Systems biology

Analyzing synergistic and non-synergistic interactions in signalling pathways using Boolean Nested Effect Models

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Abstract

Motivation: Understanding the structure and interplay of cellular signalling pathways is one of the great challenges in molecular biology. Boolean Networks can infer signalling networks from observations of protein activation. In situations where it is difficult to assess protein activation directly, Nested Effect Models are an alternative. They derive the network structure indirectly from downstream effects of pathway perturbations. To date, Nested Effect Models cannot resolve signalling details like the formation of signalling complexes or the activation of proteins by multiple alternative input signals. Here we introduce Boolean Nested Effect Models (B-NEM). B-NEMs combine the use of downstream effects with the higher resolution of signalling pathway structures in Boolean Networks.

Results: We show that B-NEMs accurately reconstruct signal flows in simulated data. Using B-NEM we then resolve BCR signalling via PI3K and TAK1 kinases in BL2 lymphoma cell lines.

Availability and implementation: R code is available at https://github.com/MartinFXP/B-NEM (github). The BCR signalling dataset is available at the GEO database (http://www.ncbi.nlm.nih.gov/geo/) through accession number GSE68761.

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1 Introduction

Cells process input signals to output signals using a network of cellular signalling pathways. For example, a small molecule binds a membrane receptor. The signal is brought into the cell via structural modification of the receptor. A set of kinases and other signalling molecules propagate the signal through the cytosol. This involves both activation and repression of proteins. Often complexes of multiple proteins must form before a signal propagates. Some of the molecules are also part of different pathways linking multiple pathways together. Eventually, the signal enters the nucleus where transcription factors and chromatin remodelling enzymes become activated. Finally, the combination of activated transcription factors and regulatory co-factors leads to the transcription of a large set of genes changing the phenotype of the cell.

Understanding the structure and the interplay of pathways is crucial both for understanding the cellular mechanism and for designing novel therapies that target specific pathways.

Inferring networks from molecular profiles is a well-developed field in bioinformatics. Transcriptional data can be generated more easily compared with protein activation data. Consequently, many algorithms were developed that focus on the reconstruction of regulatory networks. For example, Gaussian graphical models (Schafer and Strimmer, 2005), Bayesian networks (Friedman et al., 2000), the PC-algorithm (Kalisch and Bühlmann, 2007) or the Algorithm for the Reconstruction of Accurate Cellular Networks (Margolin...
et al., 2006). All these methods use observational gene expression data to construct regulatory networks based on different association scores between genes.

It is no problem to quantify the expression of any gene using standard methods like qPCR, microarrays or RNAseq. Observing signalling networks is more complicated. Protein activation can operate on the levels of protein expression, cellular protein localization or protein modifications like phosphorylation, ubiquitination etc. Although there are assays to assess activation on any of these levels, those assays are more elaborate, more expensive and less generic. Moreover, for every protein a priori information on which type of modification mediates signal transduction is necessary.

Molecular biologists have been inferring pathways without formal computations for many years. Typically functional/interventional data are used. Pathways are perturbed by activation or inhibition of genes and the consequences of the interventions are observed, organized and interpreted. Also a range of algorithms have been described that formalize these types of arguments and make them accessible to bigger and more complex pathway models.

Sachs et al. (2005) use flow cytometry data from perturbation experiments to infer protein signalling pathways with a Bayesian network approach. They test for conditional independence between proteins’ states using protein inhibition experiments and direct measurement of these states.

Markowetz et al. (2005) introduced Nested Effects Models (NEMs) (Froehlich et al., 2011; Markowetz et al., 2007; Niederberger et al., 2012). NEMs infer non-transcriptional signalling pathways by transcriptional downstream effects of pathway perturbation. A pathway is activated in a set of cellular assays where specific pathways are silenced. The silencing of a specific component blocks the flow of information in a specific branch of the pathway. As a consequence, genes that normally change expression in response to the stimulus no longer react in inhibition assays. NEMs infer the pathway structure from the nesting of these sets: if the affected genes of perturbing gene B are a noisy subset of the affected genes of gene A, then A is upstream of B. This concept has been extended to time series data (Anchang et al., 2009; Dümcke et al., 2014; Froehlich et al., 2011), evolving networks (Wang et al., 2014), and network inference with hidden confounders (Sadeh et al., 2013).

