Published in final edited form as:

J Biotechnol. 2009 March 25; 140(3-4): 203–217. doi:10.1016/j.jbiotec.2009.01.011.

Environmentally-Modulated Changes in Fluorescence Distribution in Cells with Oscillatory Genetic Network Dynamics

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Abstract

We investigated the distribution of green fluorescent protein (GFP) expression levels in a population of *E. coli* cells expressing an artificial genetic regulatory network, known as the “repressilator” (Elowitz and Leibler, 2000), which exhibits oscillations at the single-cell level. A series of shake flask experiments were performed and analyzed using flow cytometry to test how cell populations carrying this system could be controlled extracellularly using the inducers anhydrotetracycline (aTc) and isopropyl- β -D-thiogalactopyranoside (IPTG). With variation of [aTc], it exhibits bi-threshold behavior, such that the entire culture reaches one of three steady states at a quasi-time invariant “reference state.” Also, there is significant hysteresis. Transiently, the middle state shows damping oscillations, while the low and high states show a stable steady state. The addition of IPTG serves to fine-tune the characteristics of the aTc-only expression, lowering the average and CV of the distributions, and possibly perturbing the network to a different state. However, in modeling this system, the multiplicity and bi-threshold behavior are not theoretically possible according to the designed interactions. In order to explain this discrepancy, we hypothesize that one or more of the repressors has a significant nonspecific interaction with a promoter that does not contain its operator site. The new modeling results incorporating these extra interactions qualitatively match our experimental findings. After constructing plasmids to test these hypotheses, we discover that at least four of these interactions exist, which can create the low and high states and multiplicity seen experimentally. This genetic architecture has flexibility in its behavior that has not been demonstrated before, and the combination of experiment and modeling enlightened our understanding of the molecular interactions driving the network's behavior, leading us to discover the significance of nonspecific interactions.

Keywords

heterogeneity; repressilator; GFP; flow cytometry

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Introduction

Cell population heterogeneity is a term used to describe the cell-to-cell phenotypic variability that exists in all isogenic cell populations. This phenomenon is well-known, and has been studied in a variety of biological systems ever since the 1940s. A sampling of the experimental efforts include Delbrück's observations of phage burst size variability (Delbrück, 1945), Powell's studies on how the range of cell division times changes with different bacterial strains and conditions (Powell, 1956), variation in *lac* operon expression levels by Novick and Weiner (1957) and Maloney and Rotman (1973), studies on spore formation in *B. subtilis* (Chung and Stephanopoulos, 1995), and work to differentiate and find inducer-dependence of extrinsic and intrinsic sources of heterogeneity by using artificial genetic networks in *E. coli* (Elowitz et al., 2002).

This heterogeneity comes from several sources. One source is the microenvironment surrounding the cells. Cells can detect small differences in concentrations of a food source or toxin, for example, and may behave in a different way accordingly. Another source is differences in cellular content. This mainly comes from cell division, or more specifically, the unequal partitioning of cellular content from mother cell to daughter cells upon division. Division of cellular content has been experimentally shown to be unequal for most molecules other than chromosomal DNA (Sweeney et al., 1994; Tran et al., 2000; Kellehrer et al., 2000; Bellaiche et al., 2001; Orgogozo et al., 2002). Each daughter cell starts with different content, and therefore exhibits a different phenotype. This has been termed extrinsic heterogeneity. Yet, even if two cells had the exact same content, their phenotypes would differ due to another source of heterogeneity termed intrinsic heterogeneity. This refers to the random fluctuations that exist in reactions such as those between metabolites, or between DNA and proteins or other molecules, etc. This is due to the fact that molecules often exist in such low concentrations in the cell that their reactions occur randomly. Phenotype is mainly determined by gene expression, which is monitored and controlled by regulatory molecules. These molecules are often found in very low concentrations in the cell, making their actions purely stochastic. Therefore, cell population heterogeneity and regulation of gene expression share a strong connection.

Gene expression has complex regulatory mechanisms. Regulation occurs at all steps of gene expression, from before transcription to after translation (Alberts et al., 2002). Bacterial gene expression is thought to be mainly regulated at the transcriptional level (Thieffry et al., 1998; Weinzierl, 1999). At this level, regulatory molecules bind to DNA to promote or prevent gene expression.

A multitude of naturally-occurring genetic networks that control gene expression exist, but they are highly interconnected, and in many cases their functions are unknown. An alternative approach is to design a genetic network *de novo*, such that the connections are known and the elements are well-defined. These networks can be designed and combined to exhibit a particular behavior or phenotype (Guet et al., 2002), and are simpler to study than natural networks. The possibilities for design are endless, as are the biomedical and biotechnical applications that could use this technology. However, before any network can be used in an application, it must be fully characterized, which includes investigation into its relationship with cell population heterogeneity.

Several groups have already designed and characterized simple artificial genetic networks. Becksei *et al.* studied basic negative and positive feedback architectures, demonstrating the effect of various components or conditions on gene expression (Becksei et al., 2000; 2001). Two groups have been successful in designing oscillators (Elowitz and Leibler, 2000; Fung et al., 2005), and others have designed a bistable switch (Chen et al., 1993; Gardner et al.,

2000). Our focus in this work is on the genetic oscillator known as the repressilator, made by Elowitz and Leibler (2000). The design involves three promoter-repressor pairs, which form a cyclic, cascading network of negative feedback loops (see figure 1a). The three repressors are the *lac* repressor, the λ *cI* repressor from phage λ , and the *tet* repressor from the tetracycline-resistance transposon Tn10. The genes are arranged such that the *tet* repressor inhibits the λ repressor, which inhibits the *lac* repressor, which in turn inhibits the *tet* repressor. The P_LtetO-1 promoter controls expression of the λ repressor, the λ -P_R promoter controls expression of the *lac* repressor, and the P_LlacO-1 promoter controls expression of the *tet* repressor. All repressors contain a *ssrA* tag to reduce their half-life. Figure 1b shows the plasmid designs for the Repressilator plasmid holding all the regulatory genes, and for the Reporter plasmid which expresses a green fluorescent protein (GFP) mutant with a half-life of approximately 60 min (Andersen et al., 1998). GFP is a marker for the λ repressor, since both are controlled by the same promoter. Theoretically, this system can reach a stable steady state (dominant expression of one repressor) or an unstable steady state (exhibits oscillatory behavior) (Elowitz and Leibler, 2000), and Elowitz and Leibler confirmed with fluorescence microscopy that their network did oscillate in at least 40% of the cells.

In the work presented by Elowitz and Leibler (2000), the authors designed a network made from three promoter-repressor pairs via modeling, analyzed it theoretically to gain insight on how to best design the components to reach an unstable steady state, built it, and confirmed its behavior experimentally. Fluorescence microscopy was used to determine the network behavior at a single-cell level. One of the chemical inducers for the system, isopropyl- β -D-thiogalactopyranoside (IPTG), which complexes with the *lac* repressor, was used to synchronize the network oscillations at the beginning of an experiment, and was used in control experiments. Anhydrotetracycline (aTc), which complexes with *tet* repressors, is the other extracellular inducer for the system, but it was not used in experiments. The concentration of inducers is a user-controlled parameter that could provide additional flexibility in using this network biotechnologically, and this has not been investigated.

