

Model Checking Logical Regulatory Networks ^{*}

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Abstract: Regulatory and signalling networks control cell behaviours in response to environmental cues. The logical formalism has been widely employed to study these interaction networks, which are modelled as discrete dynamical systems. While biologists identify networks encompassing more and more components, properties of biological relevance become hard to verify. Here, we report on the use of model-checking techniques to address this challenge. This approach is illustrated by an application dealing with the modelling of T-helper lymphocyte differentiation.

Keywords: Systems Biology, Formal Verification, Discrete Event Systems, Biological networks, Regulatory Networks, Signalling Networks, Logical Modelling, Cell Differentiation, T-helper lymphocyte

1. INTRODUCTION

Cellular processes such as cell proliferation and differentiation are governed by molecular regulatory networks. As technological advances facilitate the identification of these complex networks, mathematical models are needed to study their functioning (de Jong (2002); Schlitt and Brazma (2007)). In this context, we focus on the formalism initially introduced by R. Thomas and collaborators (Thomas and D'Ari (1990)). In short, models are defined as Logical Regulatory Graphs that encompass nodes (the regulatory components) and directed edges (regulatory interactions). Moreover, nodes are associated with discrete variables, which embody regulatory component levels of activity. For each node, a logical function defines the target value of the corresponding variable, depending on its regulators (for further details see Thomas and D'Ari (1990); Chaouiya et al. (2003)). These models lead to discrete (a)synchronous dynamics, conveniently represented by State Transition Graphs (STG), where nodes and arcs denote states and transitions, respectively.

Properties of interest mainly relate to the attractors and their reachability. An attractor is defined as a terminal strongly connected component of the STG, i.e., a strongly connected component with no transition leaving it. At-

tractors capture potential long term behaviours of the regulatory network and refer to stable states (e.g. stable pattern of gene expression) or sustained oscillations. Not surprisingly, combinatorial explosion hinders efficient analysis of large networks dynamical properties, in particular concerning attractor reachability.

Here, we focus on the use of model-checking and related software tools for the analysis of dynamical properties of biological networks. As illustrated with a model for lymphocyte differentiation, logical models are particularly amenable to formal verification approaches (Naldi et al. (2010)). In addition to classical reachability properties, we show how novel questions concerning the structure of a regulatory network can be efficiently addressed.

2. MODEL-CHECKING

As models of regulatory networks grow in size, they tend to generate more complex behaviours. Determining whether a biological model satisfies a set of observed biological properties is thus often manually untractable. This motivates the use of automated formal verification techniques capable of verifying qualitative systems generating very large state transition graphs up to 10^{20} states (Burch et al. (1990)). One of these techniques is model-checking (Clarke et al. (2000)).

Model-checking is a computer science technique, which has been proposed 30 years ago to solve the problem of

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verification of very large systems in hardware and software industry. Since then, many improvements and variations have been developed as application fields diversified.

2.1 Application to Biological Systems

During the last decade, model-checking techniques have been successfully applied to the broad field of systems biology (Chabrier-Rivier et al. (2004); Bernot et al. (2004); Batt et al. (2005)), with variations in the mathematical modelling formalisms and/or the type of properties to be verified. Most of the formalisms considered to model biological networks are already discrete or can be discretised under a suitable abstraction criterion, therefore permitting the generation of a STG representing the transitions between the qualitative states of the system. A STG can be directly mapped into a Kripke structure, as used in model-checking, through a direct correspondance of their states and a labelling denoting the values of the components in each state. Temporal logic formulas can then be verified on such structures.

Signalling networks differ from gene regulatory networks by the consideration of input signals imposing restrictions on the dynamics. From the modelling perspective, these restrictions apply to paths in the STG that only occur for specific input valuations. As a consequence, both the characterisation of the attractors and their reachability become highly dependent on the input signals. Additionally, input variables which do not have an associated regulatory function, remain constant throughout the simulation.

From the model-checking perspective, the verification of properties is performed over Kripke structures where all the variables define the current state of the system. For models with (fixed) input variables, the STG is organised into disconnected sub-graphs, one for each complete instantiation of the inputs. Indeed, because these variables are maintained constant, no transitions allow the system to go from one state to another that differs on the values of input variables. This leads to a state-space size proportional to the number of input variables and their possible values. However, one can compress this state-space by using transition labels to specify the input values for which transitions are enabled. Only non-input variables are then explicitly denoting the states of the system (Naldi et al. (2012)).