To date NEMs can infer the upstream/downstream relations of genes in a pathway (Markowetz et al., 2005), they can distinguish activation from repression (Vaske et al., 2009) and they can resolve the flow of information (Anchang et al., 2009; Froehlich et al., 2011). However they cannot model the role of complex formation in signalling pathways. If a protein X is activated by a complex, all members of the complex must be present and in the correct activation state. The proteins in the complex operate concertedly and are linked to X by an AND gate. In another scenario, X can be activated independently by several proteins. In this case the proteins are linked by an OR gate. Boolean Networks (Kauffman, 1969) model logical gates. They have been used to simulate signalling pathways (Klamt et al., 2007) and to reconstruct them from interventional data (Saez-Rodriguez et al., 2009). Allowing for logical gates leads to identifiability problems of network structures. To resolve these ambiguities, prior knowledge on the pathway structures is used. Saez-Rodriguez et al., (2009) describe an algorithm called CellNetOptimizer to construct signalling pathways from directly observed activation states of proteins in the Boolean Network framework. They combine prior knowledge networks (PKNs), with protein phosphorylation data from interventional assays.

Here we describe Boolean Nested Effect Models (B-NEM). This method combines advantages from Boolean Network Models and Nested Effect Models. Like Boolean Networks B-NEMs distinguish between the alternative and cooperative activation of a protein, and like normal NEMs, B-NEMs do not need direct observations of protein activity. Moreover, B-NEMs can use data from assays where several pathway genes are perturbed simultaneously. In the following, we will describe the model formalism and define a pathway score together with a genetic algorithm to search for high scoring networks. We will then validate our algorithm on simulated data and use it to analyze B-cell receptor (BCR) signalling in lymphoma cells.

2 Boolean NEMs

2.1 Pathway model and score

2.1.1 Signalling pathways and deterministic boolean networks

Molecular signalling pathways can be described as Deterministic Boolean Networks (Saez-Rodriguez et al., 2009). Networks are encoded as directed acyclic hyper-graphs \( \Sigma = (S, H) \) consisting of a set of nodes \( S = \{S_1, \ldots, S_N\} \) and a set of Hyper-edges \( H = \{H_1, \ldots, H_M\} \). Every node \( S_i \) represents a signalling protein that can be either active \( (S_i = 1) \) or inactive \( (S_i = 0) \). Hyper-edges describe how the signal is propagated through the network. Every directed hyper-edge \( H_k \) connects one or more parent nodes with a single child node. Hyper-edges with one parent node specify whether the child is activated or repressed by its parent. Hyper-edges with more parents specify a unique activation pattern of the parent nodes that is required for activating the child. If a node has multiple incoming hyper-edges, it can be independently activated by all of them. Hence, every hyper-edge with more than one parent node encodes an AND gate and multiple hyper-edges with the same child form OR gates (Fig. 1). Signalling pathways form AND gates, if multiple proteins need to be jointly activated to propagate the signal to their target molecule. This is often associated with the formation of larger protein complexes. OR gates in contrast occur when signalling is organized in a redundant manner. As with Bayesian networks and NEMs, we assume that the real graph is acyclic. This limits the scope of the method to models of signalling pathways in which the signal is propagated from receptors via branching cytosolic effector pathways into the nucleus without feedback loops.

2.1.2 Experimental design and data

Our goal is to estimate the signalling pathway model \( \Psi \) from a dataset \( D \). The data consist of K gene expression profiles \( D(1), \ldots, D(K) \) from a set of functional assays with specific perturbations of the pathway. We assume that the expression data are on a logarithmic scale. Perturbations include the exogenous stimulation of pathway receptors and the inhibition of signalling components. Every profile \( D(k) \) is hence associated with a specific experimental condition \( C(k) \) that specifies which receptors were stimulated and which signalling genes were inhibited. This is the typical experimental set-up of NEMs (Markowetz et al., 2005, 2007). Following the NEM literature, we call the signalling pathway components \( S_1, \ldots, S_N \) S-Genes and the genes that show expression changes in response to perturbations E-Genes. S-genes and E-genes can but need not overlap.

