Higher order Boolean networks as models of cell state dynamics

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\textbf{Abstract}

The regulation of the cell state is a complex process involving several components. These complex dynamics can be modeled using Boolean networks, allowing us to explain the existence of different cell states and the transition between them. Boolean models have been introduced both as specific examples and as ensemble or distribution network models. However, current ensemble Boolean network models do not make a systematic distinction between different cell components such as epigenetic factors, gene and transcription factors. Consequently, we still do not understand their relative contributions in controlling the cell fate. In this work we introduce and study higher order Boolean networks, which feature an explicit distinction between the different cell components and the types of interactions between them. We show that the stability of the cell state dynamics can be determined solving

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the eigenvalue problem of a matrix representing the regulatory interactions and their strengths. The qualitative analysis of this problem indicates that, in addition to the classification into stable and chaotic regimes, the cell state can be simple or complex depending on whether it can be deduced from the independent study of its components or not. Finally, we illustrate how the model can be expanded considering higher levels and higher order dynamics.

Keywords: cellular automata, complex systems, epigenetics, transcription, protein modifications

1. Introduction

Regulation of gene expression is a complex process involving several components of different type, such as epigenetic factors, gene and transcription factors. Modeling such a complex system requires us to find the balance between the accuracy of the model predictions and our ability to interpret the model. On one side of the model spectrum, we have detailed chemical kinetics or Boolean network models Jong (2002). In these approaches the cell component heterogeneity is build in when specifying the regulatory interactions, functions (kinetic models or Boolean functions), and associated model parameters. Provided we determine all the regulatory interactions, functions and parameters correctly, these models can allow us to make accurate predictions of the cell state dynamics. However, detailed models can be queried only by means of numerical simulations, making it difficult to uncover or understand any behavior that is not known in advance. On the other end of the model spectrum we have ensemble models, which specify the statistical distributions of the regulatory interactions, functions, and associated model
parameters. While these models cannot provide precise predictions about specific cell processes, they can allow us to understand what is the typical behavior and how it can change under variation of the model parameters. Within this class of models, ensemble Boolean networks have been studied the most Kauffman (1969); Derrida and Pomeau (1986); Kauffman (1993); Aldana and Cluzel (2003); Kauffman et al. (2004).

The analysis of ensemble Boolean networks has significantly contributed to our qualitative understanding of the cell state dynamics Kauffman (1969, 1993). Different cell states can be associated with different stable attractors of the Boolean network dynamics Kauffman (1969, 1993) and we can study the breakdown of this stability following parameter changes Derrida and Pomeau (1986); Aldana and Cluzel (2003); Kauffman et al. (2004). More recently it is becoming clear that not all transcription factors regulating a given gene are equivalent. This is being modeled using Boolean functions with a biologically meaningful structure, such as canalyzing functions Harris et al. (2002); Kauffman et al. (2004) and nested canalyzing functions Kauffman et al. (2004). However, at the system level, the current ensemble Boolean network models typically comprise all elements they consider into one class of objects, within which the interactions are determined. This makes it difficult to model the general behavior and influence of different groups of elements (cell components) and the different types of interactions which systematically occur between elements of these components.

We introduce a more general class of Boolean networks with an explicit distinction between epigenetic factors, genes and transcription factors and the types of interactions among them. We call this class of Boolean networks
higher order Boolean networks (HOBN), in the sense that we specify wiring diagrams both within the three groups and between them, determining type-level interactions. We use HOBN to investigate the relative contribution of the different cell components to the cell state dynamics.

2. Higher order Boolean network model

We model the interaction of three different types of cellular components determining the cell state (Fig. 1): a set of epigenetic factors $\mathcal{E}$, a set of genes or transcripts $\mathcal{G}$, and finally a set of transcription factors or proteins $\mathcal{P}$.

Epigenetic factors form the most basic elements of our system, representing chemical modifications of the DNA and histone tails [Jaenisch and Bird (2003)]. The list of all such factors for a cell genome is the set $\mathcal{E}$. They are distributed along the DNA forming a linear graph. Within this topological graph they have neighbors influencing their states. Each epigenetic factor $e \in \mathcal{E}$ thus comes with a neighborhood $\mathcal{E}_e$ of other elements in $\mathcal{E}$, describing a linear order. The elements in this neighborhood influence the epigenetic state of the system. Epigenetics can be influenced also by single and composite gene products which can alter for example methylation patterns. We model this by assigning to each factor $e \in \mathcal{E}$ not only its direct neighbors within $\mathcal{E}$, but also elements in the set of genes $\mathcal{G}$ and in the set of transcription factors $\mathcal{P}$. Thus an element $e \in \mathcal{E}$ has three neighborhoods $\mathcal{E}_e$, $\mathcal{G}_e$ and $\mathcal{P}_e$ which regulate its state. The epigenetic factors are assumed to be in two possible states 0 (e.g. not methylated) and 1 (e.g. methylated). We model the control of the epigenetic state by a distribution of Boolean functions $f_\mathcal{E}$.
on the set $\mathcal{E}$ which each take as input the states of all three neighborhood elements of a given epigenetic factor, and give as output the updated state 0 or 1. We think of epigenetics as the most elementary units of the system since these factors act upon genes (and consequently on transcription factors) in a dominant fashion: an adequate epigenetic state is a prerequisite of all other transcription and translation activities, including up- or down-regulation of transcription by various factors.