In the model-checking community, a structure combining labels on both states and transitions is called a Kripke Transition System. Pecheur and Raimondi augmented the classical CTL semantics to account for the existence of actions, called Action Restricted CTL (ARCTL) (Lomuscio et al. (2007)). ARCTL includes the same temporal operators as CTL, except that the paths can be restricted with a given action formula α . The syntax and semantics of ARCTL is described in Table 1. This was implemented as an extension to the known NuSMV model-checking tool¹, being particularly useful for the verification of the behaviour of models influenced by fixed/varying input variables.

Table 1. Syntax and semantics of canonical ARCTL temporal operators used in this work (for a complete description see Lomuscio et al. (2007)); α is a path restriction defined by input valuations; ϕ and ψ denote internal component valuations at a given state.

Syntax	Semantics
$EAF(\alpha)(\phi)$	ϕ has to hold eventually at some future state for some α -restricted path
$AAF(\alpha)(\phi)$	ϕ has to hold eventually at some future state for all α -restricted paths
$EAG(\alpha)(\phi)$	ϕ has to hold along the subsequent path for some α -restricted path
$AAG(\alpha)(\phi)$	ϕ has to hold along the subsequent path for all α -restricted paths
$EA(\alpha)[\phi \cup \psi]$	ϕ has to hold along the subsequent path until ψ holds, for some α -restricted path
$AA(\alpha)[\phi \cup \psi]$	ϕ has to hold along the subsequent path until ψ holds, for all α -restricted paths

Due to the range of existing model-checkers and temporal logics, the adoption of this technique for the analysis of biological systems remains a difficult task for non-expert users. As a consequence, in the field of formal verification, recurrent properties have been grouped into high-level patterns to help non-expert users formulate temporal-logic queries (Dwyer et al. (1999)). In systems biology, Chabrier-Rivier et al. (2004) enumerated properties on reachability, pathways and stability of a discrete system. Bernot et al. (2004) tested necessary conditions leading to a particular state of a bistable system. Batt et al. (2005) also tested conditions leading to a given state, imposing restrictions on sequences of events along the path. Although different, these properties all share the same conceptual form: they correspond to reachability properties verifying the existence of a path between a (set of) initial state(s) and a (set of) reachable state(s). This has motivated the definition of patterns for recurrent biological properties to ease their formal verification (Monteiro et al. (2008)).

2.2 Modelling and verification tools

Here, we survey the main model-checking tools that have been applied to biological networks.

NuSMV is a symbolic model-checker based on Binary Decision Diagrams that provides a description language to specify generic finite state machines, supporting modules and processes, and verification through a set of temporal logic CTL or LTL formulas (Cimatti et al. (2002)).

Construction and Analysis of Distributed Processes (CADP) is a toolbox for the design of asynchronous concurrent systems (Garavel et al. (2007)), containing a powerful model-checker for temporal logics with highly expressive power, like CTRL (Mateescu et al. (2011)) and others. It allows on-the-fly model-checking and diagnostic generation over labeled transition systems, i.e., system information is represented on the transitions rather than on the states.

Analysis of Networks through TEmporal-LOGic sPEcifications (ANTELOPE) is a model-checker for the analy-

¹ NuSMV-ARCTL is freely available for download at <http://1v1.info.ucl.ac.be/Tools/NuSMV-ARCTL-TLACE>.

sis of Boolean gene regulatory networks (Arellano et al. (2011)). It supports Hybrid CTL, an extension of standard CTL with a special binder temporal operator, capable of selecting partially characterised states (e.g. unknown stable states, or cyclic attractors).

The Biochemical Abstract Machine (BIOCHAM) has been initially developed for the qualitative modelling of biochemical processes and currently supports both qualitative and quantitative models (Calzone et al. (2006)). It provides a simple rule-based language for model specification and integrates the NuSMV model-checker for their verification. Users can encode biological properties into CTL formulas directly into BIOCHAM, without having to export the model and run NuSMV externally.

Selection of Models of Biological Networks (SMBioNet) is a tool for the selection of all fully parametrised models of a given regulatory graph that satisfy a set of CTL formulas (Bernot et al. (2004)). It is based on the (multivalued) logical formalism and integrates the NuSMV model-checker.

