Towards interactive model checking of biological pathway dynamics

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Abstract. Biological systems such as regulatory or gene networks can be seen as a particular type of distributed systems, and for this reason they can be modeled with the same tools that were developed in the computer science context. However, tools designed to model distributed systems often require a computer science background, making their use less attractive for biologists. ANIMO (Analysis of Networks with Interactive MOdeling) was built with the aim to provide biologists with access to the powerful modeling formalism of Timed Automata in a user friendly way. Continuous dynamics is handled by discrete approximations.
This brings computational support closer to the biological laboratories, where large amounts of data are generated on a daily basis. Thanks to computational models, biological data can be analyzed in different ways, helping biologists to formulate new hypotheses, drive experimental research and share knowledge on biological processes.
In this paper we introduce an improved modeling approach that allows us to considerably increase ANIMO's performances, opening the way for the analysis of bigger models. Moreover, this improvement makes the introduction of model checking in ANIMO a realistic feature, allowing for reduced computation times. The user interface of ANIMO allows to rapidly build non-trivial models and check them against properties formulated in a human-readable language, making modeling a powerful support for biological research.

1 Introduction

To study the possible causes of a disease and design effective cures it is necessary to closely study the behavior exhibited by biological cells under particular conditions. A *signaling pathway* describes the chain of interactions occurring between the reception of a *signal* and the response with which the cell reacts to such signal. A signal is typically represented by a substance which can bind to specific receptors on the cell surface, activating them. *Active* molecules relay the signal inside the cell by activating other molecules until a target is reached. The target of a signaling pathway is usually a transcription factor, a molecule

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with the task of controlling the production of some protein. Such regulation is considered to be the response of the cell to the received signal.

The current knowledge on signaling pathways (mostly organized in databases such as KEGG [1] or PhosphoSite [2]) suggests that the interactions involved in a cellular response assume more often the shape of a network than that of a simple chain of signal relays. Such networks are typically highly connected, involving feedback loops and crosstalk between multiple pathways, making it difficult to grasp their dynamic behavior. For this reason, computational support is required when studying non-trivial biological networks.

A number of software tools are available for modeling complex networks of biochemical interactions [3–7]. These tools significantly contribute to the process of formalizing the knowledge on biological processes, rendering them amenable to computational analysis. However, a lack of familiarity with the formalisms underlying many available tools hampers their direct application by biology experts. ANIMO (Analysis of Networks with Interactive MOdeling, [8–10]) is a software tool based on the formalism of Timed Automata [11] that supports the modeling of biological signaling pathways by adding a dynamic component to traditional static representations of signaling networks. ANIMO allows to compare the behavior of a model with wet-lab data, and to explore such behavior in a user-friendly way. In order to achieve a good level of user-friendliness for a public of biologists, the complexity of Timed Automata is hidden *under the hood*, presenting ANIMO as an app for Cytoscape [12], a tool specifically developed for visualizing and elaborating biological networks. Signaling networks are represented in ANIMO using the nodes-edges notation normally used in the context of biology (see Fig. 1a).

Previously, ANIMO supported only interactive exploration of network dynamics based on simulation runs. Model checking queries could be answered through the UPPAAL tool [13], but the required knowledge of temporal logic together with the usually long response times slowed down the investigation process. In order to encourage the use of model checking on non-trivial models of signaling networks, we updated ANIMO with a new Timed Automata model (presented here). This marks a relevant improvement in terms of performances with respect to the model previously used in ANIMO. Moreover, consistently with the intents of our tool, we implemented also a user interface for the definition of model checking queries in ANIMO. This allows a user to interrogate an ANIMO model without requiring previous experience in temporal logics. These new features are available in the latest version of ANIMO, which was recently reimplemented as a Cytoscape 3 app [14]: this lets users profit of the additional analysis features made available by the other apps in the Cytoscape environment.

The paper continues as follows. After introducing the basic concepts in Section 2, we illustrate in Section 3 a new way of using Timed Automata in ANIMO. In Section 4 we present a comparison between the new modeling approach and the one previously used in ANIMO, focusing on model analysis performances. In Section 5 we describe how model checking was made accessible to ANIMO users. We conclude the paper with Section 6, discussing future work.

2 Preliminaries

2.1 Signaling pathways in biology

A signaling pathway is an abstract representation of the reactions occurring inside a biological cell when, e.g., a signaling substance comes in contact with the cell surface receptors. In this setting, a reaction is the interaction between two components: the upstream enzyme (the molecule holding the active role in the reaction) and the downstream substrate (the passive molecule). The enzyme can be for example a kinase, which attaches a phosphate group to its substrate, performing a phosphorylation: this determines a change in the shape of the substrate and consequently a new function (Fig. 1). The new state reached by the substrate is often called *active*: if the substrate of the reaction is itself a kinase, it can then proceed in passing on the signal by activating its own target molecule, continuing a chain of reactions leading to the target of the signaling chain. Such target is usually a transcription factor, i.e. a molecule that influences the genetic response of the cell, for example promoting the production of a particular protein.

