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HYSTERETIC PROPERTIES OF A MODULAR POSITIVE
FEEDBACK-BASED GENE AMPLIFIER

BY

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THESIS

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ABSTRACT

Positive feedback is a common mechanism in genetic circuits and can be used to achieve amplification, bistability, and hysteresis. Positive feedback mechanisms have been employed in numerous synthetic biology applications, including in the development of a modular positive feedback-based gene amplifier by Nistala et al. [1]. The modular design potentially enables use as a component in more complex synthetic gene networks, thus helping to achieve the oft-stated goal of designing well-characterized components that can be used in diverse synthetic biology applications. The positive feedback-based gene amplifier provides amplification of the maximum expression level of a gene product, and increased sensitivity to the inducer. However, the initial analysis by Nistala et al. did not demonstrate that this particular positive feedback mechanism can produce a hysteretic response to an inducer, i.e. exhibit memory of a prior stimulus. Positive feedback in a genetic circuit potentially, but not necessarily, leads to bistability, and a bistable circuit will produce some degree of hysteresis [2]. Therefore, the purpose of this research was to determine under what conditions, if any, the modular positive feedback-based gene amplifier will produce a hysteretic response. A modular gene amplifier with well-characterized hysteretic properties will potentially be a useful asset for synthetic biologists designing more complex networks out of simpler components.

A simple mathematical model of gene expression in a positive-feedback system is used to predict the conditions for bistability and hysteresis versus a simple graded response. Experiments are performed on the positive feedback-based tetracycline sensor in *E. coli* using green fluorescent protein (GFP) to measure expression level of the gene product. In the experiments, cells are placed in solutions of various inducer concentrations and grown to a steady-state level of GFP expression. Cells are then resuspended in solutions of lower inducer concentrations and allowed to reach steady state. Hysteresis is observed if the steady-state GFP level depends on the prior induction level of the cell culture.

This research shows that the modular positive feedback-based gene amplifier is capable of producing a hysteretic response to a stimulus. The magnitude of the hysteretic response depended on the dilution ratio used to inoculate the cultures. Additionally, the magnitude of the hysteretic response depended partially on the final induction level of the culture, as higher induction levels resulted in a larger proportion of cells exhibiting a high level of GFP fluorescence. The cultures with induction history exhibited bistable GFP expression at all inducer concentrations. Cells exhibiting low GFP fluorescence grew at a faster rate than cells exhibiting high GFP fluorescence. This suggests that low dilution ratios provide more time for cells expressing at a low level to grow before the culture is saturated, and therefore make up a larger proportion of the final culture producing a smaller

hysteretic response. These results provide a deeper understanding of the properties of the modular positive feedback-based gene amplifier and the conditions for hysteresis.

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LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|----------------|--|
| 68 | positive feedback-type cells; abbreviation for pGN68 plasmid |
| 69 | non positive feedback-type cells; abbreviation for pGN69 plasmid |
| aTc | anhydrotetracycline – inducer chemical |
| <i>E. coli</i> | <i>Escherichia coli</i> |
| FI | fluorescent intensity |
| GFP | green fluorescent protein |
| IC | initial conditions |
| LB | Luria-Bertani – cell growth media |
| NPF | no positive feedback |
| OD | optical density |
| PBS | phosphate buffered saline |
| PF | positive feedback |
| RF | relative fluorescence |
| RFU | relative fluorescence units |
| g | gene product concentration |
| k_1 | linear induction rate |
| k_2 | degradation rate |
| k_3 | maximum expression level of the self-inducible promoter |
| k_4 | concentration of g needed to achieve half-maximum response |

| | |
|-------|--|
| s_0 | inducer concentration |
| r | dimensionless model parameter, a function of k_2 , k_3 , and k_4 |
| s | dimensionless model parameter, a function of k_1 , k_3 , and s_0 |
| x | dimensionless form of gene product concentration |

CHAPTER 1: INTRODUCTION

Positive feedback is a common mechanism in natural and synthetic gene networks. In the context of a gene regulatory network, positive feedback refers to the ability of a gene product to promote its own expression [1, 3]. In synthetic networks, positive feedback can be used to achieve amplification, increased sensitivity, bistability, and hysteresis [4-7]. Amplification due to positive feedback may be described as a heightened steady state level of gene expression in response to a signal or an inducer [5]. In some cases, positive feedback can also lead to an increase in sensitivity, where gene expression is initiated at lower concentrations of an inducer chemical. Many additional applications, examples, and properties of positive feedback in natural and synthetic networks are reported in literature. For example, Maeda and Sano [8] showed that positive feedback increases rise time in gene expression, and is capable of producing either a hysteretic or graded response to a stimulus. Pomerening and co-workers [4] demonstrated that a bistable positive feedback network combined with a negative feedback loop can produce sustained oscillations. Ingolia and Murray [3] showed that simple mutations can cause a positive feedback system to produce either a bistable, inducible monostable, or constitutive monostable response.

One of the most studied applications of positive feedback is its role in implementing bistability [2, 4, 9]. A bistable system has two stable steady-state levels of gene expression, conventionally named high / low or ON / OFF states. Individual cells within a bistable system express at either the high or low steady-state level, and not at any intermediate state [2]. In general, a cell's initial conditions determine whether it expresses at a high or low level [9], and switching between the two levels can occur due to a change in the level of some input [10], e.g. an inducer. Within a population of cells, as the concentration of the inducer chemical changes, stochastic mechanisms cause the individual cells to switch discretely between the high and low states over a range of inducer concentrations [11]. Although positive feedback can in principle produce a bistable response to a stimulus, bistability is not a guaranteed outcome of positive feedback [4, 9, 11]. For instance, a circuit may default to a monostable high state if the background expression level of gene expression is higher than the threshold that discriminates high and low states.

Bistability implies that the system has some degree of hysteresis [9, 12].

Hysteresis is the ability of the system to “remember” previous exposure to an inducer, such that the system's output level at a given inducer concentration depends on the previous state of the system [6, 11, 13, 14, 15]. A system might switch from the low to high state at one inducer concentration, but not switch

from the high to low state until a lower inducer concentration. The range of inducer concentrations over which this switching takes place is called the bistable or hysteretic region of the system. A system that switches from low to high at one inducer concentration but does not switch back to the low state even when the inducer concentration is lowered to zero is referred to as an irreversible system [14]. Systems that show an irreversible response to a stimulus can be a desirable property of a synthetic system, and are found in natural systems, especially in developmental processes such as cell differentiation. For example, Xiong and Ferrell showed that the p42 mitogen-activated protein kinase (MAPK) and the cell-division cycle protein kinase (CDC2) show an irreversible response to an inducer in the maturation of *Xenopus* oocyte [7], and is an important step in the development of the embryo.

Positive feedback mechanisms have been employed in numerous synthetic biology applications, including in the development of a modular positive feedback-based gene amplifier by Nistala and coworkers [1]. The modular design potentially enables use as a component in more complex synthetic gene networks, thus helping to achieve the oft-stated goal of designing well-characterized components that can be used in diverse synthetic biology applications [16, 17, 18]. Figure 1 shows that the positive feedback-based gene amplifier provides amplification of the maximum expression level of a gene product, and increased

sensitivity to the inducer. However, the initial analysis by Nistala and coworkers did not demonstrate that this particular positive feedback mechanism can produce a hysteretic response to an inducer. In order to better characterize this system and increase its utility as a module in more complex networks, this study provides an in-depth analysis of the hysteretic properties of the modular positive feedback-based gene amplifier. I proposed three primary questions. 1) Can this particular positive feedback amplifier produce a hysteretic response to a stimulus? 2) What are the conditions for hysteresis? 3) Can hysteresis be manipulated experimentally?

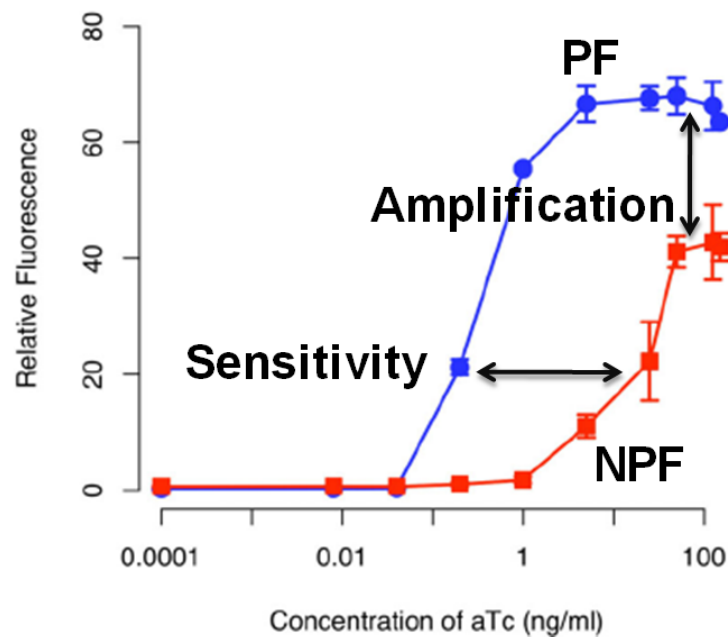


Figure 1: The positive feedback-based gene amplifier increases the sensitivity to the inducer and amplifies the response [1]

To address these questions, a mathematical model for the system of interest was developed. Theoretical stimulus-response curves were generated to visualize the effect of model parameters on hysteresis. A bifurcation diagram was used to gain a better understanding of the region of bistability within the parameter space. Experiments were performed on the positive feedback-based tetracycline sensor in *E. coli* [1] using green fluorescent protein (GFP) to measure expression level of the gene product. In the experiments, cells were placed in solutions of various inducer concentrations and grown to a steady-state level of gene expression. Cells were then resuspended in solutions of lower inducer concentrations and allowed to reach steady state. Hysteresis was present if it was observed that the steady-state expression level depended on the prior induction level of the cell culture. GFP measurements were recorded using a microplate reader (fluorescent intensity and optical density) and flow cytometry to measure gene expression at both the population and single-cell levels.

The findings show that the modular positive feedback-based gene amplifier is capable of producing a hysteretic response to a stimulus, but will not necessarily do so under all conditions. The system exhibits different gene expression behavior depending on its history, corresponding to different regions of the bifurcation diagram. Similar findings have been reported for the lactose utilization network of *E. coli* [11]. Additionally, we show that the dilution ratio used to induce cultures

at a high initial state affects the proportion of cells at both the high and low steady state levels, and therefore affects the overall magnitude of the hysteretic response.

The proportion of cells at each state is affected by the growth rate difference between ON and OFF cells, and the number of ON cells that switch OFF due to dilution of their GFP and LuxR during rapid growth.

CHAPTER 2: MODEL DEVELOPMENT

2.1 Model presentation

To model the positive feedback system, we applied a model originally presented by Lewis and co-workers in 1977 [19, 20]. The model demonstrates how positive feedback can lead to bistability and memory; it is presented in Eq. 1, and is given in dimensionless form in Eq. 2. Definitions of the dimensionless parameters are given in Eq. 3-6. All modelling was performed in Wolfram Mathematica 7.0.1, and differential equations were solved numerically using the built-in *NDSolve* function.

$$\frac{dg}{dt} = k_1 s_0 - k_2 g + \frac{k_3 g^2}{k_4^2 + g^2} \quad (\text{Eq. 1})$$

$$\frac{dx}{d\tau} = s - rx + \frac{x^2}{1 + x^2} \quad (\text{Eq. 2})$$

$$s = \frac{k_1 s_0}{k_3} \quad (\text{Eq. 3})$$

$$r = \frac{k_2 k_4}{k_3} \quad (\text{Eq. 4})$$

$$x = \frac{g}{k_4} \quad (\text{Eq. 5})$$

$$\tau = \frac{k_3 t}{k_4} \quad (\text{Eq. 6})$$

The model shows that an inducer s_0 activates transcription of a gene G in a linear fashion. The gene product g promotes its own expression giving the system positive feedback. The degradation rate of g depends on its own concentration and is a linear function with degradation constant k_2 . The model constant k_1 is the rate at which g is expressed by the inducer s_0 . The constant k_3 is the maximum expression level of the self-inducible promoter. Finally, k_4 is the concentration of g needed to achieve half-maximum response of the self-inducible promoter. The sections below give a detailed description of how the positive feedback model was expressed in dimensionless form, and how the parametric equations for the bifurcation diagram were derived.

2.2 Model in dimensionless form

This section shows the process for putting the model into dimensionless form.

Start with the model in full dimensioned form.

$$\frac{dg}{dt} = k_1 s_0 - k_2 g + \frac{k_3 g^2}{k_4^2 + g^2}$$

First, divide the entire equation by k_3 .

$$\frac{1}{k_3} \frac{dg}{dt} = \frac{k_1}{k_3} s_0 - \frac{k_2}{k_3} g + \frac{g^2}{k_4^2 + g^2}$$

Next, define x , the dimensionless parameter for the gene product concentration, g .

Express g in terms of x and differentiate with respect to time.

$$x = \frac{g}{k_4}$$

$$g = k_4 x$$

$$\dot{g} = k_4 \dot{x}$$

Substitute these terms into the equation and then simplify.

$$\frac{k_4}{k_3} \frac{dx}{dt} = \frac{k_1}{k_3} s_0 - \frac{k_2 k_4}{k_3} x + \frac{k_4^2 x^2}{k_4^2 + k_4^2 x^2}$$

$$\frac{k_4}{k_3} \frac{dx}{dt} = \frac{k_1}{k_3} s_0 - \frac{k_2 k_4}{k_3} x + \frac{x^2}{1 + x^2}$$

Define the parameter τ to replace time, t . Differentiate t with respect to τ .

$$\tau = \frac{k_3}{k_4} t$$

$$t = \frac{k_4}{k_3} \tau$$

$$\frac{dt}{d\tau} = \frac{k_4}{k_3}$$

Differentiate x with respect to τ .

$$\frac{dx}{d\tau} = \frac{dx}{dt} \cdot \frac{dt}{d\tau} = \frac{k_4}{k_3} \frac{dx}{dt}$$

Define constants r and s for the dimensionless model.

$$s = \frac{k_1 s_0}{k_3}$$

$$r = \frac{k_2 k_4}{k_3}$$

Simplify the model to express it in dimensionless form.

$$\frac{dx}{d\tau} = s - rx + \frac{x^2}{1+x^2}$$

2.3 Parametric equations for bifurcation diagram

To identify steady-state points and determine regions of bistability (regions in the parameter space with 2 stable and 1 unstable points), set the derivative $dx/d\tau = 0$.

$$0 = s - rx + \frac{x^2}{1 + x^2}$$

$$rx - s = \frac{x^2}{1 + x^2} \quad (\text{Eq. 7})$$

Steady-state points are values of x for which the left hand side of Eq. 7 equals the right hand side, i.e. the intersection points of the two functions. The parametric equations divide regions of monostability (one real steady-state value) with the bistable region (three real steady-state values, two stable and one unstable). The division between these two regions occurs when the two functions are equal and lie tangent to each other, as shown in Figure 2. Differentiate both sides of Eq. 7 with respect to x to identify tangent points. Define r in terms of x .

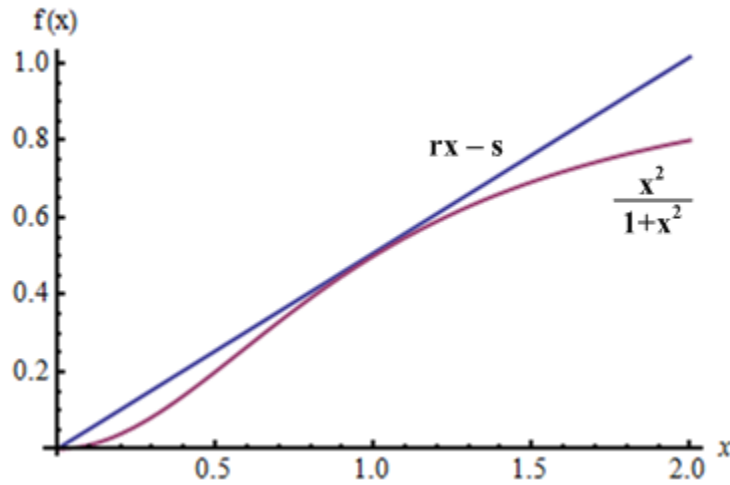


Figure 2: Division between regions of bistability and monostability occur when functions lie tangent

$$\frac{d}{dx}[rx - s] = \frac{d}{dx}\left[\frac{x^2}{1+x^2}\right]$$

$$r = \frac{(1+x^2)(2x) - (x^2)(2x)}{(1+x^2)^2} = \frac{2x}{(1+x^2)^2}$$

Define s in terms of r and x . Substitute the equation for r in terms of x into this and simplify to express s in terms of x only.

$$s = rx - \frac{x^2}{1+x^2}$$

$$s = \frac{2x^2}{(1+x^2)^2} - \frac{x^2}{1+x^2}$$

$$s = \frac{2x^2 - x^2(1+x^2)}{(1+x^2)^2}$$

$$s = \frac{x^2 - x^4}{(1+x^2)^2} = \frac{x^2(1-x^2)}{(1+x^2)^2}$$

Thus, the parametric equations for the bifurcation diagram in rs space are defined.

Model results are presented in Section 4.1.

$$s = \frac{x^2(1-x^2)}{(1+x^2)^2} \qquad r = \frac{2x}{(1+x^2)^2}$$

CHAPTER 3: EXPERIMENTAL METHODS

3.1 Positive feedback system, growth conditions, and measurements

All experiments were performed on the positive feedback-based tetracycline sensor in *E. coli* [1] using GFP to measure expression level of the gene product. This system was designed by Nistala and co-workers, and a schematic is shown in Figure 3. The presence of the inducer anhydrotetracycline (aTc) prevents the constitutively-expressed repressor TetR from binding to the $P_{\text{LtetO-1}}$ promoter and thereby repressing transcription. Therefore, the transcription of $\text{LuxR}\Delta$ proceeds in a manner dependent on the level of aTc induction. The $\text{LuxR}\Delta$ promotes its own expression by binding to the P_{luxI} promoter acting as a positive feedback. GFP is used to measure the transcriptional activity of the P_{luxI} promoter.

