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A SYNTHETIC POSITIVE FEEDBACK BASED GENE REGULATORY CIRCUIT

BY

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DISSERTATION

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Abstract

The concept of reengineering and rewiring of pathways and gene regulatory networks for novel uses, and sometimes for mimicking natural systems for various uses in various disciplines gradually acquired the name “synthetic biology”. Synthetic biology, with foundations rooted in multiple disciplines like biological engineering, molecular biology, genetic engineering, and systems biology to name a few, is a new approach towards developing applications for solving current global problems in areas of food, agricultural, environmental, health (Weber et al., 2008) and alternative energy. A synthetic biology approach was used to develop a positive feedback base gene regulatory circuit in *E.coli*.

Positive feedback is a common mechanism used in the regulation of many gene circuits as it can amplify the response to inducers and also generate binary outputs and hysteresis. In the context of electrical circuit design, positive feedback is most often employed in the design of amplifiers. Similar approaches may therefore be applied to design modular amplifier for the design of synthetic gene circuits and cell-based sensors.

A modular positive feedback circuit was developed that can function as a genetic signal amplifier, heightening the sensitivity to inducer signals as well as increasing maximum expression levels without the need for an external cofactor. The design utilizes a constitutively active, autoinducer-independent variant of the quorum-sensing regulator LuxR. The ability of the positive feedback module to separately amplify the output of a one-component tetracycline sensor and a two-component aspartate sensor was experimentally tested and validated. In each case, the positive feedback module amplified the response to the respective inducers, both with regards to the gain and sensitivity.

The advantage of this design is that the actual feedback mechanism depends only on a single gene and does not require any other modulation. Furthermore, this circuit can amplify any transcriptional signal, not just one encoded within the circuit or as inducer concentration. As our design is modular, it can potentially be used as a component in the design of more complex synthetic gene circuits.

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Chapter 1 Introduction and Literature Review

1.1 Synthetic Biology

Emerging disciplines like genomics, proteomics and metabolomics to name a few, have led to increased understanding of biology. The common underlying principle of these various disciplines is reductionism; i.e. the complex system is understood by reducing it down to its simpler constituents such as building blocks like proteins, DNA, RNA enzymes and their controlling mechanisms. The advances in recombinant biotechnology and computational biology continue to help in compiling the high-resolution 'omics' information in order to re-create bio-molecular systems and to characterize their role in their native contexts.

As we continue understanding the relationships between biological modules, we are also able to put them in novel contexts that are not seen in nature. Numerous endeavors have reengineered enzymes for the purpose of optimizing hard to produce drugs for industrial scale production (Stutzman-Engwall et al., 2005), introduced foreign enzymatic pathways into microbes to produce a desired product (Ro et al., 2006), and created novel gene regulation patterns to understand existing complex natural genetic circuits (Maeda and Sano, 2006b). This concept of reengineering and rewiring of pathways and gene regulatory networks for novel uses, and sometimes for mimicking natural systems for various uses in various disciplines has gradually acquired the name "synthetic biology".

Research in synthetic biology combines the "discovering" attitude of biology with the "building block-by-block" nature of engineering. Synthetic biology was formally described as "the design and construction of new biological parts, devices and systems and the redesign of existing natural biological systems for useful purposes" (Synthetic Biology, 2010). Synthetic biology, with foundations rooted in multiple disciplines like biological engineering, molecular biology, genetic engineering, and systems biology to name a few, is a new approach towards developing applications towards solving current global problems in areas of food, agricultural, environmental, health (Weber et al., 2008) and alternative energy (Lee et al., 2008).

1.1.1 Philosophy of Synthetic Biology

Abstraction, standardization and synthesis are the three main components of the synthetic biology framework. Abstraction refers to a method of encapsulating the information contained in complex biological structures by means of simpler linguistic or graphical notation for the purpose of reducing the amount of information displayed in order to comprehend the system. Abstraction hierarchies are used in most engineering disciplines and in object oriented programming languages like C++. The ultimate goal is to be able to design synthetic systems with predictable behavior at a high level of abstraction using engineering tools, synthesize their DNA equivalents and deploy them in living cells. Abstractions commonly used in synthetic biology, such as 'parts', 'devices' and 'systems' as defined below represent specific genetic structures that encapsulate discrete functions (Synthetic Biology, 2010).

Table 1. Abstraction hierarchy.

Layer	Description	Example
DNA	Sequence of nucleotides	AGGAGG
Part	A sequence of nucleotides with a specific function	Promoter
Device	Multiple parts with higher function	A repressor
System	Multiple devices assembled together	An oscillator

The increase in use, design and construction of genetic circuits has led to standardization with respect to interfacing simpler biological parts (Voigt, 2006) like in any other discipline of engineering. The registry of standard biological parts, BioBricks, from MIT is a collection of standardized biological parts (Registry of Standard Biological Parts, 2010).

The BioBricks Foundation's registry currently consists thousands of genetic elements that encode for promoters, terminators, enzymes, reporters, ribosome binding sites, activators,

repressors and signaling and measurement systems. The availability of standardized interfaces (DNA motifs that permit recombinant assembly) allows rapid prototyping of genetic devices and troubleshooting (Shetty et al., 2008; Arkin, 2008). Individual parts with discrete functions can be tested and characterized to build higher order systems with more complexity using a limited subset of restriction enzymes and a fixed protocol. The parts or devices that do not function as per the design requirements can be identified and repaired or replaced easily (Rosenfeld et al., 2005). Prof. Tom Knight of MIT proposed a BioBrick standard in 2003 that describes the composition of biological parts (Knight, 2003). Many students and synthetic biologists use this standard to make complex devices at “ The International Genetically Engineered Machines (IGEM) ” competition every year (IGEM, 2010). BioBricks were used to build biobrick vectors (Shetty et al., 2008). BioBricks have also been used for developing a variety of standardized parts for cyanobacteria (Huang et al., 2010), which is not as widely studied as *E.coli*. Alternative methods to standard molecular biology procedures like digestion and ligation were also developed to enable parallel assembly of higher order genetic devices (Sleight et al., 2010).

1.1.2 Directions in Synthetic Biology

Studies in synthetic biology over the last decade have taken three distinct directions and can be generically categorized as biomimetic chemistry, synthetic techniques and gene regulatory circuits.