2.1.3 Expected and observed response schemes

For a given hyper-graph \( \Psi \) and a given condition \( C(k) \), we can calculate the activation states of all nodes in \( \Psi \) as follows: (i) root nodes are initialized to zero, (ii) stimulated nodes are set to 1 and inhibited nodes are set to 0 independently of any incoming signals from parent nodes, (iii) all other nodes are determined by propagating activation states through the directed acyclic graph using the Boolean functions.
analyzing synergistic and non-synergistic interactions

Fig. 1. Hyper-graphs and their response schemes. The two matrices are an ERS of the S-genes and a hypothetical noisy continuous observed E-gene response scheme of attached E-genes for the hyper-graph left. Black matrix entries indicate up-regulation (+1), white down-regulation (−1) and gray no change (0). Each column is a response scheme of an S-gene, respectively E-gene. The rows are comparisons between two conditions. In a condition + denotes the activation of the S-gene and − the inhibition independent of the state of the parents. The set of modelled comparisons is restricted to the typical design of a NEM. Included are comparisons of stimulation versus control and stimulations − inhibitions versus stimulations only. S0 is a receptor that can be activated. The other S-genes propagate the signal and can be inhibited. The edge H4 is an AND gate with two parents. S4 is activated by H4, if S1 is active and S2 inactive. Alternatively, the inhibition of S3 can activate S4. Hence H4 and H5 implicitly form an OR gate.

2.2 Restricting the search space using prior knowledge
Like Bayesian networks and standard NEMs, also B-NEMs are affected by likelihood/score equivalence. It is possible that two different networks have the same ERS \( \Phi \). If in addition the networks have identical size, they yield identical scores no matter what the data looks like. If not the smaller network is chosen. Note that \( \Phi \) depends on the design of the set of perturbation assays \( C \). Two networks can be distinguished by one experimental design but not by another. Figure 2 gives an example, how the design can (i) affect score equivalence classes and (ii) affect the optimal scoring network.

Interestingly we need an experiment involving only S3 during stimulation of S0 to correctly identify the signalling logic of S4.

If the data cannot distinguish between competing networks it is still possible that existing domain knowledge can. Like Saez-Rodriguez et al. (2009) we represent pathway knowledge by a priori restrictions of the network search space. With these restrictions, we do not only reduce network ambiguity due to score equivalence, but also ensure that the constructed networks follow general conventions of modelling signalling pathways (e.g. the signal is propagated from receptors, via cytosolic molecules to nuclear factors). We encode prior knowledge by a directed graph \( G \) whose edges are a collection of all links between S-genes that are a priori possible. In other words, it is the missing edges of \( G \) that define the search space restriction. We refer to \( G \) as a PKN. PKNs are then extended to a Boolean network by adding hyper-edges such that all Boolean functions allowed in \( G \) are a priori possible. Hence, while B-NEMs use help from prior knowledge to estimate the network structure they infer logical gates only from data. Using prior knowledge can resolve score equivalence problems, but there is no guarantee that it always does.
known Boolean networks. We refer to underlying data generating networks as Ground Truth Networks (GTNs). GTNs and matching PKNs are generated by randomly sampling edges from a super PKN shown in the Supplementary Figure S3. The super PKN has 30 nodes and 144 edges, respectively 504 hyper-edges after extension. The nodes fall onto five layers representing ligands, receptors, membrane complexes, cytosolic and nuclear signalling. Edges connect nodes on adjacent layers. 90% of edges are stimulating and 10% are inhibiting. We first draw a PKN and then a GTN. To generate a network of \( n \) nodes we randomly choose \( n \) nodes from the super PKN, ensuring that there is at least one node at every layer. For this set of \( n \) nodes we take all hyper-edges connecting those nodes as the extended PKN. From this PKN we randomly sample 50% hyper-edges, but make sure that the network is at one point stimulated. This means we reject GTNs which do not change their state during any stimulation. Similarly we generate networks of \( n \) hyper-edges. Without restricting the GTN to a specific number of nodes. Finally 10 E-genes were attached to every S-gene. Note that the PKN is always consistent with the GTN, no existing edges are a priori excluded. For a given GTN and a set of conditions we calculated the E-gene response scheme and added Gaussian noise \( \mathcal{N}(0, \sigma) \) with \( \sigma \in \{0.5, 1, 2\} \). Every E-gene profile was generated in triplicates with independent noise. The experimental conditions consist of controls, single and double stimulations, single inhibitions and the single/double stimulations together with single inhibitions. Finally, observed E-gene response schemes were composed by the differences between controls and single/double stimulations/inhibitions and the differences between single/double stimulation and single/double stimulation with a single inhibition.