Pathways are traditionally represented in a nodes-edges form (see Fig. 1a), with nodes representing molecular species and arcs standing for reactions, where $\rightarrow$ represents activation and $\dashv$ represents inhibition (i.e. inactivation).

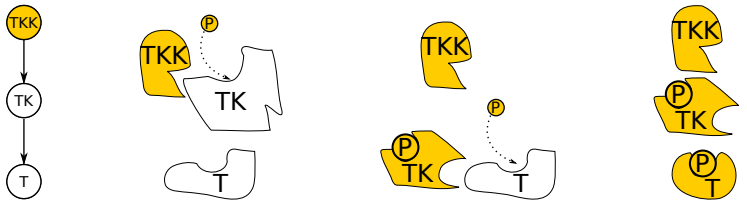


Fig. 1. A kinase signaling network represented in the nodes-edges form **(a)** and its evolution represented by abstract molecular interactions (**b-d**). T is the target of the signaling pathway, TK is the kinase that activates T, and TKK is the kinase that activates TK. **(b)** An already active (yellow) TKK can bind to an inactive (empty) TK, catalyzing its phosphorylation (i.e. binding of a phosphate group, P). This causes a change in the shape of TK, activating its enzymatic function **(c)**. The active TK can in turn activate its target T, enabling it to carry out its function **(d)**.

The current knowledge on signaling pathways [1, 2] evidences the fact that signaling interactions are rarely a simple chain of activations as represented in Figure 1a. More often, they assume the shape of a network with multiple feedback loops and crosstalk from different signaling sources. This complexity is an added difficulty for the study of such networks, reducing the possibilities to deduce the dynamic behavior of a signaling network by inspecting its static representation. For this reason, efficient computational support is essential when representing and analyzing the behavior of complex signaling networks.

2.2 Timed Automata

Timed Automata (TA) are finite-state automata enriched with real-valued clocks and synchronization channels. All clocks in a TA system advance with the same rate, and transitions between the locations of an automaton depend on conditions on clocks. In particular, a *guard* defines when a transition may be taken, while an *invariant* is the condition for permanence in a location. A transition can also allow two automata to *synchronize*, with each participant performing one of two complementary actions (*input* and *output*) on a synchronization channel. A set of clocks may also be reset by a transition, causing them to restart from 0.

The models we will present here were implemented using the software tool UPPAAL [13], which adds a number of features to the basic definition of TA. Some of these extensions include: support for integer variables in addition to clocks, broadcast synchronization channels (one sender, many receivers), definition of C-like functions to perform more operations besides clock resets. UPPAAL also allows for a special type of locations, named *committed* (marked with a C in the graphical representation). As long as an automaton is in a committed location, time is not allowed to flow. This feature can be used for example to perform immediate updates to local variables before letting the computation proceed. Examples of the listed features are found in the TA model in Section 3.2.

2.3 Activity-based models in ANIMO

ANIMO allows the definition of *activity-based* models. This means that we assume each signaling molecule in a cell to be at any time in one of two states: active or inactive. Active molecules can take an active role in reactions, changing the state of other molecules, activating inactive molecules or inhibiting (i.e. deactivating) active molecules. In a kinase-based signaling network an activation process can be a phosphorylation, and it is usually countered by the corresponding dephosphorylation. However, our models are not limited to kinase networks: other features like different post-translational modifications or gene promotion can be likewise represented, as long as their role has immediate effects on the ability of a target to perform its task.

As ANIMO is a Cytoscape app, models are defined through the Cytoscape user interface (see Fig. 2), where the user inserts a node for each molecular species and an edge for each reaction, with $\rightarrow$ indicating activation and $\dashv$ indicating inhibition similarly to traditional representations of signaling networks (Fig. 1a).