A gene or transcript $g \in \mathcal{G}$ represents any genomic region that can be transcribed, including mRNAs of genes, miRNAs and short RNAs. The active state indicates the gene presence. The state of a gene is regulated by epigenetic factors, other genes and transcription factors, however in different ways. Epigenetic factors determine the secondary DNA structure in their neighborhood and the accessibility of this region to transcription factors and the transcription machinery. Thus the aggregate epigenetic state of these factors on and nearby the DNA segment encoding for a gene can influence the gene’s transcription rate, and thus the gene state. We model this by introducing two different transcription regimes characterized by two Boolean functions $f_{-G}$ and $f_{+G}$, where $f_{-G}$ corresponds to the silent or restricted regime and $f_{+G}$ corresponds to the active or accessible regime. To model the epigenetic regulation of the transcription regime of a gene we define the set of all possible transcription regimes $\mathcal{R}$ (here, $\mathcal{R} = \{f_{+G}, f_{-G}\}$), together with an additional Boolean function $f_{\mathcal{R}}$ which controls the change of transcription regimes. This function $f_{\mathcal{R}}$ takes as input the state of the epigenetic factors associated to a gene $\mathcal{E}_g$ and the value of its present regime ($f_{-G}$ or $f_{+G}$) and determines as an output the value of the regime for the next step. Once
a regime is determined depending on the epigenetic state of the gene, the regime function will update the gene state. This update now happens depending on gene-gene interactions and transcription factor activities. Thus the inputs of the Boolean functions in $\mathcal{R}$ are taken from the set of genes and transcription factors which regulate transcription of a gene. This means that each gene comes also with a neighborhood of other genes and a neighborhood of transcription factors regulating its state. The gene neighborhood of a given gene $g$ will be called $\mathcal{G}_g$ and we denote by $\mathcal{P}_g$ its transcription factor neighborhood. The state of $g$ is now regulated by the states of elements in $\mathcal{G}_g$ and $\mathcal{P}_g$ through a transcription regime, $f_{\mathcal{G}}^-$ or $f_{\mathcal{G}}^+$, the choice of which is decided by the epigenetic state $\mathcal{E}_g$ of the gene. The model thus allows us to control epigenetic effects separately from the regulation by other transcripts and transcription factors.

The last group of cellular agents consists of the set of transcription factors $\mathcal{P}$, representing proteins and protein complexes. Each element $p$ in $\mathcal{P}$ is composed of products of a subset of genes $\mathcal{G}_p$. In order for a transcription factor $p$ to be assembled, all the transcripts in $\mathcal{G}_p$ need to be transcribed and translated. This procedure - which enables or disables the activity of transcription factors - is again best modeled by a regime switch. We assume the transcription factors can be in two different regimes characterized by the Boolean functions $f_{\mathcal{P}}^-$ and $f_{\mathcal{P}}^+$, where $f_{\mathcal{P}}^- \equiv 0$ corresponds to the not assembled complex and $f_{\mathcal{P}}^+$ to the regime of the assembled complex. The set of all regimes for protein complexes is denoted by $\mathcal{C}$. The choice of regime for a protein $p$ will depend on the states of all elements in $\mathcal{G}_p$ via a Boolean function $f_{\mathcal{C}}$. This regime switch function $f_{\mathcal{C}}$ is simply a logical
Figure 1: Higher order representation of the cell components and their regulatory interactions. The square boxes indicate the sets of Boolean functions controlling the states of the elements, the arrows entering a box indicate the input of these functions. In the case of gene expression, the choice of function is regulated by epigenetic state; in the case of transcription factors, the choice of function is regulated by expression of components (AND-function).

AND relation, since the transcription factor works under \( f_P^+ \) if and only if all its components are transcribed, i.e. if and only if all inputs of \( f_C \) are in 1-state. Here the 1-state of \( f_C \) stands for \( f_P^+ \) while 0 stands for \( f_P^- \). Within the positive regime \( f_P^+ \) the state of an element in \( \mathcal{P} \) can depend on interaction with various other elements in \( \mathcal{P} \) itself; for example via post-translational modifications [Walsh et al. (2005)]. These form the neighborhood \( \mathcal{P}_p \) of \( p \in \mathcal{P} \). The functions in the regime \( f_P^+ \) thus take as input the states of elements in this neighborhood.

The cellular components and regulatory interactions just described are
summarized in the wiring diagram shown in Fig. 1. This diagram emphasizes the level-wise organization of the different types of cellular components. From top to bottom we have regime regulation (dashed lines). From bottom to top we have state regulation, as considered by previous Boolean network models (solid lines). When both types of interactions are put together we obtain a closed system, a system with feedback, which is a distinctive property of cell regulatory networks Barabási and Oltvai (2004). Following the central dogma, we will often refer to epigenetics, i.e. the set \( \mathcal{E} \), its elements, neighborhoods and Boolean rule, as the 0-level of the system. The set \( \mathcal{G} \) together with the two Boolean rules, neighborhoods, and associated epigenetic factors constitute the 1-level, while finally the set of transcription factors \( \mathcal{P} \) together with its own functions, neighborhoods and associated genes will be called the 2-level. We will also sometimes refer to the interactions regulating state changes as primary interactions, while those regulating a choice of regimes will be called secondary interactions. This is also in reference to the existing notion of higher order cellular automata, as introduced by Baas and Helvik (2005).