Genetic Network Analyzer (GNA) is a modelling and simulation tool for genetic regulatory networks (de Jong et al. (2003)). Qualitative models are specified in the form of piecewise-linear differential equations with inequality constraints on the parameters. Besides simulations and attractor search (de Jong and Page (2008)), it permits the specification of biological properties through the use of a pattern system (Monteiro et al. (2008)) or directly into CTL and CTRL formulas (Mateescu et al. (2011)). The verification step is performed through a webservice call to a remote server capable of verifying CTL and CTRL formulas using the NuSMV and CADP model-checkers, respectively.

Gene Interaction Network simulation (GINsim) is a software dedicated to the logical modelling and simulation of regulatory networks (Chaouiya et al. (2012)). *GINsim* allows the simulation of models using the synchronous, asynchronous and priority updating policies (Fauré et al. (2006)). Additionally, it comprises analysis tools for stable state identification, construction of the reachable hierarchical transition graph, and analysis of regulatory circuits (Naldi et al. (2007); Bérenguier et al. (2013)). Models can be exported as SMV descriptions and verified externally with NuSMV (Naldi et al. (2012)), using CTL or ARCTL formulas on Kripke transition systems (Lomuscio et al. (2007)).

3. APPLICATION: T-HELPER CELL DIFFERENTIATION

To motivate the use of model-checking tools and assess their efficiency, we consider the logical model of T-helper (Th) cell differentiation defined in (Naldi et al. (2010)), as well as three specific variants of this model (which will be defined later on). Considering biological observations, we aim at discriminating between these models.

T helper (CD4+) lymphocytes play a key role in the regulation of immunity. Potentially faced with a large diversity of pathogens, naive (i.e. antigen-inexperienced) CD4+ T cells (called "Th0") orchestrate immune responses after differentiating into distinct Th cell populations expressing distinct cytokine profiles (O'Shea and Paul (2010)). Upon the stimulation of the cytokine IFN γ or IL-12, naive Th cells polarise to a Th subset secreting IFN γ (called "Th1"), whereas IL-4 induces naive Th cells to differentiate into a Th subset secreting IL-4 (called "Th2"). This differentiation is driven by pivotal transcriptional factors. While the polarization into Th1 celltype is governed by the transcriptional factor Tbet, the differentiation into Th2 is directed by Gata3.

It has been recurrently assumed that cross-inhibitions between Tbet and Gata3 would enforce mutual exclusion between Th1 and Th2 differentiation programs. However, stable phenotypes with both Tbet and Gata3 expressed have been recently experimentally observed following the polarisation of naive Th cells under a mixed environment with Th1 and Th2 polarising cytokines (Peine et al. (2013)). Moreover, Th1 polarising cytokines can direct Th2 reprogramming into a stable phenotype coexpressing Tbet and Gata3 (Hegazy et al. (2010)). These results question the role of the mutual inhibition between Tbet and Gata3, which has widely been incorporated in Th differentiation models (Mariani et al. (2004); Mendoza (2006); Naldi et al. (2010)). While there exist strong experimental data supporting a mutual inhibition between Gata3 and Tbet signalings, we still lack data demonstrating direct inhibition.

In the light of these recent experimental data, using model-checking tools, we aim to investigate the role of Tbet and Gata3 mutual inhibitions in the differentiation of naive Th cells (Th0) into Th1, Th2 and the hybrid Th subtype expressing both Gata3 and Tbet (Th1/Th2). To this intent, we verify a selection of biological properties of interest for relevant input conditions (see Table 2). In this regard, we consider the following variants of the reduced model defined in (Naldi et al. (2010)), illustrated in Fig. 1:

- model 0 as the original model, includes the cross-inhibitions;
- model 1 in which the interaction Tbet \rightarrow Gata3 is inactivated;
- model 2 in which the interaction Gata3 \rightarrow Tbet is inactivated;
- model 3 in which both interactions are inactivated.

We focus on reachability properties between four sets of states, which represent the Th cell types of interest (see Fig. 2): naive Th cells (Th0), Th1 subtype, Th2 subtype (corresponding respectively to Th0, activated Th1 and activated Th2 subtypes defined in Fig. 6 of Naldi et al. (2010)), and Th1/Th2 (defined as the set of states for which Gata3=Tbet=1). We could verify that cellular types Th0, Th1 and Th2 are stable under adequate environment conditions for all the models, whereas the hybrid Th1/Th2 type is stable only for model 3 with proTh1Th2 condition.

