Exploratory Analysis of the Biological LTM Toggle Switch

A report based upon work by Kotula *et al.*

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Forward

In this paper, I have approached an analysis of the biological LTM switch using a variety of analytical methods, focusing upon the purely mathematical analysis which we have studied over the duration of our course, while also integrating computational analysis and modeling techniques which I have studied extensively elsewhere.

I have produced an interactive, mixed-media report which effectively conveys my analysis using a combination of prose, and interactive python scripts running in the IPython Notebook — a workspace well attuned to interactive analysis. The two included notebooks ("LTM Analysis.ipynb" and "LTM Simulation.ipynb"), correspond to the two major sections of my results respectively. I have also included a supplementary PDF document containing my derivations. I hope that their distribution encourages reproducibility, modification and extension on my work. If you'd like to view my notebooks, and do not yet possess the appropriate software, please consider installing Jupyter.

Background

Since its inception, the field of synthetic biology has been expanding and accelerating rapidly. One major undertaking which has helped fuel this widespread success has been the design and characterization of reusable biological modules/components, which have then been made accessible to the entire academic community (and the public) via online repositories like the iGEM Repository of Standard Biological Parts. These standard parts can then be incorporated into the designs of novel genetic "circuitry" in labs worldwide, enabling rapid prototyping and development of synthetic biological systems via modularity.

One such biological part, the genetic toggle switch, garnered widespread interest after it was first characterized by Gardner *et al*. in a 2000 manuscript, which both established the mathematical representation and dynamics of a simple negative-feedback toggle switch. A subsequent publication by Ajo-Franklin *et al.* in 2007 further explored the concept of long-term cellular memory, developing a positive-feedback memory device in yeast. Notably, this memory module allowed for a variety of triggering/sensory elements, expanding the possible use cases. In addition they provide evidence supporting memory-longevity over multiple generations.

There are myriad applications for synthetic biological switches toggle switches, however one factor that holds common between many of the use cases, is the necessity of long-term memory. For instance, one might use a microbe to monitor environmental contamination; it's somewhat useful to know if the microbe is currently being exposed to contaminant, but more utility is derived if we can also determine exposure to contaminant going back for some time. Likewise, a radiation biosensor would be useful for monitoring exposure to high levels of UV radiation (Khalil & Collins 2010), or other kinds of high-energy EM radiation, over an extended period of time.

In this manuscript, we will review and expand upon a recent publication by Kotula *et al.* in which they describe a long-term memory (LTM) negative-feedback toggle switch for usage in monitoring environmental conditions in the mammalian gut (2014). There is a great necessity for memory in this biosensor application, given that the majority of known reporter genes would be difficult to measure directly. Fluorescent proteins would not be readily measurable unless one is able to localize a probe to the gut lumen. Gas reporters like methyl-halide transferases (MHT), however, could prove efficacious if their safety/sensitivity profile could be

verified in mammals. The Kotula group focuses in upon the usage of a LTM biological toggle switch in order to produce fluorescent sensor bacteria, which can be recovered from the stool at a later point after they are shed. Though this approach towards biosensing and reporting may not permit real-time monitoring of gut environmental conditions, continuous, delayed monitoring may be possible (Kotula *et al.* 2014).

