Book Chapter

Dissecting Cellular Phenotypes in the Tumour Microenvironment through Gene Regulatory Networks: Inference, Analysis and Dynamical Modelling

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Abstract

Gene regulatory networks (GRNs) and mathematical modelling are critical for understanding the complex regulatory mechanisms that underlie tumour development and progression, providing insights into the molecular mechanisms that drive cancer, including the identification of key driver genes/pathways, and novel therapeutic targets. In the context of the tumour microenvironment (TME), the complex interactions between immune and cancer cells give rise to a cascade of regulatory processes at different levels, defining the cellular behaviour and response to external or internal stimuli. It has been shown that, in the presence of cancer cells, several immune cells including macrophages, neutrophils or T cells undergo cell-state transitions toward pro-tumoral phenotypes or exhaustion. Therefore, the detailed molecular description of cancer cells' and immune cells' behaviour, and cell-state transitions in response to their interactions, particularly at the molecular level, remain crucial to cancer research. In this chapter, we give an overview of mathematical modelling of cancer systems through gene regulatory networks, and give a step-by-step tutorial of how to build them from gene expression data, to analyse and study their temporal behaviour through dynamical modelling.

Introduction

In various subjects, including cancer research, the term "*complex*" is usually mistaken to define "*complicated*", or non-trivial problem-solving tasks. However, the theory of complex systems provides a more precise definition of what a complex system is, and its properties. A system is considered *complex* if certain properties, such as emergent behaviour, nonlinearity, feedback loops and adaptation, emerge from the collective behaviour between the system components and the surrounding environment. From this definition, all biological systems, including the tumour microenvironment (TME), are inherently complex, and their global structure and temporal behaviour (in different scales, from molecular to cellular level) cannot be straightforwardly inferred from the local properties of their interacting components. To facilitate the study of these systems,

the intracellular interacting components can be best represented as a network, commonly visualised as a graph of *nodes (vertices)* connected by *edges (links)* (Figure 1). Depending on the types of nodes and edges, different molecular networks exist, like protein-protein networks (PPI) [1,2], gene regulatory networks (GRN) [3,4], signal transduction networks [5,6], etc. This representation enables the application of the mathematical toolbox of graph theory to study the structural properties of these networks and, furthermore, of dynamical models to study their temporal behaviour under different environmental conditions, effect of drugs, or intracellular mutations. In particular, building these networks using various sources of information constitutes an important reverse engineering process, referred to as *network inference*, and requires the combination of both a thorough biological understanding of the system, and the application of accurate and advanced computational inference methods [7]. Notably, the advanced technological improvements in measuring gene expression and the ever increasing interest in clinical applications of genomics, confer data-driven GRN inference methods with high importance and relevance to get more precise insights on gene regulation, drug action, pathway perturbation, etc.

Thanks to the technological developments of the last 20 years, inference methods have also evolved from inference based on bulk gene expression data [8–11] to single-cell transcriptomics [12–17], and extending to time-series and/or pseudotime of transcriptomics, with which more accurate knowledge on gene-gene interaction can be inferred [15,18,19].

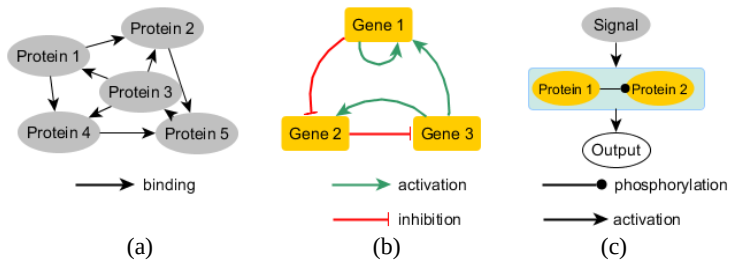


Figure 1: A simplified example of (a) protein-protein (PPI) network, (b) gene regulatory network, (c) signal transduction network.

The importance of performing such calculations relies on using these networks for getting relevant insights on the biology underlying them, aiming to make new discoveries on molecular interactions, drug targets, mechanisms of action, etc. [3]. For example, performing *in silico* experiments and verifying how a perturbation in one of the molecular pathways, genes or interactions might affect the downstream network components helps understanding which are the key intracellular mechanisms, regulators and processes that drive the appearance of particular cell phenotypes or cell states. Experimentally, we can then compare the gene abundance in the perturbed and unperturbed cells and, potentially, identify putative target genes that determine cellular dynamics.

In this chapter, we follow a *horizontal approach*, giving a description of GRNs and their topological features, describing the algorithm behind some available methods on network inference from experimental data, and giving an introduction to dynamical modelling of GRN, as a promising perspective toward bridging the computational methods to the biology of GRNs. This chapter is intended to give biologists and researchers in cancer research an overview of how to use the network approach, and the information it provides to answer their biological questions, aims and research goals. Throughout the text, we direct the readers with a higher mathematical background to consult the recommended literature, for more detailed mathematical and algorithmic details. Thus, the goals for this chapter are as follows:

- give a description of GRNs as simplified representation of regulatory interactions between molecular entities and explain the type of information provided by performing structural analysis on the GRNs;
- describe the algorithm and list some available methods on network inference from public databases and experimental data;
- give an introduction to dynamical modelling of GRNs and describe the basic principles of Boolean modelling;
- list some computational tools to simulate and analyse Boolean models, and describe some recent work on the application of Boolean modelling in cancer research.

Like other similar works, this chapter provides a partial glimpse into the domain of research that is constantly evolving. Considering the substantial amount of ongoing research in this field, it is evident that significant advancements can be expected in the coming years.

Gene Maps: Network Representation of Gene Regulation

Although a single definition of GRN does not exist, we define GRNs as topological maps representing the connection between regulatory proteins (e.g. transcription factors (TFs), RNA binding proteins) that control the expression level of a gene. These interactions can be represented as directed graphs of *nodes* and *edges*. Notably, the directionality of the edges (defining the source and the target in the interaction) represents an important feature in the case of regulatory networks, defining the direction of information flow. For example, in a GRN or metabolic network a TF can regulate the expression of a gene, or a protein can contribute to the production of another protein indirectly, but not vice versa [20]. In these networks, additional information is added by indicating specific types of interactions, represented by signed edges (Figure 1, b). In the simplest case, the interactions are categorised as *activations and inhibitions*, represented as *positive and negative edges* accordingly. GRNs are composed of *regulatory nodes* (*source/cause nodes*) and *regulated nodes* (*target/effect nodes*), generally mapped as TF-target gene networks. The structure of the network enables the calculation of various quantities that capture different features of the network topology, and reveal important information on the underlying biology of the system. In the following sections, we describe some of these features and their biological implication in the network structural organisation. We direct the reader to [21–24] for a complete overview of the topological analysis of networks, whereas a list of the basic parameters and their definition is given in Table 1.