3.1 B-NEM accurately estimate the equivalence class of networks with up to 30 S-genes
We first tested the performance of B-NEM for GTNs with 10, 15, 20, 25 and 30 S-genes. For each size we generated 10 random GTNs and matching PKNs and run B-NEM on E-gene data generated from these GTNs. The GTNs consisted of 10% of the allowed edges in the corresponding PKN, hence the PKNs were consistent with the GTN and effectively reduce the search space. We then compared the ERs of the estimated networks with that of the GTNs. Figure 3, top shows the sensitivity and specificity of the estimated networks (solid circle, dashed triangle). The corresponding computation time is shown as the dotted line connecting crosses. In this setting computation is a limiting factor for networks with 30 genes, but reconstruction accuracy is not.

3.2 Network reconstruction is sensitive to the strength of the PKN
In the previous simulation, we checked whether the algorithm finds the correct equivalence class of networks. However, equivalence classes can be large and are hard to interpret. Due to score equivalence multiple networks in the same equivalence class cannot be distinguished by data. However, equivalence classes can be shrunk effectively by strong PKNs rendering network reconstruction practical. Thus, we evaluated the accuracy of the estimated networks as a function of the strength of the PKN. For 10 random GTNs with 50 hyper-edges drawn randomly from the full PKN, we run B-NEM using PKNs of 50, 164, 277, 390, 504 a priori possible hyper-edges. Figure 3, bottom shows the sensitivity and specificity of the reconstructed networks both on the level of ERs and the actual networks. Although the performance stays very good with respect to...
response schemes (equivalence classes) it breaks down with respect to network reconstruction if the PKN becomes weak.

If we do not allow for negative regulation, the PKN needn’t be a DAG but can have cycles. Cycles in a PKN with negative regulation can lead to undefined ERSs. See Supplementary Section S2 for details.

4 Modelling B-cell signalling

We now apply the B-NEM framework to a previously unpublished dataset monitoring gene expression changes in the Burkitt lymphoma cell line BL2 after induction of the BCR. Our analysis explains how BCR signalling propagates to downstream effector pathways like the NFkB, MAP kinase, P38 or JNK pathways through activation of the intermediate messengers TAK1 and PI3K.

BCR signalling was induced in BL2 cells by cross-linking IgM with an anti-IgM antibody. S-genes were inhibited on protein level using small molecules: 5Z-7-oxozeaenol (TAK1), IKK2 inhibitor VIII (IKK2), SB203580 (P38/MAPK14), SP600125 (JNK), U0126 (ERK1/2). In addition to single inhibitions, IKK2, JNK and P38 were jointly inhibited yielding three double and one triple inhibition. All inhibitions were done in triplicate both under BCR stimulation and control conditions and gene expression profiles were generated using Affymetrix hgu133plus2 Genechips. Moreover, profiles of six negative controls (unstimulated BL2 cells) and six positive controls (BCR stimulated cells) were produced, yielding a dataset of 72 gene expression profiles in total. The dataset was made available at the GEO database under GSE68761.