1.1.2.1 Biomimetic Chemistry

Many studies attempted to recapitulate natural processes like protein translation outside the cell. Cell free systems use the catalytic machinery of the cells in order to achieve protein translation in-vitro by eliminating the cellular chassis (Jermutus et al., 1998; Jewett et al., 2008). Cell free technology is a potential protein production method for the biotechnological and pharmaceutical industry for the large-scale production of proteins and offers several advantages over in-vivo expression chassis like *E.coli* and Chinese Hamster Ovary (CHO) cells. The lack of a cell wall offers direct control over the cellular machinery and reaction conditions. Translation rates in natural cells are driven by the availability of the energy resource adenosine triphosphate (ATP). However, cell free systems are typically constrained by regeneration of ATP

through the use of expensive high-energy phosphate compounds, and they are also hindered by accumulation of inhibitory byproducts such as phosphate and changes in pH. In order to address this problem, Jewett and Swartz (Jewett and Swartz, 2004) developed a “cytomim” platform in which the cytoplasmic environment in *E.coli* was successfully mimicked with the use of pyruvate as the energy resource in order to achieve the cell free translation of a protein with higher translation efficiency. In a different study, in-vitro translation efficiency was also enhanced through the use of gold nano particle-DNA conjugates (Park and Hamad-Schifferli, 2010).

1.1.2.2 Synthetic Techniques

Since the advent of recombinant DNA technology, the synthesis of huge DNA molecules has always been an objective. A special mention should be given to the scientists at the J Craig Venter Institute who achieved the synthesis of the entire 1.1 million base pair genome of *Mycoplasma mycoides* (Gibson et al., 2010). The genome was assembled inside a yeast cell and then later transplanted into a cell of a closely related species *Mycoplasma capricolum* (Lartigue et al., 2009). The ability to propagate synthetic genomes was demonstrated by replacing the genome of one bacterial strain with the genome of a different strain (Lartigue et al., 2007). After the cell had divided and new cells contained only proteins characteristic *M.mycoides*. These achievements demonstrate that synthetic biology is the new frontier of genetic engineering and gives unprecedented opportunities to battle global problems in food, health and fuels through the possibility of genome-scale reprogramming of cellular machinery.

1.1.2.3 Gene Regulatory Circuits

Most of the initial demonstrations of synthetic biology were simple gene regulatory circuits. The objectives of these studies were bottom up design of genetic parts and circuits in order to understand gene regulatory circuits. They laid the foundation for understanding complex biological systems in a new light. It is widely agreed that the first inaugural demonstrations of synthetic biology in its current definition were the genetic toggle switch built by Collins et al., (Gardner et al., 2000) and the repressilator by Elowitz et al., (Elowitz and Leibler, 2000). Transcriptional regulatory elements were used in both these circuits to demonstrate a flip-flop

memory module that switches between two states and a ring oscillator circuit respectively in *E.coli*. Green fluorescent protein was used as an alias for the transcriptional activity for both these studies. The design and characterization of these circuits showed that gene regulatory systems could be programmed and modeled just like their non-biological counterparts using deterministic and stochastic approaches and produce systems with a degree of predictability. These two papers garnered great interest in synthetic biology, leading to many similar studies and approaches. Among the three growth areas of synthetic biology, this work is most closely related to understanding synthetic gene regulatory circuits.

1.2 Gene Regulatory Circuits

The synthetic genetic toggle switch (Gardner et al., 2000) is an elegant recapitulation of the lysis-lysogeny decision events of *E.coli* infected with the bacteriophage λ . The bacteriophage λ consists of two promoters that are mutually repressed by the gene product of the other. The Cro protein represses the *cl* promoter and the CI repressor protein represses the *cro* promoter effectively switching the organism between the lysis and the lysogeny states of bacteriophage infection (Ptashne et al., 1982). Three different promoter-repressor pairs were used to construct different combinations of mutually repressing plasmids. The P_L promoter from the bacteriophage λ was used in conjunction with *lacI* repressor to repress the P_{trc2} promoter. The P_{trc2} promoter controlled the expression of the temperature sensitive *cl-ts* gene that translates into the λ CI repressor protein which in turn represses the P_L promoter. The third variant used the $P_{LtetO-1}$ promoter in conjunction with Tet repressor. The system was manipulated using two different exogenous signals in order to activate gene production from each promoter and can be toggled between them. When one of the signals is removed the system retains or remembers its current state thus satisfying all the requirements of a toggle switch, flip-flop or a memory (Gardner et al., 2000).

The ring oscillator circuit (Elowitz and Leibler, 2000) also called a repressilator. In this system, three promoter-repressor pairs are arranged in a cyclical fashion where each protein represses the transcription under the control of the next promoter. *Lacl* protein represses the transcription of the *tetR* gene under the control of the tetracycline resistant transposon Tn10.

The tetR protein represses the transcription of the cl protein from bacteriophage λ , which in turn represses the lacI expression under the first promoter. The transcriptional activity was measured through green fluorescent protein expression on a separate reporter plasmid. The network resulted in oscillations that were slower than the cell division rate indicating that the cells carried the state of oscillation from one generation to the next. However, the cells were not synchronized and were studied in isolation (Elowitz and Leibler, 2000).

1.2.1 Network Motifs

Transcription regulation networks control the expression of genes. The transcription networks of well-studied model organisms like *E.coli* are made of a small set of recurring network motifs like feedback and feed forward loops. The same network motifs were found in different organisms ranging from simple bacteria (Eichenberger et al., 2004) and yeast to highly evolved eukaryotes like plants (Saddic et al., 2006) and humans (Boyer et al., 2005; Odom et al., 2004). Therefore, it was suggested that the network motifs are one of the foundational elements of complex regulatory networks (Alon, 2007; Milo et al., 2002). As more and more network motifs are discovered and characterized, abstractional hierarchy can be applied to the understanding of complex biological networks. Experimental testing of these network motifs will help us better identify their evolutionary significance and ultimately lead to better understanding of dynamic biological networks.