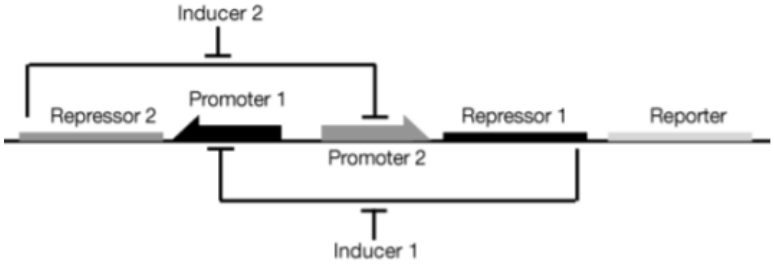
Requirements of LTM

There are many challenges associated with the development of a LTM module for use in synthetic biological systems, like coping with stochasticity, leakage of trigger element transcription, environmental fluctuations, and cell division. The properties of our bistable switch help contend with all but the last issue; mitosis introduces dilution, but we are more concerned about the effects of natural selection. Indeed, intergenerational memory requires that "the memory system should have no deleterious fitness effects" as this will cause any activated cells to perish at a higher rate in the next generation, resulting in signal attenuation (Kotula *et al.* 2014). To this end, the switch module must be engineered to have a low metabolic cost, in order to prevent mutations which would disable our circuit. The design used in this publication relies upon a switch designed by natural selection itself, the lambda bacteriophage cI/cro switch, in order to accomplish this goal. Later, I describe simulated manipulation of the characteristics of the switch in order to examine the possibility of decreasing metabolic burden or altering switch behavior. Additionally, the Kotula group inserted their genetic circuit into the host bacterial genome, rather than onto a transformed plasmid, "in order to minimize the chance of loss". They did so, appropriately enough, via P1vir phage transduction, which is a technique well tuned for usage in inserting genetic material into *E. coli*. These steps should help to ensure the long-term stability of the switch (Kotula *et al.* 2014).

Exploring Gardner's Toggle Switch

Gardner *et al.* describe the mechanics of a generic biological toggle switch circuit based upon negative feedback between two operons, one of which contains the desired reporter gene (Fig 1). A solid understanding of the dynamics of this simple case will help us to interpret the LTM case later. The following figure shows the co-repressive nature of this switch: when concentration of one switch component/repressor is high, there will be strong repression of the other component, and maintenance of this stable state. Production of repressor 1 can then be tied to a sensor promoter in order to provide an observable signal when the promoter is activated. For instance, the promoter may be a molecular sensor, like the (anhydro)tetracycline promoter (tetP), or it could be a regulatory element which responds to DNA damage following radiation exposure. We do not focus on the reporter aspect of the circuit in our analysis, as it is

tied to the behaviour of its associated repressor.



$$\frac{du}{dt} = \frac{\alpha_1}{1 + v^{\beta}} - u$$

$$\frac{dv}{dt} = \frac{\alpha_2}{1 + u^{\gamma}} - v$$

Figure 1: Gardner *et al*. present the framework for the construction of a simple switch with the structure presented in the left-hand pane, and described by the equations on the right-hand side. The components of these equations are as follows: "u is the concentration of repressor 1, v is the concentration of repressor 2, a_1 is the effective rate of synthesis of repressor 1, a_2 is the effective rate of synthesis of repressor 2, beta is the cooperativity of repression of promoter 2 and gamma is the cooperativity of repression of promoter 1." *Images and Description from (Gardner 2000).*

Results

In this report, I undertake the modeling of this system from several different analytical perspectives in order to provide a more comprehensive characterization of this genetic circuit module. This will hopefully allow greater ease of design for future synthetic biology experimenters. To this end, I have included two Python notebooks — "LTM Analysis.ipynb" and "LTM Simulation.ipynb" — which include the code used to produce my findings for the two respective segments of my results.

Molecular Reaction Network of the Bistable Switch

We begin our characterization of the LTM toggle switch genetic circuit by outlining the molecular reactions which occur therein. Our first set of equations describes the dimerization reaction, and its reverse reaction, as occurs for both repressor molecules. These equations also show the binding of these dimers to DNA (Fig 2). Notably, while both repressors dimerize, the cI protein will typically bind to only two of the three available regulatory sites on the DNA, while cro usually only binds to one (Ptashne 1980). We then show the equations describing protein production from the various DNA states; notably, the bound states produce little to no protein. Finally, each repressor component will degrade continuously due to proteolysis, and at discrete timepoints due to cell division (halving concentrations). In our ODEs to follow, we model dilution due to mitosis as a continuous process, however we model it as a discrete process in our subsequent simulations.

