” pathway information from Nature curated pathway database (from Pathway Interaction Database [101]) and from BioCarta pathway database [103]. These sources contain “canonic” pathways that are frequently used by researchers in life sciences. We use EntrezGene functional annotation data for information about gene functions and COSMIC database [104] for drug-protein interactions information. Since UniprotKB, EntrezGene and other involved resources all have different identification systems, we use information from Human Protein Reference Database [100] to link identifiers from these databases.

To our knowledge, there is not any comprehensive public source for protein expression data across different tissue types and diseases. For the purpose of this work, we use the database of protein expression patterns across hematopoietic tumors from our previous work [23] (shown as SPIRIT Protein Expression KB component in Fig. 16), supplementing it with additional data from biomedical literature and WHO guidelines [54].

Two main ontologies that we will utilize are GO [33] and Biopax [40] as shown on the top portion of Figure 3-6. GO was designed to capture the relationships among biological entities on the *gene level*. Biopax is an ontology that provides *standardized* access to many Biopax-compatible pathways repositories, including BioCarta, PID, and Reactome. The SPARQL queries and inference rules in our semantic “mashup” use the syntax and semantics of Biopax but not individual pathway-components’ ontologies. To cover the rest of the entity relationships in the system we developed custom ontology

shown as SPIRIT ontology in Fig. 3-6. For example, SPIRIT ontology describes concepts and relationships for COSMIC protein-drug interactions data (Fig. 17).

The inference model in our system employs above mentioned ontologies as well as semantic rulebases. Simple inferences like, *general-to-specific* relationships (e.g. interaction-to-biochemical reaction) are realized through ontological hierarchical (sub-class/sub-property) relationships. More complex inferences (e.g. *transitive* relations) are implemented via custom rules. We also use built-in Oracle 11g Semantic Framework rulebases such as RDFS and OWLPrime.

One of the challenges of creating RDF “mashup” is that some data are not available in RDF N3 format. We have developed a method for converting such data based on *N-ary relations modeling*. An example of this methodology for transforming tabular drug-protein data into RDF is shown in Fig. 17 below.

Figure 17 Conversion of N-ary relations data into RDF

Given drug-protein interactions' p-values in tabular format, each interaction will be modeled as a set of three RDF triples: (1) *[Drug ID] [participates] [Drug-Protein Interaction ID]*, (2) *[Protein ID] [participates] [Drug-Protein Interaction ID]*, and (3) *[Drug-Protein Interaction ID] [hasPValue] [p-value]* (p-value is shown as "0.05"^^xsd:float' in the rightmost box in Fig.17). Higher dimensionality N-ary relations will be represented similarly with "inter-entity" RDF class (e.g. "Drug-Protein Interaction" in the example above) having additional RDF type or object properties (see [25]).

3.2.4 Inferring causal relationships from RDF graphs

To infer causal links for morphoproteomic studies from SWMKB we use the fact that this knowledgebase is actually a network. Since the data structure of SWMKB is based on RDF *subject-predicate-object* triples, where the *object* of each triple can in turn be the *subject* of another triple, the SWMKB forms a graph. We use Semantic Web SPARQL query language [105] to navigate this graph and to perform set operations needed for extraction of graphoids. The central part of the algorithm is a function that finds a path between two RDF nodes. For experiments in this dissertation, we extend an RDF path search function of the Jena [106] framework. The Jena's path search function implements a Breadth First Search (BFS) algorithm. However, it searches for possible paths between nodes only in the direction from *subject* to *object*. To understand that let us look at Fig. 18.

Figure 18 Multiple paths between two RDF nodes

In the RDF path $B_1-B_2-B_3-B_4-B_5$, the *object* of each triple is the *subject* of the next adjacent triple. For example node B_2 is the *object* of the triple B_1-B_2 and the *subject* of the triple B_2-B_3 and so on. Note that direction of the edges in Fig 18 neither corresponds to ontological directionality of predicates nor indicates causal relationships between RDF resources. It just merely serves to determine *subject* and *object* of the triple (the *subject* is adjacent to the *tail* of the edge and *object* to its *arrowhead*). The Jena's path search function was not designed to find paths where the *object* of a triple can serve as the *object* of adjacent triple (e.g. path $B_1-B_2-B_7-B_6-B_5$). We extend this function to accommodate search for such paths. It however dramatically increases complexity of the search, which needs to be addressed in future research. In this dissertation we limit *search space* of the graph and demonstrate the utility of the proposed method on a subset of SWMKB.