1.2.2 Engineering Analogy

The genetic switch and the oscillator paved the path for the application of engineering inspired design to biological systems and demonstrated the programmability of biological systems and different variations of the toggle (Atkinson et al., 2003) including some based on the RNA (Bayer and Smolke, 2005; Deans et al., 2007) and the timer circuit (Fung et al., 2005; Stricker et al., 2008) have since been developed ,validating the design based genetic circuit construction approach. Various genetic circuits that mimic electrical and electronic components have since been constructed and tested including pulse generators, boolean logic gates (Anderson et al., 2007; Guet et al., 2002; Rackham and Chin, 2005), and filters (Sohka et al., 2009; Hooshangi et

al., 2005). A couple of examples of genetic circuits that mimic electrical and electronic components are described here. Basu and colleagues developed a gene network that acts like a pulse generator. It is a multi cellular system built in *E.coli* consisting of sender and receiver cells. The sender cells synthesize an inducer acyl homoserine lactone (AHL), which diffuses outside the cells to neighboring receiver cells. The pulse generator that responds to AHL is encoded in the receiver cells. A feed forward loop encoded in the pulse generator causes the cells to alternatively activate and repress the expression of green fluorescent protein (GFP) (Basu et al., 2004). Sohka and colleagues (Sohka et al., 2009) constructed a band- pass filter for cellular β lactamase activity in *E.coli*. β lactamases catalyze the hydrolysis of penicillin antibiotics such as ampicillin that inhibit cell wall synthesis. The design was a combination of positive and negative selections for β lactamase activity and analogous to an electronic band pass filter. Only those cells with β lactamase activity above certain level were able to grow in the presence of ampicillin.

1.2.3 Transcription Based Circuits

The most common form of biosensing at the cellular level is achieved through the transcription mechanism. Promoters transcribe or repress a particular gene in response to its transcription factor. Many different promoters like lac, ara and tet and their response to their associated transcription factors are very well characterized. The ability of the promoter to respond to a signal molecule can be altered by introducing mutations to the promoter site. For instance, mammalian cells have been programmed to respond to antibiotics like Streptogramin (Fussenegger et al., 2000), Tetracycline (Gossen and Bujard, 1992) and macrolides (Weber et al., 2002).

1.2.4 Translation Based Circuits

Translational and post-translational mechanisms can also be used in signal transduction. An RNA based gene regulation system was successfully demonstrated in mammalian cells where sequences of self-cleaving RNA aptamers were cloned into the sequences of genes. These ribozymes, when expressed, led to self-cleavage of the functional mRNA unit thus inhibiting the gene expression. Oligonucleotides that are complementary to the ribozyme sequences and

other small molecules inhibited self-cleavage of the mRNA (Yen et al., 2004). This technique can be used for the development of custom gene regulation systems that respond to any small molecule of interest and have huge potential in cell-based biosensors, the pharmaceutical industry and protein therapeutics.

RNA molecules could potentially act as sensors for various environmental and chemical signals and were in biosensing applications as described below. RNA aptamers were linked and bound to RNA regulatory domains. The analyte molecule is recognized and bound by the aptamer resulting in a conformational change in the molecule. The conformational change causes the aptamer to break free from the RNA regulatory domain and inhibit or activate translation of the reporter protein (Bayer and Smolke, 2005). DsrA is an *E.coli* RNA regulatory region that interacts with two different RNA regulators H-NS and RpoS by decreasing and increasing them respectively (Lease and Belfort, 2000). DsRA therefore acts a single control switch with different regulatory outcomes.

The approach to constructing synthetic biological systems has gradually moved from basic manipulation of intra-cellular transcriptional (Gardner et al., 2000) and translational (Bayer and Smolke, 2005) mechanisms to system level programming like intra-cellular communication (Basu et al., 2005; Basu et al., 2004). In both these publications, signal-processing architectures were built that can filter communication signals originating from 'sender' cells and can be programmed to form patterns. The circuits evolved from plasmid based transcriptional regulation systems into RNAi and translation-based systems (Rinaudo et al., 2007). Most of the circuits and genetic devices were designed for use in *E.coli* as the host organism or in other words "the chassis". The test bed has gradually extended from easy-to-manipulate *E.coli* to more complex chassis like yeast and mammalian cells (Deans et al., 2007; Kramer and Fussenegger, 2005). Various comprehensive reviews on the progress of synthetic biology over the last decade are available (Bhalerao, 2009; Khalil and Collins, 2010; Gore and van Oudenaarden, 2009). Though synthetic biology is a nascent discipline, its approach has been used for various applications such as in biosensing, pharmaceuticals, therapeutics and fuels.

1.3 Real World Applications of Gene Regulatory Circuits

The significant areas of contribution from synthetic biology as employed to gene regulatory circuits are metabolic engineering approaches - for manipulating existing pathways in organisms and introducing new pathways for the development of fuels and pharmaceutical products; and biosensing applications for a variety of analytes but mostly for use in diagnostics of diseases.

1.3.1 Crossover of Synthetic Biology with Metabolic Engineering

The common approaches towards making biofuel production more efficient are i) manipulating the pathways within the native organism and ii) introduction of these pathways into model organisms like *E.coli* and *S.cerevisiae*. Metabolic engineering approaches were applied to yeast for the production of ethanol from hemicellulose (Van Vleet and Jeffries, 2009) and also for the simultaneous fermentation of glucose and xylose (Jeffries, 2006). The engineered *S. cerevisiae* strain overexpressed *Piromyces XylA* xylose isomerase gene, and the native genes responsible for the conversion of xylulose to glycolytic intermediates. Pentose phosphate pathway was overexpressed by introducing fungal xylose isomerase thereby enabling xylose metabolism (Kuyper et al., 2005; Lee et al., 2008).

1.3.2 Applications in Fuels

Synthetic biology approaches are also making great strides in solving problems arising from increased energy demand. Biofuel alternatives to traditional fossil fuels are being explored. Certain microorganisms like *S. cerevisiae* have evolutionary advantage and are better suited for degrading cellulosic biomass into bioethanol. Hybrid processes that combine both biological and chemical production steps can lead to production of biofuels for use as alternative energy resources and would decrease the demand for fossil based fuels.

Butanol offers many advantages like higher energy content and hydrophobicity as a substitute for gasoline when compared to ethanol. Typically *Clostridium* is used to produce butanol in mixed product fermentation. A synthetic pathway was engineered in *E.coli* to demonstrate the

possibility of producing 1-butanol in a non-native host (Atsumi et al., 2008a). The genes responsible for synthesis of isopropanol and butanol in *Clostridium* were expressed in *E.coli* (Hanai et al., 2007). *S.cerevesiae* was also used to produce higher alcohols and esters from amino acids. A metabolic engineering approach was used to produce six different higher chain alcohols including isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol in *E.coli* from glucose (Atsumi et al., 2008b). Efforts are also being made to explore the fatty acid biosynthesis pathways of organisms like cyanobacteria and algae (Chisti, 2007).