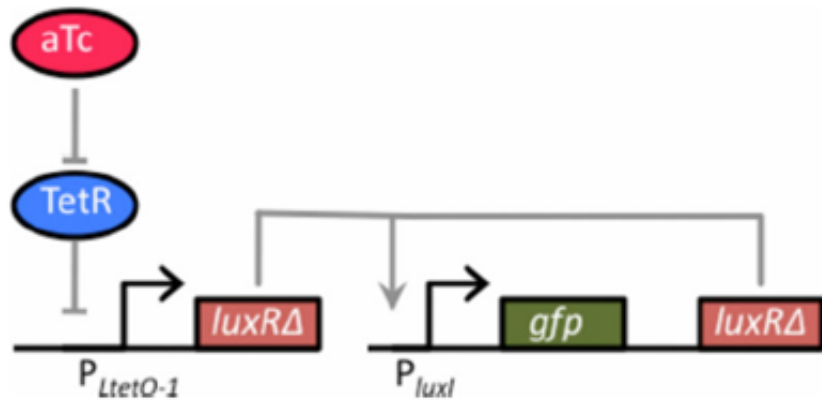


Figure 3: Schematic of the positive feedback-based gene amplifier [1]

This positive feedback (PF) system is designed using a two-plasmid system, each with antibiotic resistance to chloramphenicol and kanamycin respectively. All cultures were grown in Luria-Bertani (LB) media (Lennox) with chloramphenicol (34 $\mu\text{g/mL}$) and kanamycin (40 $\mu\text{g/mL}$) at 37°C. Before initiating experiments, cultures were started from freezer stock and grown overnight (~12 hours) in 2 mL of LB media with chloramphenicol and kanamycin. All cultures used in experiments were inoculated by diluting 1:100 from the overnight cultures into fresh solution of LB and antibiotics to ensure all cultures were started with the same cell density.

A Tecan Infinite m200 microplate reader was used to measure optical density, OD (absorbance at 600 nm, 9 reads per well) and fluorescent intensity, FI (excitation at 488 nm, emission at 520 nm, 9 reads per well). The fluorescent measurements are reported using relative fluorescence units (RFU), defined as fluorescent intensity divided by optical density. Experiments were performed using three samples (unless otherwise reported) and 95% confidence intervals are reported. For kinetic experiments, fluorescent intensity and optical density measurements were taken every hour on cultures in a 96-well plate. After measurements, the plate was shaken on the built-in orbital shaker in the microplate reader (amplitude: 2 mm) until approximately three minutes before the next

measurement. The temperature of the microplate reader was maintained at $37\pm0.5^{\circ}\text{C}$.

All flow cytometry experiments were performed using a BD LSR II flow cytometer (Franklin Lakes, NJ). Flow cytometry samples were prepared by centrifugation and resuspension of cultures from the 96-well plate in ice cold phosphate buffered saline (PBS) and stored at 4°C until measurements were taken. Thirty thousand events were recorded using the low flow setting. All analysis of flow cytometry data was performed in FCS Express Version 3.

3.2 Experimental procedure

The hysteresis experiments were performed as follows. First, eight cultures were inoculated by diluting 1:100 from the overnight PF culture into fresh solution of LB (2 mL) and antibiotics. Cultures were grown for 2 hours to an OD of ~ 0.5 and then induced using aTc concentrations of 0, 0.1, 1, 5, 10, and 25 ng/mL. Three cultures were started using an aTc concentration of 25 ng/mL due to the large volume of this sample required for the hysteresis experiments. After thoroughly mixing, 200 μL samples from the cultures were placed into wells on a 96-well plate for a kinetic experiment with FI and OD measurements taken every hour. Cultures at induction levels of 0, 0.1, 1, 5 and 10 ng/mL aTc had three 200 μL samples placed into wells, and the cultures at 25 ng/mL aTc had twenty seven 200

μL samples placed into wells. Four hours after induction, one 200 μL sample was taken from each induction level and prepared for flow cytometry using the procedure stated above. Twelve hours after induction one 200 μL sample was again taken from each induction level and prepared for flow cytometry.

Additionally, dilutions (also referred to as resuspensions) were performed twelve hours after induction. Four wells (800 μL) were collected from the culture at the high induction level (25 ng/mL). The sample was washed in PBS and resuspended in an 800 μL solution of LB, antibiotics, and 25 ng/mL aTc. This procedure was repeated five more times, each time resuspending in a solution of 10, 5, 1, 0.1, or 0 ng/mL aTc. Thus, six 800 μL samples were created. All samples were inoculated with cells previously at 25 ng/mL aTc, and all cultures were then re-induced with either 0, 0.1, 1, 5, 10, or 25 ng/mL aTc. Since 24 separate cultures (wells) of PF cells at 25 ng/mL were used to inoculate the new cultures, care was taken to ensure that the FI and OD of each 25 ng/mL culture were similar to the population. Shapiro-Wilk tests showed that the FI and OD of all 24 cultures, for each time-point measurement, were normally distributed ($p > 0.05$).

From each 800 μL sample, three 200 μL samples were placed into microplate wells. These three samples formed dilution group A. The average number of cells per well from the original four wells that formed the 800 μL sample was the same

average number of cells per well for the new three wells. Thus, the dilution ratio for group A was 1:1. From each 800 μ L sample, 60 μ L was mixed with a 540 μ L solution of LB, antibiotics, and the appropriate concentration of aTc. This solution was mixed and placed into three microplate wells (200 μ L each). These three samples formed dilution group B. One-tenth of the average number of cells per well from the original four wells that formed the 800 μ L sample was the initial number of cells for these three new wells. Therefore the dilution ratio for group B was 1:10. Finally, from each 800 μ L sample, 6 μ L was mixed with a 594 μ L solution of LB, antibiotics, and the appropriate concentration of aTc. This solution was mixed and placed into three microplate wells (200 μ L each). These three samples formed dilution group C. One-hundredth of the average number of cells per well from the original four wells that formed the 800 μ L sample was the initial number of cells for the new three wells. Therefore the dilution ratio for group C was 1:100. In summary, cells that were previously at the high induction level (25 ng/mL aTc) were washed and resuspended at six different induction levels (0, 0.1, 1, 5, 10, and 25 ng/mL aTc). Each induction level contained samples inoculated using dilution ratios of 1:1, 1:10, or 1:100. There were three 200 μ L samples at each induction level and dilution ratio for a total of 54 samples. These samples were grown in microplate wells during a kinetic experiment with measurements taken every hour.

Four hours after the dilution procedure, one 200 μ L sample was taken from each group at each induction level and prepared for flow cytometry. Twelve hours after the dilution procedure, one 200 μ L sample was again taken from each group at each induction level and prepared for flow cytometry. All flow cytometry samples were analyzed at the same time two hours after the collection of the last flow cytometry sample. In the interim, samples were stored at 4°C.

3.3 Cell sorting

Cell sorting was performed on a BD FACSAria II cell sorter. Cells that exhibited bimodal GFP expression were sorted into two populations: the ON population representing cells with a high level of GFP fluorescence, and the OFF population representing cells with a low level of GFP fluorescence. Cells were sorted directly into test tubes with fresh LB media, antibiotics, and aTc (if necessary). The cells were then grown in these test tubes for four hours at 37°C. After four hours, three 200 μ L samples of each population were placed in microplate wells and grown during a kinetic experiment in the Tecan Infinite m200 microplate reader for 8 hours with fluorescent intensity and optical density measurements taken every hour.

To perform the sorting, two populations were defined in the BD FACSDiva Version 6.1.2 software. P1 is the population exhibiting high GFP fluorescence,

and P2 is the population exhibiting low GFP fluorescence. An example is shown Figure 4. The gates to define each population varied slightly based on the particular culture being tested. In every case, an effort was made to ensure that no “intermediate” cells were sorted into either population. For each population a minimum of 100,000 cells were collected.

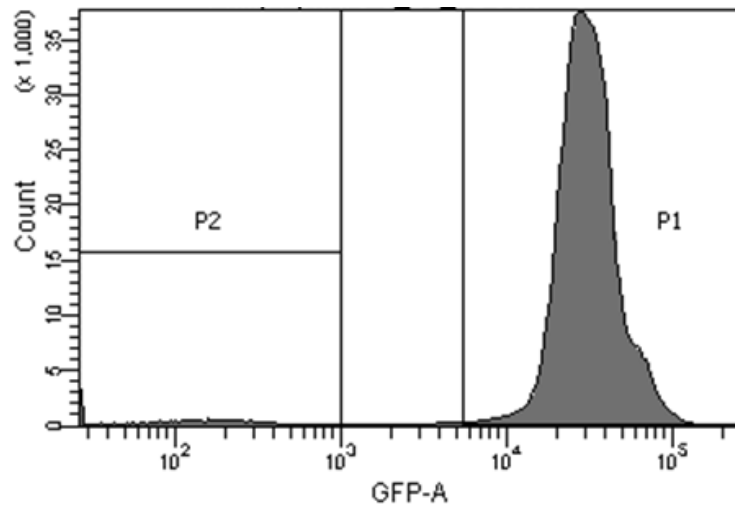


Figure 4: Cells are sorted into OFF and ON populations based on GFP fluorescence levels

3.4 Notation

Positive feedback cells are denoted by the number 68, the identification number of the plasmid (pGN68) with the positive feedback mechanism that is transformed into the dh5 α Z1 *E. coli* cell. Non-positive feedback cells are denoted by the number 69, the identification number of the plasmid without the positive feedback

mechanism (pGN69). Both cells contained a common plasmid (pGN12) which provided the aTc-inducible expression of LuxR. Notation contained in figures and the text contains two or three parts. Part 1 is a number (68 or 69) denoting whether the cell is the positive feedback or non-positive feedback type. Part 2 is the aTc concentration in ng/mL. Part 3, if given, refers to the dilution ratio (A = 1:1, B = 1:10, or C = 1:100) used to inoculate the culture. If part 3 is not given, then the culture was inoculated using cells with no prior induction. For example, the notation “68-25” refers to PF cells induced with 25 ng/mL of aTc. The notation “68-10B” refers to PF cells that were diluted 1:10 from a washed culture previously induced at a high level (25 ng/mL unless otherwise reported) into a new solution of 10 ng/mL aTc.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Model results

The model presented in Chapter 2 predicts the concentration of the gene product (g) as a function of time. Once expression is initiated, g approaches some steady-state level, indicating the system has reached an equilibrium level of gene expression and degradation. An example of this is shown in Figure 5. The model was used to predict the steady-state expression levels under a variety of conditions by varying model constants and inducer concentration. A system only exhibits hysteresis if its steady-state level depends on its induction history. Therefore, care must be taken to ensure that observations for memory are only made under steady-state conditions, and not when the concentration of the gene product is still in its growth phase.

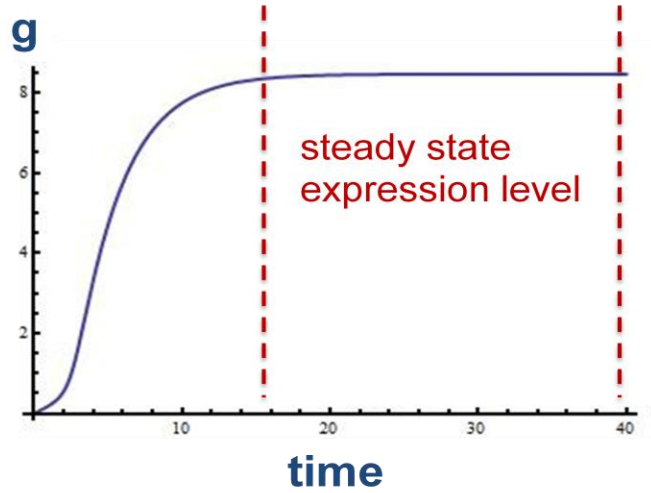


Figure 5: Steady-state level of gene expression is approached over time (a.u.)

By expressing the model in dimensionless form, the model can be simplified to include only two main parameters, r and s . Parametric equations can be defined to relate the steady-state values of x (the dimensionless form of gene product concentration, g) to r and s . The model parameter s is a function of the inducer concentration. Therefore, the dimensionless model is useful for predicting the steady-state expression level over a range of inducer concentrations, as shown in the s - x curves in Figure 6. Each stimulus-response curve shows the behavior at a given value of r . The model parameter r is a function of k_2 , k_3 , and k_4 (linear degradation rate, maximum expression level of the promoter, and activation level for half-maximum response respectively).

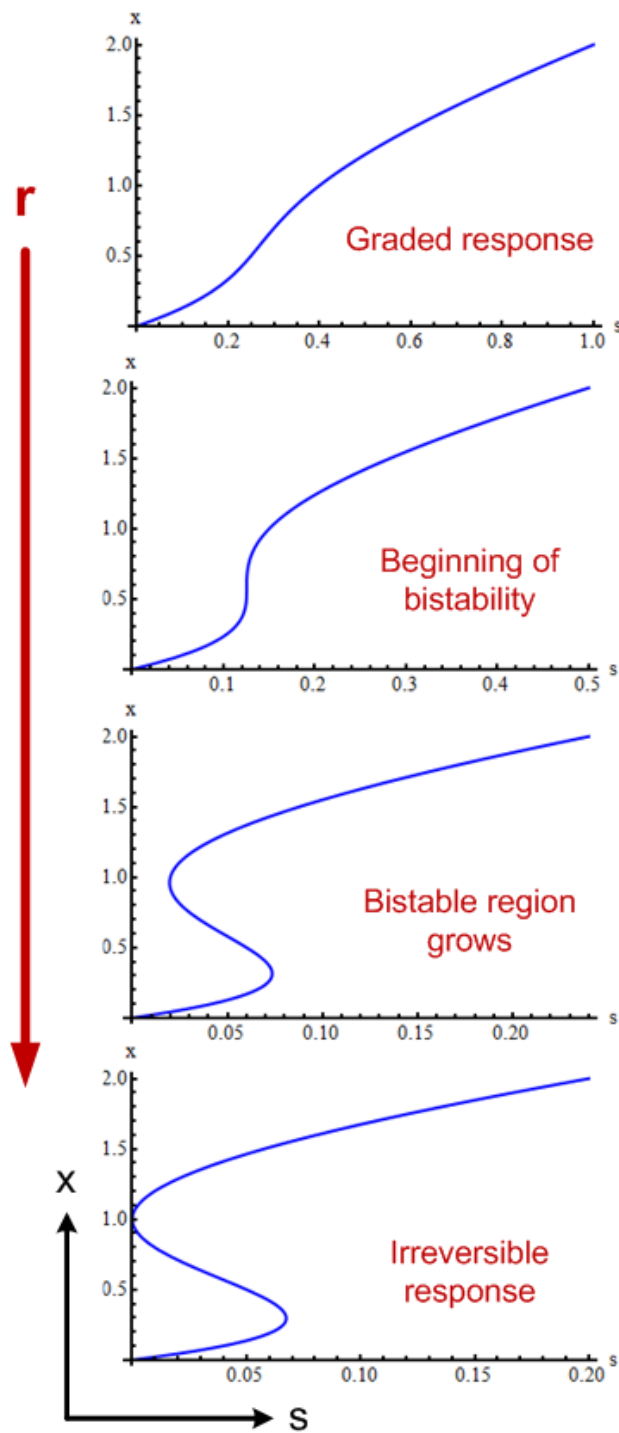


Figure 6: Stimulus-response behavior varies with r

As system conditions change, represented by variation in r , the stimulus-response behavior can be one of three distinct types. First, the system can be monostable.

In a monostable system, there is one steady-state expression level at a given inducer concentration, s . Second, the system can be bistable. In a bistable system, for a given inducer concentration, there are two possible steady-state levels of gene product concentration. The steady-state level that the system converges to depends on the initial concentration of the gene product [9]. A system that is initially at a high gene product concentration will usually converge to the high stable state, and a system initially at a low gene product concentration will usually converge to the low stable state creating “memory” of the prior induction level.

The range of inducer concentrations over which the system is bistable varies with r . A system may have a very narrow range over which bistability is possible, or a system may be bistable over the entire range of relevant inducer concentrations.

Third, a system can be irreversible. An irreversible system is one that is bistable even when no inducer is present. Thus, once the system “switches” from the low stable level to the high stable level as inducer concentration is increased, it cannot return to the low level even when the inducer concentration is lowered to zero.

This is illustrated in Figure 7.

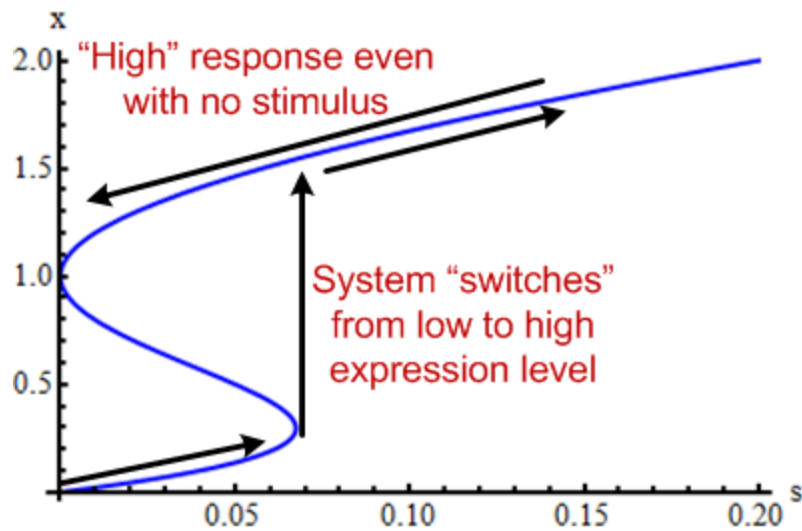


Figure 7: An irreversible system can switch from the low to high expression level, but not high to low

Instead of viewing stimulus-response curves over a range of inducer concentrations, as in Figure 6, it is useful to view the system response in 3-D parameter space. This is shown in Figure 8. At high values of r , the system exhibits a monostable graded response where only one steady-state expression level, x , exists regardless of the value of s . However, as r decreases the bistable region emerges at high s values and there are two stable steady-state expression levels at these (r, s) locations. As r continues to decrease, the response becomes irreversible. When r is in the range of irreversibility, the system will always exhibit either a high monostable response (at high s values), or a bistable response (at low s values).