We consider a *molecular species* (also called *reactant*) to include all the molecules of the same substance in both their active and inactive state inside the cell. In order to distinguish between the two activity states in which each molecule can be, we define the *activity level* to represent the percentage of active molecules over an entire molecular species. In an ANIMO model, this value is discretized on a given integer interval, to adapt to the quality of available experimental data and (for large models) allow for a trade-off between performance and precision. The user can choose the granularity for each molecular species

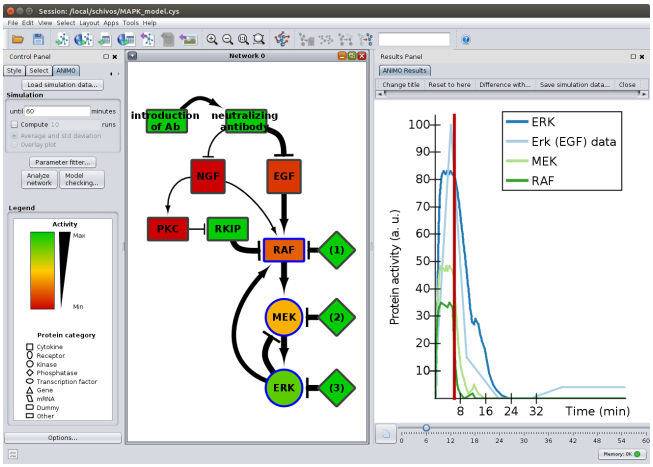


Fig. 2. The Cytoscape 3 user interface running the new ANIMO app (model from [9]). The *Network* panel in the center contains the nodes-edges model of the cross-talking signaling pathways of NGF and EGF, with colors indicating node activity levels and shapes representing different protein categories (see the *Legend* on the left). The *Results Panel* on the right contains a graph plotting activity levels of selected nodes during the first hour of evolution of the model. The slider under the graph allows the user to select the time instant (marked as a vertical red line in the graph) on which the colors nodes in the *Network* are based. The edge thickness is used to give an idea on which reactions are occurring more often at the selected time instant. The series Erk (EGF) data in the graph is the experimental data from [15] for the 100 ng/ml EGF treatment.

separately, on a scale between 2 (the reactant is seen as either completely inactive or completely active) and 101 levels (allowing to represent activity as $0, 1\%, 2\% \ldots$ 100%). The activity level of a molecular species is represented in the ANIMO network by coloring the corresponding node according to the scale shown in the Activity legend in Figure 2, where the minimum indicates that all molecules of the given species are inactive.

The occurrence of a *reaction* modifies by one discrete step the activity level of its target reactant, making it increase or decrease depending on whether the reaction is defined, respectively, as activating or inhibiting. The rate with which a reaction occurs depends on a formula selected by the user. Choosing one of three available scenarios allows the user to make the reaction rate depend on the activity of one or two reactants. The rate of each reaction can be scaled by modifying the value of one kinetic constant k, possibly using a qualitative measure from a predefined set (very slow, slow, medium, fast, very fast). The approximation allows us to reduce the dependence of a model from often unavailable quantitative parameters for biochemical reaction kinetics, while keeping a precision level that is still precise enough to be useful. For a more precise explanation on how reaction rates are computed in ANIMO, we recommend [9], where the previously used TA model is presented. The reader interested in the current methods for parameter setting in ANIMO can refer to [16].

3 A new way of modeling signaling pathways with TA

We present here a novel model to represent signaling pathways with TA in ANIMO. We define the model previously used in ANIMO to be *reaction-centered*, as for each reaction in the network an instance of a TA template is generated to mimic the occurrences of that reaction. Observing that signaling networks tend to be highly connected, containing noticeably more reactions than reactants, we shift the focus on reactants instead, achieving what we call a *reactant-centered* model. This change of view is inspired by the classical way in which biological events are modeled with ordinary differential equations (ODEs) [17].

3.1 The reactant-centered approach

The reactant-centered model presented here is based on the concept of *net effect* of a set of reactions on a reactant: instead of considering each reaction in isolation, we consider their combined influence on each reactant. As an example, consider a reactant A activated by reactions R_1 and R_2, and inhibited by reaction R_3 (see Fig. 3a). The net effect of these three reactions on A defines the *net reaction* $R_A = R_1 + R_2 - R_3$. Applying a concept similar to the definition of an ODE, where the rate of change of each reactant depends on the rate of the reactions influencing it, the rate of R_A is computed as the sum of the rates of the reactions influencing A:

$$r_A = r_1 + r_2 - r_3$$

where r_i is the rate of reaction R_i and is defined as follows. Consider R_1 to be the reaction $B \rightarrow A$ with kinetic constant k_1. Suppose the settings in the ANIMO network for R_1 make its rate depend only on the activity level of B. Then we compute the rate of R_1 as $r_1 = [B] \times k_1$, with $[B]$ the current activity level of B.