Table 1: A list of basic network parameters.

Parameter		Definition
n	total number of nodes	
m	total number of edges	
k	degree of a node	The number of incoming and outgoing edges connected to the node
z	mean degree	The average of node degree, calculated among all the nodes in the network
l	mean distance	The average shortest path between two nodes, calculated among all the nodes in the network
λ	scale coefficient	The exponent of degree distribution if the distribution follows a power law
r	degree correlation coefficient	The Pearson correlation coefficient between the degrees found at the two end of the same link
d	diameter of the network	The shortest distance between the two most distant nodes in the network
C	clustering coefficient	The level to which nodes in a graph tend to cluster together

Basic Concepts of Networks

First, let's describe the concept of *centrality*, as a measure for identifying the most important or central nodes in the network. Notably, what defines a node as important is relative to the type of centrality measure, therefore several nodes can be identified as such. Further analysis (such as TF activity estimation) can help validate the results.

The basic mathematical presentation of a network, either directed or undirected, is the adjacency matrix. By definition, the adjacency matrix is a $n \times n, n - number\ of\ nodes$ matrix, whose elements take values

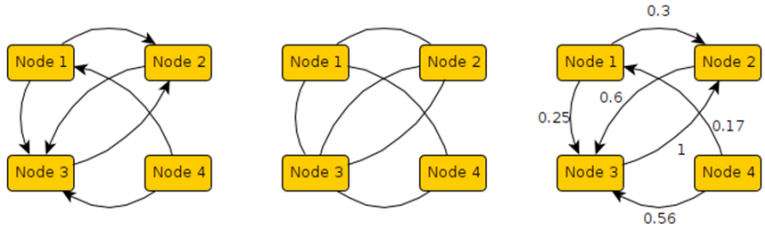
$$A_{ij} = \begin{cases} 1 & \text{if there exists an edge between node } i \text{ and node } j \\ 0 & \text{otherwise} \end{cases} \quad (1)$$

From its definition, it is important to note that in directed networks, such as GRNs, the adjacency matrix is not symmetric. For example, the adjacency matrices of the small networks in Figure 2 (a),(b), are going to be

$$A = \begin{pmatrix} 0 & 1 & 1 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 1 & 0 & 0 \\ 1 & 0 & 1 & 0 \end{pmatrix}$$
$$B = \begin{pmatrix} 0 & 1 & 1 & 1 \\ 1 & 0 & 1 & 0 \\ 1 & 1 & 0 & 1 \\ 1 & 0 & 1 & 0 \end{pmatrix} \quad (2)$$

Additionally, we notice the binary nature of the adjacency matrix ($A_{ij} \in \{0,1\}$); however, in many cases, as well as in GRNs, the elements of the adjacency matrix can take non-binary values, representing the strength or weight of the interaction between two nodes. We refer to these networks as *weighted networks*. For example, in a GRN, the weights might indicate the strength of the interaction or regulation of a TF on a gene, or the effect that a protein might have in regulating the expression of another gene.

Notably, the weights are usually positive numbers, but in regulatory networks they can also take negative values, indicating the *type* of the interaction. The positive values would then denote positive regulation (activation), and the negative values would indicate negative regulation (inhibition).



(a) (b) (c)

Figure 2. An example of a regulatory network composed of 4 nodes. (a) directed network, (b) undirected network, (c) directed weighted network.

Degree Centrality

Given a network, the *degree k* of a node is defined as the number of links connected to it. The term degree centrality is usually used to emphasise the usage of the degree as a centrality measure. In directed networks, such as GRNs, nodes can have both incoming and outgoing edges, therefore we distinguish between *in-degree* (k_{in}) and *out-degree* (k_{out}) of a node. In large networks, the degree distribution $P(k)$ defined as the fraction of nodes having degree *k* might be highly informative on the network organisation, identification of network *hubs* as nodes with particularly high degree, its connectivity, etc.
Given the definition of the adjacency matrix, the degree of node i can be simply calculated as the sum of the i^{th} row in undirected networks, or the sum of the i^{th} row plus the sum of the i^{th} column in directed networks.

Eigenvector Centrality

Eigenvector centrality can be defined as an extension of degree centrality. In principle, it measures the connectivity of *important nodes* with each other: considering a node connected with several neighbouring nodes and their downstream networks, we can note that not all the nodes are equivalently significant, and the node's importance is increased either by having many connections or by it being connected with other important nodes, or both. The eigenvector centrality score is therefore proportional to the sum of the centralities of its j neighbouring nodes:

$$x_i = \sum_j A_{ij} x_j \qquad i = 1,2,\dots n \tag{3}$$

It can be proven that the limiting vector of centralities is proportional to the largest eigenvector λ_{max} of A_{ij}, from which it takes the name [23]. It is important to note that the eigenvector centrality scores are all non-negative values, obtained from the multiplication of a positively defined matrix with a positively defined vector. Careful attention should be paid in the case of directed networks, where the asymmetry in the adjacency matrix

raises additional questions. The asymmetric nature of A_{ij} gives two leading eigenvectors, defined as *left* and *right* eigenvectors, representing the *outgoing* and the *incoming* edges respectively. Generally, the centrality score is defined from the right eigenvector - however, this might affect the calculation of eigenvector scores for the other nodes. For example, in the network of Figure 2 (a), Node 4 does not have any incoming edges, therefore its score is equal to zero. Node 1 however has an incoming edge from Node 4 and 2 outgoing edges, but since Node 4 has a score equal to zero, from Eq. 4 Node 2 will also have a score equal to zero. By following this logic, many nodes will be defined by an eigenvector centrality score equal to zero, and only the nodes with a large in-degree will be distinguished by a non-zero score.