$$2\,\mathrm{cro} \underset{k_{-dcro}}{\overset{k_{dcro}}{\rightleftharpoons}} \mathrm{cro}_2;\ \mathrm{U_{cI}} + \mathrm{cro}_2 \underset{k_{-BcI}}{\overset{k_{BcI}}{\rightleftharpoons}} \mathrm{B_{cI}}$$

$$2\,\mathrm{cI} \underset{k_{-dcI}}{\overset{k_{dcI}}{\rightleftharpoons}} \mathrm{cI}_2;\ \mathrm{U_{cro}} + \mathrm{cI}_2 \underset{k_{-Bcro}}{\overset{k_{Bcro}}{\rightleftharpoons}} \mathrm{B_{cro}};\ \mathrm{B_{cro}} + \mathrm{cI}_2 \underset{k_{-BBcro}}{\overset{k_{BBcro}}{\rightleftharpoons}} \mathrm{BB_{cro}}$$

$$\mathrm{U_{cI}} \xrightarrow{\alpha_{UcI}} \mathrm{cI};\ \mathrm{B_{cI}} \xrightarrow{\alpha_{BcI}} \mathrm{cI}$$

where: $\alpha_{UcI} >> \alpha_{BcI}$

$$\mathrm{U_{cro}} \xrightarrow{\alpha_{Ucro}} \mathrm{cro};\ \mathrm{B_{cro}} \xrightarrow{\alpha_{Bcro}} \mathrm{cro};\ \mathrm{BB_{cro}} \xrightarrow{\alpha_{BBcro}} \mathrm{cro}$$

where: $\alpha_{Ucro} >> \alpha_{Bcro} >> \alpha_{BBcro}$

$$\mathrm{cro} \xrightarrow{\gamma_{cro}} \emptyset;\ \mathrm{cI} \xrightarrow{\gamma_{cI}} \emptyset$$

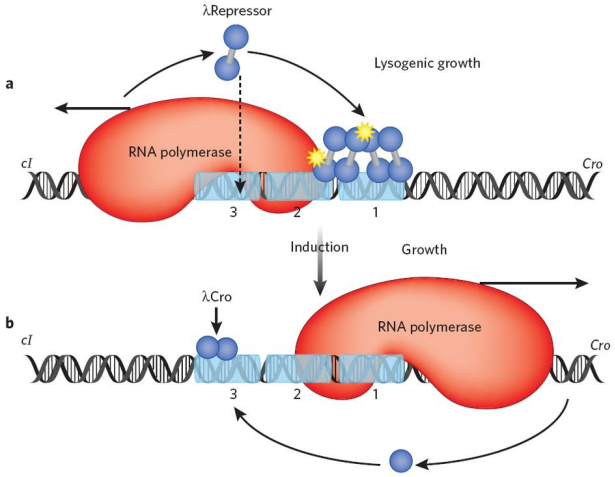


Figure 2: On the left-hand side, are a set of chemical reactions which represent the mechanics of the bistable LTM switch. The first two rows of equations dictate the dimerization and DNA binding behaviour of each of the two repressors in this system *cro* and *cI*. The next 4 rows dictate the rates of repressor production. The final row shows the degradation of each repressor. On the right-hand side is a diagram sourced from (Ptashne 2011) which helps to visualize this process. The top half shows a situation in which *Cro* expression is repressed by binding of two *cI* molecules. The bottom depicts the reciprocal situation in which cI expression is repressed. Notably, both may occur simultaneously.

Modeling Bistability of the Toggle Switch

The LTM toggle switch is bistable in nature, which implies that it has two states wherein the two ODEs are both equal to 0. We can explore this principle further by mathematically analyzing the stable states of the system. I take inspiration from the stability analysis performed in the Gardner paper to perform an analysis in an IPython Notebook using a combination of libraries, namely: Sympy for symbolic algebra, and Bokeh for interactive plotting. I begin by setting up the ODEs (Supp Doc 1) which describe the dynamics of the two repressor proteins (using the Generalized Hill formulation; Fig 3A,B), and then set these two equations equal to zero in order to find their equilibrium states (Supp Eq 2) across the range of concentrations of the other repressor. Plotting these two ODEs on opposing axis yields a graph (Fig 3C), which allows us to determine where equilibrium points exist in the system (intersections of the two lines), and if the system is bistable (two stable equilibrium states). There are three equilibrium states present in our system, however only two are stable — those at the extreme concentrations for one of the two repressors. The equilibrium state in the middle is unstable, and may be imagined as if holding a light switch between on and off. The system may hold in equilibrium, but at the slightest disturbance, it will descend the energy gradient to one of the two lower energy stable states.