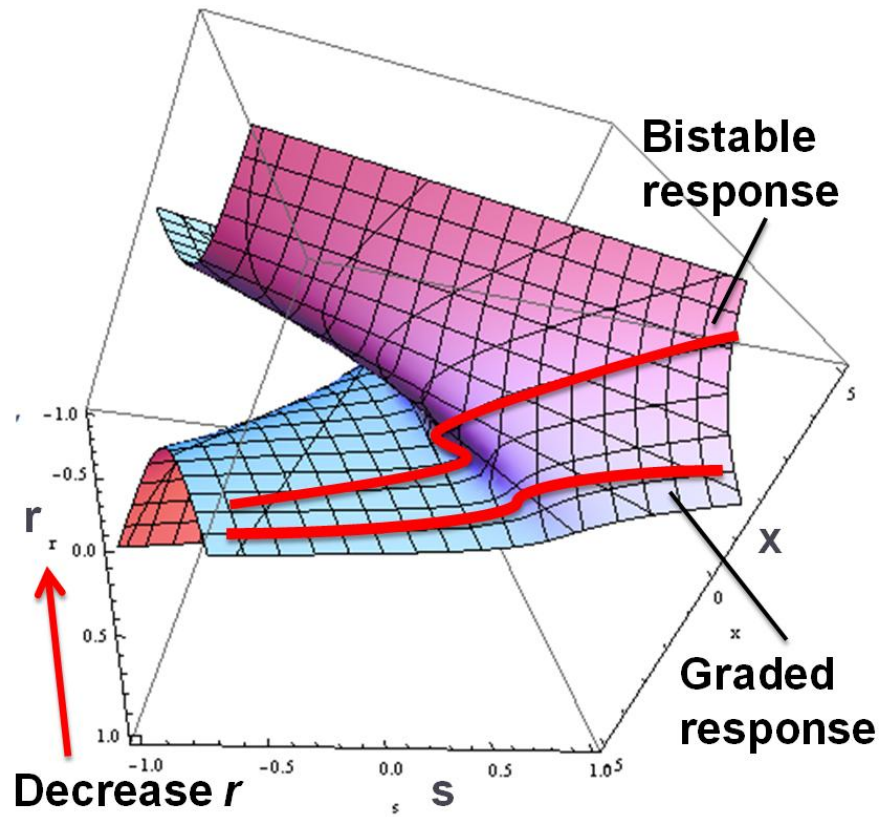


Figure 8: As r decreases, system response shifts from monostable to bistable

To show more clearly where the bistable regions of the system are, a bifurcation diagram is presented in Figure 9. The bifurcation diagram is a projection of the 3-D plot in Figure 8 onto the r - s plane. The bifurcation diagram clearly shows the regions of the three primary system responses: monostable low, bistable, and monostable high. At high r values, the system always produces a low monostable response. As r decreases, the bistable region emerges at higher values of s and increases in size over a larger range of inducer concentrations (s) until the system response becomes irreversible.

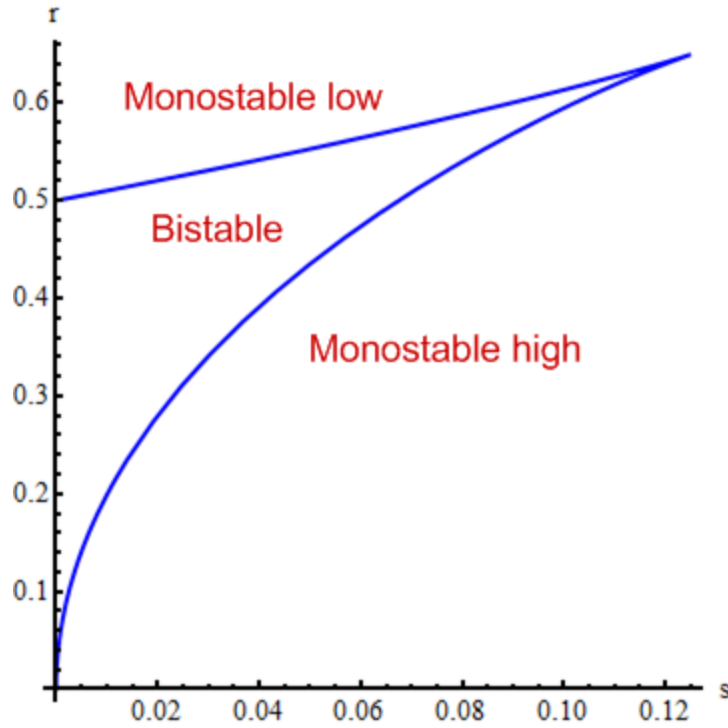


Figure 9: System bifurcation diagram shows three distinct system responses

If the system is operating in the bistable range, then there are three real steady-state values of x . Two of the steady-state values are stable, one is unstable, and the system converges to one of the two stable points. The point of convergence is determined by the initial conditions of x , or the initial concentration of the gene product in the system. An example in dimensioned form is illustrated in Figure 10. In this example, the model parameters were $k_1 = 0.235$, $k_2 = 0.345$, $k_3 = 2.76$, $k_4 = 1.06$, and $s_0 = 0$ (for demonstration purposes only, these values were not derived experimentally). The stable steady-state values are 0 and 7.86, and the unstable steady-state value is 0.14. This is an irreversible system because even

with no inducer concentration ($s_0 = 0$) the system remains bistable. When the initial conditions are greater than 0.14, the system approaches the high steady-state value of 7.86. When the initial conditions are lower than 0.14, the system converges to the low steady-state value of 0. A system that is initially uninduced ($s_0 = 0$) will exhibit no expression ($g = 0$). However, if some inducer is added and g begins to increase, and if g increases beyond the value of the unstable point, then lowering the inducer concentration back to zero will not cause the system to return to the $g = 0$ expression level. Instead, the system will converge to the high expression level, $g = 7.86$, despite the absence of any inducer. Therefore, this system is capable of producing memory, but memory will only be observed if the initial conditions of the system are sufficient.

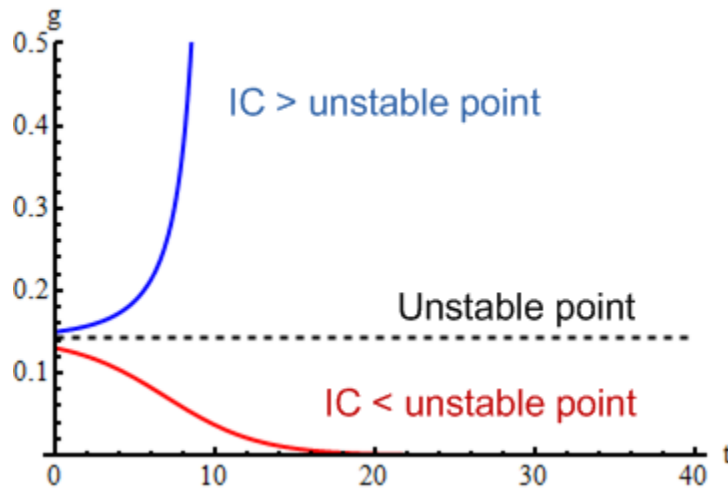


Figure 10: Convergence to a stable steady-state expression level depends on initial conditions (IC)

4.2 Experimental results and discussion

Cultures of positive feedback (PF) cells displayed memory of their prior induction level, but the magnitude of their hysteretic response usually depended on the dilution ratio used to inoculate the culture. Additionally, the range of inducer concentrations over which the PF system exhibited bistability was dependent on induction history. This section provides a detailed overview and description of these results.

4.2.1 GFP fluorescence over time

Positive feedback cells were grown at 6 different aTc induction levels to a steady-state level of expression, as shown in Figure 11. Cultures with aTc induction levels of 0, 0.1, and 1 ng/mL showed almost no GFP fluorescence (~60 RFU). The culture with an aTc induction level of 5 ng/mL showed some GFP fluorescence (~500 RFU), and the cultures with aTc induction levels of 10 and 25 ng/mL showed high levels of GFP fluorescence (>3000 RFU). The relative fluorescence (RF) of the 68-10 and 68-25 cultures continued to increase with time. This occurred because the fluorescent intensity (FI) of the cultures remained approximately constant after 8 hours, but the optical density (OD) began to decrease as shown in Figure 12.

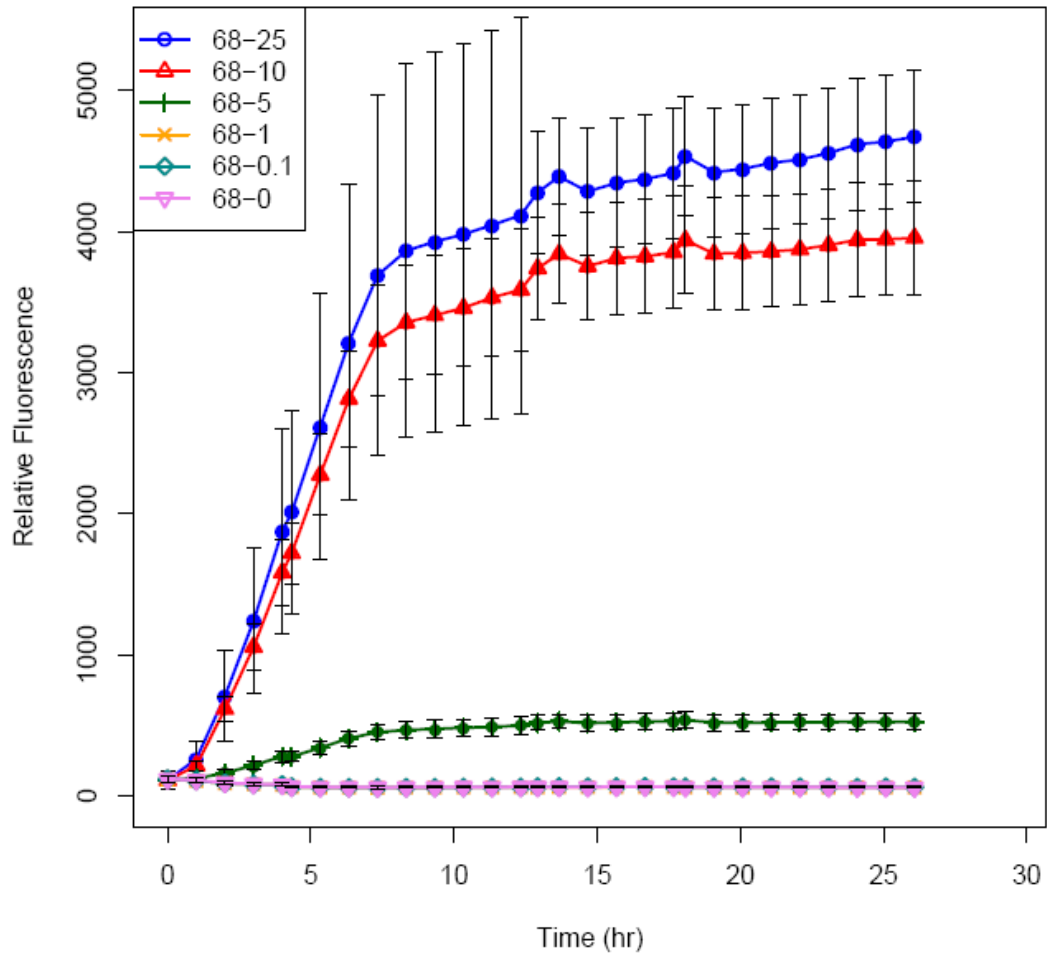


Figure 11: GFP expression in PF cells over time at 6 induction levels; 68-1, 68-0.1, and 68-0 are all at approximately the same level (~60 RFU) and are therefore obscured on the plot; error bars represent ± 2 standard deviations

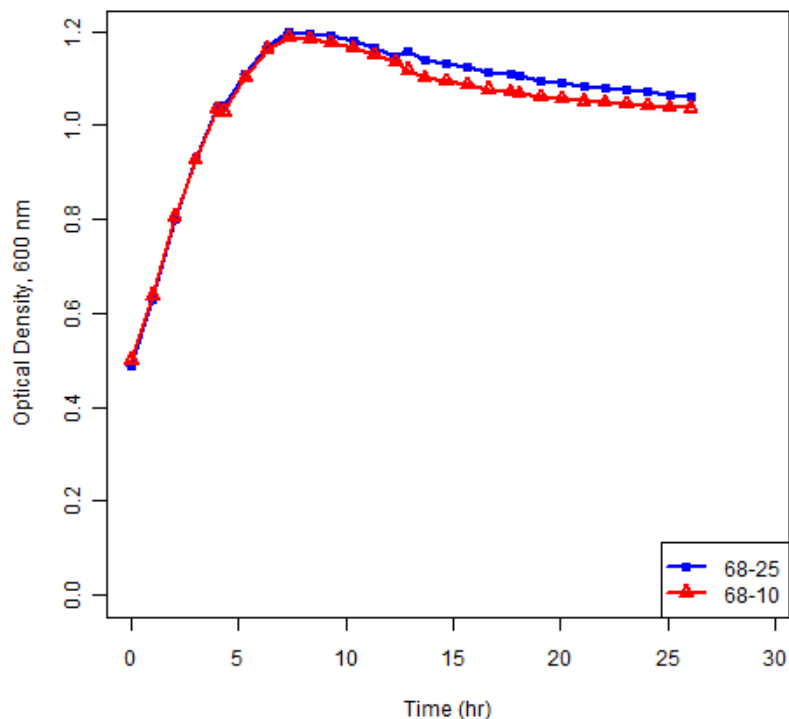


Figure 12: Optical density of the cultures begins to decrease after approximately 8 hours

Despite the continuous change in RF, flow cytometry data (Figure 13 and Figure 14) showed that these cultures were actually at a steady-state level of expression, and the populations were bistable. The GFP fluorescence levels of the two sub-populations (the location of the modes in the histograms) were constant from 12 to 26 hours after induction. Therefore, changes in RF were due to changes in cell density, not in GFP fluorescence. Figure 13 and Figure 14 also show that at 26 hours after induction, a larger proportion of cells are at the “off” state (the low GFP mode) than 12 hours after induction. Despite the change in proportion of

cells at each fluorescence level, the steady-state expression levels did not change within the time interval.

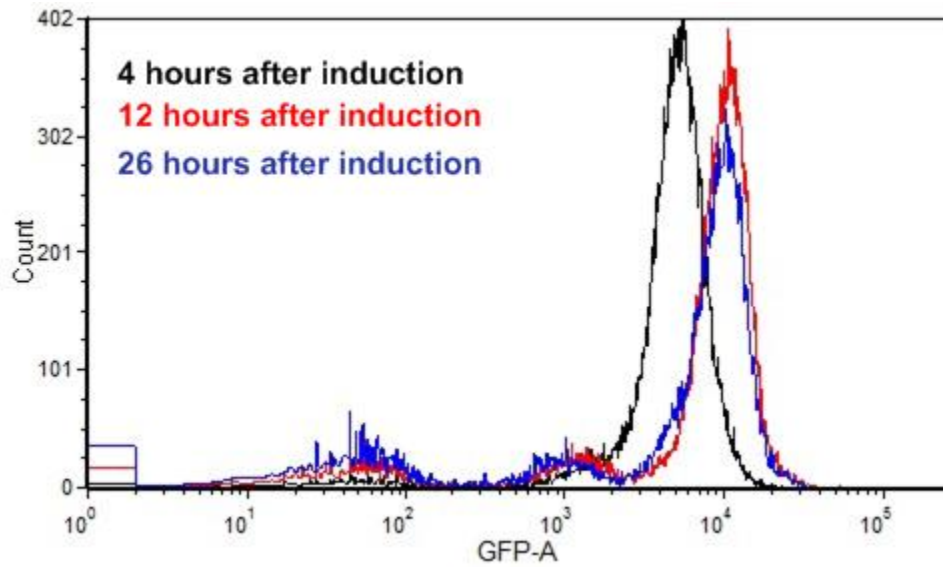


Figure 13: The 68-10 culture was bistable and at a steady-state level of GFP expression 12 hours after induction

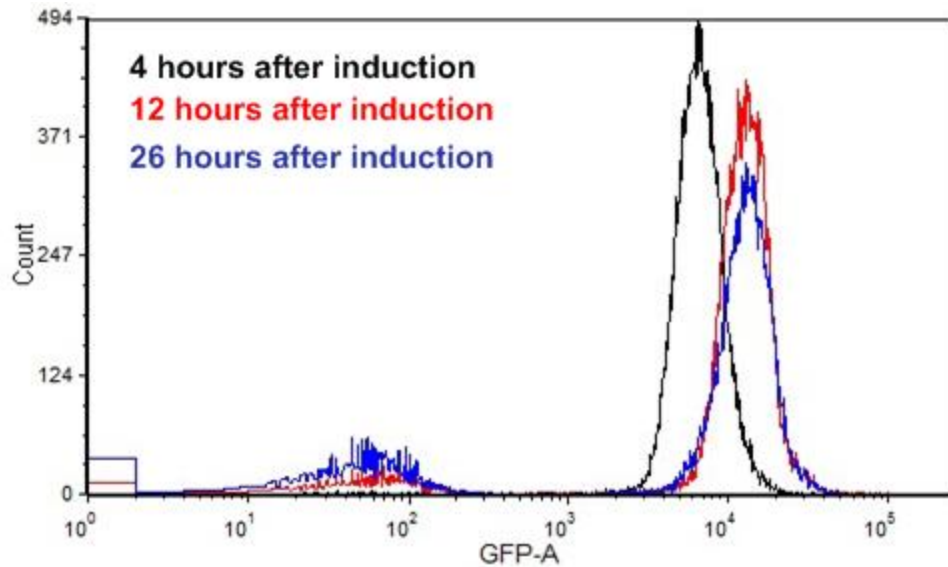


Figure 14: The 68-25 culture was bistable and at a steady-state level of GFP expression 12 hours after induction

The GFP fluorescence data over time for the four other cultures (aTc induction levels of 5, 1, 0.1, and 0 ng/mL) is shown in Appendix A.1. For cultures 68-25, 68-10, and 68-5, the populations were bistable, i.e. cells within each culture were expressing GFP at either a “high” or “low” level, where the low level represented no significant GFP production. Each of these cultures reached a steady-state level of expression within 12 hours after induction. Additionally, the proportion of cells expressing at the high GFP levels decreased with time from 12 to 26 hours after induction. For cultures 68-1, 68-0.1, and 68-0, the populations were monostable, i.e. the cells within each culture were expressing only at one level, and that “low” level represented no significant GFP fluorescence. The GFP fluorescence for

these cultures remained stable at all time points tested (4 to 26 hours after induction).

4.2.2 Cultures with a prior stimulus

Twelve hours after the initial induction of the 68-25 cultures, these PF cells were washed and resuspended using one of three dilution ratios into new LB media with 6 different aTc concentrations (25, 10, 5, 1, 0.1, and 0 ng/mL). Figure 15 shows GFP expression (measured as RF on a microplate reader) over time for cultures resuspended into media with no aTc. The magnitude of GFP fluorescence depended on the dilution ratio used during resuspension. Twelve hours after resuspension, the culture resuspended using a 1:1 dilution ratio (68-0A) showed the highest level of GFP fluorescence, and the culture resuspended using a 1:100 dilution ratio (68-0C) showed the lowest level of GFP fluorescence. The three resuspended cultures differed in GFP fluorescence, but each showed significantly higher fluorescence than the 68-0 culture with no induction history. Additionally, the three resuspended cultures each showed significantly lower fluorescence levels than the 68-25 culture used for inoculation. Therefore, hysteresis was observed because these three cultures all displayed memory of prior stimulus, but the magnitude of the hysteretic response depended on the dilution ratio.