PageRank

From the definition of eigenvector centrality, we see that a node with high centrality score increases the centrality of all the nodes it points to. Consequently, if a high-centrality node points to many other nodes, all the nodes will have a high centrality too, and the effect might be distributed throughout the network. To avoid this effect, *PageRank* calculates the centrality of a node as proportional to the centrality of its neighbouring nodes, divided by their out-degree. In this way, nodes pointing to many other nodes have a small contribution to the centrality of the nodes they are pointing to. The mathematical formula of PageRank is thus written as

$$x_i = \alpha \sum_j A_{ij} \frac{x_j}{{k_j}^{out}} + \beta_i, \quad i = 1,2,\dots n \tag{4}$$

where α and β are positive constants, representing a normalising factor and the centrality of the node with zero in-degree respectively. Importantly, β_i enables the zero in-degree nodes to have a non-zero centrality, therefore the nodes they point to derive some advantage from being pointed to. Eq. 5 however raises a problem if ${k_j}^{out} = 0$. In this case, the easier solution is to set ${k_j}^{out} = 1$, as the nodes without-degree equal to zero should not contribute to the centrality of other nodes.

Betweenness Centrality

Comparably from the measures represented above, *betweenness centrality* is a measure of the extent to which a node is located on the path connecting other nodes. Therefore, betweenness centrality might be an important measure to identify the nodes with the highest influence in the network, by quantifying their control on the spread of information. Consider, for example, a GRN with a perturbation applied on one node. We can think of the perturbation being an "information" flow, diffused from node to node by cascades of interactions. In the long term, we can suppose that the information has reached every node in the network, and define the betweenness centrality as *the number of times a node lies in the information flow path between other nodes*. Mathematically, letting ${l^i}_{j\to m} = 1$ if node i lies in path between j and m, and 0 otherwise, the betweenness centrality is calculated as

$$x_i = \sum_{j,m,j\neq m}^{n} {l^i}_{j\to m} \qquad i = 1,2,\dots n \tag{5}$$

Importantly, contrary to the other measures presented above, betweenness centrality is not always an indicator of the connectivity of a node. For example, a node connecting two clusters of nodes might have a small degree but it is essential for transmitting the information from one cluster to another and will thus have high betweenness centrality). The interpretation and generalisation of betweenness centrality however depends on the definition of information flow along the edges and the type of the network itself.

We refer the reader to [23] for a broader description of betweenness measures.

Computational Tools for Structural Network Analysis

Numerous computational tools for performing structural and topological analysis of networks have been developed and are available in different programming languages. Here, we mention some of them, focusing on the main libraries used for the

analysis of biological networks. In Table 2 we list the tools, altogether with their features and computational characteristics.

Table 2: A list of network analysis tools and their features.

Tool	Language	Features
Cytoscape [25]	Java	● Finding a set of differentially expressed genes ● Retrieving relevant networks from public databases ● Integration and visualisation of experimental data ● Topological network analysis ● Network functional enrichment analysis ● Exporting network visualisations ● Extensions according to a long list of available apps, including community detection, etc.
igraph [26]	Python and R packages Code written in C and C++	● Creating graphs from scratch or generating graphs ● Setting and retrieving attributes ● Calculating various structural properties of graphs ● Querying vertices and edges based on attributes ● Treating a graph as an adjacency matrix ● Plotting and visualisation
NetworkX [27]	Python Code written in C, C++, and FORTRAN	● Studying structure and dynamics of social, biological, and infrastructure networks ● Standard programming interface and graph implementation that is suitable for many

		applications • Rapid development environment for collaborative and multidisciplinary projects • Optimal for large datasets
gephi [28]	Java	• Intuition-oriented analysis by network manipulations in real time • Revealing the underlying structures of associations between objects • Representing patterns of biological data
R packages Tidygraph ggraph network visNetwork networkD3 WGCNA	R	• Node and edge list • Creating network objects • Centrality measures analysis • Graph exploration • Network layouts • Highlighting aspects of the network, specific nodes or links • Interactive and animated network visualisation

Gene Regulatory Network Inference

By definition, the process of building the network structure of a biological system through a reverse engineering process is referred to as GRN inference, usually describing the use of experimental data to predict the causal relationships between molecular entities. More precisely, GRN inference involves various forms of reasoning and use of evidence from different sources, including literature, public repositories of experimental data, etc. In this way, the GRN inference approaches can be classified in two groups[1]: (1) *bottom-up approach*, and (2) *top-*

[1] This classification of approaches is usually used when building Boolean models of interacting genes, which we will discuss more in detail in section 3.1. In this case the two approaches are used not only to infer the causal interactions between genes, but also the Boolean formalism governing these interactions.

down approach. The first category, also known as knowledge-driven approach, consists of an extensive literature evaluation and the use of biological pathway databases and text-mining algorithms to build the functional networks of genes of interest. The second category, known as data-driven approach, identifies differentially expressed molecular entities from 'omics-based analyses and bioinformatics tools to perform functional annotation and biological pathway mapping. Each approach has limitations that can result in the omission of important nodes and pathways within the biological system of interest. Literature-based networks may overlook crucial features or require choosing between conflicting information. On the other side, data-driven approaches can fail to identify differentially expressed components depending on experimental design, statistical methods, timing, and biological variability. Ideally, a hybrid approach that combines knowledge and data-driven methods should be employed to construct interaction networks. We will discuss more on the current challenges of each approach in the following sections.

Inference from Databases

A common approach to build a GRN is to exploit TF-target interactions annotated in public databases, which can be of different kinds, depending on the type of information they contain [29–31]. Some databases will provide information on functional interactions, that is evidence that two genes/proteins, in this case a TF-target pair, can be related based on a any type of link between them, evidenced by gene expression correlation, co-evolution of the genes, co-mention in scientific abstracts etc. These functional interactions can be found in databases such as String [32], Reactome (the functional interaction network [33], TRRUST [34] or RegNetwork [35], the latest selecting information from around 25 databases. Moreover, there are efforts to map more specific TF-target interactions based on experimental evidence, either collecting results of CHIP-seq experiments, which show in which target gene promoters the binding peak of the TF can be found, or also combining these experimental results with a crosscheck of the presence of the TF binding motif in the peak regions. Among the databases

including these interactions we mention ChEA [36] or ReMap [37]. Additionally, other databases, generally used when GRN inference is performed on single-cell RNAseq datasets, the validation of inferred interactions is performed on data on the same or similar cell types. Databases providing such information include ENCODE [38], ESCAPE [39], or CHIP-Atlas [40].