A

$$H = \frac{1}{[(x/c)^{\beta}+1]^{\rho}}$$

B

$$[\dot{cro}] = -[cro]\gamma + \frac{\alpha_{cro}}{\left(\frac{[cI]^2}{c_{cI}^2}+1\right)^2}$$

$$[\dot{cI}] = -[cI]\gamma + \frac{\alpha_{cI}}{\frac{[cro]^2}{c_{cro}^2}+1}$$

C

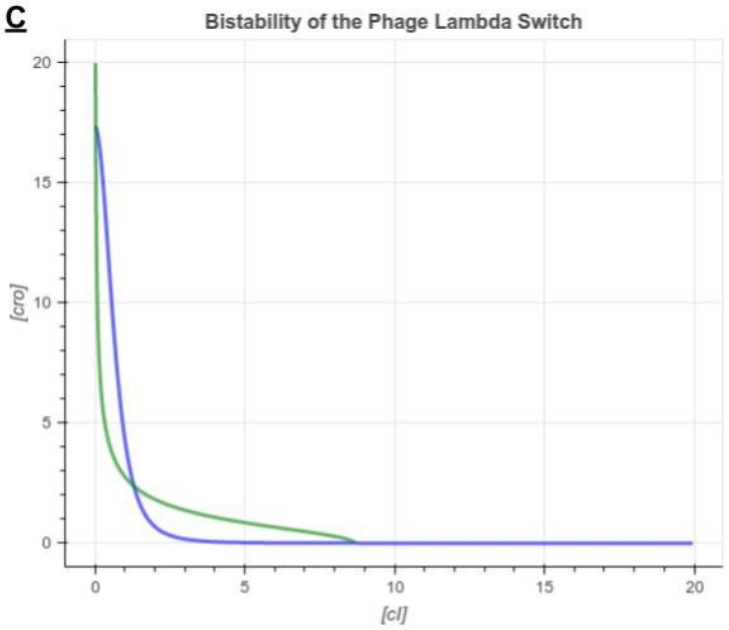


Figure 3: We model the molecular reaction network described above using a generalized Hill equation as seen in Equation A. These equations are realized for each of the repressors in the phage-lambda Cro/cI switch in Equation System B. A realization of this system using arbitrary parameters, resulting in bistability, is shown in Plot C.

Simulation of LTM Switch Using ODEs and SDEs

The aforementioned mathematical descriptions of our system are useful for outlining general characteristics and behaviour of the system, but do not account for the accumulation of stochasticity over time, nor large perturbations. These two factors are more readily and interactively explored via simulation methods, which I have developed in Python. Incorporating the same ODEs as previously noted, we are able to perform incremental adjustments to our two repressor concentrations (from initial states of cro=2, cI=25), allowing the construction of a time-series which illustrates the behaviour of the switch (Fig 4). I am able to demonstrate a simulated switch (alpha_cro=.09, alpha_cI=.07, c_cro=8, c_cI=2, gamma_cro=.001, gamma_cI=.004) which conforms with the expected behaviour of a bistable toggle switch both in the absence (Supp Fig 1) and presence of stochasticity (Fig 4B). Furthermore, this switch appears robust for storage of information over the long-term (Supp Fig 2). Using our simulated models, I have also show that switching of the circuit can be induced by a variety of signalling conditions, including both rapid and gradual increases in repressor concentration.