We first checked if naive Th cells could polarise into Th1 cells under proTh1 environmental conditions and into Th2 cells under proTh2 conditions (see Table 2). This property

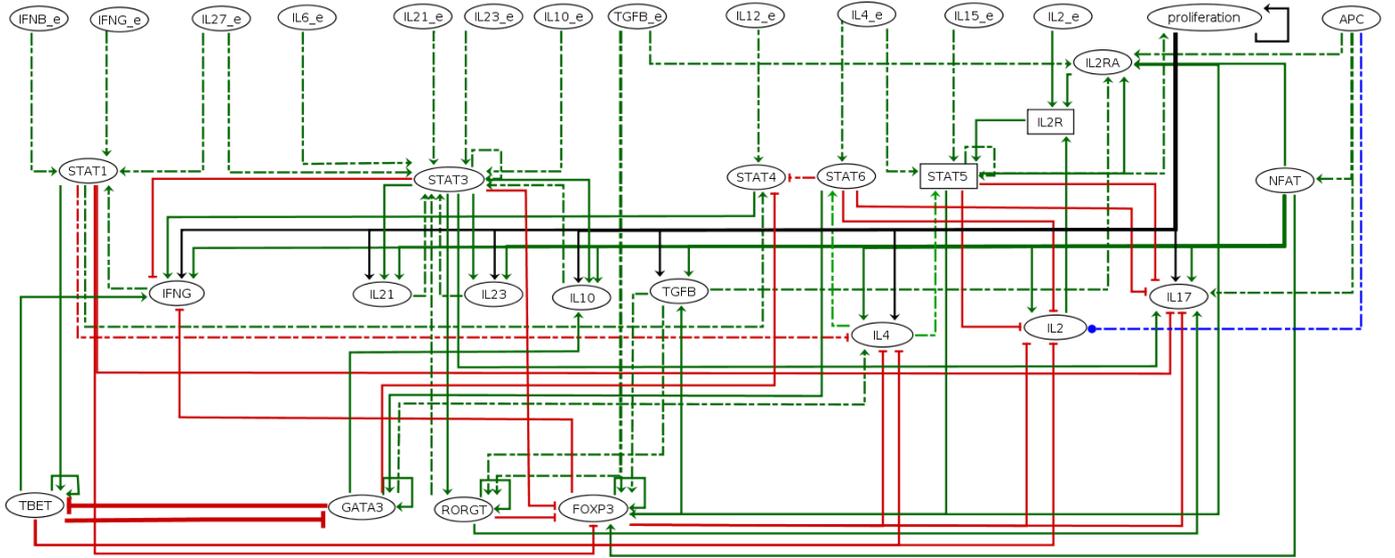


Fig. 1. Reduced regulatory graph for Th differentiation (adapted from Naldi et al. (2010)). This model contains 13 input components (APC and external input cytokines) represented on top. Oval nodes denote Boolean components, whereas rectangular nodes denote ternary components. Green arrows denote activations, red arcs denote inhibitions and the blue arc denotes a dual interaction. The two interactions modified in the model variants are emphasised with thicker arcs.

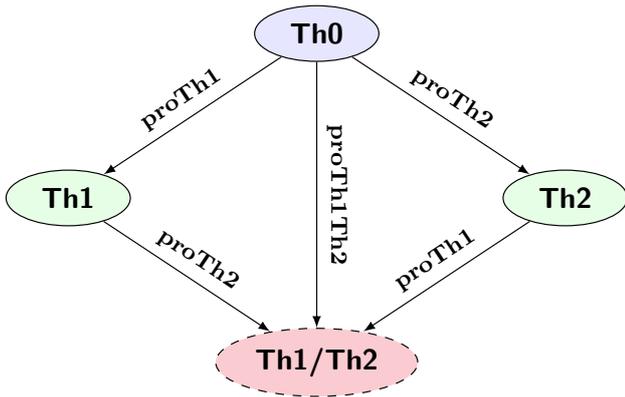


Fig. 2. Reachability graph between cellular types for the polarisation from Th0 to Th1, Th2 and the hybrid subtype Th1/Th2. Nodes represent the four cellular subtypes considered here. The dotted contour for Th1/Th2 subtypes denotes the fact that this state was found unstable for any input conditions in the original model. An arrow linking two cellular states denotes the existence of a path connecting them for given fixed input condition.

Table 2. Combinations of APC with seven different cytokine inputs defining the environmental conditions considered (see Fig. 6 in Naldi et al. (2010)).