The Repressilator plasmid has a copy number of 3-4, and the Reporter plasmid has a copy number of 50-70 (Lutz, 1997). It is unknown exactly why the authors chose to have two plasmids at these particular copy numbers. We suspect that they wanted to keep the number of repressors tightly regulated by keeping the copy number of the regulatory genes low. However, in order for GFP expression to be visible on the microscope, GFP had to be expressed on a higher copy number plasmid. For several reasons, we decided to combine the two-plasmid system into one plasmid. First, it is more difficult for cells to carry two plasmids than one. It is more of a burden to the cell, and it requires two antibiotics (which becomes expensive). In choosing which antibiotic marker to use, the kanamycin marker was chosen. Since the ampicillin resistance gene encodes for an enzyme that can function in the extracellular media, cultures have been known to exhibit plasmid instability when carrying this marker. The kanamycin marker expresses an intracellular protein, so it does not have this problem. Furthermore, keeping the concentration of repressors low in the cell contributes to stochastic, intrinsic heterogeneity. It was noted by the authors that cells often lost the ability to oscillate after division. We thought that, perhaps, if the number of regulatory molecules was increased, and partitioning was not very unequal, then the cells might be more likely to continue oscillating after cell division. Spudich *et al.* also applied this reasoning when considering synchronization of cell growth (1976): they hypothesized that the quick asynchrony they observed was due to low concentrations of enzymes, and that significantly increasing the concentration of rate-limiting enzymes involved with the cell cycle could allow cultures to remain synchronized. There are stochastic modeling studies that also support the idea that redundancy can help establish a consensus among cells (Rao et al., 2002; McAdams and Arkin, 1999). For these reasons, a high-copy plasmid was used for the construct. In this way, GFP would still have high expression levels. As a side note, in the two-plasmid system, the number of *tet* operator

sites (binding sites for repressors) was more than 10-fold higher than the number of *lac* and λ operator sites due to the inequality of the two plasmids' copy numbers. Although this ratio of operator sites was a "perturbation" on the system due to the titration effect on *tet* repressors (Elowitz and Leibler, 2000), the ratio must have helped, not hindered, the ability of the network to oscillate. In combining the two plasmids, the number of operator sites becomes symmetrical, so we may need to titrate the *tet* repressors in order to achieve oscillation, assuming that this behavior is still possible. The one-plasmid construct is called pSCP102, and its map is found in figure 1b. Its construction is outlined in the Materials and Methods section.

After making the one-plasmid construct, pSCP102, we performed experiments to better understand the potential of this system. We investigated this network's full range of expression by running a series of shake flask experiments with both a single inducer, aTc, and with mixed inducers, using aTc and IPTG. Flow cytometry was used to measure the distribution of GFP in the cells. This high-throughput method quantifies light scattering and fluorescence properties of individual cells. The relevant parameters to this study are the two light scattering properties, forward scatter (FSC) and side scatter (SSC), and green fluorescence (FL1).

Materials and Methods

Strain

E. coli strain MC4100 (*lac*⁻) was used. The cells were made chemically competent and transformed with plasmid pSCP102 as described in RbCl Transformation Procedure for Improved Efficiency (1994). This procedure was used for all plasmids tested, always in MC4100.

Plasmid construction for main experiments

To raise the copy number of the regulatory genes and to use a kanamycin resistance marker, the regulatory genes from the Repressilator plasmid were inserted into the Reporter plasmid. Since compatible restriction enzyme sites were not found in the locations desired, PCR amplification was used for both plasmids to add the desired restriction enzyme site at one end of the fragments to be combined. Standard methods were used for construction of the plasmid and polymerase chain reaction (Miller, 1992; Sambrook and Russell, 2001). The regulatory genes were PCR amplified from the Repressilator plasmid, from P_LlacO-1 to cI, ending before the terminator. The following primers (obtained from Integrated DNA Technologies, INC.) were used: N-terminus, 5'-CAC CTC GAG AAT TGT GAG CGG -3' (all bases match template, *Xho*I is in italics), C-terminus, 5'-GCA CGT ACG CTC TAG ATC AAG CTG CTA AAG CG -3' (bases in bold match template, added a *Bsi*WI site (in italics) upstream of *Nco*I). In the Reporter plasmid, the entire plasmid was replicated between the beginning of the *gfp*aav gene and after KmR, excluding the P_LtetO-1 sequence, since *gfp*aav would be placed directly after the cI gene. The following primers were used: N-terminus, 5'-GCA CGT ACG AGG AGA AAG GTA CCG C -3' (bases in bold match template, added a *Bsi*WI site (in italics) before the *gfp*aav, keeping the *gfp*aav RBS sequence), C-terminus, 5'-GGA CTC GAG GTG AAG ACG -3' (all bases match template, *Xho*I is in italics). Platinum *Pfx* DNA polymerase (Invitrogen) was used for DNA amplification with a RoboCycler Gradient 96 Temperature Cycler (Stratagene). The PCR products were cleaned using Wizard PCR Preps (Promega). The products were then cut by restriction digest with *Xho*I and then with *Bsi*WI. After digestion, the fragments were isolated from a 0.8 % NuSieve GTG agarose (ISC BioExpress) tris-borate-EDTA gel using a Qiagen, Qiaex II Gel Extraction Kit. The fragments were then ligated together using T4 high concentration DNA ligase (New England Biolabs). The ligation reaction was transformed into TOP10 SuperCompetent Cells (Invitrogen) using the "One-Shot Transformation Reaction" procedure in TOPO TA Cloning Kit. Transformants were selected on (Luria-Bertani) LB agar plates containing kanamycin. After selection, plasmid was isolated

from LB broth cultures and screened with *HpaI*. The sequences around *XhoI* and *BsiWI* were confirmed by sequencing (Lone Star Labs, Inc.), and the plasmid was named pSCP102. The plasmid was then transformed into MC4100 chemically competent cells using the RbCl procedure (1994).

Plasmid constructions for testing interaction hypotheses

Since appropriate restriction enzyme sites were not available in most cases, PCR (using either Platinum *Pfx* DNA Polymerase or PfuUltra High-Fidelity Polymerase, Stratagene) was necessary for nearly every construct. The resulting fragment(s) was either digested with appropriate restriction enzymes or underwent a kinase reaction (T4 Kinase, New England Biolabs). Either a unimolecular or bimolecular ligation followed with T4 DNA Ligase or Quick T4 DNA Ligase (New England Biolabs). Colonies were transformed either into TOP10 SuperCompetent Cells or chemically competent DH5 α cells before being transformed into MC4100 (RbCl procedure, 1994). All promoters, genes, and terminators were confirmed by sequencing.