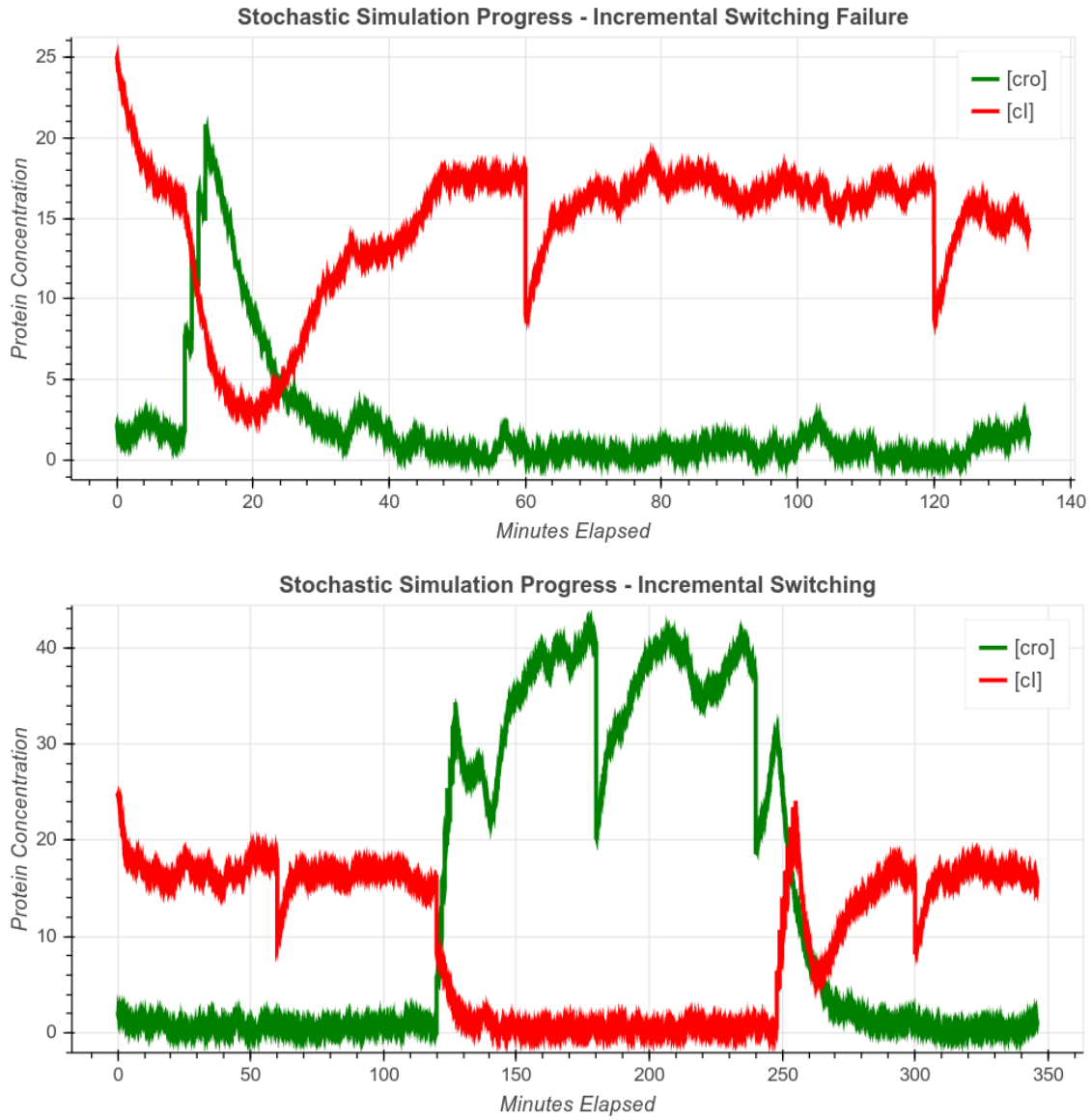


Figure 4: We model the molecular reaction network described above using a generalized Hill equation as seen in Equation A. These equations are realized for each of the repressors in the phage-lambda Cro/cI switch in Equation System B. A realization of this system using arbitrary parameters, resulting in bistability, is shown in Plot C.

Discussion

In this report, I have discussed the literature regarding the genetic bistable toggle switch circuit, as well as the specifications of a switch which would be suitable for long-term memory (LTM). It is clear that such switches may be a useful component in many synthetic biology projects, as LTM is important for discontinuous monitoring applications. In order to investigate LTM design, I have developed a basic mathematical model which describe the dynamics of our two repressor concentrations with respect to time using a pair of QSSA ODEs. These ODEs assume that we are relatively close to equilibrium conditions, which will typically be true of fast reactions like dimerization and DNA binding. However, there are certainly conditions, in which

this will not be an accurate representation of the system, causing inaccuracies. These errors will likely be largest at times of high repressor flux.