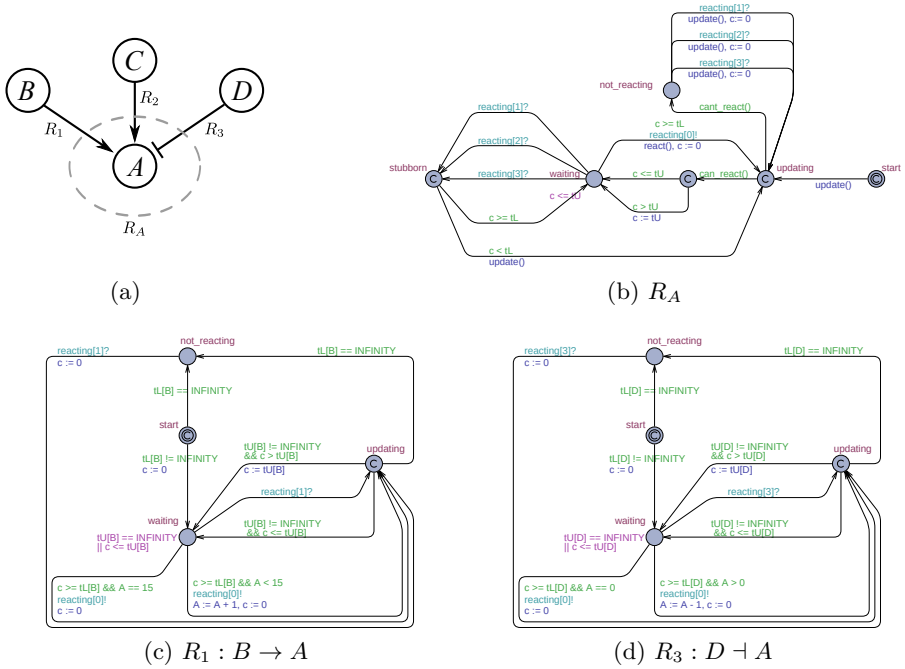
As with ODEs, if r_A is positive the activity level of A will increase; otherwise, A will decrease its activity level. The absolute value of r_A determines the speed with which such change happens. The value of the reaction rate is thus translated into a time value to be used as time bound in the TA representing R_A (see Fig. 3b) by computing

$$T_A = \frac{1}{\text{abs}(r_a)}$$

with $\text{abs}(r_a)$ the absolute value of r_a. In order to represent a natural uncertainty or variability in reaction timing, we relax the exact value of T_A by defining bounds of $\pm$ 5% (which can be changed by the user):

$$\mathsf{tL} = T_A \times 0.95 \qquad \mathsf{tU} = T_A \times 1.05$$

Analogously to the reaction-centered model of [9], we will call tL the *lower time bound* and tU the *upper time bound*. So we replace an exact reaction time by an interval of possible reaction times, implicitly assuming a uniform distribution over this interval.



(a) (b) R_A

(c) $R_1 : B \to A$ (d) $R_3 : D \dashv A$

Fig. 3. (a) A signaling network where one node is influenced by three distinct reactions. The (virtual) reaction R_A is defined in the reactant-centered model as the algebraic sum of the reactions influencing A. (**b-d**) TA templates generated by ANIMO for the reactant-centered **(b)** and reaction-centered (**c-d**) models of the network in **(a)**. The templates have been edited for an easier visual comparison; while the template for R_2 ($C \to A$) is qualitatively identical to the one for R_1, and was left out for the sake of space. Functions can_react() and cant_react() in (b) have the same content as the corresponding guards in (**c-d**), while function update() in (b) replaces the references to the pre-computed tables tL[], tU[] with on-the-fly computation (see Sect. 3.3).

Differently from what presented in [9], we do not define pre-computed tables with all possible values for tL and tU: this is because the number of variables for the computation of a reaction rate can be arbitrarily high, and the size of such tables grows exponentially with the number of variables. In the example of Figure 3, a table to represent the time bounds for reaction R_A would have four dimensions, depending on the activity levels of reactants A, B, C and D. Assuming that all four reactants are discretized on 101 levels (activity levels from 0 to 100), each of the two tables tL and tU would contain $101^4 = 104\,060\,401$ elements. Adding one more reaction $E \to A$ to the pool would make this approach exceed the memory limits of most of the currently available computers For this reason, the reaction rates are computed on the fly (see Section 3.3).

3.2 Timed Automata reactant-centered model

The behavior of the TA representing the net reaction R_A is defined as follows: after a number of seconds variable inside the continuous interval [tL, tU] (mea-

sured with the local clock c), the activity level of A will increase or decrease by one step, depending on the sign of r_A; then new values for r_A, tL and tU will be computed based on the (possibly) changed conditions.