To extract graphoids from the RDF graph of SWMKB we utilize a number of structure learning algorithms including PC (named after Peter Spirtes and Clark Glymour) [53] implemented in TETRAD package [107] and Inferred-Causation ICS [93] implemented in Weka [108]. In all cases we replace a function in these libraries that tests for conditional independence with our *IsIndependent()* function, the pseudo code of which is presented in Fig. 19.

IsIndependent(X,Y|Z)

Input: RDF path between X and Y

Output: True or False

1. Get next RDF path segment
2. Check if current edge junction is a collider
3. Non-collider: If the middle node is in Z return *True*
4. Collider: If the middle node or any of its descendants
5. is in Z, then return *False*, otherwise return *True*
6. If no more segments go to Step 6 otherwise go to Step 1
7. Return *False*.

Figure 19 Pseudo code of IsIndependent() function

Different RDF nodes in the knowledge base can represent the same biological entity. When checking if a node a is a member of the set S , we also need to check all other nodes that represent the same biological entity as node a to see if any of them is in S . For instance, in Fig. 20 node B_5 is not in S , but node B_6 (let us call it *alias* of B_5) that represents the same biological entity (through RDF predicate “XREF” in BioPax) is in S . We will, therefore, consider that node B_5 is a member of S .

Figure 20 RDF graph without entailments

Figure 21 RDF graph with entailments

To check if a node a is a member of S requires finding all *aliases* of node a . To reduce the execution time, we pre-compute entailments of the set S and get an expanded set S^* . The entailments include all aliases of all nodes in S . We then check each RDF node against the expanded set S^* . Fig. 21 illustrates this process. The set S^* includes node B_5 as an entailment. To avoid finding all aliases of B_5 during the query time, as shown in Fig. 20, we utilize a pre-computed set S^* that will already have B_5 as an alias of B_6 , which will lead to the same outcome in terms of conditional independency test but will execute much faster.

3.3 Results and Discussion

To assess the utility of the proposed approach for inferring casual links from RDF graphs of online knowledgebases, we have conducted three types of experiments. First, we have designed a “synthetic” model with predefined causal relationships and tested the validity of the algorithm to see if it can “recover” those relationships from an RDF representation of the model. Second, we have conducted validation experiments to see if our findings match results of morphoproteomic studies reported in the literature. And finally, we performed a comprehensive analysis that identifies additional causal links discovered by our algorithm in morphoproteomic studies. To do this a systematic literature review was conducted to support or reject new findings. The results of these experiments are presented in Sections 3.3.1, 3.3.2 and 3.3.3. In the end of Section 3 we discuss the benefits of our approach and implications of it for the field of computational morphoproteomics in particular and the biomedical domain in general.

3.3.1 Simulation Tests

The main goal of the simulation experiments was to test whether the implementation of *Conjecture 1* can correctly extract a set of *predefined causal links*. It must be said here that our simulation tests are not exhaustive, since the reversed process of constructing of a synthetic RDF graph for a set of predefined casual links may not be trivially performed for all cases.

In traditional probabilistic settings, information about causal relationships among entities of some physical process can be recovered from a probability distribution P that captures frequencies of events associated with these entities. Simple probabilistic tests $P(X,Y/Z)=P(X/Z)$ can be used to extract graphoids $I(X,Y|Z)$, which then can be used to extract causal relationships using constrained-based algorithms [53]. A synthetic dataset for a model with predefined causal links in this case can be constructed by simulating frequencies of data items that are consistent with the probabilistic tests and corresponding graphoids that are compatible with the predefined causal links.

In case with knowledgebases, we use *Conjecture 1* to extract graphoids from RDF graphs of these knowledgebases. Therefore, to generate a synthetic RDF graph of a model with predefined causal links we need to make sure that all RDF graph components are constructed in such a way that after applying *Conjecture 1* we can extract graphoids that are compatible with the original set of causal links. It has to be noted that there could be several different RDF graphs that are consistent with the same set of causal links. In other words, a set of causal links is not RDF-graph-isomorphic.

For the purpose of simulation tests we defined a simple model of protein and pathway entities with two causal links that are modeled using a directional RDF predicate: (a) a protein B_1 causes an activation of a pathway B_5 , and (b) a protein B_4 causes an activation of the pathway B_5 . A possible RDF graph that is consistent with these links is shown in Fig. 22.