In my simulations, I used a set of parameters to generate a bistable system which should resemble the general behaviour of the lambda-phage switch; nonetheless, my predictions are not accurate descriptions of the real system. The protein production parameters (alphas) were arbitrarily chosen, along with the repression (c) and degradation (gamma) constants. The rho and beta exponents in these ODEs from the Generalized Hill Function family (Fig 3) were chosen based upon the system's structure, however it is possible that adjustments to these coefficients may better describe the cooperativity of DNA binding in this system (particularly with regard to cI). Ultimately, I believe that the approximations I have made in my analysis are reasonable, and will result in a fairly accurate description of a theoretical biological system.

This project lays groundwork for an exciting area of inquiry, and I feel I have only begun to scratch the surface of what can be explored: theoretically and experimentally. I envision toggle switch simulations which will allow for intelligent design of toggle-switch modules with a variety of different properties: namely sensitivity to on/off switching, switch set points, and other regulatory dynamics. Manipulation of each component of the ODEs used in our simulations can be tied directly to a physical characteristic of our genetic switch, and alteration of these characteristics may yield important changes in the switch behaviour (Supp Fig 3). For instance, we may design additional regulator binding sites into our switch; or insert the switch at a set number of locations in the genome; alternatively, intentional mutations may be introduced via a variety of methods (gBlock design, or site-directed mutagenesis) in order to alter repressor binding efficiency or cooperativity. Given more time and resources, this initial analysis could result in significant innovation, the publication of may allow for the incorporation of the LTM switch into Synthetic Biology experiments globally.

References

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2. Khalil, A. S., & Collins, J. J. (2010). Synthetic biology: applications come of age. *Nature Reviews Genetics*, *11*(5), 367-379.
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4. Ptashne, M., Jeffrey, A., Johnson, A. D., Roberts, T. M., & Sauer, R. T. (1980). How the λ Repressor and Cro Work.
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Software References

- **Bokeh -** Bokeh Development Team (2014). Bokeh: Python library for interactive visualization. URL http://www.bokeh.pydata.org.
- **Numpy** - Stéfan van der Walt, S. Chris Colbert and Gaël Varoquaux. The NumPy Array: A Structure for Efficient Numerical Computation, Computing in Science & Engineering, **13**, 22-30 (2011), DOI:10.1109/MCSE.2011.37
- **Sympy** - SymPy Development Team (2016). SymPy: Python library for symbolic mathematics. URL http://www.sympy.org.

Supplemental Materials

$$u_s = \frac{\alpha_1}{\gamma\left(v^{\beta_1} + 1\right)} \qquad [cro]_s = \frac{\alpha_{cro} c_{cI}^4}{\gamma\left([cI]^2 + c_{cI}^2\right)^2}$$

$$v_s = \frac{\alpha_2}{\gamma\left(u^{\beta_2} + 1\right)} \qquad [cI]_s = \frac{\alpha_{cI} c_{cro}^2}{\gamma\left([cro]^2 + c_{cro}^2\right)}$$

Equation 1: These equations describe the steady state behaviour of an arbitrary toggle switch, and a constrained version of this equation tuned to approximate the phage-lambda switch dynamics (left and right-hand columns respectively). There are clear parallels between these solutions.