The process described in Section 3.1 to compute the values of tL and tU is an instance of the function called update() in the TA template of Figure 3b. The computation of update() is the first step taken when initializing a new model taking the transition from the initial location start (identified by two concentric circles) to updating. Location updating is then used to decide whether R_A can be executed or not (functions can_react() and cant_react() used as guards for the transitions exiting from updating). This decision is based on the computed value for r_A and the current activity level of A: if $r_A > 0$ and A is already completely active (or $r_A < 0$ and A is completely inactive), no update to A can be applied, so the automaton switches to location not_reacting. Moreover, r_A can be 0: this means that the reactions influencing A balance each other to complete cancellation, so also in this case R_A cannot occur and the next location will be not_reacting. Otherwise (i.e. if can_react() is true), the activity level of A can be changed, so the next location will be the one labeled waiting, which is reached after having made sure that its clock invariant is respected (committed location between updating and waiting).

When in location waiting, the local clock c is let run until enough time has passed for the reaction to occur: this is obtained by using c >= tL as guard for the transition from waiting to updating. Because of the invariant c <= tU on waiting, we ensure that the reaction occurs after no more than tU seconds have passed. The transition to updating leads to an update to the activity level of A (call to react()), together with the computation of new values for r_A, tL and tU (function react() calls update()). Moreover, we see in Figure 3b that a communication is sent on channel reacting[0] (0 is the index assigned to reactant A): this is used to warn the other automata that the activity level of A has just changed, possibly changing the conditions for other reactions.

On the other hand, one of the reactants influencing a reaction on which A depends may change its activity level before R_A can occur: this event may have an influence on the value of r_A, so a re-computation of r_A, tL and tU is needed. In the template of Figure 3b, the indexes of the influencing reactants are 1, 2 and 3, corresponding respectively to B, C, D in Figure 3a. Whenever a communication on one of the channels corresponding to an influencing reactant is received while in location waiting, the automaton moves to location stubborn. This location was introduced to avoid unwanted non-deterministic behavior[1] in the model. In the example, this could happen when the activity level of B is updated concurrently (in the same instant) with the activity level of A. As B influences A through reaction R_1, any change in the activity level of B causes a change in r_1, thus requiring a recomputation of r_A, tL and tU. Due to the interleaving nature of TA semantics, only one automaton may change location for the TA system to change state. This means that, should the activity level of B be changed first,

[1] The term *unwanted* refers to a non-determinism that is not related to actual biological concepts, but to the semantics of the modeling paradigm.

the conditions for R_A would be recomputed, possibly preventing the update to A to occur. Vice versa, should the activity level of A be changed first, both A and B could change their activity levels. In such a situation, the non-deterministic order in which the updates are computed decides the behavior of the model. To avoid this, we ensure that if a reaction can occur in a given instant (i.e. c >= tL), it will not consider the concurrent updates to influencing reactants (in the example, having the clock reach tL ensures that R_A occurs, independently from any concurrent update to B). Performing a check on the current value of the clock c with respect to the reaction lower time bound allows an automaton to detect the case of a concurrent update, remaining "stubborn" in its resolution of reacting with the current configuration: the guard c >= tL on the transition returning from stubborn to waiting is the same as the guard on the transition from waiting to updating and implies that the transition could occur in the current time instant. If no conflict is found, i.e. c < tL, a new time limit for the reaction is computed via function update() while moving to location updating. Note that the clock c is not reset when moving from stubborn to updating: should the new conditions only change the value of tL and tU without disabling the reaction, the "partial work" of the reactions is not discarded.

Finally, location not_reacting is used to avoid reacting when not necessary (see the description of cant_react() above), and to respond to changes in those reactants which can influence the value of r_A. Should one of such reactants change its activity level, the conditions are updated and the local clock is reset, moving to updating to check whether the reaction can now occur.

3.3 Computations on the fly

Reaction rates are all computed at run-time by the function update(), and are based on the user-chosen scenarios, their kinetic constant k and the activity level of the involved reactants. As such computations require floating-point precision but UPPAAL only provides integer variables and operators, we use a significand-and-exponent notation with 4 significant figures, which allows for an error in the order of 0.1% while avoiding integer overflow in UPPAAL's engine. For example, the floating point number $a = 1.23456$ will be represented as the pair $\langle 1235, -3 \rangle$, which is translated back as $a = 1235 \times 10^{-3} = 1.235$. The interested reader can find the UPPAAL definitions and functions needed to compute rate and time values for the TA templates inside any UPPAAL model file generated by ANIMO with a reactant-centered model type [2].