Shake flask experiments

There were four sets of experiments, two with aTc only, and two with aTc and IPTG as inducers. All of them kept the cells in exponential phase in a narrow OD₆₀₀ range with frequent subculturing steps. The basic procedure is common between them. First, cells were grown overnight at 32°C, shaking at 250 rpm in an orbital shaker (New Brunswick Scientific), in 5 ml of LB medium (4 g l⁻¹ NaCl, 10 g l⁻¹ BactoTryptone (BD Biosciences), 5 g l⁻¹ yeast extract (Fisher BioTech), 50 mg l⁻¹ kanamycin (Sigma-Aldrich)) for 12 h and then subcultured at an OD₆₀₀ of about 0.0001 (~200,000 cells ml⁻¹) in 50 ml of prewarmed and aerated LB medium without inducers in a 250 ml Erlenmeyer flask capped with foam to allow oxygen transfer. In order for the culture to reach exponential phase, it was grown at the same conditions until it reached an OD₆₀₀ of approximately 0.01. Then the culture was exposed to inducers. It was subcultured 1:9 in 45 ml fresh, warmed, aerated LB with the appropriate concentration of aTc (Acros Organics) and IPTG (Fisher BioTech). Subsequent dilutions in the same ratio were made in fresh media (4 to 6 times total) every time the OD₆₀₀ reached approximately 0.01. Samples were withdrawn about three times per flask (about once a generation) until the culture's average fluorescence (as determined by flow cytometry) did not change. Cell density was measured using a spectrophotometer (Bausch & Lomb, Spectronic 1001) at 600 nm. Cells were immediately washed twice in cold PBS (58 mM Na₂HPO₄, 17 mM NaH₂PO₄, 68 mM NaCl, in Milli-Q H₂O, pH adjusted between 7.3 and 7.4, filter sterilized). The samples were kept on ice and shielded from light before being measured with the flow cytometer.

aTc-only experiments

The culture was either started in an uninduced state or an induced state. In the first case, after reaching exponential phase, it was exposed to aTc at concentrations between 1 and 1000 nM. In the second case, it was first grown at 1000 nM aTc for two subculturing steps before being subcultured into media with a lower concentration of aTc, between 1 and 850 nM. Triplicate experiments were performed.

aTc-IPTG experiments

In the third set of experiments, cells were grown in media containing aTc (at 500 or 1000 nM) and IPTG (0 M to 600 μ M) immediately after reaching exponential phase. In the fourth set, they were grown in media containing only aTc (1000 nM) for two subculturing steps before being subcultured into media containing the same concentration of aTc with the addition of IPTG (50 to 600 μ M).

Control experiments

Experiments were set up to confirm that the oscillation in average fluorescence that was observed was not an artifact of the successive dilutions of the culture. Duplicate experiments were performed – once the culture was returned to exponential phase after being grown overnight, it was diluted by 10 in one flask, and by 20 in another, such that the culture in the second flask would be subsequently diluted one generation later than the first. This yielded identical evolutions of fluorescence over time, showing that the culture was not affected by the act of dilution.

Interaction hypothesis experiments

The same general procedure described above in “Shake flask experiments” was followed, except no inducers were used. Only 2 dilution steps into fresh media were used for these experiments, instead of 4 or 6, long enough for the cultures to reach steady averages and distributions of green fluorescence. Triplicate experiments were performed when a non-negligible interaction was detected.

Flow cytometry

The flow cytometer used was a FACScalibur (BD Biosciences), with a 15 mW, 488 nm, air-cooled argon-ion laser. All parameters were logarithmically amplified, with the following settings: FSC E01, SSC 381 V, FL1 700 V, FL2 500 V, FL3 675 V. A side scatter threshold was applied to gate out much of the noise (at channel 130). During acquisition, all events were saved until the software counted 5,000-10,000 events with a minimum FSC channel value of 512 (assumed to be cells) for each sample. BD Calibrite Beads (BD Biosciences) were used to check that there were no significant changes in the flow cytometer performance over time. In the interaction hypothesis experiments, the FL1 setting was lowered to 500 V, due to the strength of the GFP expression.

Gating data

Gates were applied in the post-processing of the flow cytometer files according to light scattering. Since *E. coli* cells are so small and close to the size of the debris being sensed by the cytometer, gates were applied to better identify the cells. After observing the change in light scattering values while the culture was in exponential phase, FSC and SSC gates were applied to include events above a linear value of 80 (channel value of 487) and 40 (channel value of 410), respectively.

Software

First, the listmode files from the flow cytometer were converted from fcs format to ascii format with MFI software (Martz, 1992), after confirming that FACScalibur data can be successfully converted using MFI. Data were then processed with a FORTRAN code we developed to apply the aforementioned gates and calculate average fluorescence per cell and number density functions, excluding events in the first or last channel (Leary, 1998). Graphs were constructed with Kaleidagraph (Synergy Software, Version 3.52). Plasmid maps were constructed using the Savvy version 0.1 online program (2001).

Experimental Results

In order to make meaningful comparisons between the fluorescence distributions for the various conditions, it is important to determine when the cells are in a similar physiological condition. Although comparisons are often made between cultures at a particular point in time (Gardner et al, 2000; Khlebnikov and Keasling, 2002), factors such as growth phase, inducer concentration (which in our case changes the doubling time from approximately 30 to 50

minutes), and cell concentration can affect the physiological characteristics of cultures. The most preferable state at which to compare the cultures is that of balanced growth, a state reached during exponential phase when the number density functions of every characteristic describing the culture are non-changing over time (see Fredrickson et al., 1967 for a thorough discussion). This theoretical state is not possible to confirm experimentally, but the idea of reaching a stable state was mimicked: our requirements for the “reference state” are that the light scattering averages, green fluorescence averages, and green fluorescence distributions are relatively time-invariant.

The shake flask procedure that was established for these experiments (see Materials and Methods section) facilitates reaching this state. By repeatedly subculturing the cells, thereby keeping cells in the exponential phase at similar OD₆₀₀ values during the entirety of the experiment, the FSC and SSC values stay constant (except near points of perturbation). Once the fluorescence properties stabilize, the reference state is determined. It can be reached in a few generations or after many generations, depending on the experimental conditions. This is in contrast to our previous work, in which light scattering and fluorescence parameters plateau during the same period of time (Portle et al, 2007).

Using shake flask experiments and applying the requirements to find reference states, the oscillatory system was characterized by varying the concentration and order of inducers. The system was found to have many interesting features. The aTc-only experiments revealed three steady states with hysteresis, population level average dynamics that show oscillatory behavior and other interesting features, and distinct number density functions for each state. In the mixed aTc-IPTG experiments, greater flexibility is seen in the ability to control average fluorescence and coefficient of variation, and the order of addition of IPTG determines the inducer's effect on the dynamic behavior of the culture.

Experiments with aTc reveal three steady states with hysteresis

In the single-inducer experiments, aTc was chosen instead of IPTG. Without any inducer, expression of GFP is low. In order to differentiate the GFP expression under varying conditions, higher levels of GFP needed to be reached. As the aTc concentration increases, more *tet* repressors become bound to aTc. This depletes the *tet* repressor population bound to the *tet* operator sites, which, according to the network architecture (refer to figure 1a), increases the frequency of GFP expression.

The aTc concentration was varied, starting from both uninduced and induced conditions, and the average fluorescence at the reference states, average fluorescence over time, and corresponding number density functions were determined.

First we look at the data at the reference states for each condition.

As expected, as [aTc] increases, the average fluorescence of the culture increases. However, the increase is not monotonic; instead, as shown in figure 2, the system exhibits bi-threshold behavior, and the cultures reach one of three steady states. The low state is near a relative fluorescence value of 10, the middle state near 80, and the high state near 240. No other artificial genetic network yet made has been shown to reach three distinct states.

It is clear from figure 2 that there is a considerable amount of overlap between the three states. Hysteresis is seen between the low and middle states at [aTc] = 100-150 nM, and is seen between the middle and high states at [aTc] = 500-850 nM. The variations in ending state were found when experiments were performed on different days or with different initial states (uninduced or induced). Starting from different initial states allowed us to view more complete, expanded ranges of multiplicity.