3. Cell state dynamics

Previous studies of Boolean network models indicate the existence of two dynamical modes. An ordered mode where two different trajectories in the cell state space will converge to the same trajectory, and a chaotic mode where the trajectories will instead diverge Derrida and Pomeau (1986). Later on it was shown that the ordered mode implies a nearly static system behavior where most elements (stable core) are not changing state Flyvbjerg (1988).
Here, we follow the latter approach. The total state of our system is expressed by five state variables: the epigenetic state $e(t)$ for $e \in E$, the gene state $g(t)$ for $g \in G$, the transcription factor state $p(t)$ for $p \in P$, and furthermore the transcription regime state $r(t)$ and the protein regime state $c(t)$, where in the latter two cases the $-$ and $+$ states are represented by 0 and 1 respectively. The basic set-up for the system dynamics thus consists of five equations expressing the probability of changes to happen in all of the five state variables. We can think of the total probability of change of state of the system as a five-dimensional vector $\vec{q}(t) = (q_0(t), q_1(t), q_2(t), Q_1(t), Q_2(t))^t$, where $q_0(t)$, $q_1(t)$, $q_2(t)$, $Q_1(t)$ and $Q_2(t)$ are the probability that a given epigenetic, gene, transcription factor, transcription regime, and protein regime state respectively, will change from step $t$ to step $t + 1$.

In the general case $\vec{q}(t + 1)$ is a nonlinear function of $\vec{q}(t)$, which depends on the detailed definition of the Boolean model. Nevertheless, in the nearly static, ordered mode, where most elements do not change state, this function can be linearized in good approximation: in this range the absolute value of the total probability for change in the system is very close to 0, i.e. $|\vec{q}| \to 0$, which allows us to neglect higher exponent terms. Note that this linear approximation of the system dynamics breaks down outside the ordered mode and cannot predict any behavior there, other than the fact that the system is in an unstable mode. In the near-static range we thus obtain

$$\vec{q}(t + 1) = A\vec{q}(t),$$

where $A$ is a five by five positive definite matrix. The entries in the matrix $A$ reflect the regulatory patterns indicated in Fig. 1 and therefore it is of the
form

\[
A = \begin{pmatrix}
    a_{11} & a_{12} & a_{13} & 0 & 0 \\
    0 & a_{22} & a_{23} & a_{24} & 0 \\
    0 & 0 & a_{33} & 0 & a_{35} \\
    a_{41} & 0 & 0 & 0 & 0 \\
    0 & a_{52} & 0 & 0 & 0
\end{pmatrix}
\] (2)

Each set in Fig. 1 which is acted on or regulated creates one state dimension in \( \vec{q} \). Each arrow indicating regulation is represented by one non-trivial entry in the matrix. Notice that the regime control interactions (dashed lines) are represented in the off-diagonal blocks, while the state control, or ordinary interactions (solid lines) appear in the upper triangular submatrix.

In the following we focus on the stability of the linear map (1). Specifically, the map is said to be stable when \( |\vec{q}| \to 0 \) as \( t \to \infty \), and it is called unstable otherwise. The stability of a linear map can be deduced from the properties of the eigenvalues of the corresponding matrix, in this case \( A \). When the largest eigenvalue has absolute value less than one the system is in stable or ordered mode and \( \vec{q}(t) \) converges to zero. If however the largest eigenvalue becomes larger than one, this indicates that the system is in unstable mode where the linearization (1) is not a suitable approximation to the actual system anymore. Using the linearization (1), we can thus analyze the model within its ‘stable range’, i.e. in its near static mode. We can furthermore determine the conditions on parameter space which distinguish between stable and unstable range, by calculating the largest eigenvalue and setting it equal to one. Finally, we can analyze the derivative in time direction to determine the influence of certain parameters on the growth of the
Figure 2: Effective dynamic modes

largest eigenvalue.

Since $A$ is derived from a strongly connected graph (see Fig. 1), $A$ is irreducible. Furthermore the entries of $A$ are non-negative. In this case we can apply the Frobenius Theorem for non-negative irreducible matrices Gantmacher (2000). This theorem guarantees that $A$ has a real positive eigenvalue $\Lambda$ such that any other eigenvalue $\lambda$ of $A$ satisfies $|\lambda| \leq \Lambda$.

The largest eigenvalue of $A$ can be obtained finding the roots of the characteristic polynomial $P(\lambda) = \det(\lambda I - A)$, where $I$ is the identity matrix. In our case $P(\lambda)$ is given by the quintic polynomial