Inference from Expression Datasets

Based on input data used, data–driven GRN inference methods can be categorised in two types: (i) *steady state gene expression*, and (ii) *time-series gene expression* inference methods. In the first category, gene regulatory network (GRN) inference typically involves perturbing the system or studying different instances to estimate gene expression levels once the system reaches equilibrium. In the second category, the input data consists of gene expression measurements taken at multiple time points following a perturbation, enabling the observation of the temporal evolution of expression profiles. As a result, time-series inference methods can provide more comprehensive information compared to static data, allowing for the inference of gene functionalities, interactions, causal relationships, and potential clinical implications based on the dynamics of gene expression [41]. However, both methods have limitations due to technical issues inherent in experimental protocols, such as limited sampling time points, cost constraints, difficulties stemming from lack of cell-cycle synchronisation, and sparsity of gene expression data (in the case of single-cell RNAseq). To address these limitations, various computational methods have been developed that combine steady-state and time-series approaches. Moreover, the emergence of single-cell transcriptomics technology has prompted the development of inference methods specifically tailored to single-cell data analysis [42].

Problem Definition

Let's define the dataset as D_S, a matrix with dimensions $N \times S$, where N is the number of genes and S is the number of samples in which their expression is measured:

$$D_S = \{\boldsymbol{X^1}, \boldsymbol{X^2}, \ldots, \boldsymbol{X^S}\} \quad (6)$$

where $\boldsymbol{X^s}, s = 1,2,\ldots,S$ is a vector of $\boldsymbol{N}$ genes with their expression for each sample s. A similar problem definition follows in the case of time-series transcriptomics, in which case the expression dataset D_S is given as a function of expression levels at different time points [43]. The main goal of inference methods is to assign a weight $w_{j,i} \geq 0, i, j = 1,2,\ldots,N$ to any putative interaction between gene i (target) and j (source), representing a regulatory interaction in the biological system. To this purpose, different inference methods use various regression tools to model the expression of a gene as a function of its regulators. Independent of the method chosen, the goal is to reconstruct the GRN that would produce the observed profile of expression, in the form of a directed graph, in which each edge is associated with its characteristic weight.

Based on the inference model they use, inference methods can be grouped into 7 categories, namely (i) mutual information (MI), (ii) dynamical Bayesian, (iii) Granger causality, (iv) Boolean, (v) ordinary differential equation (ODE), (vi) graphical Gaussian and (vii) regression. Importantly, many methods apply a combination of different models and approaches in order to increase the accuracy to gain more insights from the inferred network. We refer the reader to [43] for a mathematical description of these inference algorithms based on bulk and single-cell RNAseq time-series.

Validation of the inferred network is a crucial step in the GRN inference process. It is important to have a validation protocol that can assess the quality of each proposed model, enabling the selection of the optimal inference procedure from the available algorithms. Despite progress in this area, evaluating the effectiveness of inference methods remains challenging, primarily due to limitations in ground truth or gold standard datasets. Existing repositories of experimental data provide reference data for only a limited number of interactions, thereby restricting validation to a subset of the inferred network. Consequently, interactions without reference data are often considered non-existent, raising questions about potential biases

and the ability to infer novel interactions and regulators. Relying solely on prior knowledge for validation may hinder the identification of new regulatory pathways within the system under investigation, especially when gold standard references with high scores are absent or scarce.

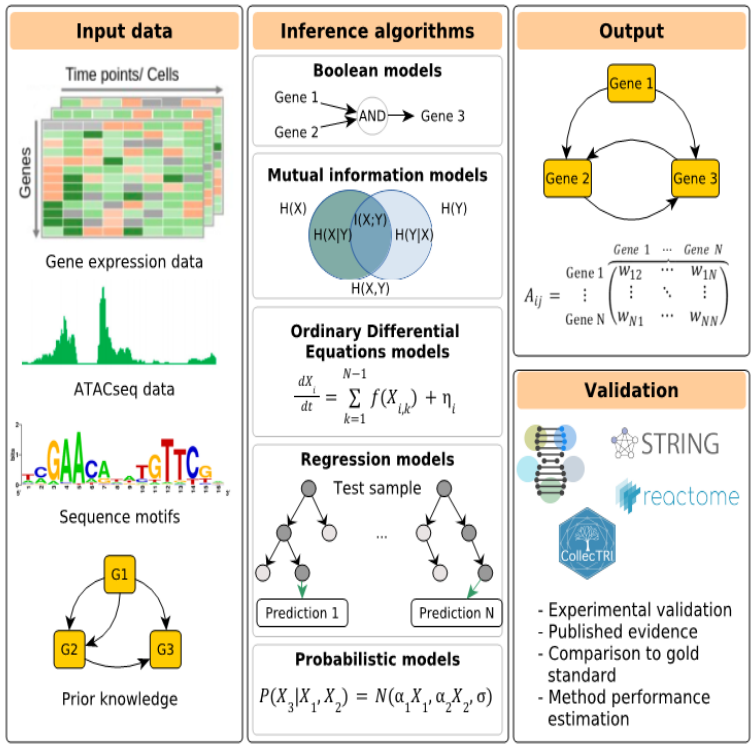


Figure 3: General schema of network inference from expression datasets.

Another possibility for network validation comes from using simulated data, which can be engineered to include several conditions and measurements [9] - yet the extent of coverage of the inferred interactions remains limited to small network size. Other than validating the inferred network with a gold-standard one, another important point in GRN inference is comparison between networks obtained by the different inference methods, on the same dataset, which can provide an estimate of the "robustness" of the inferred network. Additionally, one may

perform this benchmark analysis in order to choose the algorithm/method that best suits a given dataset.

Quantitatively, the algorithm's performance can be evaluated as for any prediction by two metrics typically used in prediction tests: (i) area under precision-recall curve for estimating the performance of a certain algorithm, and (ii) area under receiver operating characteristics curve for comparing the GRN inferred by the algorithm against a gold standard network.

Computational Tools for GRN Inference from Expression Data

When performing GRN inference, the scope is to deduce which are the key molecular entities, whose interactions determine and explain a cell state (phenotype), or a cell type (in differentiation processes). Therefore, depending on the type, and amount of data available, the focus of GRN inference can be either to build a general GRN (i.e., covering all the possible scenarios of cell states/types), or a cell-state/type specific GRN (i.e. a specific GRN for each of the cell states/types identified during the experiment).