Conditions	Inputs							
	APC	IL2_e	IL4_e	IL6_e	IL10_e	IL12_e	TGFB_e	IFNG_e
proTh1	1	1	0	0	0	0	0	1
proTh2	1	0	1	1	0	0	0	0
proTh1Th2	1	1	1	1	0	0	0	1

was shown to be **true** using the NuSMV-ARCTL model-checker for each of the four models (see Table 3). Therefore, the four models reproduce the expected polarisation of naive Th cells into Th1 (resp. Th2) cells under proTh1 (resp. proTh2) environmental conditions.

As Tbet is the master transcriptional regulator governing the polarisation of Th1 cells secreting IFN γ , we asked whether IFN γ activation was necessarily preceded by Tbet activation during Th1 polarisation. For this, we changed the ARCTL formula to include a sequential activation restriction along the path:

$$AAF(e)(t \ \& \ AA(e)[t \ U \ ti \ \& \ AA(e)[ti \ U \ c]]) \quad (1)$$

where e corresponds to proTh1 environmental condition, t to any state having Tbet=1 and IFN γ =0, ti to any state having Tbet=1 and IFN γ =1, and c to the target Th1 cellular type.

This property was shown to be **true** by the model-checker for each of the four models, supporting the hypothesis

Table 3. Reachability analysis considering the cellular types and environmental conditions defined in Table 2, for each of the four models. Each line corresponds to a CTL formula specification, having the pattern-like CTL temporal formula $EAF(e)(c \ \& \ AAG(e)(c))$, where e corresponds to an input condition and c is the desired reachable stable state.

Initial State	Input Condition	Target State	Model			
			0	1	2	3
Th0	proTh1	Th1	true	true	true	true
	proTh2	Th2	true	true	true	true
	proTh1Th2	Th1Th2	false	false	false	true
Th1	proTh2	Th1Th2	false	false	false	true
Th2	proTh1	Th1Th2	false	false	false	true

that Tbet activation would be necessary for $\text{IFN}\gamma$ to be expressed.

We then asked whether Th0 can polarise to the hybrid subtype Th1/Th2 under the mixed cytokine environment proTh1Th2 (see Table 2). This property was shown to be true only for model 3 (see Table 3).

Finally, we checked whether activated Th2 cells could be reprogrammed into Th1/Th2 cells under proTh1 condition. This property was also shown to be true only for model 3 (see Table 3).

From this analysis, among the four model variants, model 3 (with no inhibition between Gata3 and Tbet) thus appears to be the most consistent with the biological properties considered (see Table 3).

4. DISCUSSION

In this paper, we illustrate the verification of models of signalling-regulatory networks, relying on the GINsim modelling software tool together with the NuSMV-ARCTL model-checker. As a biological application, we consider four variants of a logical model for Th differentiation (Naldi et al. (2010)) under specific environmental conditions.

The consistency of these variants was assessed regarding the differentiation and plasticity of Th1 and Th2 cell types, along with a newly described hybrid subtype expressing both master regulators Tbet and Gata3. The model with no cross-inhibition between Gata3 and Tbet appears to be the most coherent with experimental observations to date. Other model variants could have also been considered in our analysis, for example, where one and/or the other cross-inhibition is refined by introducing an additional level for Tbet and/or Gata3.

The Th differentiation full model has 65 components and a space of $\sim 2.8 \times 10^{20}$ states. Here, we considered the reduced version specified by Naldi et al. (2010) with 34 components and $\sim 3.86 \times 10^{10}$ states. It is worth noting that all trajectories of the reduced model have their counterparts in the complete model, whereas the reverse is not true (Naldi et al. (2011)). Therefore, to be fully conclusive, we would need to check whether properties that are not verified in the reduced model are still not verified in the full model. This will require further developments of the NuSMV-ARCTL model-checker to enhance its capacity to handle such huge state spaces.

Despite availability of well established model-checking tools, and their integration in systems biology modelling tools, model verification remains difficult for non-expert users because of the need to specify temporal logic formulas. Previous work by Dwyer et al. (1999) and later by Monteiro et al. (2008) tried to close this gap through the definition of a set of patterns covering the most used formulas in a given domain of application. However, as models of regulatory networks encompass ever more information, in particular restrictions imposed by environmental cues, it becomes evident that the existing patterns are not sufficient and need to be extended. Hence, we are currently defining a set of patterns covering most

dynamical properties to be checked for cellular networks, and plan to incorporate them into GINsim.

Finally, logical models of cellular networks present interesting challenges to the field of discrete event systems, with original properties to be verified over large and complex dynamics.

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