The number density functions (ndfs) are unique and consistent in shape for each state, regardless of the initial condition. Figure 3 shows sample ndfs for each state: 3a from experiments starting with uninduced cultures, 3b from experiments starting with induced cultures. One can see how the two sets almost mirror each other, and ndfs within each state overlap each other.

Comparing the characteristics of the number density functions for each state leads to several observations. For one, the middle state is not a combination of the low and high states. The average fluorescence values of the middle state seen in figure 2 are not the result of some cells in the population being at the high state and the rest being at the low state – the cell population is centered in between the high and low states. A related statement that may seem obvious, but has deeper implications, is that, since the number density functions for one state are the same and unimodal, *all* cells in the culture choose one path or another. Whatever combination of factors exist that cause a cell to reach one state or another, it is felt in the same way by the entire population.

Something that sets the middle state number density function apart is that, while the low and high state distributions look symmetrical on this logarithmic scale, the middle state distribution does not. It looks flattened and has a long right tail. The distribution of green fluorescence (and therefore that of GFP expression in individual cells) is more heterogeneous than in the low and high state.

Transient studies reveal middle state is oscillatory while others are stable

Although cultures at many different aTc concentrations end up in the same state, the path they take to get there varies. Figure 4 shows the average fluorescence over time exhibited when aTc is first introduced to the system. Figures 4a, 4b, and 4c include dynamics that asymptotically reach the low, middle, and high state, respectively. The middle state is oscillatory, even at the population level, while the low and high states are not. We suspect that the middle state corresponds to an unstable steady state, and the low and high states correspond to stable steady states. aTc serves the same purpose as the extra *tetO* sites on the Reporter plasmid in the original two-plasmid system – it titrates out free and DNA-bound *tet* repressors, and, in a certain range, allows the network production of GFP to oscillate.

The single-cell behavior of cells in the middle state may help explain the wider distribution of this state. When aTc is added at time = 0 min, the culture is synchronized to an extent, and therefore oscillations, though damped over time, can be observed on average. The damping effect is an inevitable result of heterogeneity. Since there is no external control to synchronize the oscillations, individual cells may become desynchronized and still oscillate, or some cells may partition the regulatory components so unequally that oscillation ceases completely. If some cells are still oscillating at later time points (but desynchronized), even after the population average fluorescence is unchanging, then this variation in single-cell fluorescence levels could account for the larger CV of the middle state. For the low and high states, since the GFP expression is steady, the distribution of fluorescence could be narrower.

Each state has average fluorescence dynamics that give insight into the relationship between aTc concentration and network behavior.

For instance, figure 4a shows different typical trajectories for the low state: at 1 and 10 nM, there is very little change in average fluorescence over time, but at 50 and 100 nM, there is an initial overshoot in fluorescence. At low aTc concentrations, there is basically no change from the GFP expression with no aTc. Once the aTc concentration gets high enough, it will complex with enough *tet* repressors that GFP expression is increased. However, the concentration is not

high enough to sustain oscillations, so once the network adjusts to the lower amount of free *tet* repressors, fluorescence decreases.

As shown in figure 4b, almost all dynamics of the middle state show a damping oscillation. At the network level, there is enough aTc in the cell to allow λ repressors to initially dominate. As the λ repressor level increases, the *tet* repressor expression becomes less inhibited and *tet* repressors start to accumulate. Even though some of the *tet* repressors complex with aTc, enough are present to downregulate λ repressor expression, which in turn upregulates *lac* repressor production. *Lac* repressors reduce *tet* repressor expression, and the cycle continues. The internal aTc concentration in this state serves as a balancing weight, making sure that the *tet* repressor concentration remains low enough that, in the turn of the cycle, *lac* repressors can deplete the *tet* repressor level in order to allow λ repressor expression. Even if the oscillatory behavior does not present itself initially (for [aTc] > 500 nM), it emerges by the end of the experiment. For example, as seen at 750 nM aTc, the asymptotic average fluorescence level is overshoot more than it is at lower aTc concentrations, but then it slowly returns to damped oscillations after the network adjusts to the level of free *tet* repressors.

The high state dynamics are shown in figure 4c. They show a typical first order response – a swift rise to high fluorescence. This response is the same for all experiments resulting in the high state. There is a saturating amount of aTc, depleting the concentration of free *tet* repressors to such an extent that the λ repressors are dominating in the system, even though the expression of *lac* repressors is relatively uninhibited.

The dynamics of the average fluorescence of the cultures that were fully induced at 1000 nM aTc at time = 0 min are also shown in figure 4, parts d through f. One can see in figure 4f that lowering the aTc concentration as low as 750 nM aTc provided no perturbation in GFP expression, so the cultures remained at the high state for the duration of the experiment.

The middle state dynamics, shown in figure 4e, are similar in nature to those from cultures that were uninduced at time = 0 min. The overshoot (in this case to lower fluorescence) is still present, as is the oscillatory nature. One noticeable trend is that as [aTc] increases, the oscillations are lower in amplitude and are damped more quickly.

The low state dynamics (figure 4d) are relatively slow compared to the high state dynamics for cultures starting at an uninduced state (the fall to low fluorescence is slower than the rise to high fluorescence). However, the dynamics still appear to be a first order response, with an initial lag. The dynamics at 150 nM aTc are different than the rest; they appear almost oscillatory, but in contrast to the middle state dynamics, they tend toward the low state.

The initial lag shown in the low state dynamics is also seen in the middle state. The difference in dynamics between initially uninduced (no lag) and induced cells may be explained by aTc dynamics. Starting with uninduced cells, assuming that aTc enters the cell quickly, there should be little lag in the fluorescence change, as the network will immediately be affected (since the GFP maturation is quick). In contrast, when decreasing the aTc concentration, first the cells must divide to dilute out the existing aTc-*tet* repressor complexes before the network will experience any noticeable change in the complex concentration.

With aTc, the user can extracellularly control this three promoter-repressor architecture to achieve three different steady states, and the middle state is characterized by damped oscillations at the population level.

Using aTc and IPTG as co-inducers allows finer control over distribution characteristics

Since IPTG is also an extracellular inducer of the network, its effect on the network was tested in conjunction with aTc. IPTG was not used alone because GFP expression without any inducers is at a low level, and IPTG would decrease expression further: referring to figure 1, IPTG depletes the number of *lac* repressors bound to *lacO* sites, which increases the expression of *tet* repressors, which in turn suppresses GFP expression. Testing IPTG with aTc allowed its effect on the network to be seen through comparison with aTc-only experiments.

Two concentrations of aTc were used: 500 nM (at middle state) and 1000 nM (high state), and [IPTG] was varied from 0 M to 600 μ M. We also explored the two scenarios of exposing the system to both inducers concurrently, or introducing IPTG after cells were already exposed to aTc (1000 nM only).

First we examine the results of introducing IPTG with aTc from the beginning of the experiment. At the reference point, we see how IPTG influences the average fluorescence as well as the coefficient of variation (CV). Figure 5a shows a linear decrease in fluorescence as [IPTG] increases. The CV has a similar trend, shown in figure 5b.

At 1000 nM aTc, one can see that the CV decreases between 0 and 50 μ M IPTG, even though the average does not change. IPTG first affects the shape of the distribution before it affects the average. In the opposite way, at high IPTG, even though the average decreases between 300 and 600 μ M IPTG, the CV plateaus. This implies that there is a limit to how compact the distribution can become.