1.3.3 Applications in Pharmaceuticals and Medicine

Malaria affects three to five hundred million people annually (Marsh, 1998). The parasite *Plasmodium falciparum* is multi-drug resistant. Artemisinin, a sesquiterpene lactone endoperoxide extracted from *Artemisia annua* is very effective against multi-drug-resistant Plasmodium. It is in short supply however, and unaffordable to most malaria sufferers. The chemical synthesis of the end product artemisinin is difficult and expensive. However, the semi-synthesis of artemisinin from its precursor artemisinic acid could be cost effective and reliable. Keasling et al. used a metabolic pathway reengineering approach to modify *E.coli* to produce higher levels of hard to produce drug artemisinic acid. They engineered *E.coli* to produce high titers of upto 100mg/L of artemisinic acid using an engineered mevalonate pathway, amorphaadiene synthase and cytochrome P450 monooxygenase from *A.annua* that performs a multi- step oxidation of amorpha-4, 11-diene to artemisinic acid (Newman et al., 2006b; Chang et al., 2007; Newman et al., 2006a) .

1.3.4 Single Cell Biosensor (SCB)

The cell can be considered as a signal-processing unit that is capable of distinguishing between different signals and integrating them in a biologically relevant manner and producing an output based on predefined logic (Bhalerao, 2009). 'Single-cell biosensors' (SCBs) are cells engineered to sense specific analytes and to produce a phenotypic response. This response can be interpreted as the presence or absence of the analyte or its concentration range. Because an SCB typically uses a biological or biochemical process to detect the analyte, the response can be

interpreted as a measure of the bioavailability of the analyte. This is an important class of application in synthetic biology, and one that is very closely related to this project. Therefore the topic of SCBs has been considered in detail in its own section below.

1.4 Design of a Single Cell Biosensor (SCB)

A biosensor is usually made up of a detection layer followed by the transduction of the detection signal to the display or the reporting layer. Internal sensors of a control system that are not for human interaction, like the error in a cruise control usually are missing the display layer. In a transcriptional biosensor, the detection mechanism can be compared to the activation of the promoter by the analyte, the transduction mechanism to the transcription of the mRNA, and the reporter mechanism to the translation of the reporter gene into a protein (Figure 1).

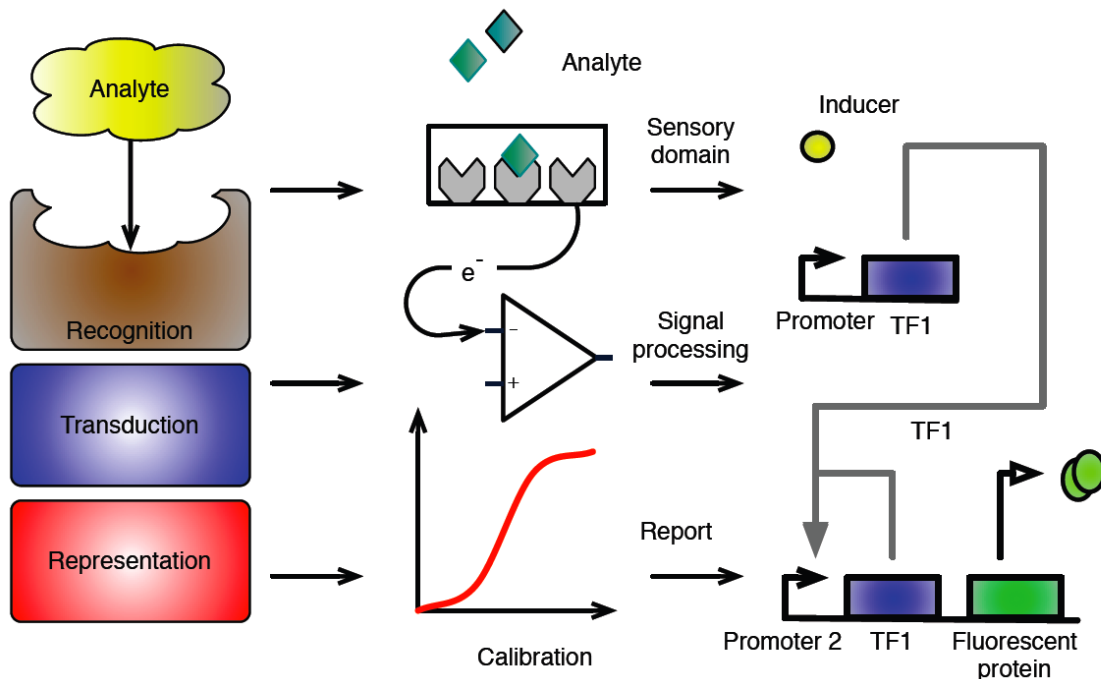


Figure 1. Layers of a biosensor and relevance to transcriptional gene circuit.

Biosensors can be abstracted to have three layers, a sensory detection layer, signal transduction and the reporting layer. In a transcriptional biosensor, the detection mechanism can be compared to the activation of the promoter by the analyte, the transduction mechanism to the transcription of the mRNA, and the reporter mechanism to the translation of the reporter gene into a protein.

1.4.1 Examples of Single Cell Biosensors

Synthetic biology approaches have also been used for constructing cells capable of sensing their environment. Such systems have applications in drug discovery and protein based therapeutics for diseases. *E.coli* was engineered to respond to their environment and selectively invade cancer cells without affecting healthy cells (Anderson et al., 2006). The *inv* gene that encodes invasins from *Yersinia pseudotuberculosis* was expressed in *E.coli*. Invasin binds to surface of β 1-integrins on mammalian cell surfaces and induces cellular uptake and was used to invade mammalian cells through adhesion. *E.coli* was designed to invade the mammalian cells at high cell density after anaerobic growth through the use of the quorum sensing mechanism of the *lux* operon and through induction of the arabinose operon. A synthetic platform was developed for the purposes of screening of small molecules that could increase the effectiveness of ethionamide (Weber et al., 2008). Ethionamide is a mycobacterium tuberculosis antibiotic used in the treatment of “superbugs” like multidrug resistant tuberculosis. The repressor protein *ethR* represses the transcription of enzyme *ethA* rendering ethionamide-based treatment ineffective. An *ethR*-based transactivator of a reporter gene was used to screen cells with *ethR* repressors and effectively repeal them thus making the drug more effective. Such an approach could be effectively applied to other disease causing drug resistant superbugs.

Genetic circuits were built to monitor the redox balance as an indicator for nutrient and oxygen availability (Weber et al., 2006) and other novel metabolites like nicotine (Malphettes et al., 2005). Genetic circuits in which genes could be regulated by addition of acetaldehyde in the gaseous form (Weber et al., 2004) and light sensitive *E.coli* (Levskaya et al., 2005) were developed and tested. These approaches can be seen in both bacterial based systems and mammalian systems where transcription factors are fused to a transactivation domain in order to regulate the gene expression through inducer dosage. The ability to create custom sensory domains that respond to a signal of interest could have far reaching implications in transgene therapy and biopharmaceuticals and synthetic biology allows us to do so.