Bulk transcriptomics, either steady-state or time-series, come as a low cost sequencing solution compared to single-cell RNAseq, but comes with limitations, such as providing only an average gene expression level at a given time, estimated from a population of cells of different states/types. Nonetheless, this type of data can be more adapted when the focus is to build a general GRN of cell fate decisions. This evolving field has resulted in the development of various algorithms and computation approaches, from sparse candidate models, module networks, ensembles of networks, and furthermore methods that combine prior knowledge and TF activity estimation with data-driven network inference [30,44].

For some biological processes, like cell differentiation or phenotypic reprogramming, a higher resolution of the temporal dynamics of gene expression is necessary to identify major phenotypic transitions in complex tissues while characterising

the phenotypic spectrum of individual cells. In this regard, single-cell RNA-sequencing technology enables deeper investigation of the molecular interactions and identification of novel molecular mechanisms that orchestrate biological processes at the single cell level. Computationally, this technological revolution has led to the development of several algorithms to analyse single-cell RNA-seq, and - as a part of it - inference of GRNs. Intuitively, a single-cell resolution of genes' dynamics would lead to an increased accuracy in inferring the functional interactions between genes that define the biological process. However, due to the limitations in some of the most widely available single-cell technologies, the heterogeneity and sparsity of single-cell data lead to limitations and challenges for GRN inference methods and put reliability of these approaches in question.

An important limitation in using single-cell RNA-seq data for GRN inference is usually the lack of time-resolved expression measurements. Instead, many inference methods exploit the multiplicity of RNAseq profiles at one single time point across cells as a proxy for temporal evolution of the phenotype, assuming ergodicity of this system, as is the case for trajectory inference based on pseudo-time ordering of the cells [45]. In this process, the pseudo-temporal trajectory is generated by linearly ordering the single-cell profiles from a specific time point based on their transcriptional similarity, thus enabling the identification of gene patterns along the developmental trajectory of continuously ordered cells [46]. Subsequently, to extract discrete time-points from pseudo-temporal ordering, different techniques can be followed: cell cluster time-point assignment (Slingshot [47], TSCAN [48] or Palantir [49]), partition of pseudo-temporal trajectories into discrete time-points or differential gene expression time-point assignment (Monocle [50]). However, it is important to note that the temporal representation obtained from pseudo-temporal analysis in single-cell lacks the equivalence between pseudo-time and real time. In addition, this pseudo-temporal representation is better suited in developmental systems, in which cells undergo differentiation processes recognised by the presence of bifurcation points in the pseudo-temporal trajectory. This is not always the case, in which

occasion the trajectory inference may lead to inaccurate results. Nonetheless, this representation helps implement the GRN inference algorithms in modelling the expression levels at a given (pseudo)time-point as a function of gene expression at the previous (pseudo)time-point(s). Accordingly, a subset of the inference methods require specific information about the pseudo-temporal ordering of the cells, having a significant difference in performance when such information is not available. Other methods, like GENIE3 [51], GRNBoost2 [52], or PPCOR [53] do not require a temporal ordering of the cells as input and have relatively good performance when tested on some published curated models [54]. However, the incomplete equivalence between bulk gene expression time courses and pseudo-time time-series implies that these two types of inference cannot always be performed by the same tools.

Several benchmarking papers on the performance of single-cell RNA-seq inference methods have been published, facilitating the comparison of different inference methods. We refer the reader to [12,13,16,55] for an extensive review and comparison of single-cell RNA-seq inference methods, [14,54,56] for some benchmarking libraries and to [43,57,58] for an algorithmic review. For a user, the choice between all the different inference methods will depend primarily on the type of data available, the methods' overall performance, the type and amount of information they require, and the type of reconstructed network they provide.

Some of the inference methods to use for bulk and single-cell RNAseq are recapitulated in Table 3. We note that this list is not exhaustive and many other methods are mentioned in the benchmarking and review references mentioned above.

Table 3: List of some GRN inference methods for bulk and single-cell RNAseq datasets.

	Method	**Source**	**Reference**
Bulk transcriptomics	ARACNE TimeDelay ARACNE	https://github.com/califano-lab/ARACNe-AP	[59]

	Method	Source	Reference
	Minet CLR, MRNET		[60] [61]
	GRENITS	https://bioconductor.org/packages/release/bioc/html/GRENITS.html	[62]
	GeneNet	https://strimmerlab.github.io/software/genenet/	[63]
	Inferelator 3.0	https://github.com/flatironinstitute/inferelator	[64]
	TSNI		[65]
	GENIE3 dynGENIE3	https://github.com/vahuynh/GENIE3 https://github.com/vahuynh/dynGENIE3	[51] [19]
	Jump3	https://github.com/vahuynh/Jump3	[58]
	SWING	https://github.com/bagherilab/SWING	[66]
	CellNet	https://github.com/pcahan1/CellNet	[67]
	TIGRESS	https://github.com/jpvert/tigress	[68]
Single cell transcriptomics	SINCERITIES	https://github.com/CABSEL/SINCERITIES	[69]
	SCODE	https://github.com/hmatsu1226/SCODE	[15]
	GRISLI	https://github.com/	[18]

	Method	Source	Reference
		PCAubin/GRISLI	
	SCENIC	https://github.com/aertslab/SCENIC	[45]
	WASABI	https://github.com/eliasventre/cardamom	[70,71]
	CARDAMON	https://github.com/ulysseherbach/harissa	[72]
	CellOracle	https://github.com/morris-lab/CellOracle	[73]
	Pando	https://github.com/quadbiolab/Pando	[74]
	SCRIBE	https://github.com/cole-trapnell-lab/Scribe	[75]
	PAGA - partition-based graph abstraction	https://github.com/theislab/paga	[76]

It is important to note that, although inferring regulatory networks is a trending topic and despite the multitude of different algorithms available, data-driven GRN inference methods struggle to reach a high performance in real-world studies, on both bulk and single-cell RNA-seq data, as reported in [42]. Therefore, their application to biologically relevant datasets remain limited. Nevertheless, different applications of inferring GRNs for novel discoveries in biology have been presented in most of the cited works on GRN inference methods, including studies on cancer, cell development, and cell fate decision. Importantly, combining information from multiple data sources to improve predictions is a current challenge in several research areas. Several recent papers have addressed this issue, including [77,78], where gene expression data are combined with

DNA methylation, copy number variation, or genome-wide binding data. As a consequence, many inference methods introduced above combine multiple data sources to improve the predictions of GRN inference and provide more insightful results on the regulatory processes. For example, in SCENIC [45], results of a GRN analysis are combined with transcription factor binding motif information from RcisTarget to identify a subset of high-confidence interactions. In CellOracle [73] and Pando [74], single-cell RNAseq time-series is combined with ATACseq data. These papers illustrate diverse strategies for integrating multiple data sources to enhance predictions and gain a deeper understanding of complex biological systems.