$$P(\lambda) = \lambda^2(A_\xi - \lambda)(A_\zeta - \lambda)(A_P - \lambda)$$

$$- \lambda(A_\xi - \lambda)B - \lambda(A_P - \lambda)C + D \quad (3)$$

where

$$A_\xi = a_{11} \quad A_\zeta = a_{22} \quad A_P = a_{33} ,$$

$$B = a_{23}a_{35}a_{52} \quad C = a_{24}a_{41}a_{12} ,$$

$$D = a_{24}a_{41}a_{13}a_{35}a_{52} . \quad (4)$$

The direct inspection of this equation tells us about the basic dynamic cy-
cles of the system (Fig. 2). There are three minimal self-controlling cycles, represented by the type A cycles in Fig. 2. The gene self-cycle represents the standard type of dynamics in ordinary Boolean network models, where genes are regulated by other genes in a regulation network. This is demonstrated in more detail in Section 5. Similarly, the epigenetics self-cycle models how epigenetic factors update their states based on their previous states and the states of their neighbors along a DNA segment. The transcription factor self-cycle models changes in the transcription factors' state due to interactions between them and therefore represents regulation at the post-translational level. There are two cycles composed of three components, represented by the cycles B and C in Fig. 2. B represents a dynamic mode where a change in gene state alters the protein regime state and thus the state of transcription factors. This in turn alters genes' states, while epigenetic factors and the transcription regime remain unvariable. C involves state changes in epigenetic factors, transcription regime and genes. Finally, cycle D is composed of five elements, representing a dynamic mode where changes in the epigenetic states result in changes of transcription regime, leading to changes in the genes' states, thus altering the transcription factor regime and transcription factor states, which then go on and change the epigenetic factor states.

Although we cannot explicitly solve the quintic polynomial equation determining the roots of (3), we can derive some general results. For example, the boundary separating the stable from the unstable regime is given by the equation $P(1; A_G, A_P, B, C, D) = 0$. We investigate also the relative influence of the different cycles calculating the partial derivatives of $\Lambda$ with respect to the effective coefficients in (4) (see Supp. Material). It can be
shown that in the stable range ($\Lambda < 1$), $\Lambda$ is most sensitive in the direction of $D$. In other words the easiest way to make a transition to the unstable regime is to increase $D$. This result indicates that the dynamic mode $D$, which makes full use of all regulatory mechanisms, dominates over the other modes. Consequently all regulatory mechanisms are coupled together and their influence in the cell state dynamics cannot be analyzed independently of each other. This type of structural analysis can provide insight into the regulation of the system as a whole without even breaking the calculations down to the actual “microscopic parameters” characterizing the neighborhoods, memberships and the Boolean functions. Furthermore, provided we have quantitative estimate of the model parameters we can always numerically compute the largest eigenvalue of $A$ and determine whether the system is or is not in an stable regime.

4. Neighborhoods, memberships, Boolean functions and updating schemes

The matrix elements of $A$ can be derived from the properties of neighborhoods, memberships and Boolean functions. In this way we can also investigate the influence of “microscopic” parameters on the cell state dynamics. For example, let us assume that, given a type of elements and neighborhoods, all neighborhoods have the same size (in an ensemble network one will use the estimated mean value). In this case there are three neighborhood parameters $K^0_0$, $K^1_0$ and $K^2_0$ for the three types of neighborhoods ($\mathcal{E}_e$, $\mathcal{G}_e$, $\mathcal{P}_e$) on the 0-level (epigenetics). On the 1-level (genes) there are two of those, $K^1_1$ and $K^2_1$, and finally on the 2-level (transcription factors) there is only...
one $K^2_2$. These parameters also give the input lengths for the Boolean rules determining change of state on the three levels. Furthermore the change of regime rule $f_R$ on the 1-level has input length $M_0$, the number of “members” constituting the epigenetic state of a gene. Finally, the rule $f_C$ changing transcription factor regime on the second level has input length $M_1$, the number of transcription factor members.

The Boolean functions can be characterized by the probability that two different inputs result in a different output (sensitivity Shmulevich and Kauffman (2004)) and the probability that the output is 1 for a randomly chosen input. We assume the Boolean functions $f_E$, $f^\pm_G$, $f^+_P$, and $f_R$ are randomly sampled from the function classes $F_E$, $F_G$, $F_P$, and $F_R$ respectively, while $f^-_P \equiv 0$ and $f_C \equiv AND$. These function classes are characterized by their expected sensitivity $s_E$, $s^\pm_G$, $s^-_P = 0$, $s^+_P$, $s_R$ and $s_C$ and probability to be in the 1-state $\rho_E$, $\rho^\pm_G$, $\rho^-_P = 0$, $\rho^+_P$, $\rho_R$ and $\rho_C$, respectively. The expected sensitivities depend on the class of Boolean function Shmulevich and Kauffman (2004). For example, when the functions are sampled from a distribution with a given $\rho$ we have $s = 2\rho(1-\rho)$. On the other hand, for $f_C = AND$ we obtain $\rho_C = \rho^M_G$ and $s_C = \rho^{-1}M_1$, where $\rho_G = \rho_R\rho^+_G+(1-\rho_R)\rho^-_G$ is the probability that a given input of the AND-rule is in state 1. Finally, the matrix elements will also depend on the specific updating scheme. In the following we will assume a synchronous updating scheme, where the state of all elements in the systems are updated simultaneously.