The raw data were normalized (see Supplement Section S3) and observed E-gene response schemes from the data were calculated for the comparisons listed in Table 1. We filtered for E-genes that respond to BCR stimulation by at least an absolute log2 fold change of 1 and to an other comparison by at least an absolute log2 fold change of log2(1.5) ≈ 0.58. This corresponds to a change in expression of at

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<td>(BCR+)</td>
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+ denotes activation of the node and – inhibition in that particular condition.

Fig. 3. Simulation results. From left to right we set $r = 0.5, 1, 2$ in each column respectively. Top: Random GTN of $n$ nodes (x-axis) and the median sensitivity, specificity of the ERS (solid circle, dashed triangle) and running time (dotted cross) for ten runs. The top axis shows the mean PKN size. Bottom: Results for ten runs each given a fixed GTN and different PKN sizes (x-axis) including the GTN. Median sensitivity and specificity of the ERS (solid circle, dashed triangle) and the hyper-edges (dotted cross, dashed-dotted x)

Table 1. Contrasts of conditions used to calculate the observed E-gene response schemes from the data
least 100%, respectively 50%, leaving us with 602 E-genes and their continuous observed E-gene response scheme.

4.1 Prior knowledge in BCR signalling
The BCR is the cell surface receptor that initiates BCR signalling upon binding of an antigen. BCR signalling leads to the activation of IKK2, P38, ERK and JNK (DeFranco, 1997; Richards et al., 2001; Schuman et al., 2009; Shinohara and Kurosaki, 2009). These four effector pathways send signals into the nucleus that affect gene expression. The two proteins PI3K and TAK1 are potential mediators of BCR induced activation of effector pathways. We do not put any restriction on the hierarchical ordering of PI3K and TAK1. PI3K and TAK1 are parts of several other pathways where they are described as activators and not as repressor of signalling. We thus assume that the same holds true in BCR induced signalling. What is not known is which activations depend on which of the two mediators, nor is it known whether they activate downstream pathways independently from each other (OR gate) or jointly (AND gate). Furthermore the combinatorial inhibitions of IKK2, P38 and JNK are a subset of downstream targets of PI3K, which is not true in our model. PI3K and TAK1 allow more freedom in the PKN and therefore we do a complete reconstruction of the sub network consisting of these three S-genes described as activators and not as repressor of signalling. We thus assume that the same holds true in BCR induced signalling.

4.2 Calibrating the sparseness parameter $\zeta$ Calibrating $\zeta$ is critical to the performance of B-NEM. We randomly split the set of E-genes in half. For various settings of $\zeta$ (exponential decrease $\zeta \in \{1, 0.64, 0.36, 0.16, 0.04, 10^{-10}, 0\}$) we learn a network using the first half of the data (training set), and then score this network using the second independent half (test set) but without employing the complexity penalty in Equation (1). We repeat this step with 100 different random splits of E-genes and take the mean of graph size, connected S-genes (both in percent) and scores of the test sets. Figure 5 shows that the score continuously improves as $\zeta$ approaches zero. For $\zeta = 0$ the test accuracy drops again. Note that for any positive zeta the smaller network wins in case of likelihood equivalence while for $\zeta = 0$ there is no size penalty operating at all. We thus set $\zeta$ to $10^{-10}$.

4.3 The role of PI3K and TAK1
We run B-NEM on this data using the PKN and the parameter settings described above. Figure 6 shows the highest scoring network. The network predicts that the activation of the JNK pathway is only PI3K dependent, while Erk is only TAK1 dependent. IKK2 activation is predicted either as redundant by PI3K or alternatively TAK1. P38 is positively regulated by PI3K via either JNK or alternatively jointly with IKK2. The signal flow to P38 can be stopped either with the inhibition of PI3K or the double inhibition of JNK and IKK2. The ORS side by side with the corresponding ERSs can be seen in Supplementary Figures S5–S11.