To visualize the changes in the distributions with the addition of IPTG, the number density functions at the reference state are shown in figure 6. The distributions with IPTG and 500 nM aTc are in figure 6a and those with IPTG and 1000 nM aTc are in figure 6b. In figure 6a, as [IPTG] increases, the fluorescence of the highest-fluorescing cells decreases, resulting in a lower average and a sharper distribution. In figure 6b, the whole distribution shifts to lower fluorescence and the number density functions become sharper as [IPTG] increases, but there is no change in shape between 300 and 600 μ M IPTG ndfs.

Although IPTG affects the shape and fluorescence of the distributions at the reference state, it was found to have no effect on the network's dynamic behavior when introduced at the same time as aTc. The oscillatory middle state expression at 500 nM aTc in figure 7a and the stable high state expression at 1000 nM aTc in figure 7b is merely attenuated in the average fluorescence value and/or amplitude of oscillation.

Experiments performed by adding IPTG after the cultures were already exposed to aTc yielded different results. Average fluorescence versus time is shown in figure 8a and the corresponding number density functions at the reference state is shown in figure 8b. The dynamics of the culture at 50 and 100 μ M IPTG were similar to that of the previous set of experiments, reaching slightly lower average values as [IPTG] increases, but qualitative behavior of steady expression is unchanged. However, the dynamics at 200 to 600 μ M IPTG were slower than in the previous experiments, and they asymptotically reach different average values (displaying the same trend of reaching lower values at higher [IPTG]). The slower dynamics were probably due to two things: the fact that GFP degrades at a slower rate than it becomes fluorescent, and slower cell growth. The inducer-initiated shock to the cells was most acutely felt after adding 600 μ M IPTG, at which point cell growth was delayed for more than 4 hours. This growth delay stalled the dynamics because the loss of GFP was not accelerated by division (which would drop the amount of GFP per cell roughly in half).

The reference state number density functions explain even more. The ndfs at 50 and 100 μM IPTG are not very different than those in figure 6b. However, the ndfs at 200, 300, and 600 μM IPTG resemble the distributions of the middle state, closer to those in figure 3 or 6a. Those distributions are not sharper, but broader than those at lower [IPTG]. The trend of the distributions sharpening as [IPTG] increases is true, but only within the 200-600 μM IPTG group. Once the [IPTG] was in this range, it constituted enough of a perturbation to the network to change its behavior from the high state to the middle state. The reason that this was not seen in the experiments introducing IPTG and aTc at the same time is because the perturbation made by IPTG was made concurrently with aTc, so the network could adjust to both at the same time.

So, IPTG can help to control characteristics of average fluorescence and CV when used with aTc, but the order of introduction makes a difference in whether the network state is affected. When added together with aTc, IPTG attenuates the average, CV, and oscillation amplitude. When added after aTc is introduced, a higher concentration of IPTG can also perturb the system to a lower state.

Discrepancy exists between known model and experimental data

The aTc-only reference point results include bi-threshold behavior as well as multiplicity. Also, the aTc-IPTG results indicate multiplicity depending on initial condition. Looking to the model developed by Elowitz and Leibler (2000), and adding the aTc-*tet* repressor interaction and the IPTG-*lac* repressor interaction to include the network's dependence on inducers, the experimental and simulation results do not qualitatively match. Theoretically, if the network behaves as designed, with each repressor interacting *only* with the promoter containing its complimentary operator site, there is a continuum of steady states and no multiplicity (El Samad et al, 2005). The results cannot be explained by that model. One could suppose that the experimental data are incorrect due to some artifact, but we believe the results are genuine, especially the features of multiplicity and bi-threshold behavior that put the results at odds with the original model. After carefully looking over the data and chronology of experiments, some factors that might have caused an artificial multiplicity result were discarded, like aTc degradation, the initial OD₆₀₀, and the exact length of the growth period without inducer. So, we then set out to find why the network behaves in this manner.

One of the possible causes that differentiate the gene expression designed at the single-cell level from the gene expression that is measured at the population level is cell population heterogeneity. Heterogeneous cells exist in different physiological states and therefore exhibit cell-to-cell phenotypic variability. The variability of cells harboring the artificial repressilator, however, must be very profound to justify such differences in the network dynamics at the single-cell and population level. Thus, instead of regarding heterogeneity as the sole cause that creates multiplicity of the repressilator gene expression at the population level, it appears more reasonable to assume that the cell population exhibits the observed bi-threshold behavior because each individual cell of the population has the ability to display the exact same behavior. From this perspective, heterogeneity can be regarded as the source of variability that drives the cells towards one of the multiple states.

The only way to see multiplicity of steady states at the single-cell level is for there to be extra interactions in the network influencing the action of the repressors, either from some external force or from within the network. For example, some molecules external to the network could influence the interactions between the repressors and operator sites. Or, the repressors themselves might act in unexpected ways, for example, by binding to promoter-operator sites without their specific operator sequences. If the interaction is strong enough, it could influence the network's behavior.

Since repressors are known to have measurable nonspecific interactions with non-operator DNA (Kleinschmidt et al, 1988; Kao-Huang et al, 1977, Lin and Riggs, 1972; and others), we continued with this idea. We modeled the inhibition of each repressor on its own expression and on the expression of the repressor upstream of it. Concurrently, we experimentally tested the existence of these interactions. The “self-promoting” interactions of each repressor binding to the promoter of the upstream repressor are: *tet* repressor- λ P_R, *lac* repressor-P_LtetO-1, and λ repressor-P_LlacO-1, as shown in figure 9 (dashed lines). The “self-inhibiting” interactions of each repressor binding to its own promoter are: *tet* repressor-P_LlacO-1, *lac* repressor- λ P_R, and λ repressor-P_LtetO-1 (dotted lines).

Modeling extra network interactions leads to qualitative match with experimental data

In order to model the dynamics of the repressilator in the presence of nonspecific repressor-promoter interactions, we modify the mathematical model derived by Elowitz and Leibler (2000) to explicitly account for a) the ability of each protein to nonspecifically complex with the promoter of the upstream repressor as well as its own promoter (the promoters not containing its operator site), b) the effect of the aTc and IPTG inducers, and c) the dilution effect due to cellular growth (Fredrickson 1976).

Let m_i and p_i ($i=lac(L), tet(T), \lambda(C)$) denote the concentrations of the mRNA transcripts and repressor proteins respectively transcribed and translated from the LacI, TetR and λ cI genes. Let, also, α_{0i} and α_i represent the synthesis rates of the mRNAs respectively transcribed in presence and absence of saturating amounts of repressors, whereas ε_i account for the specific rates (per unit transcript) of protein translation. Moreover, while k_{ji} ($j=\lambda(C), lac(L), tet(T)$) are the dissociation constants describing the interaction between promoters and downstream repressors, k_{ki} ($k=tet(T), \lambda(C), lac(L)$) are the dissociation constants accounting for the interactions between promoters and upstream repressors, whereas k_{ii} are the dissociation constants representing the interactions between the repressors and their own promoters. Furthermore, η_i and γ_i respectively denote the degradation rates of the mRNA transcripts and proteins, while μ represents cellular growth rate. Finally, aTc and IPTG represent the concentrations of the extracellular inducers, whereas K_{aTc} and K_{IPTG} are the *tet* repressor-aTc and *lac* repressor-IPTG dissociation constants, respectively. The dynamics of the artificial repressilator can then be described by the following differential equations subject to appropriate initial conditions:

$$\frac{dm_L}{dt} = \alpha_{0L} + \frac{\alpha_L}{1 + p_C^2/k_{CL} + p_T^2/k_{TL} + p_L^2/k_{LL}} - (\eta_L + \mu)m_L \quad (1a)$$

$$\frac{dm_T}{dt} = \alpha_{0T} + \frac{\alpha_T}{1 + p_L^2/k_{LT} + p_C^2/k_{CT} + p_T^2/k_{TT}} - (\eta_T + \mu)m_T \quad (1b)$$

$$\frac{dm_C}{dt} = \alpha_{0C} + \frac{\alpha_C}{1 + p_T^2/k_{TC} + p_L^2/k_{LC} + p_C^2/k_{CC}} - (\mu_C + \mu)m_C \quad (1c)$$

$$\frac{dp_L}{dt} = \varepsilon_L m_L - (\gamma_L + \mu)p_L - 2K_{IPTG} p_L^2 IPTG^2 \quad (1d)$$

$$\frac{dp_T}{dt} = \varepsilon_T m_T - (\gamma_T + \mu) p_T - 2K_{aTc} p_T^2 aTc^2 \quad (1e)$$

$$\frac{dp_C}{dt} = \varepsilon_C m_C - (\gamma_C + \mu) p_C \quad (1f)$$

Equations (1) state that the rates of mRNA transcript (1a)-(1c) and protein (1d)-(1f) accumulation are equal to the difference between the synthesis rates respectively due to the transcription and translation processes and the consumption rates due to degradation and the dilution effect. In equations (1d) and (1e) there are additional consumption rates, which account for the depletion of free *lac* and *tet* proteins due to the inducer-repressor binding. The functional form of the transcript synthesis rates includes the effect of all possible interactions. Moreover, the functional form of the inducer-repressor binding rates reflects the ability of each repressor dimer to complex with two inducer molecules. The nominal values of the parameters appearing in equations (1) are listed in table I. In employing equations (1), we assume that the λ repressor and GFP expression level dynamics are identical.

Figure 10 illustrates the steady-state λ repressor (GFP) expression levels as a function of the aTc inducer concentration computed for the aTc-only series of experiments. In qualitative agreement with the experimental data measured at the population level (refer to figure 2), the simulation results indicate that individual cells harboring the modified repressilator can indeed exhibit bi-threshold behavior. The low and the high state are stable, while the middle state can be either asymptotically stable (solid line) or oscillatory unstable (dashed line). Moreover, the model predicts multiplicity between the low and the middle state as well as the middle and the high state, as experimentally observed.

Figure 11 shows the simulated λ repressor (GFP) expression levels as a function of time for cultures started either at an induced or uninduced state in the single-inducer series of experiments (compare to figure 4). As initial conditions for equations (1) were selected the asymptotic values reached by the gene expression levels of cells grown either in absence of inducer (uninduced cells) or at 1000 nM aTc (induced cells).

At low [aTc], the repressilator dynamics are mainly controlled by the mutual inhibition through which the *lac* repressor inhibits TetR (specifically) and the *tet* repressor downregulates LacI (nonspecifically) due to the low λ repressor (GFP) expression levels. Thus, both uninduced (figure 11a) and induced cultures (figure 11d) transiently adjust to the lower stable steady state with trajectories that are essentially unaffected. Notice that, due to the choice of the initial conditions, the model is unable to capture the overshoot in the GFP expression levels experimentally observed at [aTc] between 50 and 100 nM (see figure 4a).

At intermediate inducer concentrations and depending on the initial gene expression levels, the repressilator can do one of three things. If the networks dynamics are under the control of the *lac-tet* mutual inhibition described in the above paragraph, it can a) adjust to the low steady state. It can b) exhibit sustained oscillations, if the network behavior is dictated by the basic structure designed by Elowitz and Leibler (figures 11b, e). Or it will c) settle to the stable middle state, if the network dynamics are regulated by the double negative feedback loop through which λ repressors inhibit LacI and *lac* repressors downregulate the upstream λ cI due to the low TetR expression levels.

Finally, at high [aTc], the dynamics of the network are mainly driven by the λ -*lac* mutual inhibition. Therefore, depending on the initial gene expression levels, both uninduced and induced cultures transiently either adjust to the high stable steady state with trajectories that are essentially unaffected (figures 11c, f), or settle to the middle steady state exhibiting damped oscillations.

Summarizing, our simulation results indicate that individual cells harboring the repressilator network can indeed exhibit bi-threshold behavior in presence of nonspecific repressor-promoter interactions. Moreover, the model can predict gene expression dynamics that are qualitatively similar to those measured in the shake flask experiments. Therefore, our computational analysis supports the hypothesis that nonspecific repressor-promoter interactions can have a critical role in the repressilator dynamics.

Experiments show existence of extra network interactions

With the knowledge from simulations that adding extra interactions between a repressor and its own promoter and the promoter of the upstream repressor to the model can qualitatively capture the network behavior, we set off to find the existence of these interactions experimentally.

A simple network was created to test each hypothesized interaction, in which a constitutively expressed repressor negatively controls GFP expression by (possibly) binding to the GFP promoter. The plasmid pSCP102 was simplified using standard molecular biology techniques into separate test plasmids for each interaction. Each plasmid consists of a repressor being expressed from the same promoter as in pSCP102 and GFP being expressed from a promoter with the hypothesized interaction. We test for a difference in GFP expression when the repressor protein is being expressed and when it is not.

In order to prevent repressor expression, a second plasmid was created for each hypothesized interaction by removing the promoter in front of the repressor (including its translation start site), which should significantly reduce its expression and relieve any GFP repression. This approach to knocking out repressor expression was chosen in order to minimize the difference in size between plasmids, since significantly lowering the plasmid size by removing the full repressor gene could lessen the burden on the cells and create an artificial increase in GFP expression. This approach was applied for each interaction to keep the method consistent, to avoid any inducer effects on cell growth, and to avoid the question of how much inducer is required to reach maximum GFP expression.

The gene order on each plasmid was kept consistent (gfpaav upstream of repressor gene) to prevent any leaky expression of the repressor due to promoter activity in the area upstream of the first promoter (Alper et al, 2005). The plasmids retain a ColE1 origin of replication and kanamycin resistance, just like pSCP102. The test plasmids are summarized in table II with the arrangement of their respective promoters and genes. If the hypothesized interaction exists, there will be an increase in GFP expression when the promoter for the repressor is removed. If there is no interaction, there will not be any difference in GFP expression level.

lac repressor interactions (II and V). The interaction between the *lac* repressor and $P_{LtetO-1}$ is tested with plasmids pSCP142 and pSCP152. The first plasmid contains the λP_R promoter in front of LacI, while in the second one it is deleted. A similar growth method to that used in the main repressilator experiments was used, repeatedly subculturing cells containing each plasmid in media without inducer. A difference is observed between GFP levels. With expression of LacI, the relative GFP expression level is 147.7 ± 6.1 . When the promoter for LacI is removed (uninhibited GFP expression), the expression level rises to 264.5 ± 10.9 . The

increase is significant (79%), and can easily be seen when comparing the number density functions (figure 12a).