In cancer research, special focus has been set onto identifying driver genes in cancer progression, and drug resistance. For example, in [79] the authors perform GRN inference from time-series RNAseq in gliomas to build sensitive and resistant networks, found to exhibit significant differences with respect to network topology, local entropy and gene expression dynamics. Based on the node importance score, they developed a differential regulatory network-based biomarker model to identify the most influential genes in the differential network for predicting and controlling drug resistance. Going a step further, [80] use time-series of single-cell RNAseq to study epithelial–to–mesenchymal transition, following a multi-layer network approach, and linking the intracellular gene regulation to cell-cell communications in ovarian cancer cell lines. Other applications of GRN inference to cancer research focused on identifying putative key regulators and gene modules in PDAC disease progression [81], building cancer cell expression networks in liver hepatocellular carcinoma and bladder urothelial carcinoma (BLCA) [82], analysing the functional components by extracting subnetworks and investigating the local landscape of prostate cancer genes [83], etc.

Other applications have been focusing on differentiation processes, like cell differentiation and development [45,70]. In addition, research efforts have been directed towards integration of different layers of information in GRN inference. For example, in [84] time-series of RNAseq data is combined with

ATACseq data to derive dynamic gene regulatory networks for human myeloid differentiation, specifically promyelocytes differentiating into macrophages, neutrophils, monocytes, and monocyte-derived macrophages. In Bocci et al [85], single-cell transcriptomics is fed with mRNA splicing data to identify the key molecular drivers leading to different final states when starting from a common initial state during pancreas endocrinogenesis and epithelial/mesenchymal state transition. In another application, Thorne [86] used public time-series datasets from recount2 database [87] to infer the GRN of neural progenitor cell differentiation. Applying structural analysis on the inferred GRN, they identified key genes which were experimentally observed to influence neuronal differentiation. In another approach, Kamimoto et al [73] used single-cell RNAseq and ATACseq datasets to infer cell state-specific GRNs that emerge during the differentiation process of fibroblasts. In this way, analysing the changes in the GRNs during cell reprogramming or development can help understanding how the TF interactions regulate and define cell identity. In cell fate decision studies, Fleck et al [74] used multi-omics datasets including RNAseq and ATACseq combined with transcription factor binding sites analysis to infer a GRN describing brain organoid developments, leading to the identification of key regulators of cell fate.

Some applications include building molecular disease maps [88,89], phenotypic characterization of a cell in a given microenvironment [90,91], identifying predictive or prognostic biomarkers [92], performing extensive studies on performance of the available methods on different datasets/conditions [17,93,94], and many more.

Despite the exciting progress in the application of data-driven GRN inference methods in biological research, significant challenges remain, as discussed in [43]. Gene expression is a process determined from different levels of regulation, from chromatin to post-translational modifications and signal transduction levels, and including all these levels consist of a non-trivial computational and conceptual task. In addition, these networks are highly context-specific (to a given cell type, tissue, condition, and furthermore to each individual), for which, in

most of the cases, the GRN is unknown. Another major challenge lies in accurately modelling the non-linear dynamics and stochastic nature of gene regulation. Similarly, inferring causality in gene-gene interactions remains a difficult problem to solve due to the observational nature of the data.

Moving forward, a promising direction is the integration of multi-omics data, such as combining transcriptomic, proteomic, and epigenomic information, for a more comprehensive understanding of cellular mechanisms. Transitioning into the realm of artificial intelligence, these computational tools are progressively enhancing our capacity to interpret intricate biological data, especially in the realm of GRNs.

Dynamical Modelling

In addition to the types of analysis represented in the previous sessions that one can perform on the inferred GRNs, dynamical modelling plays a crucial role in analysing the system's behaviour with temporal resolution and across various conditions. For example, having performed GRN inference and validation, one can study what is going to be the *state* (a vector of gene expression of the nodes composing the GRN) of the GRN after multiple time steps, when starting from a certain initial condition (gene expression at the initial time). Dynamical modelling of gene regulatory networks is a computational approach used in systems biology to study the complex interactions between genes and their targets (proteins and RNAs) with the primary goal of understanding how these GRNs evolve over time in response to various internal/external stimuli, predicting their dynamic responses, and gaining insights into the underlying biological processes.

Methods for dynamical models of GRNs cover a spectrum of approaches as wide as the GRN inference methods do, ranging from continuous quantitative ordinary differential equation (ODE) models to discrete logic qualitative models). In continuous models, the temporal evolution of the state X_i of gene i $(i = 1,2,\ldots,N)$ in the network is given by continuous mathematical functions of its regulators, such as ODEs,

stochastic differential equations, etc. and the regulatory interactions between genes are usually modelled as chemical reactions, or species interactions in ecological models [95,96]. In this way, the temporal evolution of the system is described at the level of individual reactions. However, their usage in dynamical modelling of GRN remains limited because of the detailed and complete mathematical and parametric description they require. On the other side of the spectrum, in discrete models, such as multivariate logic models [97], Petri nets [98], or Boolean models [99,100], a discrete logic of interactions is applied and the temporal evolution of expression of each gene is given by a discrete (and often logic) function of their regulators (example given below). Contrary to continuous models, discrete models can be applied with no or considerably fewer parameters and fragmented mechanistic description, making them suitable for large networks. However, the system behaviour is only described (semi)quantitatively. In the following section, we will elaborate more in detail on the basic principles of Boolean modelling of GRNs.

For a modeller, the type of questions being asked about the system, the type of description of the system dynamics available (quantitative or qualitative), the type and amount of available data, prior knowledge, etc. will all influence the modelling approach to use. The basic objective, regardless of the dynamical model employed, is to pinpoint the phenotypic alterations that occur in a cell in response to medications, specific extracellular environmental factors, cellular interactions in the microenvironment, or experimental knockout/overexpression. According to Shah et al. [101], these phenotypic changes may be represented graphically as the system's steady states or attractors, which are states (a vector of expression values for each gene) that remain constant despite perturbations. A GRN often has numerous attractors, each of which represents a potential phenotype or state that may be attained given a set of initial conditions. In this situation, extra analysis must be done to examine the attractors' stability, biological significance, or their classification into biologically interpretable phenotypes.