3.4 Comparison with the previous TA model

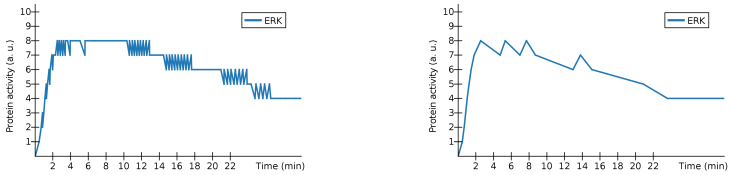
With reference to Figures 3b and 3c-d, we propose a qualitative parallel between the reaction- (old) and reactant-centered (new) TA models. The first remark is

[2] Models generated by ANIMO are saved in the system's temporary directory. Further details are available in the ANIMO manual at `http://fmt.cs.utwente.nl/tools/animo/content/Manual.pdf`

that equally-named locations have the same function in the automaton: so for example location waiting is used to wait before updating the reactant.

Being based on the same abstractions, the two models can be analyzed with the same UPPAAL queries as long as no explicit reference is made to TA locations. Such an analysis would normally not be necessary if considering the model as a whole and using it for what it is, i.e. an abstract representation of a set of biological processes.

We note that replacing all the existing reactions with the net reactions relative to each reactant in a model does change the behavior of the model in the sense that continuous oscillations of activity levels around an equilibrium point are less frequent. However, this does not mean that the behavior of two models using the two different approaches will diverge: the equilibrium points are still the same. Two opposite reactions occurring with the same (or similar) frequency will (nearly) cancel each other out: the behavior of the single reactions is explicitly represented in the reaction-centered model and gives rise to more oscillations. On the other hand, in the reactant-centered model, the trajectory of a reactant's activity level is determined taking into account also the canceling effect of opposite reactions, so the simulations will generally contain less oscillations. From the user's point of view, graphs will look smoother while still exhibiting the same qualitative behavior, as can be seen in Figure 4. The TA templates in Figure 3 show that the main difference between the two approaches is indeed due to the choice of lumping all the reactions influencing A into the single reaction R_A. In the next session we will show that having less oscillations leads in practice to better analysis performances.



(a) Reaction-centered (b) Reactant-centered

Fig. 4. Comparison of two ERK activity graphs generated from the model based on the case study from [9] with treatment of 50 ng/ml NGF. ERK was set to 10 levels of granularity to evidence the oscillations in the graphs.

4 Reaction-centered VS reactant-centered

We will now apply some basic model checking queries to the case study presented in [9], measuring the performances of the two modeling approaches. This will allow us to evaluate the benefit brought by the shift in perspective from a reaction- to a reactant-centered model.

All experiments were carried out on an Intel® Core™ i7 CPU at 2.80GHz equipped with 4 Gb RAM and running Ubuntu GNU/Linux 14.04.1 64bit. UPPAAL version 4.1.19 64bit was used to compute the result of the queries, asking for "some trace" with random depth-first search order when an execution trace was expected to be produced. For the simulation queries using the statistical model checking engine, we left all options at their default values.

The case study we use as a testbed is the network model shown in the *Network* panel in Figure 2, which represents signaling events downstream of growth factors EGF (epidermal growth factor) and NGF (nerve growth factor) in PC12 cells (a cell line used to study neuronal differentiation). The model topology proposed in [15] was analyzed with an ANIMO model based on the reaction-centered approach, reproducing the experimentally observed ERK (extracellular signal-regulated kinase) activity changes [9]. In particular, a 10 minutes stimulation with EGF resulted in transient behavior (i.e. peak-shaped, see also the graph in Fig. 2), while NGF stimulation led to sustained activity (see Fig. 4).

4.1 Simulation cost

We start by evaluating the cost of simulation with the two different models. This is a particularly important aspect to consider, as during the model building phase a user may need to perform a large number of simulations, continuously adapting the topology or quantitative parameters of a network model. In order to make the modeling approach in ANIMO as interactive as possible, it is desirable to decrease dead times, and this translates into reducing the computational cost of model analysis as much as possible. UPPAAL's statistical model checking engine [18] is based on the generation of random walks (i.e., simulation runs) from a TA model. In this experiment, we query UPPAAL for simulation runs on the models generated by ANIMO applying the reaction- and reactant-centered modeling approaches to the case study. To define the initial state of the model, we consider the starting condition to be the treatment with 50 ng/ml NGF, which translates into setting the activity level of node NGF to 15/15, while changing EGF to be at 0/15 activity. This configuration was chosen as it generates a more interesting behavior from the biological point of view, also w.r.t. the model checking queries in Section 4.2; the treatment with 100 ng/ml EGF was also tested and gave similar performance results. Table 1 illustrates the computation time and memory usage when performing 100 simulation runs on each of the two considered models. Computing the simulation runs took about 91% less time with the reactant-centered model, using 97% less memory. This decrease in computation time for long simulation runs brings the approach nearer to the idea of interactive exploration of a network.