Similarly, testing the interaction between the *lac* repressor and λ P_R with pSCP146 and pSCP156 shows a difference between the GFP expression levels (figure 12b). The expression level of GFP increases from 111.4 ± 7.9 to 235.3 ± 32.3 when the promoter in front of LacI was removed, an increase of 111%. Therefore, the *lac* repressor affects the expression of its two nonspecific promoters to an extent that we can experimentally verify.

λ repressor interactions (III and IV). The interaction between the λ repressor and P_LlacO-1 is tested with pSCP113 and pSCP123, with and without the promoter for λ cI, respectively. The results, shown with comparative number density functions in figure 12c, demonstrate a jump in GFP expression once the promoter is removed, from an average expression level of 279.2 ± 10.3 to 874.9 ± 60.9 , an increase of over 200%. Moreover, there is a distortion in the shape of the number density function: with the λ repressor being expressed, the distribution of fluorescence is much wider than it is when the repressor is not expressed. This experiment provides evidence that a relatively strong interaction exists between the λ repressor and P_LlacO-1, stronger than either of the *lac* repressor interactions.

pSCP114 was created to test the interaction between the λ repressor and P_LtetO-1, along with the control plasmid without the promoter in front of λ cI, called pSCP124. These plasmids are similarly tested, and again a difference in GFP expression is found – rising from 396.9 ± 3.0 with the λ cI promoter to an average without the promoter of 666.5 ± 21.6 , an increase of 67%. Number density functions are shown in figure 12d. Clearly, an interaction between the λ repressor and P_LtetO-1 also exists, though not as strong as that with P_LlacO-1.

tet repressor interactions (I and VI). Plasmids pSCP141 and pSCP151 were created to test the interaction between the *tet* repressor and λ P_R. Cells with pSCP151, not producing *tet* repressors, grow at a normal rate; however the cells containing pSCP141, expressing TetR highly, experience stunted growth both overnight and after dilution into fresh media. Not knowing whether the growth problem is due to *tet* repressors or some other mutation, cultures containing pSCP141 are grown overnight with varying amounts of aTc. It is determined that higher amounts of aTc removed the inhibition of the cells' growth to an extent, indicating that high expression of TetR was likely the culprit for pSCP141's reduced growth. A similar problem was reported in 1984, when it was found that high expression of TetR from a plasmid in bacteria led to growth arrest (Oehmichen et al). The growth problem prevented us from testing this interaction.

Knowing that high expression of tetR inhibits growth, the *tet* repressor interaction with its own promoter, P_LlacO-1 (hypothesis VI), was not tested. Even though *tet* repressor interactions could not be found, it would not be surprising if the interactions did exist, at least to the extent that the *lac* repressor's interactions do.

Results for the interaction hypothesis experiments are summarized in table III.

According to our modeling studies, the λ repressor-P_LlacO-1 interaction helps to explain the existence of the high state and the multiplicity between middle and high states. The interaction between the λ repressor and the P_LtetO-1 promoter has only a marginal effect on the stationary behavior of the network and solely brings the high state expression level closer to that of the middle state, better resembling our experimental results than if only the self-promoting interaction with P_LlacO-1 was found. Incorporating the *lac* repressor interactions into the model can add the lower steady state and multiplicity, though only for a narrow range of parameters. However, if the suspected *tet* repressor interactions exist, particularly that with λ P_R, the range of parameters is significantly extended for which we can match modeling to

experimental behavior. Even if the *tet* repressor interaction does not exist, and the region of [aTc] where the low state is found would be quite small, cell population heterogeneity could expand the region to the range seen experimentally.

Summary and Conclusions

We investigated the interaction between cell population heterogeneity and the repressilator architecture with the aid of flow cytometry to discover the distribution of phenotypes. We explored the ability of the repressilator system, a cyclic negative feedback loop of three promoter-repressor pairs, to be controlled by the extracellular inducers aTc and IPTG. The original two-plasmid system designed by Elowitz and Leibler (2000) was combined into a one-plasmid system, purposely raising the intracellular concentration of the repressors. With this new plasmid, four sets of shake flask experiments were performed: 1) starting at an uninduced state, growing cells in media with aTc only; 2) starting at an aTc induced state, growing cells in media with a lower aTc concentration; 3) starting at an uninduced state, growing cells in media containing either both aTc and IPTG; and 4) starting at an aTc induced state, adding IPTG to the media while keeping the aTc concentration constant.

In the aTc-only experiments, it was discovered that, at the reference state, the one-plasmid system exhibited bi-threshold behavior (three steady states), with significant hysteresis regions. The number density functions demonstrated that the middle state is not a bimodal combination of the low and high states, but a new state. The average fluorescence values and CV's are extremely consistent within each state. The middle state is unsteady while the low and high states are steady. The ability of the cultures in the middle state to oscillate in GFP fluorescence may explain the larger CV value of the middle state.

The aTc-IPTG mixed inducer experiments demonstrated that when IPTG is added concurrently with aTc, up to 600 μ M, it fine tunes the qualities of the GFP distribution in comparison to growing cultures in aTc only. IPTG lowers the average and CV, though there is a limit to how compact the distribution can get. However, this fine tuning does not affect the corresponding behavior that the culture has with aTc alone – in the middle state, IPTG attenuates the average and amplitude of oscillation, and in the high state, IPTG simply lowers the average. If IPTG is added after the culture is already stable in the high state, then IPTG can perturb the system enough for the network to reach the middle state if added in a high enough concentration.

The bi-threshold behavior and multiplicity of the system observed experimentally are not features that are possible with the original model accompanying the repressilator system, in which each repressor only interacts with the promoter containing its respective operator sites (Elowitz and Leibler, 2000). In order to obtain these features with a model, we must include extra interactions, namely interactions between a repressor and the nonspecific promoters of the other two repressors. Two nonspecific interactions between a repressor and the nonspecific promoter of the upstream repressor are needed to create the high and low states, as well as the multiplicity observed. Experimentally, four significant nonspecific interactions (between the *lac* and λ repressors and their nonspecific promoters) are observed, which according to our model can create the high state and perhaps the low state. We suspect that significant interactions also exist between the *tet* repressor and its nonspecific promoters (further solidifying the low state), but could not verify them due to toxicity of high *tet* repressor concentration. Cell population heterogeneity could expand the low and high state regions to match the experimental results. Finding the extra interactions in this network is quite significant, for it shows that even small nonspecific interactions can have a major effect on network behavior. Moreover, it lends credibility to our results, since it helps to explain why the network did not behave as expected from its design.

There is a large amount of flexibility in the behavior of this genetic architecture, and it can be controlled extracellularly. The fine tuning that can be achieved when IPTG and aTc are used together shows that it is possible to reach two distributions with the same average fluorescence value but different CV values, and this could have use in biotechnology. We also saw the importance of the order in which the culture is exposed to various levels of aTc or IPTG in impacting the culture's asymptotic behavior, since hysteresis was observed after starting the cultures at an aTc-induced high state. Looking at the distributions of fluorescence as well as the averages gave a more complete view of the nature of the three states that are reached with this system, giving us a better understanding of how this genetic architecture functions under different inducer conditions. Moving beyond the singular oscillatory behavior previously observed, this paper shows that this system can behave in a much richer manner than previously discovered.

Acknowledgements

The authors would like to thank Michael Elowitz for the Repressilator and Reporter (pZE21-GFPAAV) plasmids, T. Bryce Causey for help in designing the construct pSCP102, and Mary Harrison and Blair Doneske for advice with the constructs to test for extra interactions. Partial support by grants NSF (BES-0331324, BES-0420840), NIH (R01GM071888) and an NSF IGERT Training Fellowship to SP (DGE0114264) is also gratefully acknowledged.