1.4.2 Two-component Cell Signaling Systems and Framework

In the context of this study, the cell is considered as a signal-processing unit (Bhalerao, 2009).

Typically, the sensory elements used in SCBs are two-component signal transduction systems. They are composed of an outer signal receptor that is exposed to the environment and responds to changes in the native environment of the cell like chemical concentration, osmolarity and light intensity, to name a few. The receptor is linked to an inner cytoplasmic kinase through a transmembrane protein. A variety of hybrid receptors were developed using two component signal transduction systems, where the signal receptor of one system is fused to the transmembrane and the cytoplasmic unit of a different system, including light (Levskaya et al., 2005), various amino acids (Jin and Inouye, 1993; Michalodimitrakis et al., 2005) and sugars (Dueber et al., 2003; Dattelbaum et al., 2005).

Taz is a chimeric transmembrane receptor comprising of the periplasmic region of Tar, an aspartate receptor and the cytoplasmic domain of EnvZ, an osmosensor (Jin and Inouye, 1993). In the presence of aspartate, Taz can modulate the kinase to phosphatase ratio of OmpR. The phosphorylated OmpR-P binds to the OmpC promoter and switches on any genes under its control (Figure 2).

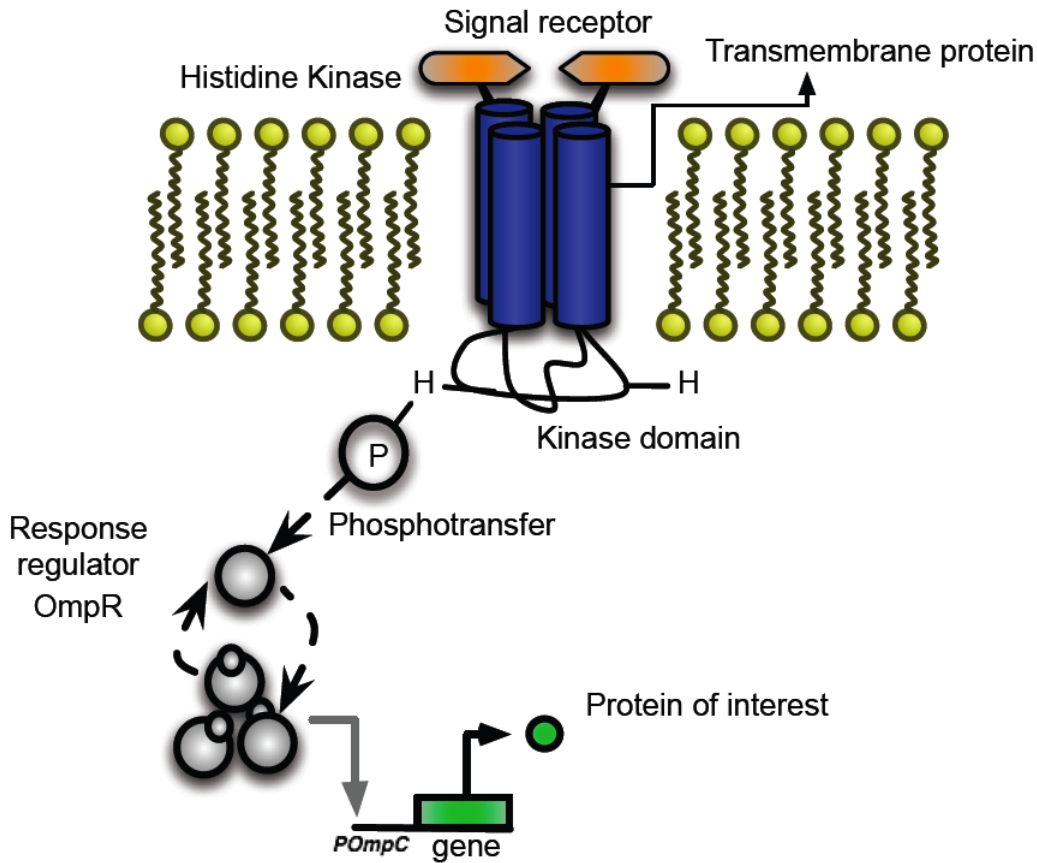


Figure 2. Two-component signaling systems with hybrid receptors.

Two-component-signaling systems are composed of an outer signal receptor that responds to changes in the native environment of the cell. Hybrid receptors have the signal receptor of one system fused to the transmembrane and the cytoplasmic unit of a different system. Taz is a chimeric transmembrane receptor containing periplasmic region of aspartate receptor Tar and the cytoplasmic domain of osmosensor EnvZ. In the presence of aspartate, Taz modulates the kinase to phosphatase ratio of OmpR. The phosphorylated OmpR-P binds to the OmpC promoter and switches on any genes under its control.

1.5 Amplification in Biosensors

“Biosensor” is a term broadly used to refer to sensor systems that are designed to detect or quantify the presence of a biological analyte. Furthermore in a biosensor, the recognition element of the sensor, which discriminates between the analyte from its environment, frequently exploits biochemical specificity. In addition to biochemical recognition, a biosensor system typically consists of transduction and reporting elements. Frequently, signal-processing

elements such as filters and amplifiers are also included in the biosensing system.

Whole organism biosensors are a special case of biosensors where all the elements of the sensor are integrated into one organism, which can be uni- or multi-cellular. Multi-cellular biosensors like the daphnia used for toxicity testing of water bodies are non-quantifiable and typically take a long time (a few days to attain a mature stage) thereby resulting in low sensitivity (Leung and Bulkley, 1979). SCBs, based on easily available platforms like various strains of *E.coli* or *S.cerevisiae*, are also inexpensive to culture, have a rapid generation rate and the reporting mechanism is faster relative to a multi-cellular system.

Computational approaches were also used to design receptors to specific analytes (Fazelinia et al., 2007; Tang et al., 2008). Computational approaches to designing signal receptors not only increase the range of analytes for which biosensors can be developed but also provide exogeneous sensing capabilities where the analyte does not enter the cell. A variety of signal receptors were found in probiotic *lactobacilli* (Wall et al., 2003) and were used as biosensors to investigate the binding to specific A-antigen expressed in the intestinal mucosa (Uchida et al., 2006).