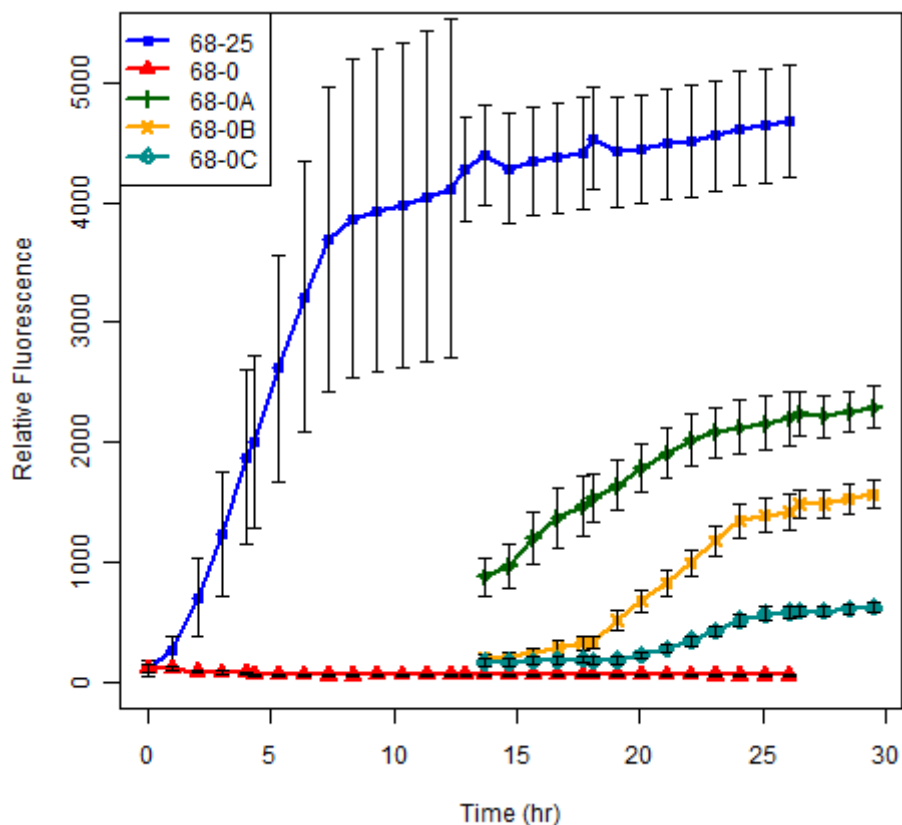


Figure 15: PF cultures previously at 25 ng/mL induction were resuspended into media with no aTc using three dilution ratios (A, B, and C)

Flow cytometry data was also collected for the three resuspended cultures (68-0A, 68-0B, and 68-0C) at four and twelve hours after the dilution procedure. Figure 16 (68-0A) shows that the ON population (the cells with high GFP fluorescence) continued to fluoresce at approximately the same level as the original 68-25 culture. However, over a time period of 8 hours, the proportion of ON cells decreased from 45% of the culture to 23% of the culture. Figure 17 (68-0B) shows that the GFP fluorescence level 4 hours after the dilution procedure is

lower than the level 12 hours after dilution. Twelve hours after dilution, the GFP fluorescence level has returned to the original fluorescence level of the 68-25 culture. During that same time period, the proportion of ON cells decreased from 47% of the culture to 29% of the culture. Figure 18 (68-0C) shows that again the GFP fluorescence level of the culture is lower 4 hours after dilution than 12 hours after dilution. After twelve hours, the fluorescence level has returned to the same level as the original 68-25 culture. In that time period, the proportion of ON cells decreased from 49% of the culture to 12%. Each of the three resuspended cultures exhibited bistability, despite the fact that the 68-0 culture was monostable. Additionally, each of these cultures displayed hysteresis.

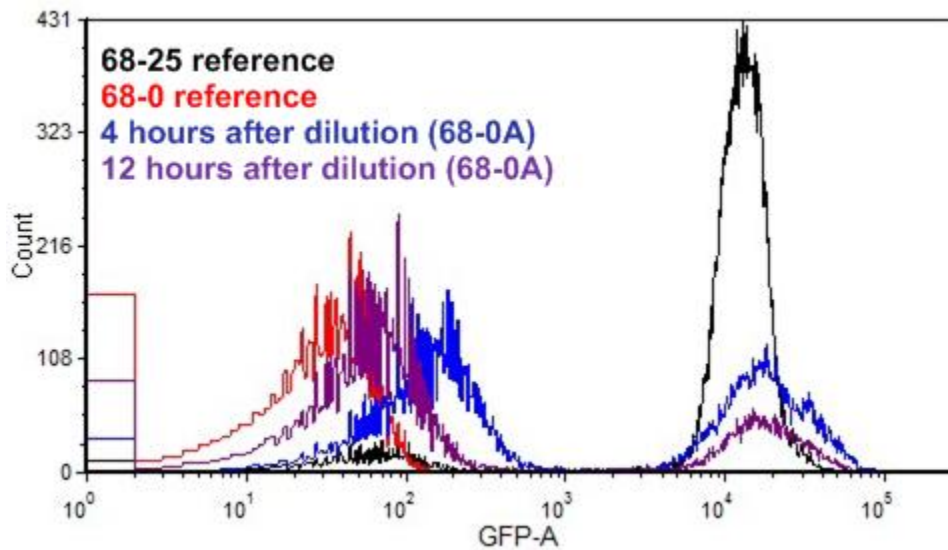


Figure 16: Cells initially induced using 25 ng/mL aTc then resuspended into media with 0 ng/mL aTc using a 1:1 dilution ratio (68-0A)

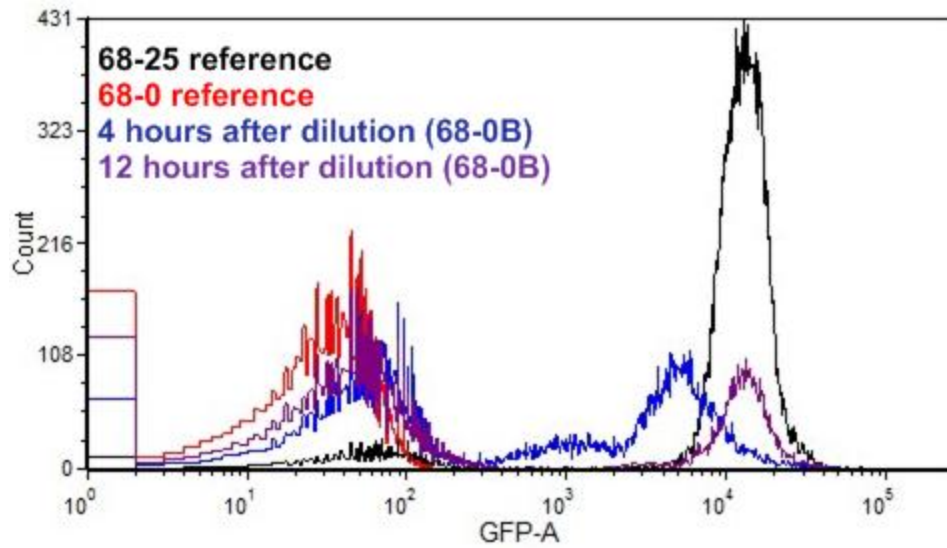


Figure 17: Cells initially induced using 25 ng/mL aTc then resuspended into media with 0 ng/mL aTc using a 1:10 dilution ratio (68-0B)

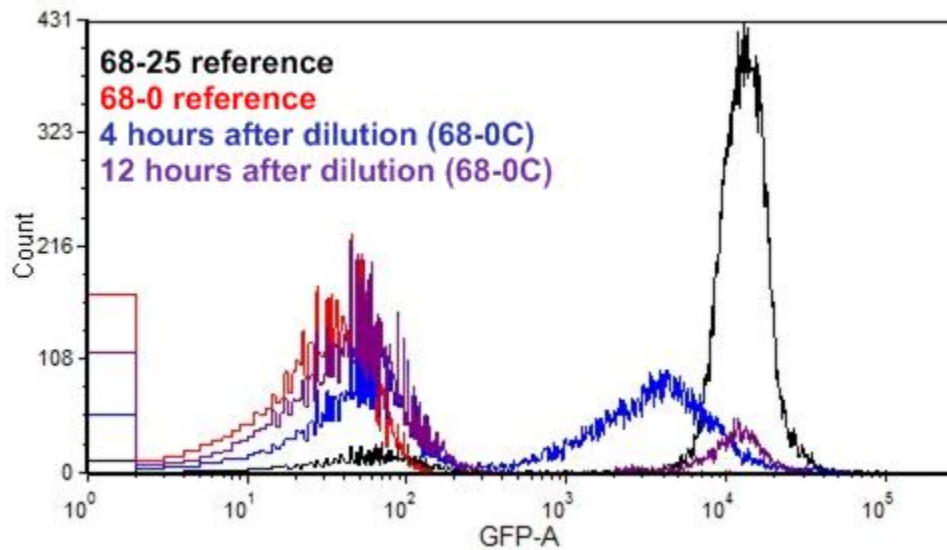


Figure 18: Cells initially induced using 25 ng/mL aTc then resuspended into media with 0 ng/mL aTc using a 1:100 dilution ratio (68-0C)

A similar trend is shown for cells resuspended into media at a non-zero aTc concentration. Figure 19 shows GFP expression (measured as RF on a microplate reader) over time for cultures resuspended into media with 5 ng/mL aTc. The magnitude of GFP fluorescence again depended on the dilution ratio used during resuspension. Twelve hours after resuspension, the culture resuspended using a 1:1 dilution ratio (68-5A) showed the highest level of GFP fluorescence, and the culture resuspended using a 1:100 dilution ratio (68-5C) showed the lowest level of GFP fluorescence. The three resuspended cultures differed in GFP fluorescence, but each showed significantly higher fluorescence than the culture initially induced with 5 ng/mL aTc (68-5). Additionally, the three resuspended cultures each showed significantly lower fluorescence levels than the 68-25 culture used for inoculation. Once again, hysteresis was observed because these three cultures all displayed memory of prior stimulus, but the magnitude of the hysteretic response depended on the dilution ratio.

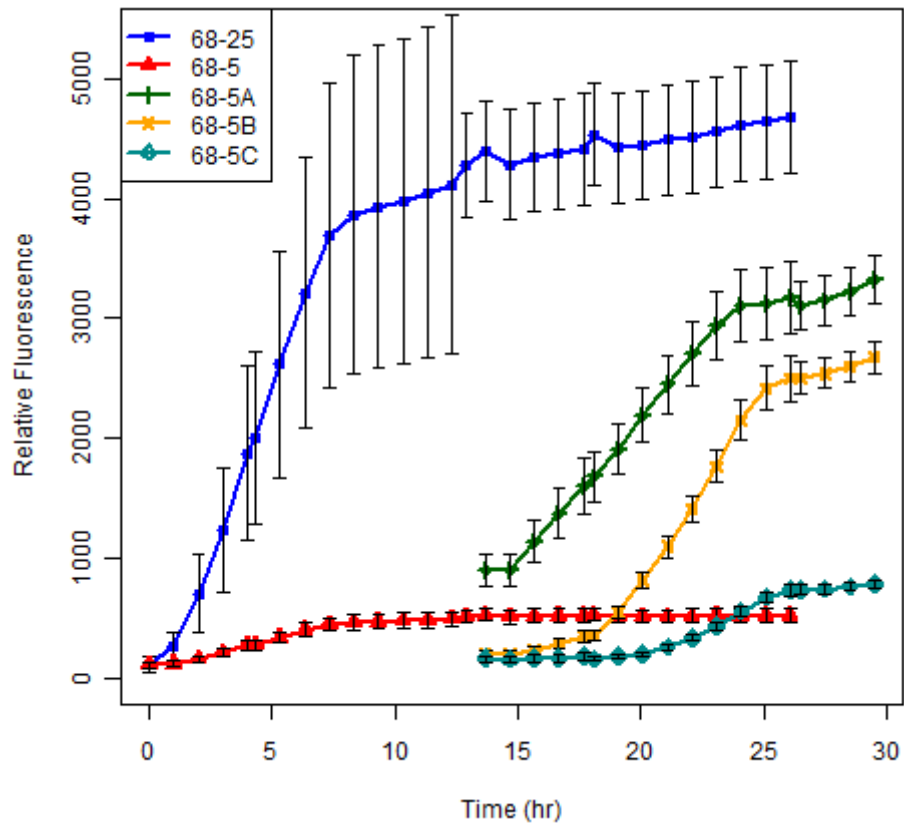


Figure 19: PF cultures previously at 25 ng/mL induction were resuspended into media with 5 ng/mL aTc using three dilution ratios (A, B, and C)

Flow cytometry data was also collected for the three resuspended cultures (68-5A, 68-5B, and 68-5C) at four and twelve hours after the dilution procedure, and was compared to the cultures with no induction history (68-5 and 68-25). Figure 20 (68-5A) shows that the ON cells fluoresced at approximately the same level as the 68-25 culture used for inoculation. This level was slightly higher than the fluorescence level of the 68-5 culture with no other induction history than its initial induction into media containing 5 ng/mL aTc. Over a time period of 8

hours, the proportion of ON cells decreased from 81% to 54% of the culture.

Figure 21 (68-5B) and Figure 22 (68-5C) show that four hours after dilution, the GFP fluorescence levels of the ON populations are lower than the 68-5 and 68-25 cultures, but twelve hours after dilution the levels have increased to the fluorescence level of the 68-25 culture. During that same time period, the proportion of ON cells decreased from 81% to 59% of the 68-5B culture, and from 82% to 19% of the 68-5C culture. Each of the three cultures resuspended into 5 ng/mL aTc displayed bistability and memory (hysteresis) of their prior induction level to 25 ng/mL aTc. The ON population of each culture fluoresced at the same level as the 68-25 culture, and at a higher level than the ON population of the 68-5 culture with no other induction history. Additionally, in each case the proportion of ON cells within the culture decreased over time from 4 to 12 hours after resuspension.

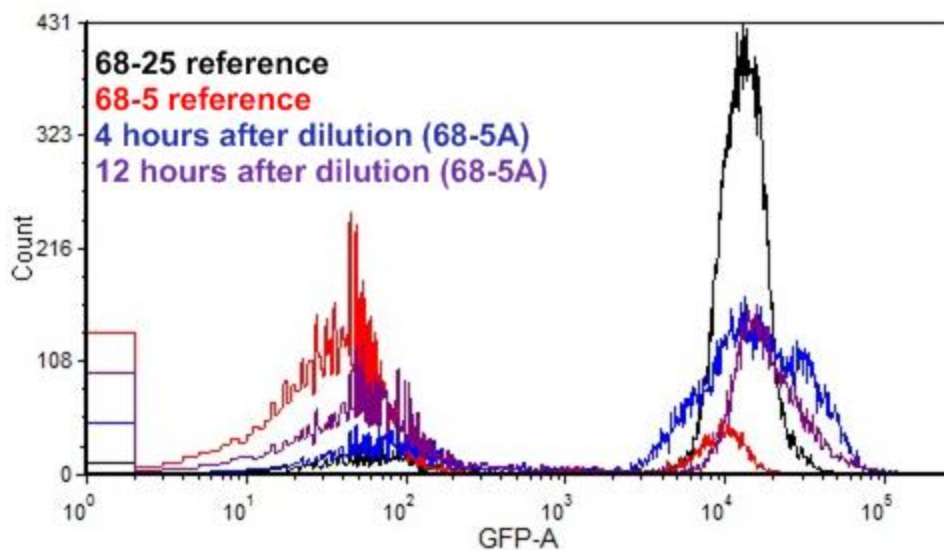


Figure 20: Cells initially induced using 25 ng/mL aTc then resuspended into media with 5 ng/mL aTc using a 1:1 dilution ratio (68-5A)

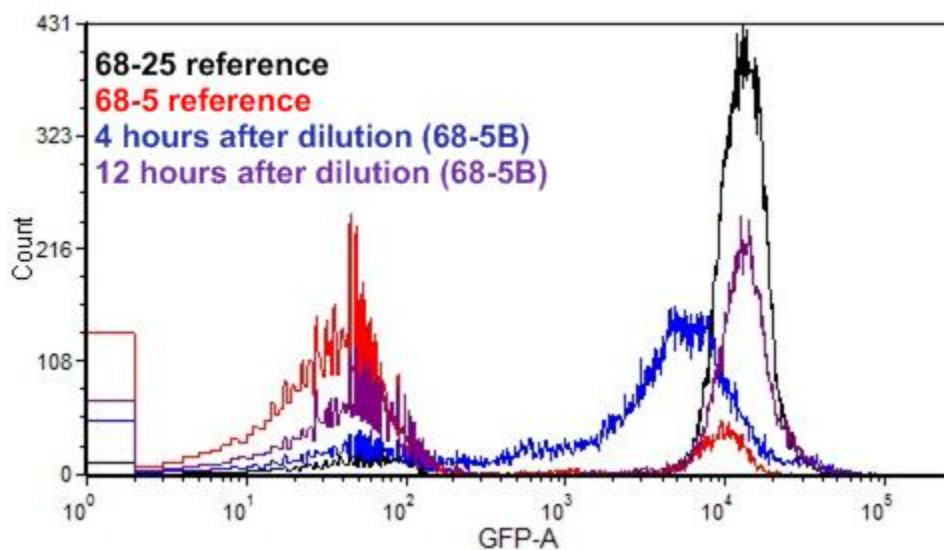


Figure 21: Cells initially induced using 25 ng/mL aTc then resuspended into media with 5 ng/mL aTc using a 1:10 dilution ratio (68-5B)

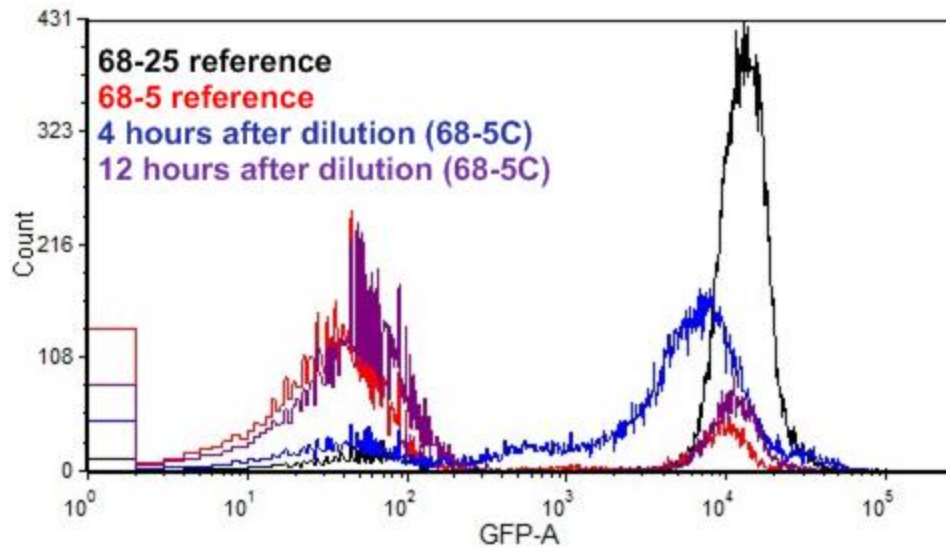


Figure 22: Cells initially induced using 25 ng/mL aTc then resuspended into media with 5 ng/mL aTc using a 1:100 dilution ratio (68-5C)

The overall trend demonstrated in these experiments is that four hours after cells are resuspended into media at a new induction level, their ON population usually fluoresces at a lower level than it did at its previous “high” induction level (25 ng/mL). However, twelve hours after resuspension the fluorescence level of the ON population returns to the level of the previous induction level. Over that time period, the proportion of ON cells in the culture decreases, and the greatest decrease occurs with cultures induced with a 1:100 dilution ratio (C). Cultures were resuspended to six different induction levels using three different dilution ratios, and each culture exhibited bistability and memory of its previous induction level. Therefore, the system was bistable over the entire range of inducer

concentrations, 0 – 25 ng/mL. The data for the other induction levels tested (0.1, 1, 10, and 25 ng/mL) is shown in Appendix A.2.