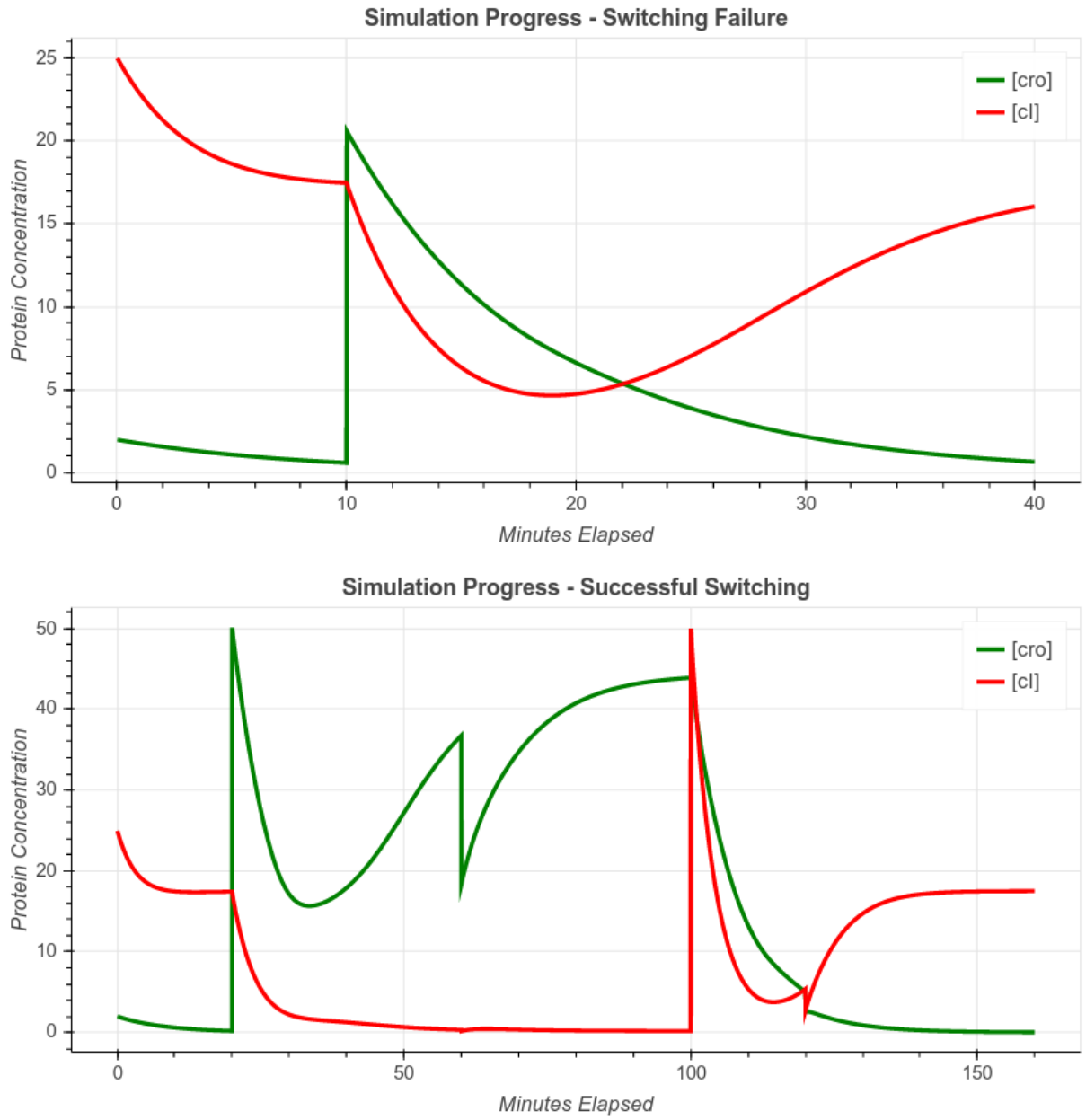

Figure 1: The above plots demonstrate proof-of-concept simulations, performed without stochasticity, in order to demonstrate the switching behaviour of the switch. The first time series plot shows a situation in which there is a spike in *cro* expression, but it is not sufficient to cause switching. In the subsequent plot, we show successful switching between the two states.