Overall, we have a system with 15 parameters. The coefficients $a_{ij}$ can be derived from these parameters. Most of them can be determined as in previous Boolean network models, consisting of the product of the sensitivity
and the neighborhood size. This is the case for $a_{1i} = s_{E}K_0^{i-1}$ ($i = 1, 2, 3$), $a_{2i} = s_{G}K_1^{i-1}$ ($i = 1, 2$), and $a_{33} = s_{P}K_2^2$, where $s_{G} = \rho_{R}s_{G}^+ + (1 - \rho_{R})s_{G}^-$ and $s_{P} = \rho_{C}s_{P}^+$ are the expected sensitivities of the respective Boolean functions after accounting for regime changes. The coefficients characterizing the change in regimes can be calculated in a similar way, but replacing neighborhood sizes by membership sizes. This is the case for $a_{41} = s_{R}M_0$ and $a_{52} = s_{C}M_1$. Finally, the coefficients representing state changes following regime changes are calculated differently. What matters in this case is the probability that two Boolean functions from different regimes result in a different output given the same input. This is the case for $a_{24} = s(f_{G} \leftrightarrow f_{G}^+) = \rho_{G}^+(1 - \rho_{G}^-) + \rho_{G}^-(1 - \rho_{G}^+)$ and $a_{35} = s(f_{P} \leftrightarrow f_{P}^+) = \rho_{P}^+(1 - \rho_{P}^-) + \rho_{P}^-(1 - \rho_{P}^+)$. Taking these results together we obtain

$$
\begin{align*}
  \text{a}_{11} &= s_{E}K_0^0, \quad \text{a}_{12} = s_{E}K_0^1, \quad \text{a}_{13} = s_{E}K_0^2, \\
  \text{a}_{22} &= s_{G}K_1^1, \quad \text{a}_{23} = s_{G}K_1^2, \quad \text{a}_{24} = \rho_{G}^+(1 - \rho_{G}^-) + \rho_{G}^-(1 - \rho_{G}^+), \\
  \text{a}_{33} &= s_{P}\rho_{G}^M_1K_2^2, \quad \text{a}_{35} = \rho_{P}^+ \\
  \text{a}_{41} &= s_{R}M_0, \quad \text{a}_{52} = M_1\rho_{G}^M_1^{-1}
\end{align*}
$$

(5)

From the Frobenius Theorem it follows that the largest eigenvalue $\Lambda$ is a monotonic increasing function of the matrix elements $a_{ij}$ Gantmacher (2000). Therefore we can always investigate the contribution of any of the parameters listed above by analyzing their influence on the matrix elements $a_{ij}$. For fixed sensitivities, the increase of any neighborhood size $K_j^i$ always result in the increase of at least one matrix element, pushing the system towards the unstable regime. In contrast, the number of members of a transcription
factor $M_1$ influences $a_{33}$ and $a_{52}$, which are both decreasing functions of $M_1$ provided $M_1 \geq 1$. Therefore, the larger the protein complexes are, the more stable is the system.

The Frobenius Theorem also provides bounds for the largest eigenvalue, namely by the minimum and maximum of the row sums of the matrix $A$:

\[
\min_i(\sum_j a_{ij}) \leq \Lambda \leq \max_i(\sum_j a_{ij})
\]

Gantmacher (2000). In the current example we obtain

\[
\sum_j a_{ij} = \begin{cases} 
    s_E(K_1^0 + K_0^1 + K_0^2), & i = 1 \\
    s_G(K_1^1 + K_1^2) + s(f_G^- \leftrightarrow f_G^+), & i = 2 \\
    s_P \rho_G^{M_1} K_2^2 + s(f_P^- \leftrightarrow f_P^+), & i = 3 \\
    s_R M_0, & i = 4 \\
    M_1 \rho_G^{M_1-1}, & i = 5 
\end{cases}
\]

These sums put together all the contributions regulating the units states at each level. When all of them are smaller (or larger) than 1 we can guarantee that $\Lambda < 1$ ($\Lambda > 1$) and the system is in a stable state (or unstable state, respectively). However, when some are smaller and other are larger than 1, we are forced to compute $\Lambda$ to determine the system stability. In other words, the system exhibits non-trivial complex behavior, where fundamental properties of the system as a whole are not directly coupled with the corresponding properties of its subsystems.

5. Examples

To illustrate the concepts introduced above we discuss a few examples, allowing us to emphasize the flexibility of this modeling framework and the importance of including the regulatory structure at the level of components.
Figure 3: **Reduced versions of the HOBN depicted in Fig. 1**

a) Standard Boolean network model. b) Boolean network model accounting for the formation of protein complexes and regulation at the protein level.

**Standard Boolean network:** Here we show how we can reduce our model to compare directly to the standard Boolean network models considered in the literature so far, with the reduced wiring diagram shown in Fig. 3a. In this case, there is no regulation at the epigenetic level and at the transcription factor level, nor at the transcription regime level. The only dynamic mode is the self-cycle $A_G$ in Fig. 2 and the characteristic polynomial (3) is reduced to $P(\lambda) = \lambda^4(\lambda - A_G)$. In this case, the largest eigenvalue is $\Lambda = A_G$ and therefore the effective parameter controlling stability is $\theta = A_G$. In particular, taking into account that $A_G = a_{22}$ (4), and assuming constant neighborhood sizes and a synchronous update (5), we obtain

$$\theta = s_G K_1^1.$$  \hspace{1cm} (7)

This is precisely the result obtained for the classical standard Boolean net-
work model [Kauffman (1969, 1993)]. Thus, our approach contains the standard Boolean network model as a special case.