4.2.3 Sorting cell populations

A consistent trend shown in these experiments is that over time the proportion of cells that are ON (exhibiting high GFP fluorescence) decreases. There are two possible reasons for this. First, cells that are ON might be switching to OFF over time. Second, the OFF cells might be capable of growing at a faster rate than the ON cells because they are not burdened by having to produce LuxR and GFP. Over time, the proportion of OFF cells relative to ON cells would increase due to the higher growth rate.

To determine the cause of the proportion change, the 68-5C sample was taken four hours after resuspension and sorted into OFF and ON populations using the BD FACS Aria II cell sorter. The samples were grown in test tubes for four hours and then transferred to the microplate reader for an 8 hour kinetic experiment with OD and FI measurements taken every hour. Figure 23 shows the optical density of both populations over time. The growth rate was estimated by taking the derivative of the OD curves with respect to time, and the result is shown in Figure 24. Initially, the growth rate of the ON population is greater. Two hours after the start of the kinetic experiment, the growth rate of the OFF population surpassed

that of the ON population, and remained greater for the duration of the experiment. Therefore, the cells that are not expressing GFP grow slightly faster than cells that are expressing GFP.

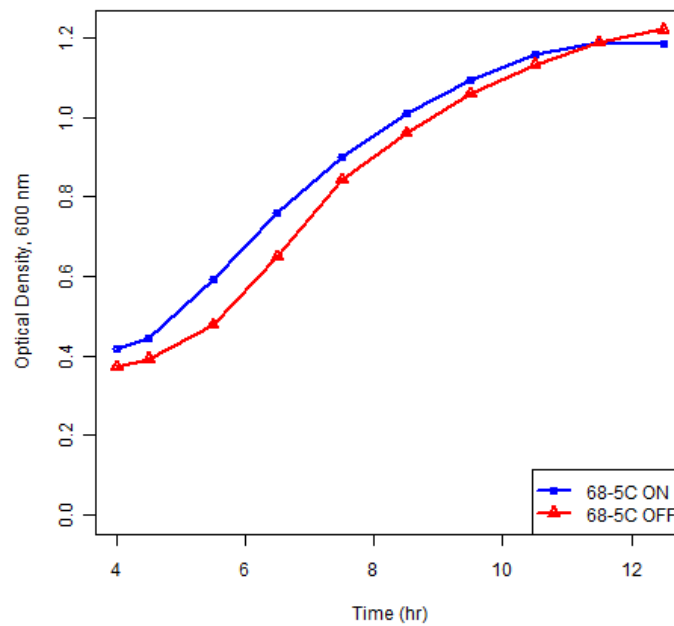


Figure 23: The 68-5C culture was sorted into OFF and ON populations and both populations were grown separately in a kinetic experiment

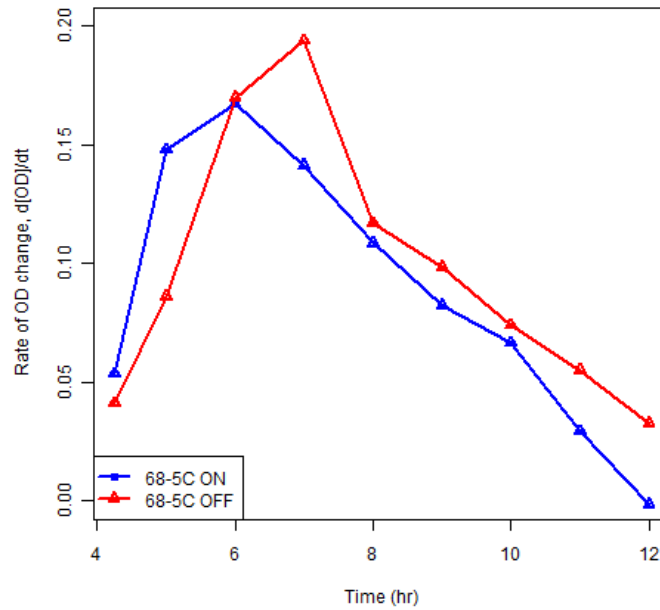


Figure 24: For most of the experiment, the 68-5C OFF population grew at a faster rate than the 68-5C ON population

Additionally, flow cytometry data was recorded for the OFF and ON populations at both the start and end of the kinetic experiment (4 and 12 hours after sorting). Figure 25 shows that the cells in the ON population were initially fluorescing at a low level. This is because the act of resuspending cells in new media provides excess nutrients, and the cells begin to divide rapidly. Each time a cell divides, the GFP contained in it decreases by half. Under nutrient rich conditions, the cell division rate exceeds the GFP synthesis rate, and ON cells express at a low level due to the dilution of their GFP. However, during the kinetic experiment many of the cells began to return to a high level of fluorescence. At the start of the kinetic experiment, less than 1% of the ON cells were actually fluorescing at a high level.

Eight hours into the kinetic experiment, 72% of the ON population had returned to the high fluorescence level. The model predicts that despite the low initial fluorescence level, the concentration of LuxR and GFP in the cells was just above the threshold (i.e. the unstable critical point) required for switching ON once the division rate slowed down and GFP could begin to once again accumulate in the cells. Additionally, Figure 26 shows the GFP histograms for the OFF population at the start and end of the kinetic experiment. In that time frame, 3% of the cells switched to a high level of GFP fluorescence.

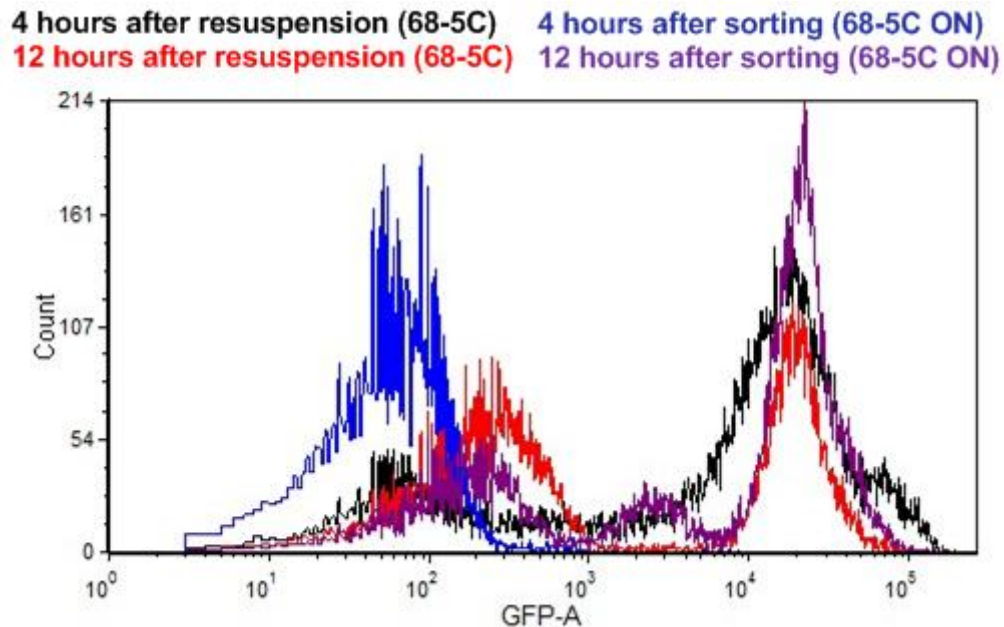


Figure 25: After sorting, the 68-5C ON population fluoresces at a low level, but over time 72% of the population returns to a high fluorescence level

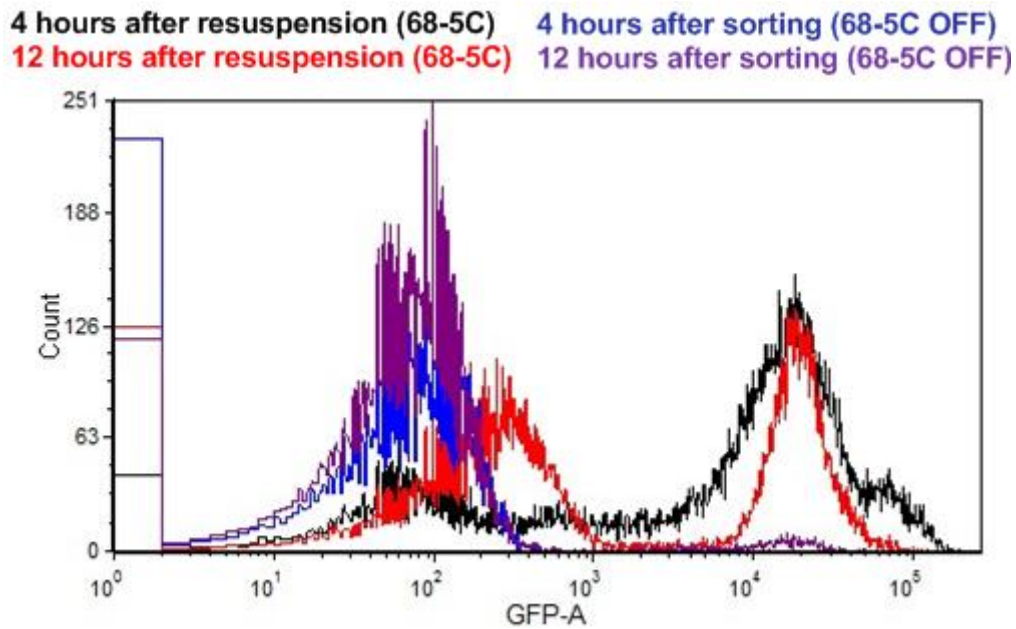


Figure 26: The 68-5C OFF population remains mostly at a low fluorescence level over time

These results show that the act of resuspending cells into new media causes ON cells to fluoresce at a low level. Over time, many of these cells will return to high fluorescence, but some will remain at a low expression level because they switched OFF when their GFP was being diluted, i.e. their GFP/LuxR concentration fell below a critical threshold. Additionally, the data suggests that the OFF cells grow at a slightly faster rate than the ON cells. One common trend seen among the resuspended cultures is that 12 hours after resuspension a higher proportion of the cells are OFF than 4 hours after resuspension. The evidence suggests that the reason for the increasing proportion of OFF cells is due to some

ON cells switching OFF after resuspension, and due to the higher growth rate of the OFF cells relative to the ON cells.

4.2.4 Hysteresis

The main result of this experimental work is that the modular positive feedback-based gene amplifier is capable of producing a hysteretic response to a stimulus, i.e. the expression behavior of this system depends on its induction history. The overall magnitude of the hysteretic response, as given by the culture's relative fluorescence, depends on the dilution ratio used when resuspending cells into new media at a different level of aTc induction. Figure 27 shows that dilution ratio A (1:1) tended to produce the greatest hysteretic response, and dilution ratio C (1:100) tended to produce the lowest hysteretic response, although this response was still significant. For each dilution ratio, the response was irreversible. Even when cells were reintroduced into media with no induction (0 ng/mL aTc), the cells retained some memory of prior induction.

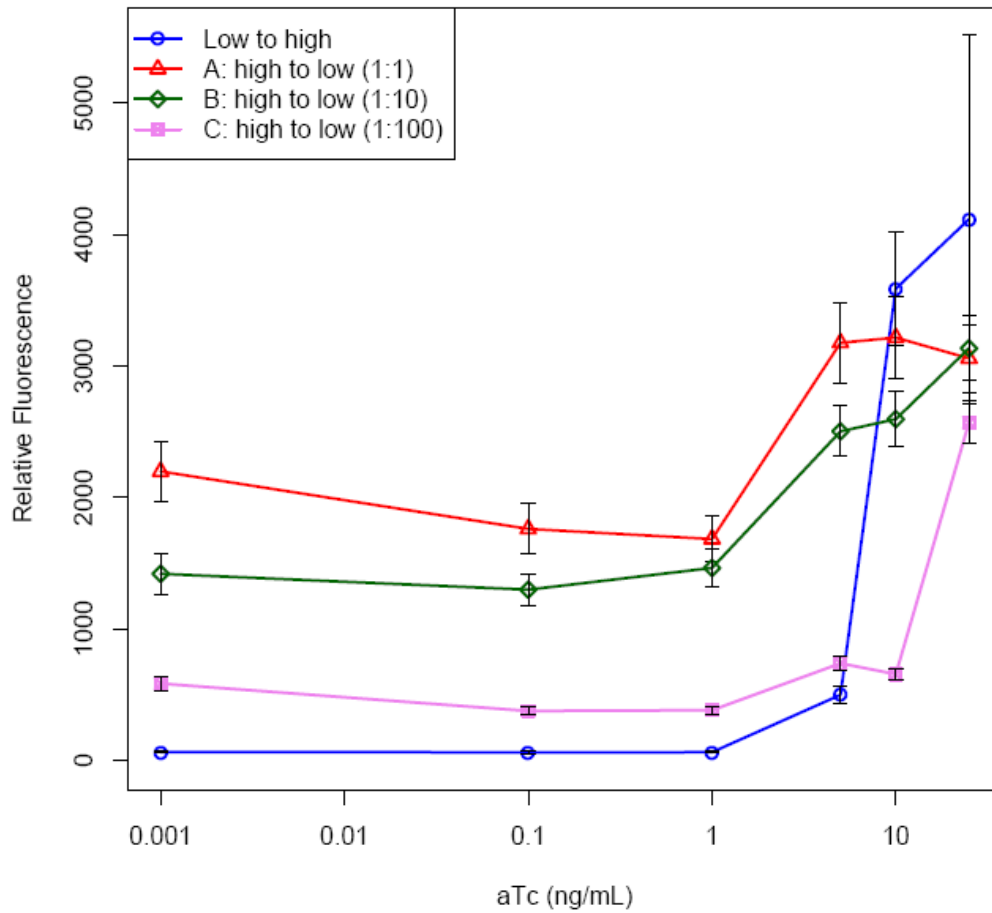


Figure 27: The magnitude of the hysteretic response depends on the dilution ratio used when introducing cells into new media at a different aTc induction level

It is also useful to view hysteresis using flow cytometry data. Figure 28 shows GFP histograms for cells initially induced to six different induction levels (black) and histograms for cells initially at a high induction level (25 ng/mL) and resuspended into fresh media at the same six inducer concentrations (red) using a 1:1 dilution ratio (A). Cultures with no induction history (black) exhibited bistability at induction levels of 5-25 ng/mL. Cultures with a “high” induction

history (red) exhibited bistability over the entire inducer range of 0-25 ng/mL.

The fluorescence level of the OFF population tended to be slightly higher for the resuspended cultures than the cultures with no induction history.

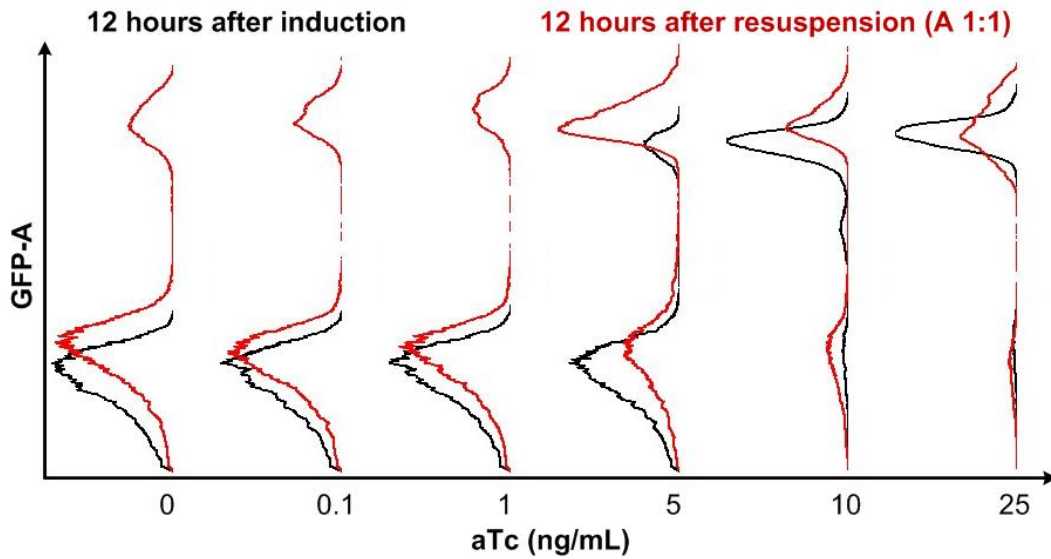


Figure 28: Hysteresis and bistability over the entire tested range of inducer concentrations for cultures resuspended using a 1:1 dilution ratio (data has been smoothed for clarity)

Figure 29 shows GFP histograms for cells initially induced to six different induction levels (black) and histograms for cells initially at a high induction level (25 ng/mL) and resuspended into fresh media at the same six inducer concentrations (red) using a 1:10 dilution ratio (B). As in the previous case, the resuspended cultures exhibited bistability over the entire range of inducer concentrations.