4.2 Model checking performances

Next, we set out to test the model checking performances on the two versions of the TA model, comparing the execution times and memory requirements for a number of interesting queries:

Model type	Time (s)	Memory (peak KB)
Reaction-centered	30.72	291 576
Reactant-centered	2.86	9 768

Table 1. UPPAAL processor time and memory usage for reaction- and reactant-centered modeling approaches when computing the query `simulate 100 [36000] { R1, R2, ..., R11 }` on the model from [9] with starting condition NGF = 15/15, EGF = 0/15, corresponding to a treatment with 50 ng/ml NGF. The query asks for 100 time series of the activity levels of all reactants in the model over the first 60 minutes of execution.

- (1) and (2): `A[] not deadlock`. The model continues to execute indefinitely (`[]` refers to all possible paths in the transition system of the model, and `A` asks the property to always hold along a path).
- (3): `RKIP < 10 --> ERK >= 40`. After RKIP (Raf kinase inhibitory protein) activity has been lowered, ERK activity increases. As in the model RKIP has 20 levels of granularity and ERK has 100 levels, `RKIP < 10` means that RKIP is less than half active, and `ERK >= 40` means that ERK activity is at least 40%.
- (4): `E<> RKIP < 10`. Find a point when RKIP is low (`<>` asks for the existence of at least one path for which the property holds, while `E` requires the property to hold at least once in a given path). This query is expected to generate a trace, the last point of which will be used as initial configuration for model checking queries (5) and (6).
- (5): `A[] ERK < 70` and (6): `A[] ERK > 35`. Once RKIP activity has significantly decreased, ERK activity is sustained at an intermediate level.

The initial conditions are:

- (1): EGF = 15/15 and NGF = 0/15, all others as the original configuration, corresponding to the treatment condition with 100 ng/ml EGF.
- (2) - (4): EGF = 0/15, NGF = 15/15, all others as the original configuration, corresponding to the treatment condition with 50 ng/ml NGF[3].
- (5) and (6): all activities as in the last state of the trace computed from query (4).

We note that performing model checking means dealing with state space explosion problems, and model reduction is recommendable in order to obtain any result within adequate time limits. One of the most user-accessible ways available in ANIMO to reduce the size of a model is setting the "natural uncertainty" defined in Sect. 3.1 to 0 before performing model checking queries. In order to still consider some biological variability, the user can manually perform multiple model checking queries with changed interaction parameters. The tests performed in this section have an uncertainty level set to 0, instead of the 5% recommended for simulation-based experiments, to make model checking feasible within seconds.

[3] In the laboratory experimental setting, NGF is used at a lower concentration than EGF, but it is still enough to saturate all NGF receptors, which are rarer than EGF receptors.

Query	Reaction-centered		Reactant-centered		Improvement	
	Computation time (s)	Memory usage (peak KB)	Computation time (s)	Memory usage (peak KB)	Time (n-fold)	Memory (n-fold)
(1)	126.56	523 448	1.04	9 236	122	57
(2)	159.29	436 496	1.73	11 480	92	38
(3)	146.09	439 484	1.04	10 384	140	42
(4)	0.74	293 508	0.06	7 484	12	39
(5)	581.79	448 764	6.86	16 880	85	27
(6)	561.01	449 248	6.42	15 852	87	28

Table 2. UPPAAL processor time and memory usage for reaction- and reactant-centered modeling approaches when computing the given queries on the case study from [9].

The queries were used with both the reaction- and reactant-centered TA models, and returned the same results as expected: in particular, query (1) returned false and all other queries returned true. From the biological point of view, the answer to query (1) confirms that under EGF treatment no other activity is observed in the model after the initial peak, while query (2) confirms that with NGF activity continues indefinitely. Moreover, queries (3) - (6) confirm the result of the simulations shown in [9], with NGF treatment leading to sustained ERK activity.

The results of the model checking performance test are shown in Table 2.