**Regulation at the protein level:** In the next example we study regulation on the transcription factor level Fig. 3b. As opposed to the first example, here we have proteins and protein compounds regulating transcription; this includes the assembly rule, i.e. the regime change on transcription factor level. The only non-zero matrix elements are those corresponding to arrows in Fig. 3b and the only relevant dynamic modes are the cycles $A_P$ and $B$ in Fig. 2. The characteristic polynomial (3) now reduces to $P(\lambda) = -\lambda^2(\lambda^3 - A_P \lambda^2 - B)$. While finding the roots of this cubic polynomial can be cumbersome, finding the stability condition is in this case straightforward. At $\lambda = 1$ we obtain the stability condition $B + A_P = 1$. Furthermore, since the largest eigenvalue $\Lambda$ of $A$, $A_P$, and $B$ are all continuous increasing functions of the associated matrix elements of $A$, then $\Lambda < 1$ for $B + A_P < 1$ and $\Lambda > 1$ when $B + A_P > 1$. So in this case, the effective control parameter for stability is given by $\theta = B + A_P$. In particular, assuming constant neighborhood and membership sizes and synchronous updates, from (4) and (5) we obtain

$$\theta = s_G K_1 \rho P^+ M_1 \rho G^{M_1 - 1} + s_P K_2 \rho G^{M_1}$$

(8)

Notice that this formula consistently solves the following subtlety: instead of speaking of gene-gene regulation networks as in the previous example, one could actually distinguish between the gene and its products (proteins), and construct a network where single gene products regulate gene transcription. This would be a more accurate description of the biological reality, should, however, lead to the same results, since de facto we do not change any inter-
actions. In our model, this set-up would mean that we set \( M_1 = 1 \), i.e. all protein complexes are single gene products; furthermore \( K_2^2 = 0 \), i.e. there is no protein-protein regulation (only proteins regulating genes), and lastly \( K_1^2 = K_1^1 \), since we have replaced the number of neighbor genes regulating a gene by the same number of their proteins. We obtain \( \theta = s_g K_1^2 = s_g K_1^1 \), which coincides perfectly with (7). Therefore, unless we account for the formation of protein complexes or some regulation at the protein level, we obtain the same effective control parameter of the standard Boolean network model.

The addition of protein-protein interactions results in the second term in the r.h.s. of (8). Here - just as for the case of gene regulation - increasing the neighborhood size \( K_2^2 \) increases \( \theta \), pushing the system towards the unstable mode. On the other hand, \( \theta \) decreases exponentially with increasing the protein complex size \( M_1 \), making the system more stable. We see from the above formulas how the stability conditions change drastically depending on primary and secondary interaction parameters. These corrections emphasize the importance of considering the right structure in the modeling framework. Furthermore, although there is an increase in model complexity, we can still derive analytical results allowing us to obtain a better qualitative understanding of the cell state dynamics.

6. Beyond three levels

The system we analyzed is an example of a Higher Order Cellular Automata (Baas and Helvik 2005), or even more general, a higher order network (HON). The first ingredient of a HON is a hierarchical structure, the idea
Figure 4: **Beyond three levels:** General scheme to construct a HON with up to second order dynamics. The sets $\mathcal{L}_i$ represent a hierarchy of types, where higher level elements correspond to groups of lower level elements (members). Their states are regulated by regimes $f_i$ (e.g. Boolean functions) taking as input states of neighbor elements at the same of higher levels (black arrows). The transition between the regimes is controlled by second order dynamics $r_i$ taking as input the members state at level $i - 1$. 
being that *groups* of agents can act *together* as an entity. This hierarchy is modeled by creating a new agent on the next higher level, as illustrated in Fig. 4. Thus we have a collection of sets $\mathcal{L}_0, \mathcal{L}_1, \ldots, \mathcal{L}_n$ of agents of different type, one set on each level. The hierarchical structure is expressed by assigning to each element on level $n + 1$ the set of its *members* on level $n$. Note that this is a fixed structure which will not change over time. The second ingredient consists again of *neighborhoods*. Neighbors of an element on level $i$ are other agents who can take influence on the state of the element. They can be of the same type as the element itself (that is, level $i$-agents) or they can be higher level agents. So the neighborhood of a level $i$-agent consists of subsets in levels $i$ and higher. The state of an element on level $i$ is regulated by the states of its neighbors, which serve as input for a Boolean regime. In a higher order network we assign sets of regimes $\mathcal{R}_i$ on each level, in other words, the system now has *regime states*, or *first order derived system states*. The latter name corresponds to the fact that the rules describe change of state. The choice of regimes is regulated by *second order rules*. Input for these second order rules can be chosen depending on the context of the model. With this type of wiring we create type-level feed-back loops containing primary interaction through direct state control (neighbors) and secondary interaction through regime control (members).

The configuration of the regime change rules at a given time step $t$ can be thought of as *second order derived system state*: it describes the second order derivative of the state function. Naturally, if we allow *choice* again for these rules, we can extend this to third order. We would then pick another rule determining the “change of regime switch” and depending on states of
Figure 5: **Third order dynamics**: Extension of the regulatory structure to include third order dynamics. We could also imagine a situation where there are more than one regime switch rule associated with the elements at one level, as illustrated at the third level (box $R'_2$). The transition between these regime switchers can be controlled by a third order dynamics taking as input the state of elements at a lower level.
certain subsets of elements (see Fig. [5]). Our example concerning the cell state stops at the second order regulatory structure, but one can think further. For instance, as the chromatin strand folds within the nucleus, certain regions of DNA can become inaccessible to both epigenetic state modifiers and transcription factors Grewal and Moazed (2003). This process does not affect the epigenetic state of the folded regions. However, the procedure does cause silencing of the genes within the folded regions. In our model, this would mean that the folding process has locally eliminated epigenetic influence on transcription. In other words, it has turned off the epigenetic switch between silent and accessible regimes. The epigenetic states are still there, but whatever they are, the transcription regime is silent. Thus the switching mechanism has been exchanged for a constantly silent one by a master-switch which depends on the folding structure.