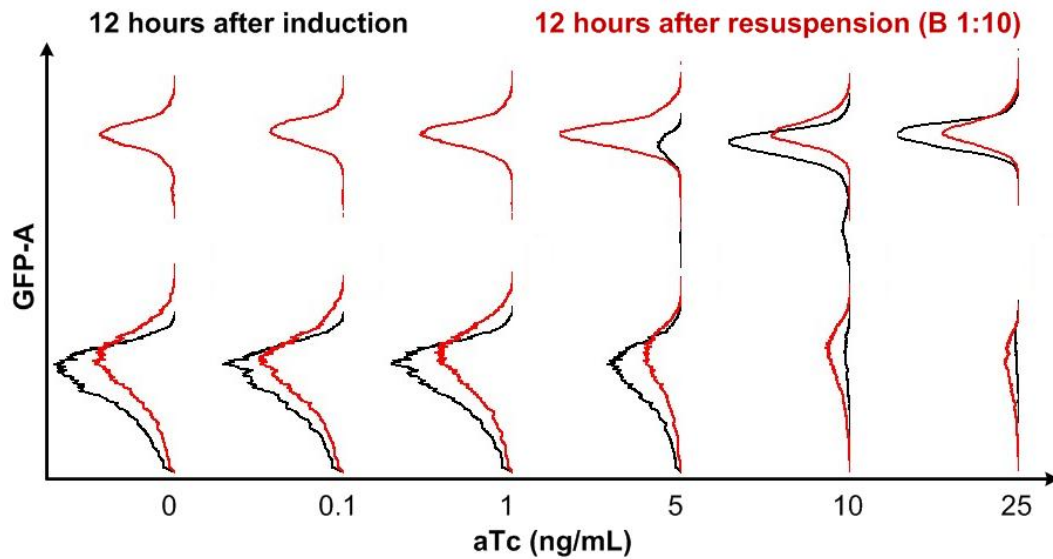


Figure 29: Hysteresis and bistability over the entire tested range of inducer concentrations for cultures resuspended using a 1:10 dilution ratio (data has been smoothed for clarity)

Figure 30 shows GFP histograms for cells initially induced to six different induction levels (black) and histograms for cells initially at a high induction level (25 ng/mL) and resuspended into fresh media at the same six inducer concentrations (red) using a 1:100 dilution ratio (C). As in the case of dilution ratios A and B, these resuspended cultures exhibited bistability over the entire range of inducer concentrations.

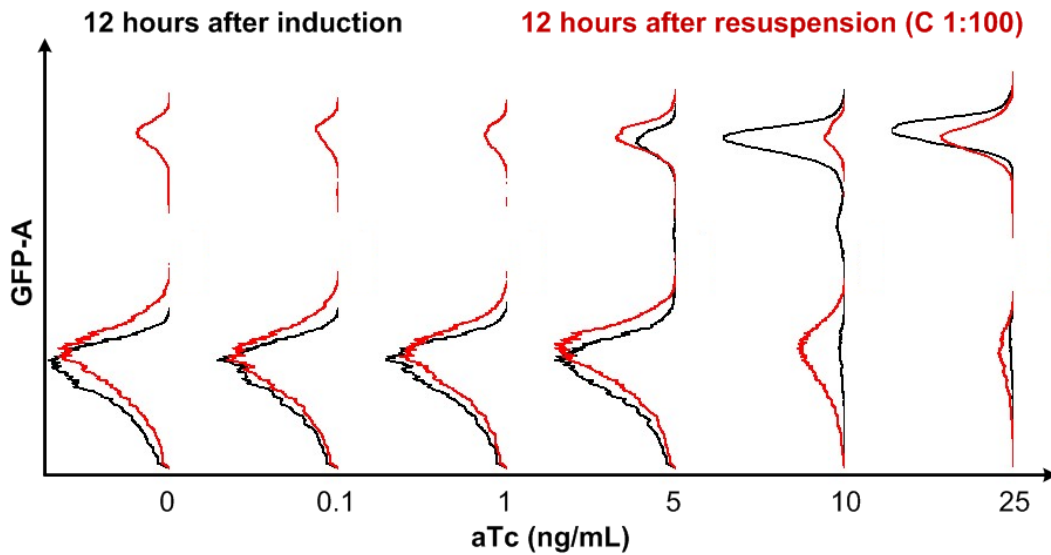


Figure 30: Hysteresis and bistability over the entire tested range of inducer concentrations for cultures resuspended using a 1:100 dilution ratio (data has been smoothed for clarity)

Although the resuspended cultures exhibited bistability and hysteresis for each dilution ratio, the overall magnitude of the hysteretic response varied with inducer concentration. This is demonstrated by the relative fluorescence hysteresis plots in Figure 27. Additionally, this effect can be seen in the flow cytometry data by looking at the proportion of cells that are ON. Figure 31 shows the proportion of cells that are ON after twelve hours at various inducer concentrations for the cultures with induction history (A, B, and C) and the cultures with no induction history. The relative fluorescence plot and the proportion of ON cells plot show a remarkably similar trend for each of the three dilution ratios. At low induction levels a smaller proportion of the cells in the culture are ON, and therefore the

relative fluorescence is small. At high induction levels, a larger proportion of the cells in the culture are ON, and the relative fluorescence is large. Although the trend is similar for each dilution ratio, the relative magnitudes between the dilution ratios are not.

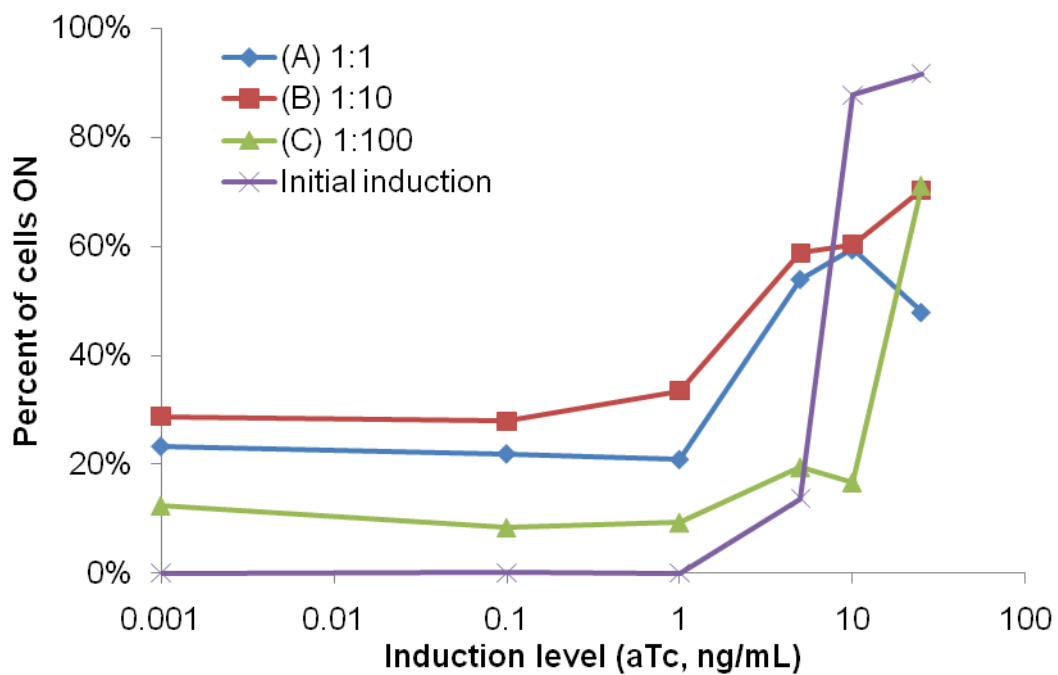


Figure 31: Proportion of cells that are ON after 12 hours for cultures with no induction history and cultures resuspended using dilution ratios A, B, and C

4.3 Mathematical description of hysteresis and bistability

For cultures with no induction history, bistability was observed at inducer concentrations of 5-25 ng/mL aTc. At high inducer concentrations of 10 and 25 ng/mL aTc, the system was bistable but only a small proportion of cells were in the OFF state (12% and 8% respectively). At 5 ng/mL aTc 86% of cells were in the OFF state. Therefore, the ON/OFF switching of the system occurs over a relatively small range of inducer concentrations. However, cultures with an induction history exhibited bistability over the entire range of inducer concentrations (0-25 ng/mL aTc) leading to irreversibility. Additionally, the proportion of cells that were ON at high inducer concentrations (10 and 25 ng/mL) was never as high as the proportion for the cultures with no induction history. Similarly, the proportion of cells that were ON at low inducer concentrations (0-5 ng/mL) was always higher than the proportion for cells with no induction history.

The model is useful for explaining the different regions of bistability for cells with an induction history versus cells with no induction history. The difference in bistable expression can be interpreted mathematically as a shift in the system's operating region of the bifurcation diagram, as shown in Figure 32. Initially, the system was operating in a region where varying inducer concentration caused the system to shift from monostable expression to bistable expression. After

resuspension, the system was operating in a region where there was bistable expression for all inducer concentrations. Similar interpretations have been reported previously [11].

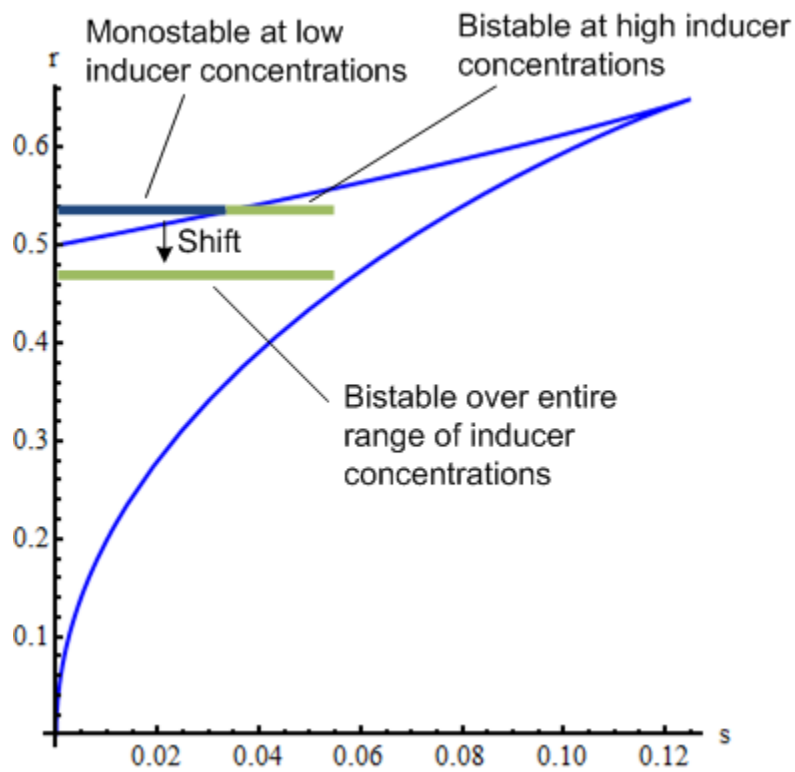


Figure 32: Differences in bistable expression for cultures with and without induction history can be interpreted as a shift in the system's operating region of the bifurcation diagram

CHAPTER 5: CONCLUSION

The modular positive feedback-based gene amplifier is capable of producing a hysteretic response to a stimulus. Cultures with no induction history exhibited bistability at high inducer concentrations (5- 25 ng/mL aTc), and cultures previously induced with 25 ng/mL aTc exhibited bistability regardless of their new induction level after resuspension. Therefore, the positive feedback cultures “remembered” their prior stimulus level. The underlying cause of this hysteretic response can be interpreted mathematically using the system bifurcation diagram. Cultures with no induction history operate in a region of the bifurcation diagram with a low monostable response at low inducer concentrations and a bistable response at high inducer concentrations. Cultures with induction history operate in a region of the bifurcation diagram with a bistable response at low inducer concentrations and a high monostable response at high inducer concentrations.

The overall magnitude of the hysteretic response depended on the dilution ratio used to inoculate the cultures with induction history. Large dilution ratios (1:1 and 1:10) produced higher GFP fluorescence corresponding to a higher proportion of cells in the population expressing at a high level. The small dilution ratio (1:100) produced a lower overall amount of GFP fluorescence corresponding to a smaller proportion of cells in the population expressing at a high level. Even at low

dilution ratios, the magnitude of the response was significantly greater than for the cultures with no induction history. Additionally, for each dilution ratio the magnitude of the hysteretic response varied with the final inducer concentration of the resuspended culture. Cultures resuspended to a higher induction level exhibited greater GFP fluorescence corresponding to a higher proportion of cells in the ON state than cells at low induction levels.

After resuspension, the proportion of cells that are ON decreases over time. This is primarily due to the higher growth rate of the OFF population relative to the ON population, which is burdened by producing LuxR and GFP. High dilution ratios provide less time for the culture to saturate because of the high initial concentration of cells, and there is less time for the OFF cells to grow and form a larger proportion of the population. Low dilution ratios provide more time for the ON and OFF populations to compete before the culture saturates, resulting in a higher proportion of cells in the OFF state. Additionally, the longer the cells experience exponential growth due to nutritional surplus after resuspension, the more GFP and LuxR get diluted inside individual cells, and a larger number of cells in the ON population will switch to OFF.

These results point to the importance of population dynamics on hysteresis. The overall magnitude of the hysteretic response a culture exhibits is dependent upon

different growth characteristics between ON and OFF cells. Smaller dilution ratios provide more time for high growth rates within a culture, during which GFP in ON cells becomes diluted and OFF cells grow faster than ON cells. An observable hysteretic response can potentially disappear if the population has sufficient time for the effects of dilution and growth rate difference to manifest.

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APPENDIX A: ADDITIONAL EXPERIMENTAL DATA

This appendix presents microplate and flow cytometry data not shown in the main text.

A.1 GFP fluorescence over time

This section demonstrates the GFP fluorescence levels over time of the cultures at aTc induction levels of 5, 1, 0.1, and 0 ng/mL. For the 68-5 culture (Figure 33), the population was bistable and steady-state expression was obtained between 12 and 26 hours after induction, even though the proportion of cells at each steady-state level changed during that time. For the 68-1 (Figure 34), 68-0.1 (Figure 35), and 68-0 (Figure 36) cultures, the populations were monostable and fluorescence was at steady state for all time values tested.

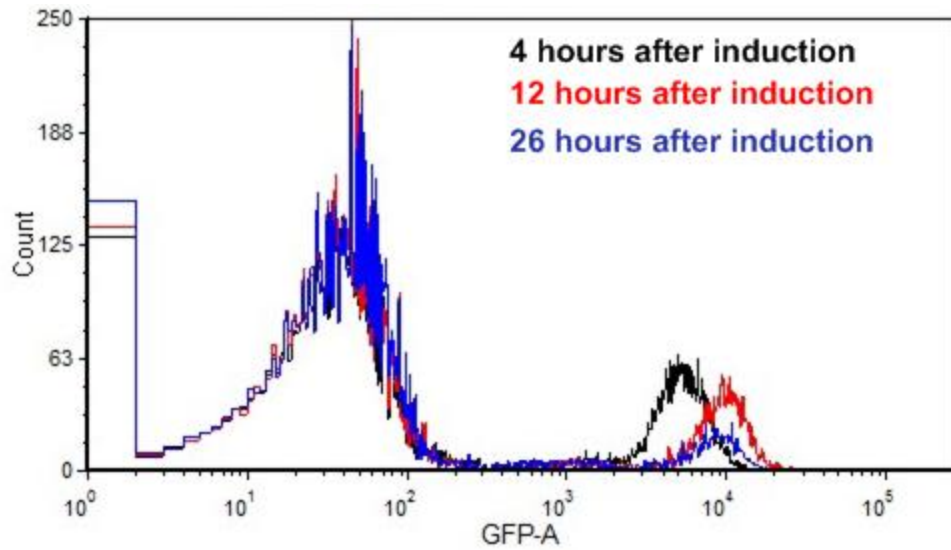


Figure 33: The 68-5 culture was bistable and obtained a steady-state level of GFP expression 12 hours after induction

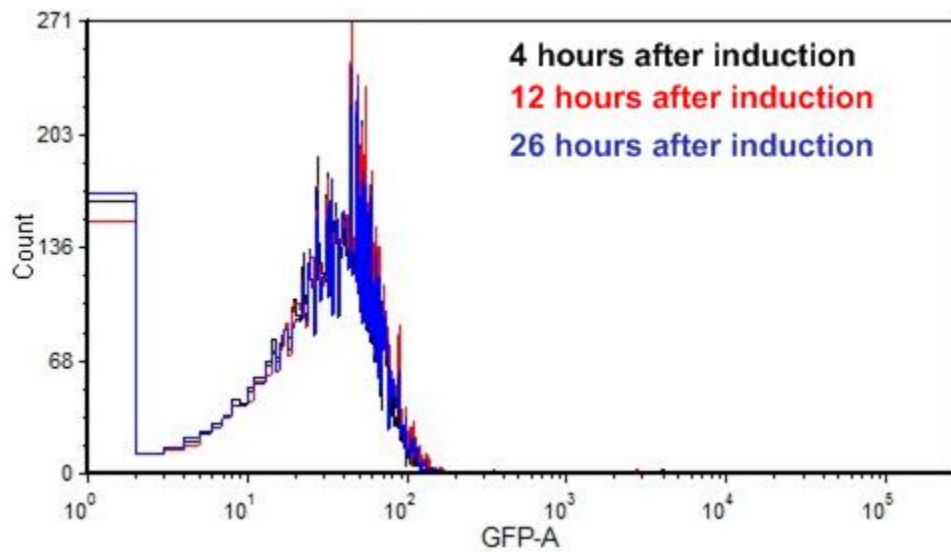


Figure 34: The 68-1 culture was monostable and expressed at a steady-state level for all time values tested

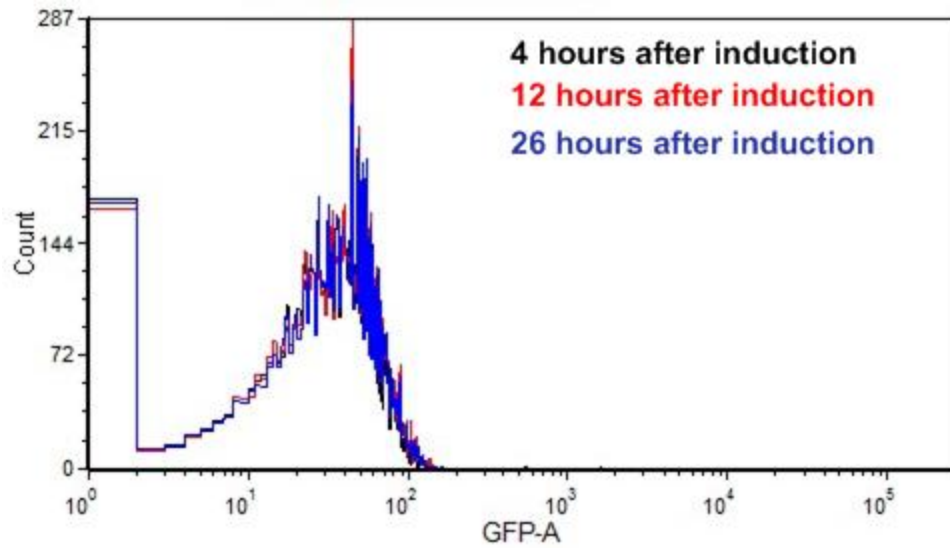


Figure 35: The 68-0.1 culture was monostable and expressed at a steady-state level for all time values tested

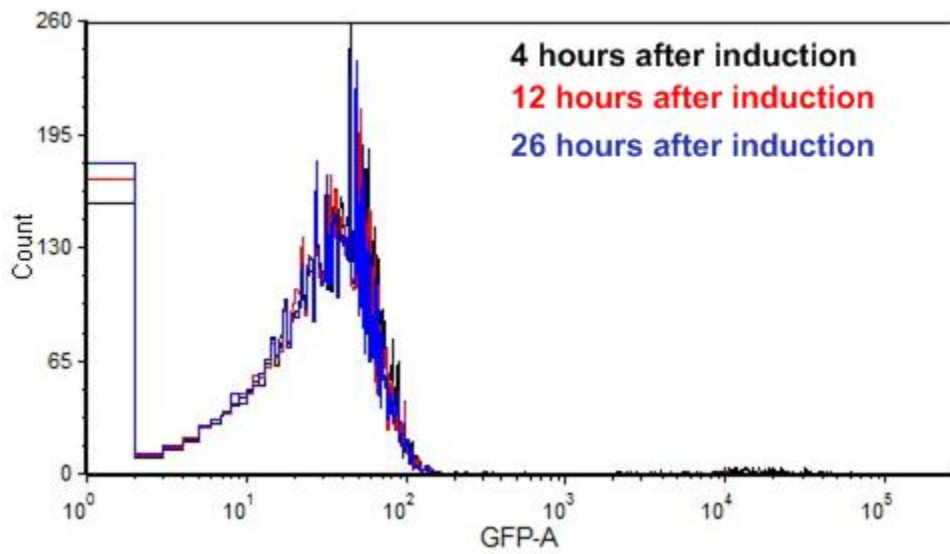


Figure 36: The 68-0 culture was monostable and expressed at a steady-state level for all time values tested

A.2 Cultures with a prior stimulus

The cultures resuspended into media with 0.1 ng/mL aTc showed the same trends as described in Section 4.2, and the data is shown in Figure 37 through Figure 40.