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Figure 1.

The oscillatory network. a) The network architecture, consisting of three promoter-repressor pairs, forming a cyclic cascading network of negative feedback loops. Network is controlled by two inducers, aTc and IPTG. b) Relevant plasmid maps. Repressilator and Reporter are the original two-plasmid system. They were combined to form pSCP102, which is the plasmid used in this work.

Figure 2.

Average green fluorescence as a function of aTc concentration at reference state. Three states are reached, with hysteresis between the high (squares) and middle (circles) states as well as between the middle and low (diamonds) states. Included are data from experiments starting with uninduced cultures (closed markers) and induced cultures (open markers).

Figure 3.

Typical number density functions at the reference state for low, middle, and high states, including data at various aTc concentrations. The initial culture was grown at a) 0 M aTc or b) at 1000 nM aTc, yet both sets are consistent with the other. The middle state ndf is broader than that of the other two states.

Figure 4.

Average green fluorescence as a function of time at various aTc concentrations. Plots are grouped by initial and final states. Cultures that start uninduced and end in the a) low state; b) middle state; c) high state. Cultures that start induced, ending in the d) low state; e) middle state; f) high state. The low and high state trajectories appear stable while the middle state trajectories appear oscillatory.

Figure 5. Change in fluorescence properties with IPTG. a) Average green fluorescence vs. IPTG; b) coefficient of variation vs. IPTG. IPTG decreases GFP production and makes expression less heterogeneous.

Figure 6.

Number density functions at the reference state for mixed inducer experiments when inducers are added simultaneously. Trends of decreasing averages and CV's are shown as IPTG increases a) at 500 nM aTc and b) at 1000 nM aTc.

Figure 7.

Average green fluorescence as a function of time for different IPTG concentrations when inducers are added simultaneously. a) An aTc concentration of 500 nM aTc demonstrates the trend for the middle state, and b) an aTc concentration of 1000 nM aTc demonstrates the trend for the high state.

Figure 8.

Fluorescence properties for cultures at 1000 nM aTc that start initially with no IPTG. a) Average green fluorescence as a function of time and b) ending number density functions. Note how addition of 200 μ M or more IPTG perturbs an induced culture without IPTG to the middle state, in contrast to the effect of IPTG shown in the ndfs in figure 6b.

Figure 9.

The regulatory repressor-promoter interactions that define the structure of the artificial repressilator. We hypothesize the existence of six nonspecific interactions. Three are “self-promoting” interactions due to the binding of 1) the *tet* repressor to the λ - P_R promoter, 2) the λ repressor to the P_{Lac01} promoter, and 3) the *lac* repressor to the P_{tet01} promoter (dashed lines). The other three are “self-inhibiting”, due to the binding of 1) the *lac* repressor to the λ - P_R promoter, 2) the *tet* repressor to the P_{Lac01} promoter, and 3) the λ repressor to the P_{tet01} promoter (dotted lines).

Figure 10.

Theoretical dependence of the stationary λ (*gfp*) expression levels on the aTc inducer concentration after incorporating the six nonspecific interactions. Solid line: stable steady-states; dashed line: unstable steady-states; circles: maximum and minimum levels of the oscillations. Notice the similarity of the states to the experimental results in figure 2.

Figure 11.

Theoretical λ (*gfp*) expression levels as a function of time at various aTc concentrations. Plots are grouped by initial and final states. Cultures that start uninduced and end in the a) low state; b) middle state; c) high state. Cultures that start induced, ending in the d) low state; e) middle state; f) high state. Results are qualitatively similar to experimental results in figure 4, except for a few overshoots in the experimental results that the model did not capture.

Figure 12.

Comparative number density functions for the four confirmed nonspecific interactions. The interactions include that of the *lac* repressor with the promoters a) $P_{LtetO-1}$ and b) λP_R , and of the λ repressor with the promoters c) $P_{LlacO-1}$ and d) $P_{LtetO-1}$. When the promoter responsible for repressor expression is removed, GFP expression becomes less inhibited, resulting in higher green fluorescence. Note the increase in GFP expression and sharpening of the peak (particularly in part c) between the red (inhibited) and blue (uninhibited) distributions.

Table I

Nominal model parameters for the modified repressilator dynamics

Parameter	Value
α_{0L} (nM/min)	$1.5 \cdot 10^{-3}$
α_{0T} (nM/min)	$3.0 \cdot 10^{-3}$
α_{0C} (nM/min)	$1.2 \cdot 10^{-3}$
α_L (nM/min)	15
α_T (nM/min)	45
α_C (nM/min)	36
k_{CL} (nM ²)	0.9
k_{LT} (nM ²)	0.7
k_{TC} (nM ²)	1.2
k_{LC} (nM ²)	15
k_{TL} (nM ²)	10
k_{CT} (nM ²)	8
k_{LL} (nM ²)	200
k_{TT} (nM ²)	150
k_{CC} (nM ²)	100
ε_L (1/min)	20
ε_T (1/min)	20
ε_C (1/min)	20
η_L (1/min)	$\ln(2)/2$
η_T (1/min)	$\ln(2)/2$
η_C (1/min)	$\ln(2)/2$
γ_L (1/min)	$\ln(2)/10$
γ_T (1/min)	$\ln(2)/10$
γ_C (1/min)	$\ln(2)/10$
μ (1/min)	$\ln(2)/40$
K_{aTc} (1/min/ nM ³)	$6.9 \cdot 10^{-9}$

Table II

List of plasmids used in the hypothesis experiments

Hypothesis	Regulatory elements of each hypothesis plasmid, in order					name
I	λ P _R	gfpaav	terminator	P _L lacO-l	TetR	pSCP141
	λ P _R	gfpaav	terminator		TetR	pSCP151
II	P _L tetO-l	gfpaav	terminator	λ P _R	LacI	pSCP142
	P _L tetO-l	gfpaav	terminator		LacI	pSCP152
III	P _L lacO-l	gfpaav	terminator	P _L tetO-l	λ cl	pSCP113
	P _L lacO-l	gfpaav	terminator		λ cl	pSCP123
IV	P _L tetO-l	gfpaav	terminator	P _L tetO-l	λ cl	pSCP114
	P _L tetO-l	gfpaav	terminator		λ cl	pSCP124
V	λ P _R	gfpaav	terminator	λ P _R	LacI	pSCP146
	λ P _R	gfpaav	terminator		LacI	pSCP156
VI	P _L lacO-l	gfpaav	terminator	P _L lacO-l	TetR	
	P _L lacO-l	gfpaav	terminator		TetR	

Table III

Results summary of the interaction hypothesis experiments

Hypothesis	Interaction	Strength of Interaction	Increase in Average Fluorescence
I	$\text{TetR} - \lambda P_R$	not tested, inhibited growth	n/a
II	$\text{LacI} - P_{L\text{tetO-1}}$	weak	79%
III	$\lambda cI - P_{L\text{lacO-1}}$	relatively strong	213%
IV	$\lambda cI - P_{L\text{tetO-1}}$	weak	68%
V	$\text{LacI} - \lambda P_R$	weak	111%
VI	$\text{TetR} - P_{L\text{lacO-1}}$	not tested	n/a