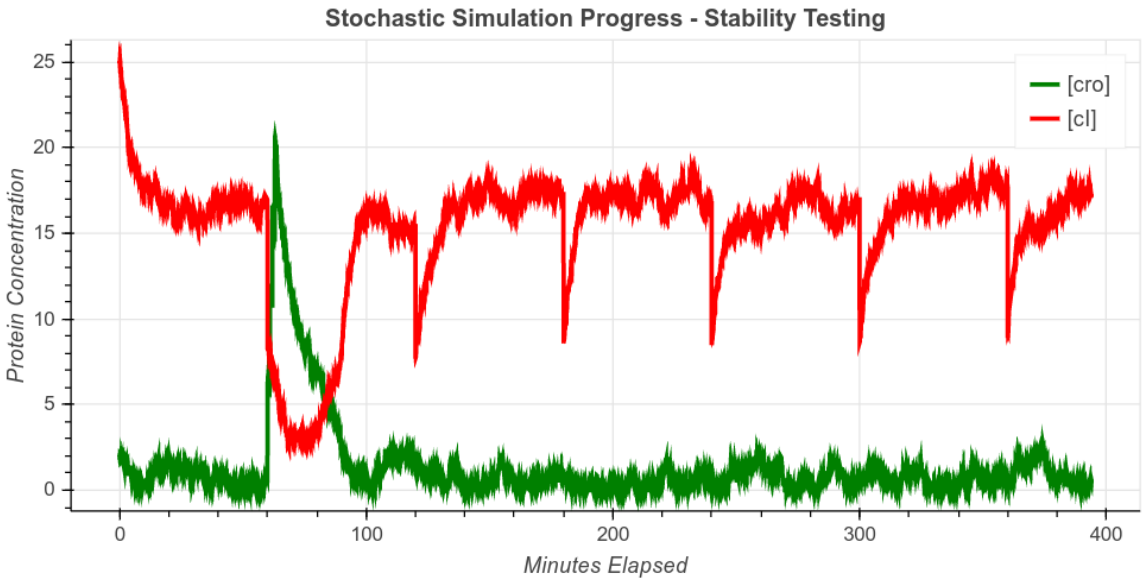

Figure 2: In the above plot, I demonstrate that the LTM switch is robust to fluctuations in repressor concentration, in addition to the effects of cell division. In other words, the switch will not activate randomly due to environmental changes.

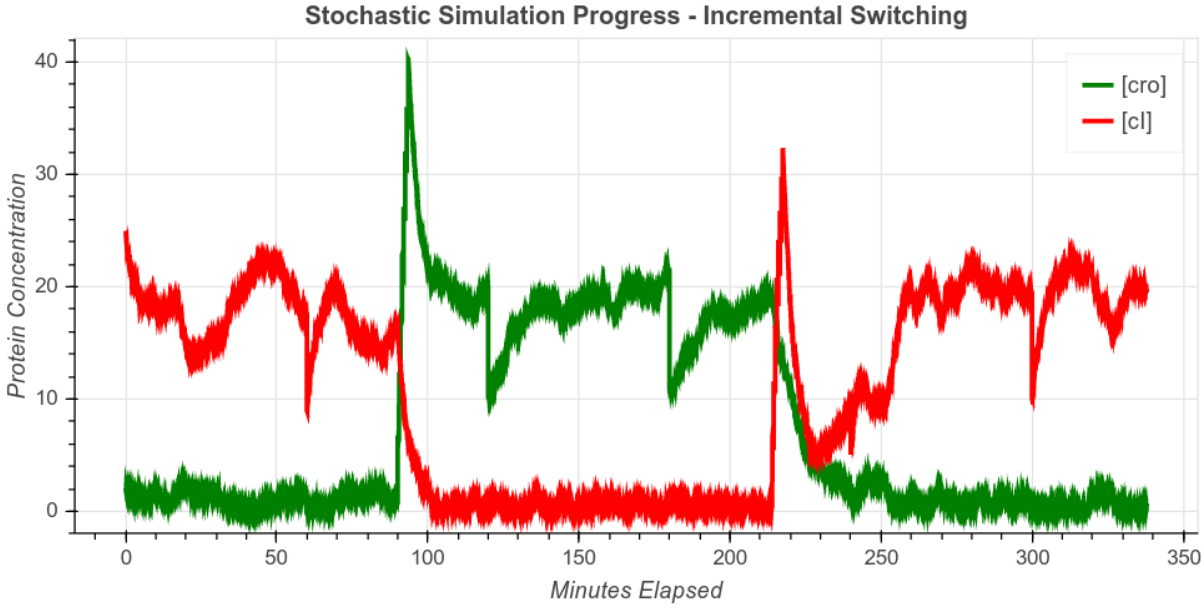

Figure 3: Tuning the parameters of the simulated LTM switch is possible in order to achieve different behaviours from this genetic circuit. In the above figure, I have tuned the parameters of the switch in order to have bistability at a low concentration of both repressors (~20 arb. unit), yet still require a robust signal for switching.