Boolean Model Formalism of Gene Regulatory Networks

To study the temporal behaviour of this system, it is necessary to associate each gene with a dynamical function, which will explain how its *state* (expression, activity state) will change with *time*, given the types of interactions with its regulators. Boolean networks, referring to Boolean modelling of regulatory networks, were first introduced by Kauffman [102] and Thomas [103] to model the metabolic stability of epigenetics networks, displaying a *sigmoidal* behaviour, which could then be approximated to a switch-like behaviour. For example, let us consider the production of mRNA of a gene (X) as a function of the concentration of a transcription factor (A), usually described with a Hill function: $\frac{dX}{dt} = T_{max} \frac{[A]^n}{K^n + [A]^n}$ [104,105], where T_{max} is the maximal transcription rate, n is the Hill exponent, and K is the concentration of transcription factor at which the synthesis of mRNA of X is at half maximal rate). If the Hill coefficient is high, the synthesis term can be approximated by a Boolean step function, $[X] = 0$ if $[A] \leq threshold$, and $[X] = 0$ if $[A] > threshold$ (Figure 4).

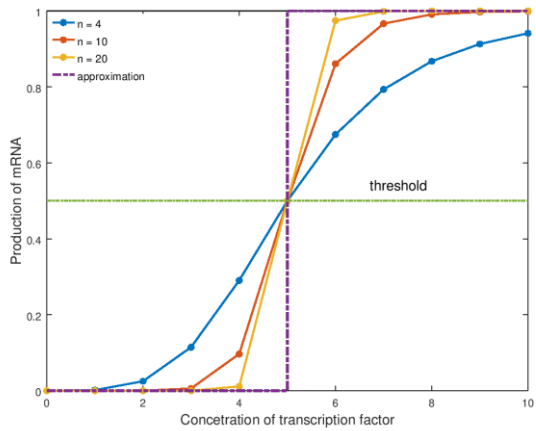


Figure 4: Illustration of the transition between a continuous and a Boolean description of a process through which transcription factor A positively regulates the mRNA production of gene X. If the Hill coefficient is high, the mRNA synthesis can be approximated by a Boolean function, $[X] = 0$ if $[X] \leq 0.5$, and $[X] = 0$ if $[X] > 0.5$.

In this formalism the interactions between the nodes are represented by *Boolean transition functions (Boolean rules)*, usually expressed via the logic operators: AND and OR for positive interactions, and NOT for the negative ones. Special care should be put on the choice between AND and OR operators, as they indicate a simultaneous synchronisation of the expression of the regulators of a certain target gene, or an independent regulation of two or more regulators on the target gene (see example below). In Boolean modelling *time is implicit* and *discrete* and each function at a given time point will take as input the variables' expressions at the previous time point. Most importantly, as mentioned above, the state of each node in a GRN will be characterised by discrete values (1 - expressed or 0 - not expressed), there will therefore be *finite combinations of possible states of the system* ($2^N, N$- number of nodes in the GRN) (binary vectors, each element of which representing the state of a node in the GRN, for example $state\{0,0,1\} \equiv \{G1\ (not\ expressed), G2\ (not\ expressed), G3\ (expressed)\}$). As a consequence, the system will eventually fall in one of the states it has previously been to, usually referred to as *stable states*, *fixed points* or *attractors*. Graphically, the set of all system's states and their possible transitions (defined by the Boolean rules) can be represented in the form of a *state transition graph* (special care should be taken to distinguish between the GRN and the state transition graph, where nodes are not genes but states of the entire system described by vectors of 0/1 values), in which the fixed-point attractors are usually identified by the presence of self-loops.

While the transition functions of a Boolean model specify the rules for calculating the future states of the nodes in the GRN (usually referred to as update), the order in which the updates are performed needs to be specified. In this context, various update methods can be implemented, which are broadly classified into *synchronous* and *asynchronous*. In the synchronous method, the states of all nodes in the GRN are updated simultaneously considering the state of the system at the previous time step, implicitly assuming that the time-scales of all biological events in the system are similar and that the state transitions of all the different components are synchronised. To avoid this

assumption, which is rarely biologically relevant, different asynchronous methods were developed to account for timescale diversity of biological processes by updating the nodes in an asynchronous or probabilistic manner. In this case all nodes are updated according to a random sequence or with a given probability [106], or one randomly selected node is updated at each time step [107,108]. Afterwards, multiple simulations can be performed to mitigate the effect of random updates, thus considering numerous updating sequences. Most importantly, the fixed-point attractors are time-invariant, implying that the same fixed points will be obtained independent of the updating method. Therefore, choosing between the updating methods will depend mostly on the computational question a modeller wants to answer, and other aspects, such as the size of the GRN (the size of the state transition graph increasing exponentially with the number of nodes), the availability of additional information regarding the *timing* of specific interactions in the GRN, the interest in following the system's dynamics before reaching the fixed points, etc.

Example: Let's consider the GRN in Figure 5, (a) as being the output of a given inference method, either from following a data-driven approach or provided from the available public databases. We can imagine it as the network illustration of a cell, whose behaviour is represented by the interactions of these 3 genes. Here, Gene 2 (G2) is positively regulated by Gene 1 (G1) and negatively regulated by Gene 3 (G3), thus the state of G2 can be a result either of a cooperation of G1 with G3 (AND operator), or each of these genes can regulate the expression of G2 independently (OR operator). In these cases, additional experimental or biological information will be necessary to determine the Boolean operators to be used.

Let's write the Boolean functions for each node, as in Figure 5. (b). Finding the system's attractors implies solving the system of Boolean equations by considering all the possible combinations of the states of the nodes ($2^3 = 8$ states) and calculating their future states, which can be represented as a *truth table*. In our case, the system displays a single fixed-point attractor (110, Figure 5, (c)), in which G1 and G2 are both expressed, and G3 is

not expressed. What is the biological meaning of this? Here the attractor will represent a phenotype of this virtual cell, or a cell state, characterised by the repression of G3.