Figure 22 A “synthetic” RDF graph

To model causal links (a) and (b) we notice that both proteins B_1 and B_4 are causes of activation of the same pathway B_5 . In other words, the pathway B_5 is a *common effect* for proteins B_1 and B_4 . We could therefore use the *explaining away* technique [52] and model these causal links using a *collider*. We added protein nodes B_2 and B_3 to bring some complexity to the RDF graph without altering relationship among entities B_1 , B_4 and B_5 . The reason behind this addition was to check the correctness of the algorithm because proteins B_2 and B_3 should not change conditional independence of entities B_1 , B_4 , and B_5 according to *Conjecture 1*. The actual collider here is an edge junction $B_1 \rightarrow B_2 \leftarrow B_3$, but since B_2 is the cause of B_5 , and B_3 is “correlated” with B_4 , we should expect the extraction of the original causal links using *Conjecture 1*. The corresponding graphoids are $I(B_1, B_3 | B_4)$ because of *Conjecture 1 Part 2*, $I(B_1, B_4 | B_3)$ because of

Conjecture 1 Parts 1 and 2, and $I(B_1, B_5|B_2)$, $I(B_4, B_5|B_2)$, $I(B_4, B_5|B_3)$, $I(B_3, B_5|B_2)$, $I(B_4, B_2|B_3)$ because of *Conjecture 1 Part 1*.

Figure 23 Recovered causal links from “synthetic” RDF graph

After running our algorithm for RDF graph shown in Fig. 22 for proteins B_1 and B_4 and pathway B_5 , correct causal links are obtained (Fig. 23).

3.3.2 Validation of Morphoproteomic Studies

We have run experiments with the protein and pathways from two morphoproteomic cases reported by the group of pathologists in [2] and [20].

In the first case, the pathologists used morphoproteomics to find alternate drug targets for a patient who did not respond to a conventional chemotherapy. They wanted to use a *Bortezomib* drug, a proteasome inhibitor, in a combination with another drug *Dexamethasone*, to treat relapsed *Acute Lymphoblastic Leukemia* (ALL). The goal was to construct a morphoproteomic diagram related to the case’s biochemical processes in such

a way that appropriate drug target can be identified. After conducting *immunohistochemical* (IHC) tests for paraffin-embedded tissue, correlative expression of *AKT*, *NF-kappa-B* and *BCL-2* proteins was revealed. The task then was to find biological circuitries that were consistent with the expression of these proteins. After analysis of several signal transduction pathways, the pathologists [2] came to the conclusion that an *anti-apoptotic* mechanism had been activated in this case, which led to the disruption of *programmed cell death* and therefore abnormal cell proliferation. More specifically, the activation of AKT pathway led to the activation of the of NF-kappa-B protein that in turn resulted in up-regulation of BCL-2 protein, which is known to be an anti-apoptotic agent.

To test our approach, we ran the algorithm for BCL-2, AKT and NF-kappa-B proteins and a set of signal transduction pathways that included AKT and NF-kappa-B pathways. The resulted diagram of extracted causal links is presented on Fig. 24.

Figure 24 Uncovered morphoproteomic relationships Case 1

As it can be seen from Fig. 24, the algorithm has identified the AKT pathway as the cause of the expression of *RELA* protein, which is a subunit of NF-kappa-B protein. Our method has also correctly positioned BCL-2 downstream from NF-kappa-B signaling pathway, which explains the expression of its main effector RELA (NF-kappa-B) protein.

The second morphoproteomic case [20] involved a study of *Rapamycin* drug as a potential treatment for *Mesenchymal Chondrosarcoma*. In this case IHC studies revealed cytoplasmic expression of phosphorylated AKT (p-AKT) and *mTOR* (p-mTOR) proteins as well as nuclear expression of phosphorylated *p70 S6 kinase* (p-p70S6K). After morphoproteomic analysis the conclusion was that p-AKT causes an activation of p-mTOR, which in turn phosphorylates p-p70S6K. Since mTOR is a molecular target of Rapamycin, it was suggested this drug be used to inhibit mTOR activity and therefore disrupt activation of p70S6K and consequent *G1 cell cycle progression* in malignant *Mesenchymal Chondroblasts*.

Figure 25 Uncovered morphoproteomic relationships Case 2

To see if our method can produce the same morphoproteomic portrait, we ran the algorithm for AKT1, *FRAP1* (alias for mTOR) and *RPS6KB1*(alias for p70S6K) proteins and AKT and mTOR pathways. The morphoproteomic diagram with discovered causal links is presented in Fig. 25.