The culture initially induced to 0.1 ng/mL with no induction history was monostable, but all cultures with an induction history at 25 ng/mL exhibited bistability. Each resuspended culture displayed hysteresis, and the proportion of ON cells in each culture decreased from 4 to 12 hours after dilution. For the 68-0.1A culture (Figure 38) the proportion of ON cells decreased from 43% to 22%. For the 68-0.1B culture (Figure 39) the proportion of ON cells decreased from 48% to 28%. Finally, for the 68-0.1C culture (Figure 40) the proportion of ON cells decreased from 49% to 8% of the population.

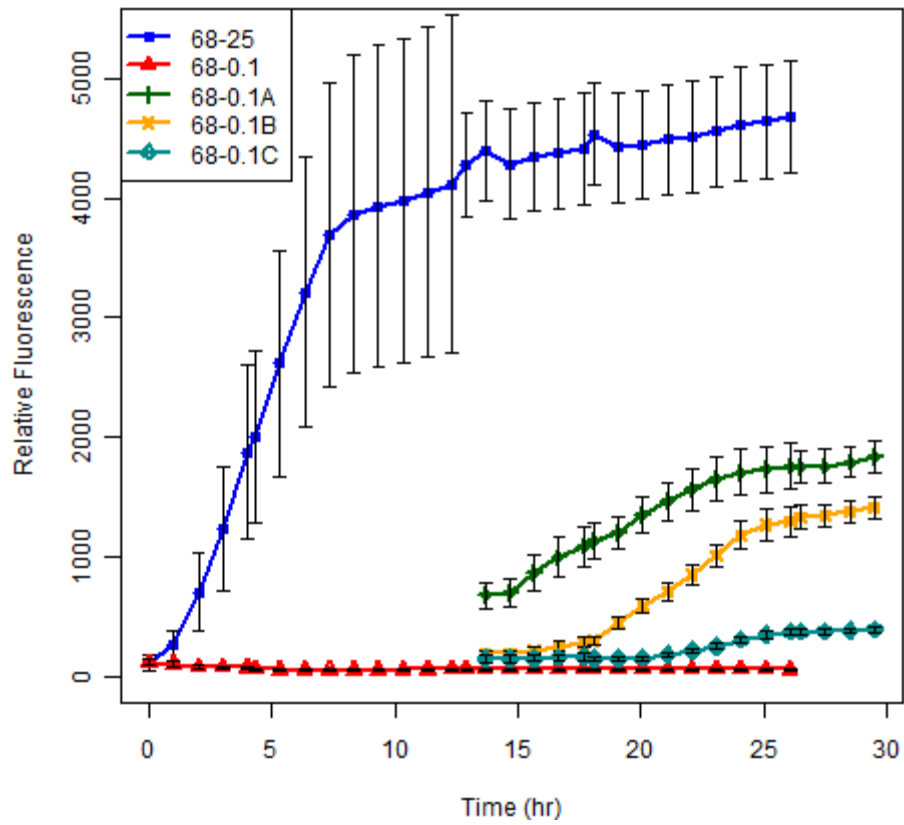


Figure 37: PF cultures previously at 25 ng/mL induction were resuspended into media with 0.1 ng/mL aTc using three dilution ratios (A, B, and C)

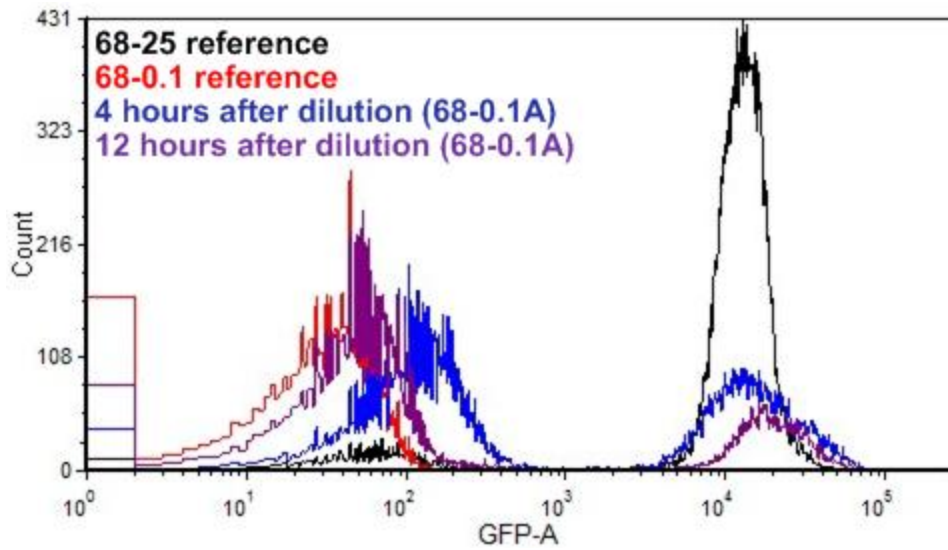


Figure 38: Cells initially induced using 25 ng/mL aTc then resuspended into media with 0.1 ng/mL aTc using a 1:1 dilution ratio (68-0.1A)

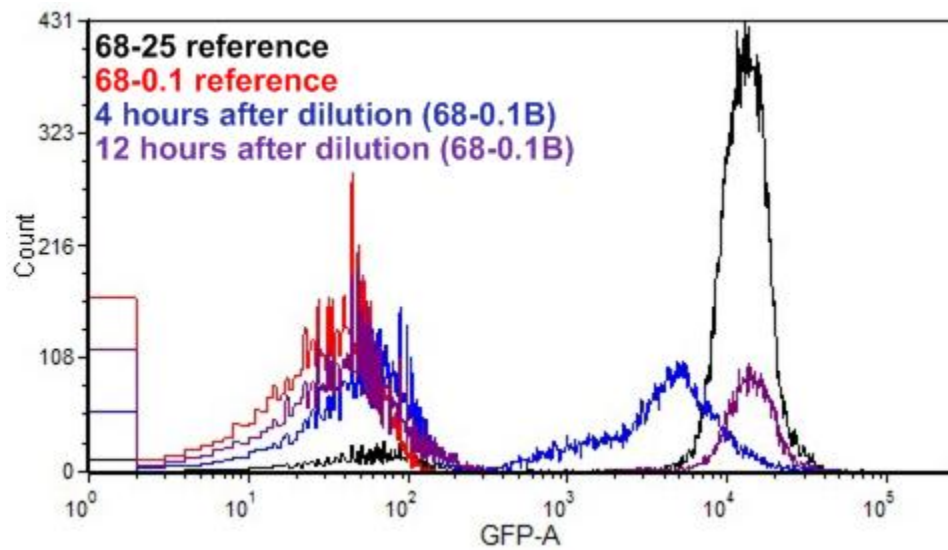


Figure 39: Cells initially induced using 25 ng/mL aTc then resuspended into media with 0.1 ng/mL aTc using a 1:10 dilution ratio (68-0.1B)

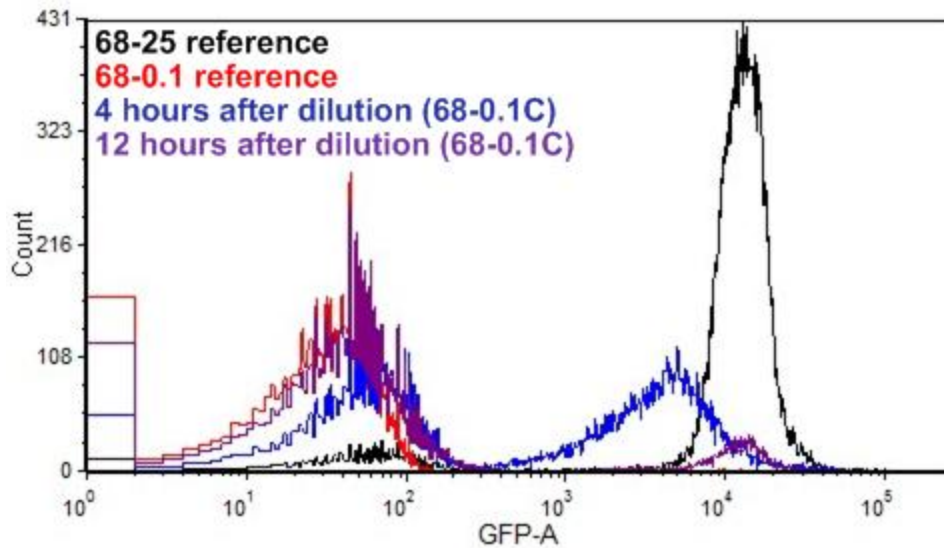


Figure 40: Cells initially induced using 25 ng/mL aTc then resuspended into media with 0.1 ng/mL aTc using a 1:100 dilution ratio (68-0.1C)

The cultures resuspended into media with 1 ng/mL aTc showed the same trends as described in Section 4.2, and the data is shown in Figure 41 through Figure 44.

The culture initially induced to 1 ng/mL with no induction history was monostable, but all cultures with an induction history at 25 ng/mL exhibited bistability. Each resuspended culture displayed hysteresis, and the proportion of ON cells in each culture decreased from 4 to 12 hours after dilution. For the 68-1A culture (Figure 42) the proportion of ON cells decreased from 40% to 21%. For the 68-1B culture (Figure 43) the proportion of ON cells decreased from 69% to 34%. Finally, for the 68-1C culture (Figure 44) the proportion of ON cells decreased from 65% to 9% of the population.

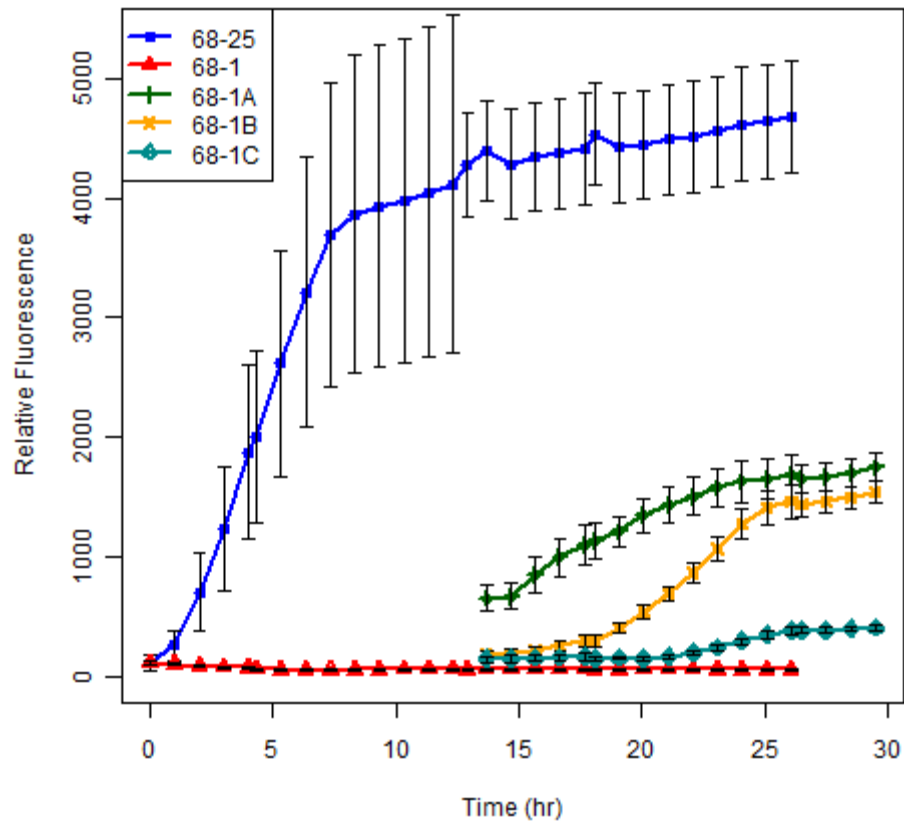


Figure 41: PF cultures previously at 25 ng/mL induction were resuspended into media with 1 ng/mL aTc using three dilution ratios (A, B, and C)

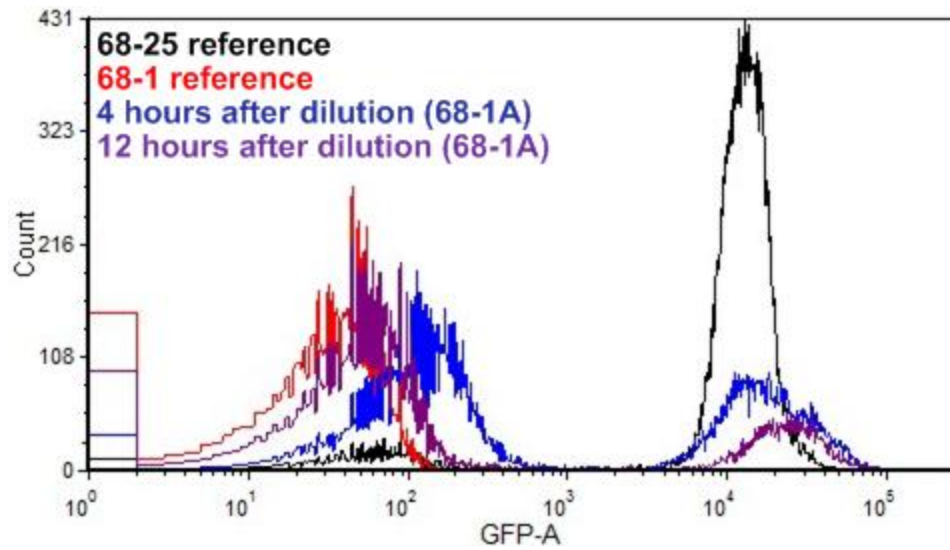


Figure 42: Cells initially induced using 25 ng/mL aTc then resuspended into media with 1 ng/mL aTc using a 1:1 dilution ratio (68-1A)

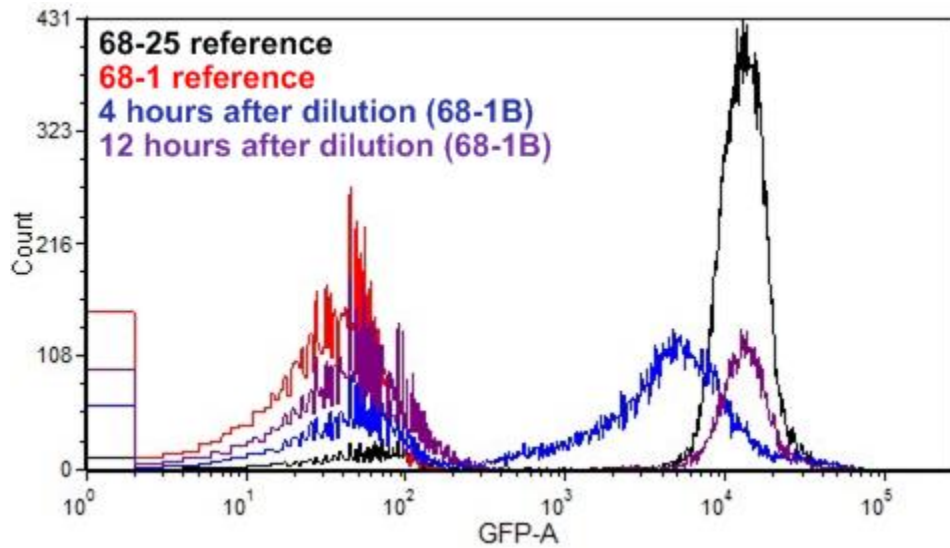


Figure 43: Cells initially induced using 25 ng/mL aTc then resuspended into media with 1 ng/mL aTc using a 1:10 dilution ratio (68-1B)

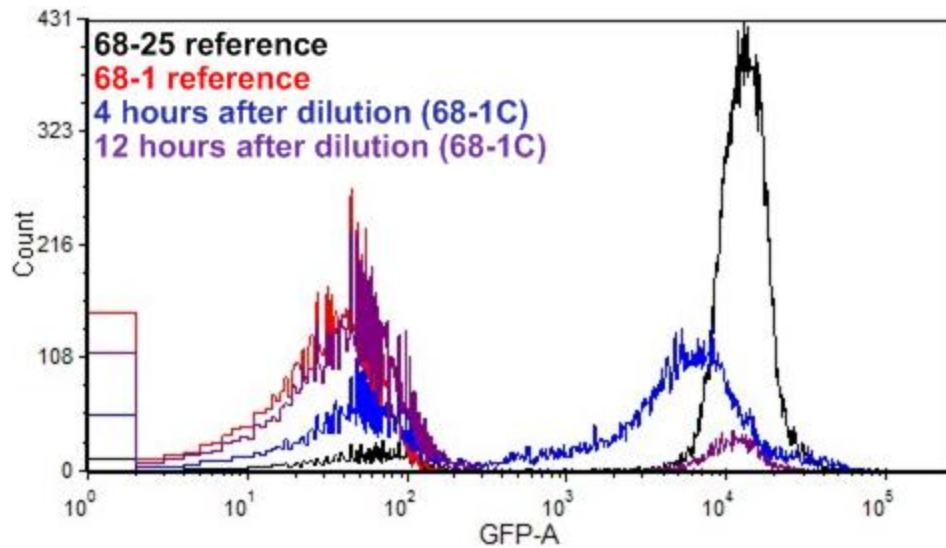


Figure 44: Cells initially induced using 25 ng/mL aTc then resuspended into media with 1 ng/mL aTc using a 1:100 dilution ratio (68-1C)

The cultures resuspended into media with 10 ng/mL aTc showed slightly different trends than the ones described in Section 4.2, and the data is shown in Figure 45 through Figure 48. Figure 45 shows that each resuspended culture displayed some degree of GFP fluorescence, but the overall relative fluorescence of the culture was not as high as the culture initially at a 10 ng/mL aTc induction level.