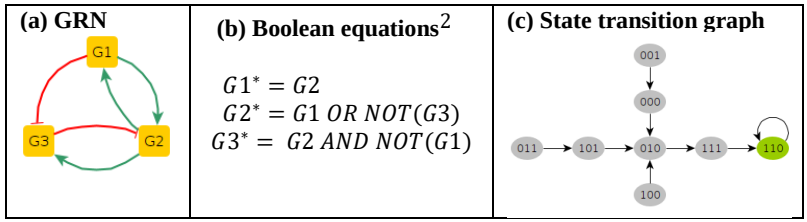


Figure 5: A toy example of a Boolean model of a GRN. Starting from a GRN (a), the next step consists of building the Boolean equations (b), by taking into consideration all the regulatory interactions and their nature (positive or negative interactions). Afterwards, the solution of the system of Boolean equations can be represented as a state transition graph. The attractors of the system can be identified by self-loops (in the case of fixed point attractors), or loops involving a subset of nodes (in the case of limit cycles). In this example, the system has only one fixed point attractor (110), coloured in green.

It is understandable that, for larger networks, solving the system of Boolean equations with the truth table quickly becomes challenging and impractical. For this reason, various computational tools have been developed to run Boolean models of hundreds of nodes (we will discuss more on these methods in the following section).

The example given above was intentionally chosen to point out that a system can display more than one attractor; in fact, the higher N, the most likely the system can display multistability (multiple possible attractors). We can imagine these different attractors being various states of a cell (e.g. a differentiating cell, a tumour cell entering apoptosis, proliferation, migration, etc.), each of which is defined by a combination of gene expression levels/states. We will discuss more on analysis of large networks and attractor classification in the following section.

[2] In these equations, the asterisk (*) indicates the expression of each gene at the next time point (i.e, *t+1*) as a Boolean function of the regulators at the current time point (i.e, *t*). Note that time is *implicit*, and is not involved as a variable when solving the system of the Boolean equations.

Beyond identifying the attractors of the system, the Boolean model can be further used to simulate internal or external perturbations on the states of the nodes, e.g. overexpression (OE - a node or a group of nodes forced into state 1) or knock-out (KO - a node or a group of nodes forced into state 0), thus mimicking the effect of a change in the system's internal or external environment (e.g. the action of a drug, a change in the extracellular stimuli, etc.). In this scope, special interest has been focused on the opposite analysis: given the possible stable states the system can have, identifying which nodes should be perturbed (either OE or KO) in order to prevent the system from reaching certain states. For example, if we consider the GRN in Figure 5, (a) to represent a cancer cell and the fixed point attractor to be a proliferative state of the cell, the interest is to find which gene or combination of genes should be ONE or KO in order to completely repress this attractor. One way to do this is to use the prior biological knowledge on the role of each gene in the GRN and perform multiple simulations with various combinations of perturbations. Alternatively, some computational tools have incorporated functions to perform this analysis, as we will see in the following chapter.

Boolean Model Inference: Applications

As mentioned in section 2, the same classification of approaches applies for Boolean model inference as for GRN inference, I,e. using the available evidence to build the Boolean functions reflecting the functional regulations between the molecular entities in the GRN. In addition to public databases mentioned in section 2.1, other databases such as Signor [109], BioModels [110], Minerva [111], NaviCell [112], etc. provide curated Boolean models, which can be further modified according to the system of interest.

In the data-driven approach, similar to using expression data for GRN inference, the information in the changes of the genes' expression is also used to infer the Boolean rules that govern these changes [113–119], which can then be studied using several available tools, integrated in the Consortium for Logical Models and Tools (CoLoMoTo) [120].

Despite the oversimplified representation of biological events in the Boolean formalism, predictions on cancer evolution and immuno-oncology, descriptions of signalling pathways activated in specific cell types (different tumour types such as bladder [121], breast [122], gastric [123], to name a few, but also T cells [124,125], macrophages [126,127], etc.), suggestions of patient-specific treatments [128,129], have flourished over the past decades [130]. More recently, patient-specific Boolean models have been developed to design targeted therapy strategies for patients based on their ‘omics profile [131]. Importantly, these intracellular models can be further integrated with other cell population models, like agent-based models or metabolic models, thus providing a multilevel description of the system dynamics, including mechanistic functionalities like cell motility, cytokine diffusion, tissue expansion and spatial organisation, etc. [132–134]. This combined approach enables addressing more complex questions, like drug design, or therapy action from the cell to the tissue scale [135–138]. For example, in [135] the intracellular Boolean models of cancer cells, stellate cells, macrophages, CD4+ and CD8+ T cells were implemented into an agent-based model of pancreatic ductal adenocarcinoma, thus describing cell type–specific molecular interactions and cytokine-mediated cell-cell communications. From model simulations, the authors suggest that the autocrine loop involving EGF signalling is a key interaction modulator between pancreatic cancer and stellate cells, and that reducing bFGF secretion by stellate cells will have a positive impact on cancer apoptosis.

Conclusions

The applications of GRNs in representation and modelling of regulatory systems has reached a mature stage in methodological research, with its applications in cancer research expanding and gaining popularity. In this chapter, we have endeavoured to provide a general overview of the field, emphasising the biological motivations behind it and the technological advancements in data collection that have boosted its recent growth. Additionally, we have presented a high-level perspective

on the statistical principles that underlie several widely used methodologies for GRN inference. Our main focus has been on establishing a foundational understanding, covering broad concepts in GRN analysis, inference and dynamical modelling. It is important to note that numerous significant contributions in developing methods in GRN inference, dynamical modelling and structural analysis exist, and we could not cover them all into this simplified summary.

Naturally, attempting to cover such a vast and diverse research area in a brief chapter is a challenging task. Our intention here is to equip the reader with the fundamentals of system biology, and its great potential to study diverse biological systems. We have endeavoured to make this chapter as self-contained as possible, hoping that it will also serve as a valuable introduction for new researchers to the field.

The technicalities of GRN inference and modelling of regulatory systems are the key motivating forces behind the surge of interdisciplinary collaborations. The intersection of bioinformatics, molecular biology, clinical research, computer science, physics and other disciplines is the core upon which these applications are built and expanded. Specialists in bioinformatics and artificial intelligence, for instance, are continuously developing powerful algorithms for network inference. However, these computational platforms should be firmly grounded in the rich experimental data provided by molecular biologists. Any given computational algorithm's outputs need to be decoded and interpreted within the correct biological context to bring meaningful insights. This process underscores the critical role of effective collaboration with biologists and clinicians. By embracing a multidisciplinary approach that merges computational methodologies with experimental validation, the aim is to unravel the complexity of biological systems. Furthermore, this synergistic approach is key to translating computational and theoretical findings into clinical applications.

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