1.5.1 Rationale

In signal receptor based biosensors, the possibility of non-detection or the dependence of threshold parameters to identify between true positives and false positives is rather high. In some cases, the signal maybe too generic or weak to characterize in a complex environment. A good example is the logical AND gate developed by Anderson (Anderson et al., 2007) that could be used to integrate multiple signals into one genetic circuit thereby rendering specificity to the signal. Genetic biosensors like this could be useful for communicating the state of a specific microenvironment within a mixture of environmental conditions, such as temperature, metabolite levels or cell density. Such biosensors could be envisioned to be of great use in bioprocessing industry for use within bioreactors.

One more approach to render specificity to a weak signal could be amplification. The presence of an amplification mechanism can be envisioned to decouple the performance of the

biosensor from sensitivity, analyte-signal receptor binding dynamics and threshold value dependence, enabling us to distinguish between true and false positives.

1.5.2 Objectives of the Study

The objective of this work was to construct and demonstrate a signal amplifier in the context of an SCB using the synthetic biology approach of abstraction, synthesis and modularity. A positive feedback motif was chosen in order to leverage the associated properties like amplification and hypersensitivity. The survey of positive feedback motifs in literature and their applications for uses in synthetic biology follows this section. The positive feedback based gene amplifier was designed to encompass all the prominent features of a positive feedback system including:

- 1) Amplification: The final level of output using the PF gene amplifier should be significantly higher than the control.
- 2) Increased sensitivity: The PF gene amplifier system should respond to inducer signals of weaker strength than the control with no positive feedback.
- 3) Bistability: The PF gene amplifier system was able to exist at two different steady states
- 4) Modularity: The PF gene amplifier should be modular and easily switchable and applicable in different scenarios.

1.5.3 Feedback Regulation

Cellular processes in living organisms are governed by interactions between various types of molecules such as proteins, DNA, RNA and enzymes. The interactions between proteins, their transcription factors (TF) and their target genes play a major role in the expression or suppression of a gene. These interactions can be studied and described as regulatory gene transcriptional networks and are ubiquitous in most of the biological processes. These networks are largely composed of a recurring set of network motifs found in bacteria, yeast, plants and animals.

Feedback is a very common network motif found in prokaryotic and eukaryotic organisms. In some cases, feedback is implemented via transcriptional control, i.e. through the action of gene products as transcription factors. When the transcriptional factor directly affects its own transcription, either in a positive or negative manner, it is said to assume an “autoregulatory” role. When an autoregulatory transcription factor enhances its own production, the circuit is said to involve positive feedback and the autoregulatory transcription factor is called an auto-inducer. Likewise, when the gene product suppresses its own production, the circuit is said to involve negative feedback and the autoregulatory transcription factor is called an auto-repressor. Autoregulatory behavior is analogous to feedback loops in electromechanical control systems.

1.5.4 Positive Feedback Systems

Positive feedback (PF) is a commonly seen mechanism in genetic circuits (Mitrophanov and Groisman, 2008). In the lac operon, LacI normally acts as a repressor and represses the transcription of the lacZYA operon. However in the presence of allolactose inducer, it cannot bind to the lac operator anymore and is derepressed, leading to the production of LacY protein. The LacY protein is responsible for the intake of lactose and its subsequent conversion to allolactose leading to the production of more LacY protein. Thus, there is a Positive Feedback Loop (PFL) involving allolactose and LacY. However there is also an inbuilt negative feedback loop in the form of LacZ that metabolizes allolactose. Therefore, the amount of allolactose available for the derepression of LacI is decreased, ultimately leading to repression of the lacZYA operon (Lewis, 2005; Jacob and Monod, 1961).

The mutual repression network seen in the phage lambda lysis-lysogeny decision-making circuit is also an example of a positive feedback loop. The CI protein is an activator for its production but also acts as a repressor at very high levels of CI. The CI protein also acts as repressor for the *cro* and the *CI* genes resulting in a negative feedback loop (NFL) embedded in a PFL (Ptashne et al., 1982; Tian and Burrage, 2004; Dodd et al., 2005). In a number of synthetic biology applications, positive feedback has been used to design switches, oscillators, and amplifiers. Besckei and coworkers (Besckei et al., 2001), for example, showed in yeast that a simple

positive feedback loop could transform a graded response to an inducer into a binary one. Likewise, Kramer and Fussenegger (Kramer and Fussenegger, 2005) showed that positive feedback could be used to generate hysteresis with respect to an inducer in mammalian cells. Maeda and Sano (Maeda and Sano, 2006b) analyzed a synthetic positive feedback loop in *E.coli* and demonstrated that it could give rise to either a graded or hysteretic response depending on the specific configuration. In terms of building circuits, Ajo-Franklin and coworkers demonstrated that positive feedback could be used to engineer memory into yeast cells (Ajo-Franklin et al., 2007). Stricker and coworkers, on the other hand, built a simple oscillator by coupling positive feedback with negative feedback (Stricker et al., 2008). In work most closely related to the present study, Sayut and coworkers demonstrated that a positive feedback loop could make the transcriptional activity of the quorum-sensing regulator LuxR more sensitive to autoinducer (Sayut et al., 2006). In these regards, their design is most closely related to how positive feedback is typically employed in electronic circuits, namely to amplify the response to a signal.

1.5.5 LuxR-Based Implementation of the Positive Feedback

Acylhomoserine lactone-mediated quorum sensing and response is very commonly found in Gram-negative bacteria. A typical example of this kind is cell density dependent gene regulation and the activation of the *Vibrio fischeri* luminescence (*lux*) operon by LuxR. LuxR function requires sufficient concentrations of the diffusible signal, N- (3-oxohexanoyl)-homoserine lactone (3-oxo-C₆-HSL) also called AHL (acyl HSL), which is a product of LuxI. A fragment of *V. fischeri* DNA that encodes all of the functions necessary for autoinducible luminescence was cloned into *E.coli* (Engebrecht et al., 1983). There are seven different *lux* genes that are required for luminescence in *E.coli*. They are organized as two divergently transcribed units. One unit contains the *LuxR* gene that encodes the LuxR protein and is required for a response to auto inducer. The other unit is activated by the LuxR protein in the presence of autoinducer and contains *luxA* and *luxB* that encode for the α and β subunits of luciferase, *luxC*, *luxD*, and *luxE* that encode for polypeptides involved in synthesis of the aldehyde substrate for luciferase and finally *luxI* that is required for synthesis of autoinducer by *E.coli* (Figure 4).