AKT is protein is known to be a key modulator of AKT pathway whose activity is closely related to mTOR pathway [85]. This fact is shown by a causal link on our diagram in Fig. 25. The second matched molecular mechanism is represented by a causal link from FRAP1 to RPS6KB1 proteins. It was reported in the literature that mTOR signaling pathway regulates FRAP1 (mTOR) protein kinase [109], which makes these two entities “interchangeable” in terms of the overall workflow of involved proteins and pathways. This demonstrates that revealed by our approach relationships confirm the findings described in [20].

The conducted experiments resulted in discovering causal links that match conclusions done by the pathologists. But unlike manual analysis by a human, these discoveries were performed by a computer, based on the information from the knowledgebase.

3.3.3 New Findings in Morphoproteomic Studies

Knowledge exploration through SPIRIT may effectively lead to the discovery of clinically useful information in regard to treatment of disease of all forms. In this dissertation, we focus on applications that identify new or novel means of treating malignancies that are advanced and resistant to the usual therapeutic modalities.

Generally, these patients have already undergone treatment with multiple standard drug regimens and perhaps even experimental drug trials. It is in such cases that morphoproteomics provides essential information for personalized medical care based on the interpretation of findings in the biopsies of the patients own tumors in contrast to basing therapy on that general class of tumors. However, the linkage of visually observable tumor features to potentially effective chemotherapy requires a deep, as well as broad, knowledge of both cancer genomics and pharmacogenomics, which is well beyond the capability of most pathologists. Nonetheless, when provided with access to an extensive genomics database and a rational approach to information retrieval based on cellular pathways and protein interactions, a pathologist or oncologist can leverage his/her basic knowledge of disease to obtain more specific and relevant data through their own iterative interactions with the knowledge base.

For example, we consider the treatment of squamous cell carcinoma (SCC) which is a very common malignancy affecting an extensive variety of tissues. Feng et al. [11] have described their experience in *marshalling* proteomic evidence for the unconventional treatment of cervical squamous cell carcinoma by identifying over-expression of proteins within the epidermal growth factor receptor (EGFR) and the mammalian target of rapamycin (mTOR) signaling pathways. These pathways were evaluated in view of their known frequent involvement in malignancies as well as their susceptibility to pharmacologic inhibition. The EGF pathway may be down regulated with erlotinib and mTOR with an analogue of rapamycin, temsirolimus. The authors conclude that such a regimen would be reasonable for the treatment of early stage cervical SCC.

In order to investigate the potential for SPIRIT to contribute new knowledge relevant to the treatment of SCC in general, we queried the knowledge base for other possible protein interactions in the EGFR signaling pathway that were not investigated by Feng et al. [11].

**Figure 26 Morphoproteomic diagram from Feng et al.
(included with permission from R. Brown and Int J Clin Exp Pathol)**

Starting with a simplified schematic illustration of the EGFR pathway in Fig. 26, several proteins were queried leading to the identification of interactions between JAK1, RASA1, STAT1 and STAT3 (Signal transducers and activators of transcription 3) which are components of the JAK-STAT signaling pathway. To check protein and pathway

relationships discovered by our method a systematic literature review was conducted using a set of queries for PubMed searches.

STAT1 and STAT3 do indeed interact. The following query returned 191 articles that support activation of STAT1 by STAT3, with [110], [111] being the representative papers.

<stat1[All Fields] AND ("stat3 transcription factor"[MeSH Terms] OR ("stat3"[All Fields] AND "transcription"[All Fields] AND "factor"[All Fields]) OR "stat3 transcription factor"[All Fields] OR "stat3"[All Fields]) AND ("Interaction"[Journal] OR "interaction"[All Fields])>

STAT3 over-expression is an important factor in SCC acting as an oncogenic transcription factor, which regulates a number of pathways important in neoplastic transformation including angiogenesis, cell cycle regulation and apoptosis [7], [112-118]. The following query returned 87 articles supporting the involvement of STST3 in SCC.