However, the flow cytometry data reveals that each resuspended culture did display hysteresis because the GFP fluorescence levels of the resuspended cultures after 12 hours were equivalent to the fluorescence level of the 68-25 culture. Additionally, the proportion of ON cells in each culture decreased from 4 to 12 hours after dilution. For the 68-10A culture (Figure 46) the proportion of ON cells decreased from 81% to 59%. For the 68-10B culture (Figure 47) the

proportion of ON cells decreased from 82% to 60%. Finally, for the 68-10C culture (Figure 48) the proportion of ON cells decreased from 83% to 17% of the population.

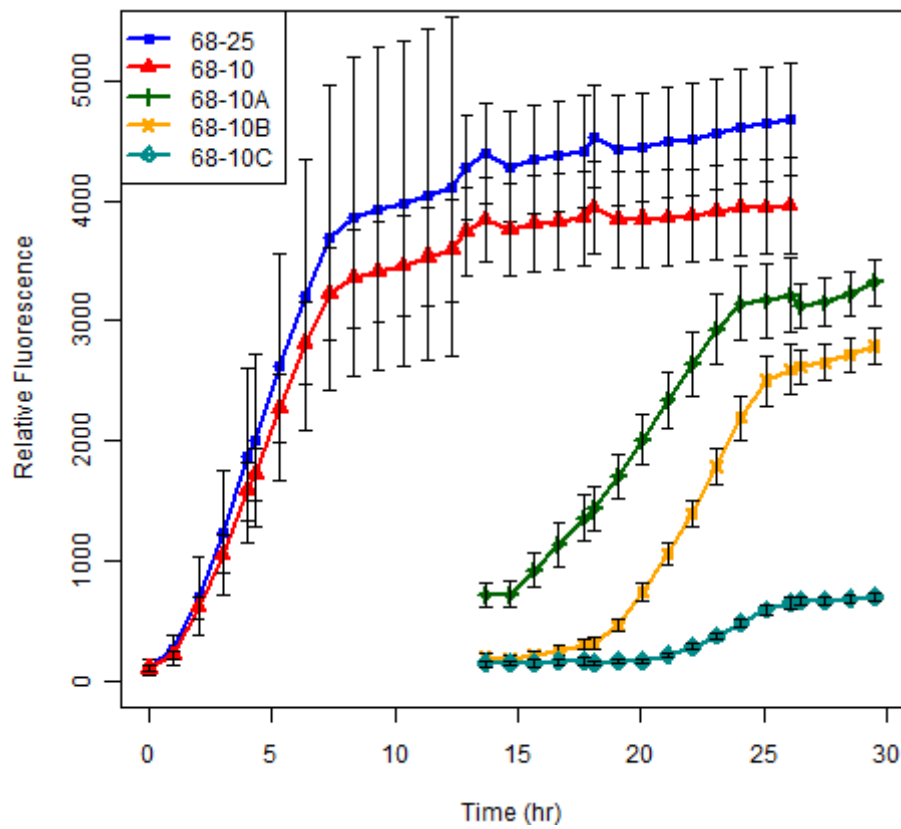


Figure 45: PF cultures previously at 25 ng/mL induction were resuspended into media with 10 ng/mL aTc using three dilution ratios (A, B, and C)

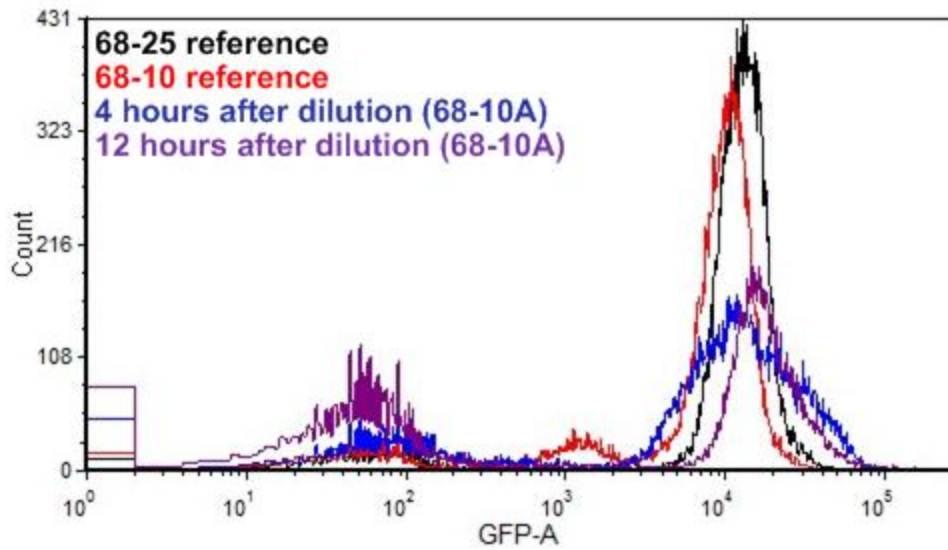


Figure 46: Cells initially induced using 25 ng/mL aTc then resuspended into media with 10 ng/mL aTc using a 1:1 dilution ratio (68-10A)

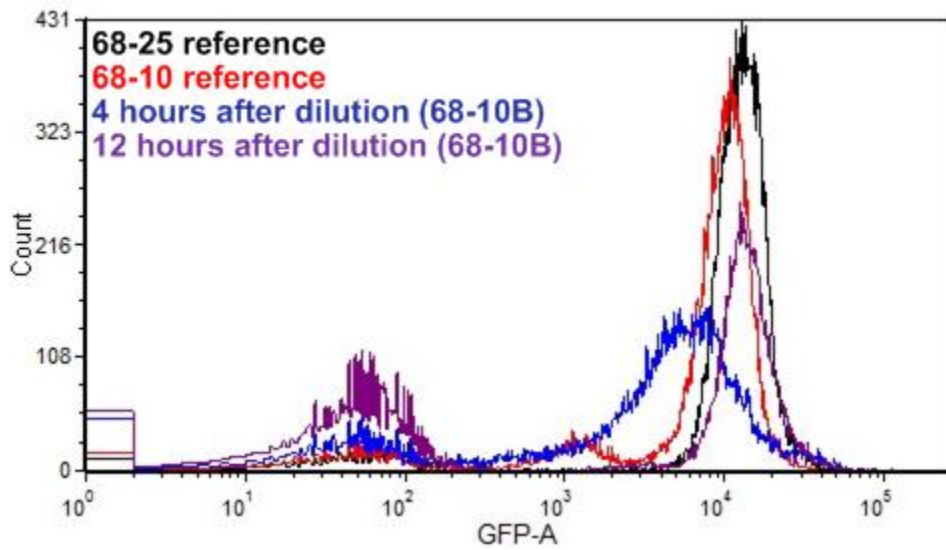


Figure 47: Cells initially induced using 25 ng/mL aTc then resuspended into media with 10 ng/mL aTc using a 1:10 dilution ratio (68-10B)

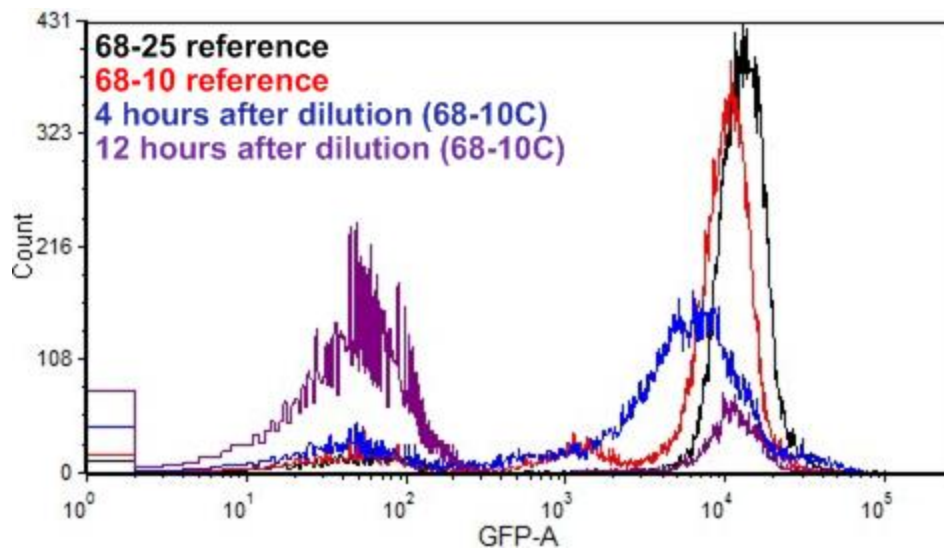


Figure 48: Cells initially induced using 25 ng/mL aTc then resuspended into media with 10 ng/mL aTc using a 1:100 dilution ratio (68-10C)

Three cultures were resuspended into fresh media with the same 25 ng/mL aTc induction level, and the data is shown in Figure 49 through Figure 52. Figure 49 shows that each resuspended culture displayed some degree of GFP fluorescence, but the overall relative fluorescence of the culture was not as high as the culture initially at a 25 ng/mL aTc induction level. The 68-25A culture (Figure 50) fluoresced at the same level as the 68-25 culture four and twelve hours after resuspension. However, during that time the proportion of ON cells decreased from 84% to 48% of the culture. The ON population of the 68-25B culture (Figure 51) fluoresced at a lower level four hours after resuspension, but twelve hours after resuspension the ON population fluoresced at the same level as the 68-25 culture. In that time period, the proportion of ON cells decreased from 83% to

70% of the culture. The ON population of the 68-25C culture (Figure 52) fluoresced at a lower level four hours after resuspension, but twelve hours after resuspension the ON population fluoresced at almost the same level as the 68-25 culture. In that time period, the proportion of ON cells decreased from 88% to 71% of the culture.

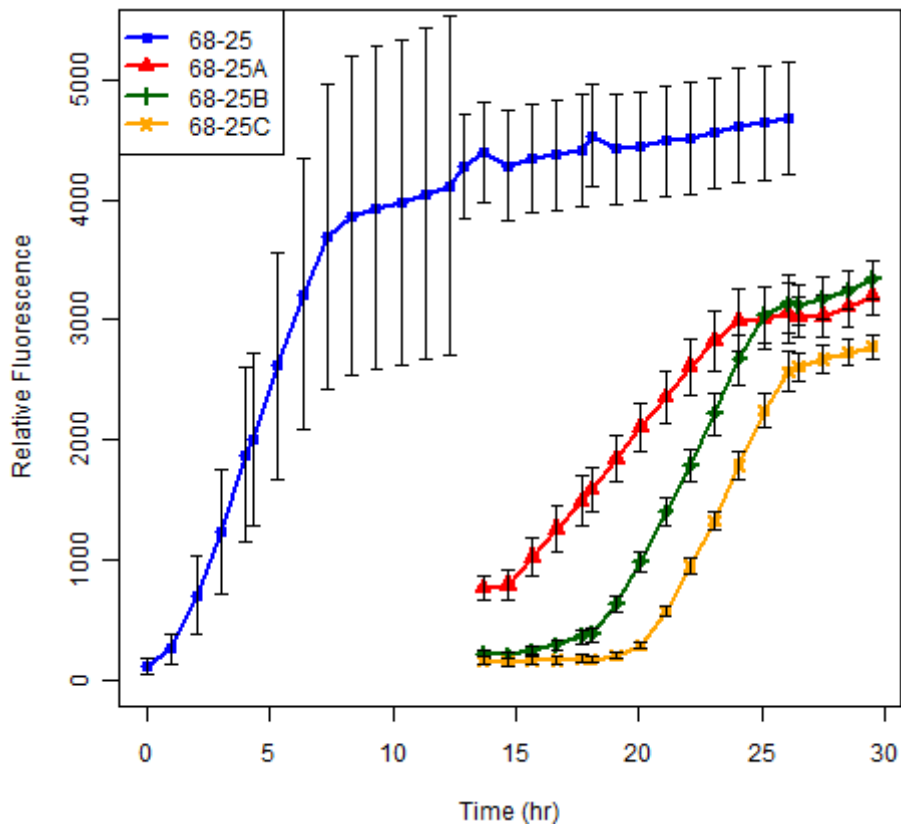


Figure 49: PF cultures initially induced into media with 25 ng/mL aTc were resuspended into fresh media at the same induction level using three dilution ratios (A, B, and C)

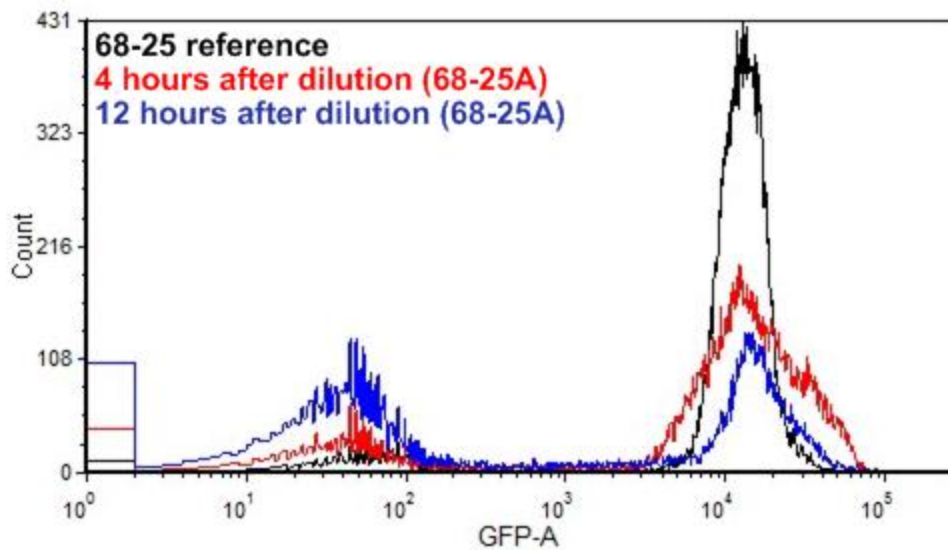


Figure 50: Cells initially induced using 25 ng/mL aTc then resuspended into fresh media with 25 ng/mL aTc using a 1:1 dilution ratio (68-25A)

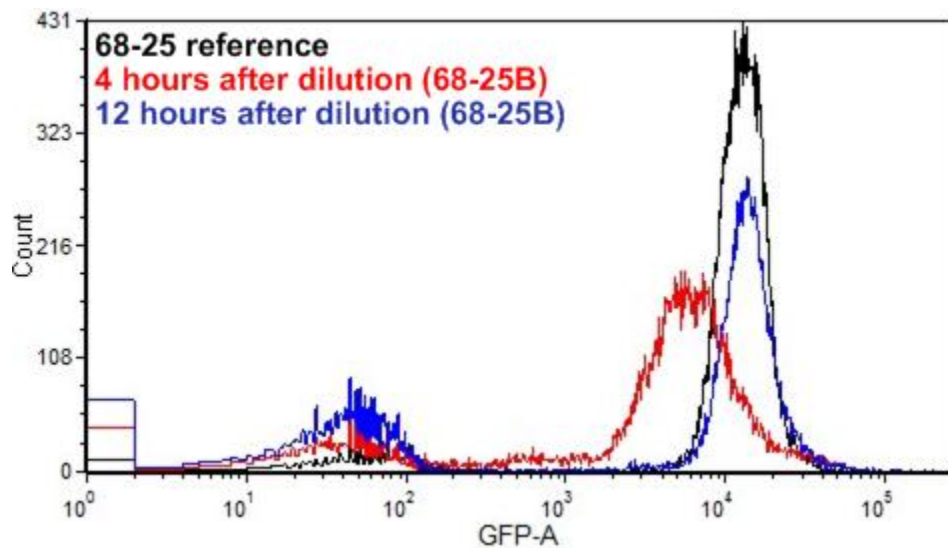


Figure 51: Cells initially induced using 25 ng/mL aTc then resuspended into fresh media with 25 ng/mL aTc using a 1:10 dilution ratio (68-25B)

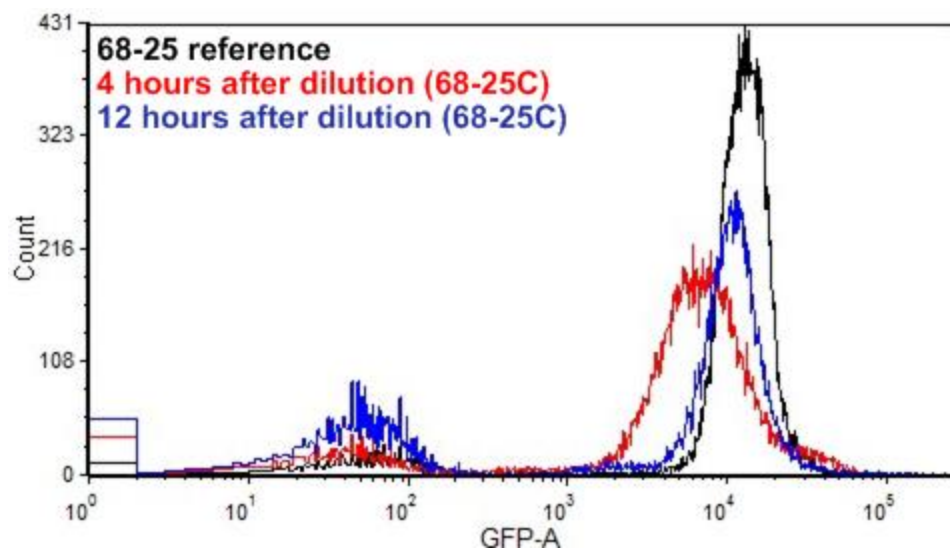


Figure 52: Cells initially induced using 25 ng/mL aTc then resuspended into fresh media with 25 ng/mL aTc using a 1:100 dilution ratio (68-25C)