1.5.6 Transcriptional Regulators Responsive to AHL

The LuxR family of proteins usually are about 250 amino acids in length and are composed of two functional domains: N-terminal domain that is involved in AHL-binding and a C-terminal transcription regulation domain, which includes a helix-turn-helix (HTH) DNA-binding motif. The LuxR-type proteins interact with DNA in a dimeric state and specifically recognize a symmetric sequence located in the regulatory regions of the target genes. Most of them are activators and the regulatory protein/DNA interaction causes the recruitment of the RNA polymerase to the promoter and, therefore, the transcription of the target genes. The LuxR protein is predicted to be 250 amino acid residues. Randomly generated point mutation studies indicate that two regions of the LuxR protein are critical for activity. Residues 79-127 are involved in autoinducer binding, and residues 184-230 constitute the highly conserved helix-turn-helix region of the LuxR family is involved in lux DNA binding (Slock et al., 1990).

1.5.6.1 Lux Regulatory Region (Consensus Binding Sequence)

The lux box (Figure 3) is a 20-bp inverted repeat centered at -42.5 bp from the start of luxI transcription and is a binding site for LuxR. Lux box-type sequences have also been identified in the binding sites of other LuxR members like TraR and LasR.

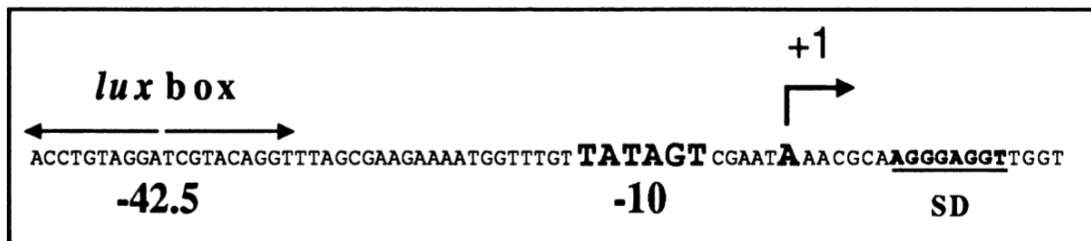


Figure 3. Lux box.

Lux box is a binding site for LuxR and is a 20-bp inverted repeat centered at -42.5 bp from the start of luxI transcription start codon.

1.5.6.2 AHL Binding Leads to Multimerisation

AHL binding causes large conformational changes and change in transcriptional activity of the regulators. For most of the activators of the LuxR family, AHL binding induces dimerization of

the protein, which is a prerequisite for the activation of transcription. TraR, LuxR, RhIR and LasR bind to their target DNA only after dimerization and in the presence of their cognate autoinducer.

1.5.7 Autoinducer Independence

Mutations within the LuxR can make it autoinducer independent. It is of significance in this study in order to make LuxR activation independent of the cell density and the autoinducer AHL.

1.5.7.1 Point Mutation

Point mutations have been identified that result in the activation of LuxR in the absence of the autoinducer. The residue Ala221 to Val 221 substitution renders LuxR capable of activating the expression of target genes independent of the presence of AHL. Since the resulting mutant Ala221 -> Val was not affected in the autoinducer binding, this residue enables the N-terminal domain to inhibit the activity of the C-terminal domain (Poellinger et al., 1995).

1.5.7.2 Deletion of N Terminal Domain

Deletion of the LuxR N-terminal domain results in a derivative that is able to interact with DNA and able to activate the transcription of target genes, in the absence of AHL.

LuxR Δ N

LuxR Δ N, a 95-residue C-terminal fragment of LuxR, binds DNA and activates transcription of the lux operon in a signal- independent manner (Choi and Greenberg, 1991). LuxR Δ N purifies as a monomer and the region required for oligomerization resides in the N-terminal domain of the full-length polypeptide. Only a small part of this oligomerization region exists on LuxR Δ N (Stevens et al., 1994). The N-terminal domain masks the C-terminal DNA binding domain in the absence of AHL and thus interferes with DNA binding. One dimer activation model states that binding of the appropriate ligand to the activator induced conformational changes, which led to the exposure of the DNA binding site.

LuxR Δ N Binding Affinity to Lux Promoter

Neither LuxR Δ N nor RNAP alone bind tightly to the luxI promoter region, but together they protect a region extending from -54.5 to +6 against DNase I digestion in vitro (Stevens et al., 1994). Because LuxR Δ N by itself does not protect the lux box from DNase I digestion, it can be assumed that LuxR Δ N binds to the lux box and RNAP to the downstream region in the DNase I protection experiments. Because LuxR Δ N is monomeric, it is possible that activation of the luxI promoter by this protein should only require a lux box half-site to be occupied if a contact with RNAP adjacent to that half-site is necessary for LuxR activity.

1.5.8 Structure of TraR

The crystal structure of TraR, a luxR type protein has been determined (Qin et al., 2000). TraR is a dimer in solution and binds target promoters in vitro as a dimer. TraR was crystallized in the presence of OOHL and a self-complementary oligonucleotide containing the tra box sequence. The crystal asymmetric unit contains two structurally similar TraR dimers, each binding two molecules of OOHL and one duplex DNA fragment. The N-terminal domain of each monomer (residues 1–162) binds one molecule of OOHL. The C-terminal domain (residues 175–234) binds to half of a tra box. A twelve-residue amino acid linker joins these domains. The N-terminal and C-terminal domains both contribute to protein dimerization. The two N-terminal domains dimerize chiefly through α -helix 9 of each protomer (the longest α -helix in the protein), forming a coiled-coil, whereas α -helix 1 of each subunit contacts α -helix 8 of the opposite subunit. The two C-terminal domains dimerize through α -helix 13 of each subunit, which form a second coiled-coil. Additional contacts are made by salt bridges between the C-terminal carboxylate of each subunit and Arg 230 of the opposite subunit (Zhang et al., 2002). Analysis of heterodimers formed between TraR and its deletion mutants localized the dimerization domain to a region between residues 49 and 156. The binding signal drives dimerization of TraR and its release from membranes into the cytoplasm.