<carcinoma, squamous cell"[MeSH Terms] OR ("carcinoma"[All Fields] AND "squamous"[All Fields] AND "cell"[All Fields]) OR "squamous cell carcinoma"[All Fields] OR ("squamous"[All Fields] AND "cell"[All Fields] AND "carcinoma"[All Fields])) AND ("stat3 transcription factor"[MeSH Terms] OR ("stat3"[All Fields] AND "transcription"[All Fields] AND "factor"[All Fields]) OR "stat3 transcription factor"[All Fields] OR "stat3"[All Fields])>

STAT3 may be down regulated by a wide range of compounds including clinically utilized small molecule inhibitors, numerous experimental drugs, small interfering RNAs, and nutrigenic substances including the green tea extract, EGCG [111], [119-123]. The

systematic literature review using the following query returned 2059 supporting the inhibition of STAT3.

<("inhibition (psychology)"[MeSH Terms] OR ("inhibition"[All Fields] AND "(psychology)"[All Fields]) OR "inhibition (psychology)"[All Fields] OR "inhibition"[All Fields]) AND ("stat3 transcription factor"[MeSH Terms] OR ("stat3"[All Fields] AND "transcription"[All Fields] AND "factor"[All Fields]) OR "stat3 transcription factor"[All Fields] OR "stat3"[All Fields])>

And finally, native and activated (phosphorylated) STAT3 proteins may be identified with routine immunohistochemical staining with localization in the cytoplasm and nucleus respectively. We executed the following query which returned 1067 supporting papers, with [113], [115], [117], [124-127] being representative.

<("stat3 transcription factor"[MeSH Terms] OR ("stat3"[All Fields] AND "transcription"[All Fields] AND "factor"[All Fields]) OR "stat3 transcription factor"[All Fields] OR "stat3"[All Fields]) AND ("immunohistochemistry"[MeSH Terms] OR "immunohistochemistry"[All Fields])>

The interactions that have been revealed by our approach are shown in Fig. 27.

Figure 27 Uncovered morphoproteomic relationships Case 3

Therefore, this experiment demonstrates the type of *expanded and newly discovered knowledge* that is facilitated by SPIRIT. Some of the factual knowledge acquired through the assistance of SPIRIT may be summarized as follows: The activation and over-expression of the transcription factor STAT3 plays an important role in the SCC genomic apparatus. The activation of this protein may be identified by the presence of the phosphorylated form and its translocation from the cytoplasm to the nucleus. STAT3 expression has been demonstrated to be down regulated by several pharmacologic agents. These agents have shown some experimental effectiveness in other malignancies including breast carcinoma and chronic myeloid leukemia.

3.3.4 Discussion of Proposed Approach and Implications to Biomedical Domain

We believe that our computational approach for discovering relationships among proteins and pathways will have broad impact on personalized medicine. Generated by our methods morphoproteomic diagrams will enable pathologists and oncologists to effectively utilize vast amounts of publicly available “omics” data to *find new drug targets*.

First of all, the central goal of the proposed method is to infer causal dependencies among biological entities *based on data from public biomedical databases*. Even though traditional bioinformatics research does involve studies of casual links in protein-protein interactions or biological pathways, we, however, found that the research community still needs significant work that focuses on inferring casual relationships from online knowledgebases. Unlike most of the current bioinformatics studies, which utilize either wet lab results or results from mining unstructured literature, our approach draws its conclusion from high quality, *curated data* from sources that are considered by the scientific community as the state-of-the-art knowledge of biochemical processes (e.g. Reactome, UniProt and EntrezGene). The result of a traditional bioinformatics experiment is greatly dependent on the conditions and type of wet lab study (e.g., study protocol, contamination, normalization method) that provides low-level “raw” data for computational processing. That explains possible heterogeneity of the results. In this research, we use high-level data that have been reviewed and accepted by the experts in the field and confirmed as a consensus for a number of wet lab experiments done by

different investigators. Moreover, since we use data sources that include citations of original articles from which a certain fact of the knowledgebase was generated, we can provide links to those articles during morphoproteomic experiments to “justify” any conclusions that our framework might make.

Second of all, given the task of finding a mechanism that explains protein expression pattern from an IHC study, we can search a large number of signal transduction pathways and proteins. In [6] Brown mentions numerous collaborations with pathologists from other institutions. The primary goal for this is to use the knowledge of other experts to be able to check the pattern against a larger set of biochemical processes, to find a better place of intervention. In our case, the SPIRIT data integration methodology does not put a cap on the number of its constituent knowledgebases. In our opinion, SPIRIT has greater chance to provide more comprehensive molecular pathology picture of what is happening in diseased tissue than even an extended team of experienced pathologists.