7. Discussion

The study of Boolean networks allows us to understand the characteristic features of the cell dynamics despite the great complexity of cell regulatory networks. A fundamental pre-requisite to achieve this goal is the use of ensembles of Boolean networks whose average properties are representative of the cell behavior. It is clear that a multi-level system such as the one described above can as well be encoded as an ordinary Boolean network. However, such a network will be a very rare realization within the entire set of Boolean networks with no pre-defined level organization. In other words, the average properties derived from the study of ordinary Boolean networks are not representative of a cellular system with its natural hierarchical struc-
ture. Higher order Boolean networks are therefore a necessary step to obtain network ensembles with pre-defined level-wise structure, a distinctive feature of cell regulatory networks.

Our main conclusion from a first qualitative calculation of a higher order model is quite striking. We can show that determining the stability of the cell state can be a simple or a complex problem depending on the stability condition for each level individually. When the stability condition is satisfied for all levels we can guarantee that the system is stable. Similarly, when the stability condition is not satisfied for any level we can assure that the system is in an unstable state. In these cases we would say that, although the system has a second order structure, it is simple, i.e. its state can be determined from the analysis of its components independently from each other. In contrast, in between the simple dynamical regimes described above, there is a third regime where some levels do and others do not satisfy the stability condition. In this latter case we cannot deduce the stability of the system from the analysis of the stability of each single level. The system is complex, i.e. we are forced to consider all levels at once to determine its stability. This evidence indicates that the cell can be in four different states: Simple stable when all levels satisfy the stability condition. This would imply that the probability for change in any of the cells components is zero or converges to zero. The system is complex stable or complex unstable when there is at least one level that satisfies and another that does not satisfy the stability condition, but the system as a whole is stable or unstable. This could represent for example somatic cells in multicellular organisms with tissue regeneration (e.g., humans), which are epigenetically stable but may
exhibit different dynamical behaviors at the gene and protein levels. Finally, *simple unstable* when all levels do not satisfy the stability condition. A potential example of this extreme case could be cancer cells, which manifest continuous transformations at the epigenetic, gene and protein levels.

We can further draw first rudimentary conclusions on the factors that influence changes between these dynamic modes based on the linear analysis of near-stable regimes. Our set-up allows us to weigh the contributions of the different cell components against each other and determine their comparative influence using the control structure given by the type-level wiring. We show that the primary factor in regulating stability is a dynamic mode involving all cell regulatory mechanisms (cycle $D$ in Fig. 2), in particular also epigenetics. To our current knowledge there are so far no ensemble models in the literature which integrate epigenetic influence into gene expression in a systematic fashion which separates the different regulation mechanisms on the system level. In our model we can distinguish epigenetic factors from other cell components and account for the special role of epigenetic transcription regulation in a biologically sensible and accessible way.

Our approach also allows us to investigate the influence of “microscopic” parameters such as neighborhood sizes, membership sizes and Boolean function properties. We obtain that the increase on neighborhood size, at any level, push the systems towards the unstable regime. In contrast, the increase in protein complex sizes makes the system more unstable. This mathematical result has important biological implications. It tell us that if, during the course of evolution, both the number of regulatory interactions and the protein complex sizes are increased, then the cell can remain in a nearly stable
regime.

For the sake of simplicity we have focused our attention on Boolean models. This framework can be generalized to the case when there are more than two states chosen from some alphabet. More generally the states can take different values in algebraic groups or fields [Jarrah and Laubenbacher (2008)]. We have freedom to choose different updating regimes as well. In the linear regime the form of the matrix $A$ is only determined by the topology of the wiring diagram, while the updating scheme just affects the actual values of the non-zero matrix elements.

Higher order structures are a powerful means of expressing intricate relations in regulatory networks of all kinds [Baas (2006)]. We have shown here that structures of this kind are natural and adequate candidates for modeling biological processes. Such models are systematically more exact than single-level models since they formally represent patterns and types of regulations in the correct way and allow us to resolve the relative contribution of the different cellular components. They are called to play an even more fundamental role when addressing problems at the multicellular level. We hope this work motivates further efforts towards the annotation of cell regulatory networks, making an explicit distinction between the different cellular components, their level organization, and feedback regulation.

**Appendix: Partial derivatives of $\Lambda$**

To compute the derivatives of $\Lambda$ with respect to $A_\varepsilon$, $A_\zeta$ and $A_\eta$, we take into account that
where $P_{ij}$ is the characteristic polynomial associated with the minor of $A$ after removing line $i$ and column $j$. Furthermore

$$\frac{\partial P}{\partial D} = -1 + \frac{\partial P}{\partial \Lambda} \frac{\partial \Lambda}{\partial D}, \quad (10)$$