4.3 Analysis of the results

Requesting a full inspection of the state space as we do when using a query of the type `A[]`ϕ returning true in cases (5) and (6), allows us to indirectly compare the state space size of the two model versions. As the computation time improvements in Table 2 show, the reactant-centered model produces indeed a noticeably smaller state space, allowing for a higher level of interactivity also when performing non-trivial model checking. Moreover, our experiments point out that the reactant-centered approach considerably lowers the memory requirements for the model. This is not only due to the absence of possibly large precomputed time tables, which can contain thousands of elements each. Indeed, this point was further investigated by implementing a reaction-centered model which avoids the use of tables and instead makes on-the-fly computations of the time bounds with the same number representation as in the reactant-centered model. This resulted in improved performances in the cases of reachability and simulation-based queries, with memory requirements closer to the ones for the reactant-centered model (0.69 s and 24 796 KB for query (4)). However, in all other cases a much larger amount of memory (around 2 Gb) was used with respect to the table-based implementation of the same model, without leading to appreciable benefits in terms of execution time: in some cases performances were noticeably deteriorated (600-800 s for queries (1)-(3)). These findings seem to support the idea that a reactant-centered models have a smaller state space.

Note that while the network we consider for the case study is not particularly large, the gain in performances is still considerable and promising in the perspective of analyzing more complex networks.

5 Model checking in ANIMO

In order to allow a non-expert user to profit from the power of model checking, we have implemented a template-based user interface to define queries directly inside the ANIMO Cytoscape App: Figure 5 shows the interface for composing a model checking query in ANIMO. The mappings between user interface templates and actual model checking queries were inspired by the ones proposed in [19], and are shown in Table 3.

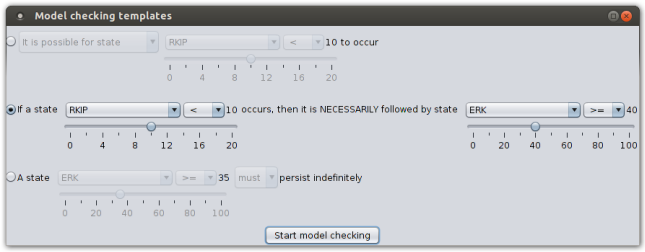


Fig. 5. The interface used in ANIMO to compose a model checking query. The settings on the three lines correspond, from top to bottom, to queries (4), (3) and (6).

ANIMO template	UPPAAL formula
It is possible for state ϕ to occur	`E<>` ϕ
State ϕ never occurs	`E<> !(`ϕ`)`
If a state ϕ occurs, then it is necessarily followed by a state ψ	ϕ `-->` ψ
A state ϕ can persist indefinitely	`E[]` ϕ
A state ϕ must persist indefinitely	`A[]` ϕ

Table 3. Mapping between queries as presented in ANIMO user interface and the corresponding model checking queries in UPPAAL syntax. State formulas indicated by ϕ and ψ are all in the form $R \bowtie n$, with R the identifier of a reactant in the model, $\bowtie \in \{<, \leq, =, \geq, >\}$ and $n \in [0, g(R)]$ a valid activity level value between 0 and the granularity (number of discrete levels) of R.

If the answer to a model checking query contains a (counter-) example trace, the trace is automatically parsed by ANIMO and presented to the user in form of a graph of activity levels, in the same fashion as is normally done with simulation

runs. Finally, a button positioned near the time slider under a simulation graph allows the user to easily change the initial activity levels of the whole network by setting them as in the currently selected time instant. This feature was used after executing query (4) to set the initial conditions for queries (5)-(6). Such an addition makes it easier to inspect the behavior of a network by using a sequence of model checking interrogations.

6 Conclusions and future work

We have presented here how the ANIMO tool was improved to provide a more interactive modeling process. Thanks to the increased performances of the new reactant-centered modeling approach, we are able to obtain answers to model checking queries in a matter of seconds. The features of model checking are made accessible without the need to directly deal with TA models. In this way, ANIMO acts as an intermediary between the biologist and a formal representation of biological signaling pathways, letting the experts concentrate on investigating the mechanisms of cellular responses.

In order to enforce the concept of user interaction as a primary focus of the tool, we plan to extend ANIMO by increasing the support for parameter sensitivity analysis and parameter fitting, as a follow-up to what was presented in [16]. Moreover, inspired by works on automata learning [20], we plan to add also the possibility to automatically derive a network topology based on experimental data and previous knowledge.

We aim at widening the available set of model checking queries, in order to allow biologists to perform in silico experiments on an already fitting model and to obtain answers to more relevant questions. This would increase the usefulness of a model as a help to drive wet-lab investigation. In order to allow for meaningful in silico experiments, we plan to purposefully introduce user-defined non-deterministic parts in our models, which would allow for drug dosage investigations through model checking. This can be done e.g. through the definition of intervals for the values of some reaction kinetic constants, adding considerable uncertainty in the timing of those reactions. More useful results can be achieved through proper application of the statistical model checking part of UPPAAL [18] to an extended version of our model to include realistically significant stochastic behavior. Finally, in order to further improve performances, the extension of ANIMO with support for a multi-core model checking approach based on the works by Dalsgaard et al. [21] is under study.

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