Next, the underlying SPIRIT Semantic Web framework allows our method to use predicate logic and entailments to flexibly make connections among different entities in the “mashup”. We utilize the Semantic Web rules and entailments to deduce new knowledge that is not presented in the KB *directly*. It provides means to solve, for instance, the *problem with aliases*. It is a known fact that in most cases biomedical databases list different names for the same entity. If a researcher wants to find an interaction using the name of a protein, he/or she may need to list all its *aliases*. We define Semantic Web rules to tie together RDF resources that represent the same biological entities. Moreover, given a protein, we expect our approach to be able to associate it with other proteins that are either from a related functional family, its sub-units

or from the same protein complex. That increases a chance of potentially discovering biochemical mechanisms that would otherwise be missed.

Our approach represents a computerized Pathologist-In-The-Loop setting which serves a hypothesis generator. This approach leverages the endurance of a computer to process vast amounts of data and the creative power of a human brain to make the final decision.

Finally, the proposed methods have great potential to be extended to other areas of biomedicine. For instance, SPIRIT can be used to generate hypotheses in the more general systems biology domain. We can alter the SWMKB's Semantic Web rules and add new ontologies with RDF data sources to tackle more general problems of inter-relationships among proteins and pathways on a systems biology level. Theoretically, the proposed formalisms of inferring casual links from RDF knowledgebases can be utilized in any field of pathology, we just need to represent the data in RDF format and supply required ontologies. One of such areas could become a forensic pathology, where *cause-and-effect* conclusions are sought in the basis of available facts and common knowledge of physiological and pathological processes.

Summary and Future Work

3.4 Summary

In this dissertation we have presented two novel informatic methods to help pathologists streamline and improve molecular pathology studies.

The ATOS tool optimizes IHC antibody test selection by employing Shannon's entropy maximization idea to select the most informative antigen at each step. This results in a more than three-fold reduction of the turn-a-round time, but most importantly dramatically decreases inter- and intra-pathologist variability in IHC studies.

The second proposed method brings the theory of causation into the area of Semantic Web knowledgebases of protein and signal transduction pathways. We extend Pearl's formalism [52], [96] to infer causal links among proteins and pathways from RDF graphs of these knowledgebases. In our opinion, this method not only makes the new field of morphoproteomics more feasible to practice by pathologists and oncologists alike, but also has great potential to be extended into molecular systems biology and other biomedical domains.

Both tools are parts of our larger computational SPIRIT framework that is intended to facilitate contribution of pathology to personalized medicine and the new emerging field of theranostics.

3.5 Future Work

We have identified three directions for the future research related to the methods discussed in this dissertation. All three are concerned with improving our computational morphoproteomics module of the SPIRIT framework, i.e. the method of inferring causal links from RDF graphs. These future research directions are presented below.

Currently, SPIRIT is designed *to only depict a possible picture of causal morphoproteomic mechanisms and to facilitate generation of new hypotheses*. However, the main morphoproteomic goal is to *search for new effective drug targets*, i.e., places in the web of inter-relationships among proteins and pathways that can be interrupted to prevent undesired physiological processes from happening. Therefore, an effective computational method to search for drug targets is needed. There are several issues associated with such a search. As one can see from Fig. 24, the AKT pathway is associated with more than one path that ends with BCL-2 protein. It is shown as a direct cause of expression of BCL-2, but can also be viewed as an indirect cause of this protein's activity through its constituent protein AKT1, RELA and NF-kappa-B pathway mechanism. In other words, our framework is set to discover *any causal link* between any two biological entities. We need a means to analyze such paths and select the ones that can serve as candidates for interruption by drugs. We also need to analyze drug targets on their clinical merits as well as their potential to cause undesired side effects.

Another challenge is related to the fact that the success of discovering causal links by constrained-based structure learning algorithms greatly depends on the sufficiency of supplied graphoids. We have noticed that adding an additional graphoid can “trigger” the algorithm to discover a causal link, which would otherwise be missed. We believe that this happens because of insufficiency of the set of constraints (graphoid) for the causal formalism to infer causality. Therefore, we need to extend the formalism to suggest possible graphoids, the addition of which can lead to a larger number of discovered causal links.

More research is needed to improve the efficiency of RDF path computations. Since RDF paths are computed on the basis of SPARQL queries, we could explore parallel processing algorithms including those based on MapReduce and Hadoop technologies [128, 129].

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