That TAK1 alone, as proposed by our model, cannot block signalling into IKK2 and JNK has been detected for toll-like receptor 8 (TLR8) signalling in mouse embryonic fibroblasts (MEF, Qin et al., 2006). TAK1 knock-out mice still showed an activated NF$\kappa$B pathway. TLR8 also seems to be causative in some lymphomas (Ngo et al., 2011). Furthermore Matta et al., (2012) show that herpes virus encoded viral FLICE inhibitory protein K13 inducted NF$\kappa$B activity is not impaired in TAK1 deficient MEFs, Chen and Debnath (2013) give evidence that the IKK complex (IKK1, IKK2, NEMO) acts independently of PI3K in mammary epithelial cells and Xue et al. (2000) that ERK can be activated independently from PI3K in nerve growth factor-dependent sympathetic neurons. Kloo et al. (2011) propose the regulation of IKK2 by PI3K in diffuse large B-Cell like lymphomas. They show in their data, that the PI3K inhibitor only partially blocks IKK2 inhibitor target genes i.e. downstream targets of PI3K are a subset of downstream targets of IKK2, which is not true in our case. In the NEM logic this either places PI3K downstream of IKK2 or PI3K and IKK2 have joint downstream targets. A third explanation is that some NF$\kappa$B activity is regulated by PI3K, but another
alternative regulation is possible as depicted in our network in Figure 6.
As a comparison we applied the original NEM to the data (Supplementary Section S4).

5 Discussion
We have introduced B-NEM a novel method to infer signalling network structures. B-NEM extends the framework of standard NEMs. Standard models infer upstream/downstream relations of signalling genes. B-NEMs in addition resolve the Boolean logic of signal propagation. They distinguishing between the independent activation of a protein by multiple upstream proteins and the joint activation by a complex formed from the upstream proteins. Moreover, B-NEMs distinguish between activating and inhibiting interactions. Due to identifiability problems associated with the estimation of Boolean networks, B-NEM modelling must build on strong prior assumptions on the network structure. Networks cannot be constructed from scratch, but unknown features of existing networks can be resolved. We demonstrated this idea by resolving the role of PI3K and TAK1 as mediators of BCR signalling. In our cancer derived cell lines signalling was perturbed. Such cancer derived changes in signalling mechanisms can be further characterized in a comparative B-NEM analysis of signalling in primary healthy cells and tumor-derived cell lines. B-NEM exploits combinatorial perturbations of multiple genes. Because it is in general not feasible to perform all possible combinations, a subset needs to be chosen. We suggest an iterative procedure. First a B-NEM is learned from single gene perturbations. In case high scoring networks are incoherent with respect to an important pathway feature, one can simulate combinatorial perturbation data from these high scoring networks aiming at identifying those combinations that resolve the incoherence.

In simulations we showed that B-NEMs identify the correct score equivalence class of a signalling pathway with high accuracy. Identifying the correct signalling topology is harder. The most critical step here is the use of prior knowledge. We implement prior knowledge by excluding hyper-edges. A full Bayesian framework allows using softer priors that specify prior distributions on the full space of network topologies. However, for large networks these are not practical with B-NEM because a reduction of the search space is needed for computational reasons. The a priori restriction of the search space is needed for at least three independent reasons. First, the search space needs to be reduced in order to achieve practical running times of the algorithm. Second, together with the regularizing penalty for overly complex networks the PKN is used to limit over-fitting. Third, PKNs limit network ambiguities due to score equivalence of multiple networks. Fortunately, these goals are not opposing. The stronger the prior assumptions, the faster the algorithm, the less over-fitting we have, and the fewer the ambiguities due to score equivalence. On the other side, incorrect a priori assumptions can be disastrous as well. They lead to biased and under-fitted network models. Therefore one must find a good balance between belief in prior knowledge from literature and the data.

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Fig. 6. Learned network. The highest scoring network (black edges). The BCR signal is propagated via PI3K into JNK and P38. IKK2 is alternatively regulated by PI3K or TAK1. PI3K and TAK1 are directly regulated by BCR. TAK1 propagates the signal into the ERK pathway. Additionally P38 is alternatively regulated by JNK or IKK2. Grey dashed edges illustrate the propagation of signals from all molecules into the nucleus to regulate transcription.