From these equations it follows that

$$\frac{\partial P}{\partial a_{ij}} = (-1)^{i+j} P_{ij}(\Lambda), \quad (11)$$

Now, since $A$ is irreducible and non-negative, the largest eigenvalue of $A$ is larger or equal than the largest eigenvalue of any submatrix of $A$ (Frobenius Theorem, Gantmacher (2000); Debreu and Hestrein (1953)). The latter result implies that $P_{ij}(\Lambda) \geq 0$. To show that $P_{ij} < 1$ we need to inspect the precise form of $P_{ij}$. For $i = j = 1$ we obtain

$$P_{11}(\Lambda) = \Lambda(\Lambda^3 - (A_G + A_P)\Lambda^2 + A_G A_P \Lambda - C). \quad (12)$$

Since $\Lambda \geq A_G$ (Frobenius Theorem) we have that

$$P_{11}(\Lambda) \leq \Lambda(\Lambda^3 - (A_G + A_P)\Lambda A_G + A_G A_P \Lambda - C) \leq \Lambda(\Lambda^3 - A_G^2 \Lambda - C) \leq \Lambda^4. \quad (13)$$

For $\Lambda < 1$ we finally obtain that $P_{11}(\Lambda) \leq 1$ and therefore
\[ \frac{\partial P}{\partial A_{\varepsilon}} = \frac{\partial P}{\partial a_{11}} \leq \frac{\partial P}{\partial D}. \] (14)

Following a similar analysis we obtain that

\[ \frac{\partial P}{\partial A_G} = \frac{\partial P}{\partial a_{22}} \leq \frac{\partial P}{\partial D}. \] (15)

\[ \frac{\partial P}{\partial A_P} = \frac{\partial P}{\partial a_{33}} \leq \frac{\partial P}{\partial D}. \] (16)

On the other hand, for the derivatives with respect to \( B \) and \( D \) we obtain

\[ \frac{\partial \Lambda}{\partial B} = \Lambda(\Lambda - A_{\varepsilon}) \frac{\partial \Lambda}{\partial D}, \] (17)

\[ \frac{\partial \Lambda}{\partial C} = \Lambda(\Lambda - A_P) \frac{\partial \Lambda}{\partial D}. \] (18)

Now again \( \Lambda \) is larger than the largest eigenvalue of any submatrix (Frobenius Theorem, Gantmacher (2000)) and thus it is larger than any matrix element. In particular \( \Lambda \geq a_{11} = A_{\varepsilon} \) and \( \Lambda \geq a_{33} = A_P \). Under the assumption \( \Lambda < 1 \), these results then imply that \( 0 \leq \Lambda(\Lambda - A_{\varepsilon}) \leq 1 \) and \( 0 \leq \Lambda(\Lambda - A_P) \leq 1 \), and therefore

\[ \frac{\partial \Lambda}{\partial B} \leq \frac{\partial \Lambda}{\partial D}. \] (19)

\[ \frac{\partial \Lambda}{\partial C} \leq \frac{\partial \Lambda}{\partial D}. \] (20)

References

Aldana, M., Cluzel, P., 2003. A natural class of robust networks. Proc. Natl. Acad. Sci. USA 100, 8710–8714.
Baas, N., Helvik, T., 2005. Higher order cellular automata. Adv. Comp. Syst. 8, 169–192.

Baas, N. A., 2006. Hyperstructures as abstract matter. Adv Comp Syst 9, 157–182.

Barabási, A.-L., Oltvai, Z. N., 2004. Network biology: understanding the cell’s functional organization. Nat. Rev. Genet. 5, 101–113.

Debreu, G., Hestrein, I. N., 1953. Nonnegative square matrices. Econometrica 21, 597–607.

Derrida, B., Pomeau, Y., 1986. Random networks of automata: a simple annealed approximation. Europhys. Lett. 1, 45–49.

Flyvbjerg, H., 1988. An order parameter for networks of automata. J. Phys. A: Math. Gen. 21, L955–L960.

Gantmacher, F. R., 2000. Mathrix Theory, Vol. II. AMS, Providence.

Grewal, S. I. S., Moazed, D., 2003. Heterochromatin and epigenetic control of gene expression. Science 301, 798–802.

Harris, S. E., sawhill, B. K., Wuenshe, A., Kauffman, S., 2002. A model of transcriptional regulatory networks based on biases in the observed regulatory rules. Complexity 7, 23–40.

Jaenisch, R., Bird, A., 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat. Rev. Genet. (Supp) 33, 245–254.
Jarrah, A. S., Laubenbacher, E., 2008. On the algebraic geometry of polynomial dynamical systems. In: The IMA volumes in Mathematics and its applications: Emerging applications of Algebraic geometry. Vol. 149. pp. 1–15.

Jong, H. D., 2002. Modeling and simulation of genetic regulatory systems: A literature review. J Comp Biol 9, 67–103.

Kauffman, S. A., 1969. Metabolic stability and epigenesis in randomly constructed genetic nets. J. Theor. Biol. 22, 437–467.

Kauffman, S. A., 1993. The origins of order: Self-organization and selection in evolution. Oxford Univ. Press, New York.

Kauffman, S. A., Paterson, C., Samuelsson, B., Troein, C., 2004. Genetic networks with canalyzing boolean rules are always stable. Proc. Natl. Acad. Sci. USA 101, 17102–17107.

Shmulevich, I., Kauffman, S. A., 2004. Activities and sensitivities in boolean network models. Phys. Rev. Lett. 93, 048701.

Walsh, C. T., Gorneau-Tsodikova, S., Gatto, G. J., 2005. Protein posttranslational modifications: the chemistry of proteome diversifications. Angew. Chem. Int. Ed. 44, 7442–7372.