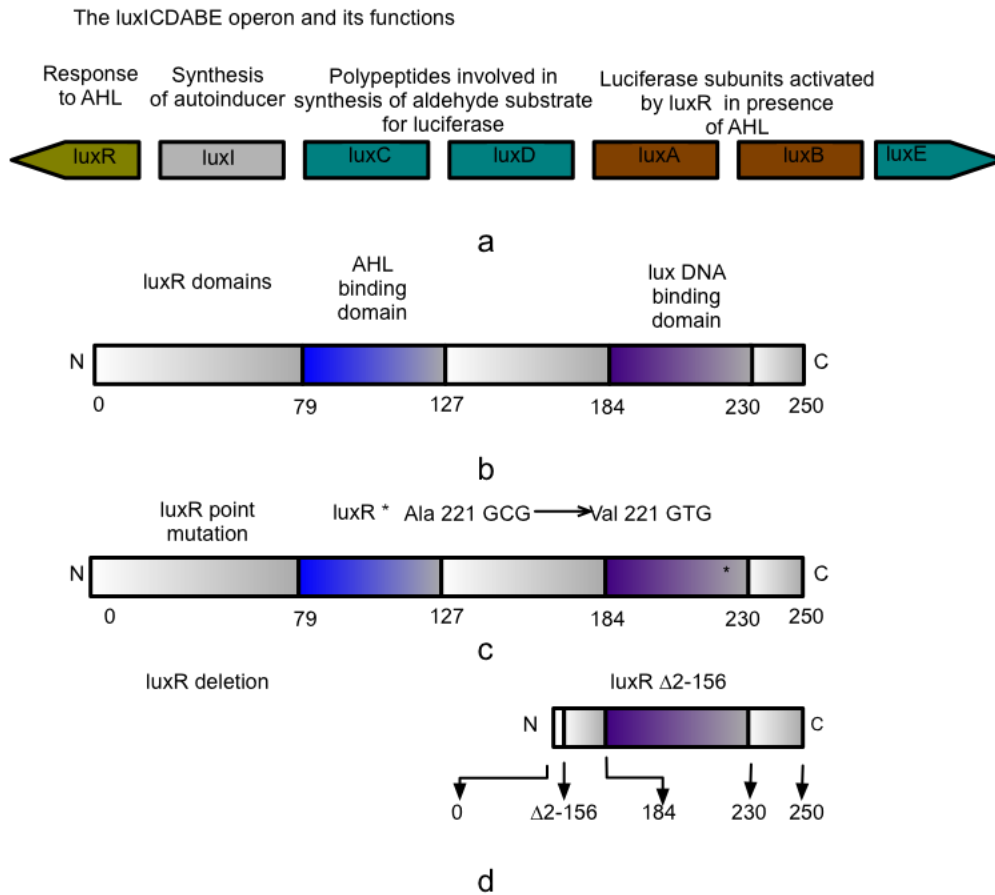


Figure 4. LuxICDABE operon and mutations for autoinducer independence.

a. *luxR* gene encodes the LuxR protein and is required for a response to AHL. *luxA* and *luxB* encode for the α and β subunits of luciferase. *luxC*, *luxD*, and *luxE* encode for polypeptides involved in synthesis of luciferase aldehyde substrate. *luxI* is required for synthesis of autoinducer by *E.coli*. The lux promoter is activated by LuxR protein in the presence of autoinducer. **b.** Residues 79-127 are involved in autoinducer binding, and residues 184-230 constitute the lux DNA binding domain **c.** Ala221 to Val substitution disables the N-terminal domain to inhibit the activity of the C-terminal domain and makes LuxR capable of activating the expression of target genes independent of the presence of AHL. **d.** LuxR Δ N, a 95-residue C-terminal fragment of LuxR, binds DNA and activates transcription of the lux operon in a signal-independent manner.

1.5.9 Testbed

Positive feedback can result in amplification behavior. LuxR can be modified to make it autoinducer independent and also act as an autoregulator. Therefore, *luxR* was chosen as the feedback component of the positive feedback based amplifier. The simplest realization of this

system was a one-component sensor in *E.coli*. The SCB responds to signal of interest. The positive feedback signal amplifier amplifies this response. The response was measured through a fluorescent protein. The anhydro tetracycline sensor was chosen because of ease of use and availability.

Once the system was developed and characterized, it was then tested for modularity. The feedback amplifier was not altered. The system was manipulated only at the sensor level and developed for use as an SCB for an analyte of interest. The two-component Tar-EnvZ fusion Taz was chosen for use as an aspartate biosensor. The details of design, development and characterization are presented in the next chapter.

Chapter 2 Materials and Methods

2.1 Media and Growth Conditions

All cultures and experiments involving the one component tetracycline sensor were performed in Luria-Bertani (LB) broth. The LB broth composition is given in Table 2.

Table 2. LB broth.

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

1000 ml of water was then added and the solution was autoclaved .

All the cultures and experiments involving the two-component aspartate sensor were performed in M9 minimal media. 10x M9 salts stock solution was prepared as shown in Table 3.

Table 3. 10X M9 stock solution.

$\text{Na}_2\text{HPO}_4 \times 7\text{H}_2\text{O}$	60 g
KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g

1000 ml of water was then added and the solution was autoclaved.

The 1x M9 minimal media was then made using the stock solution as shown in Table 4.

Table 4. 1X M9 minimal media.

Sterile water	900 ml
M9 salts 10X stock solution	100 ml
Autoclaved 1 M MgSO ₄	1 ml
Autoclaved 1 M CaCl ₂	0.1 ml

Supplements were added to the M9 minimal media as shown in Table 5.

Table 5. Supplements to M9 minimal media.

Glucose	0.4%
Thiamine	1 µg/mL
Biotin	1 µg/mL

All the experiments were performed at 37°C unless noted otherwise. Antibiotics were used at concentrations as shown in Table 6.

Table 6. Antibiotics.

Ampicillin	100 µg/mL
Chloramphenicol	34 µg/mL
Kanamycin	40 µg/mL.

Primers were purchased from IDT Inc. (Coralville, IA). Restriction enzymes were purchased from New England Biolabs Inc. (Ipswich, MA) and Fermentas Inc. (Glen Burnie, MD) and used according to the manufacturer's recommendations.

2.2 Bacterial Strains and Cell Line Construction

All the cloning steps were performed in *E.coli* strain DH5 α (F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ 80d/lacZ Δ M15 Δ (*lacZYA-argF*) U169, hsdR17 ($r_K^- m_K^+$), λ^-). The experiments involving aspartate induction were performed in GN101 (F^- *ilvG rfb-50 rph-1 Δ envZ::FRT*). Strain GN101 was constructed first by P1vir transduction of the Δ envZ::*kan* insert from JW3367-3 (The *E.coli* Genetic Stock Center, CGSC# 10509) into strain MG1655. The process of lysate preparation and the transduction are described below.

2.2.1 Preparation of the P1 Phage Lysate From the Donor Strain JW3367-3

1. An over night culture of the donor strain Δ envZ :: *kan* insert from JW3367-3 was made (The *E.coli* Genetic Stock Center, CGSC# 10509)
2. The donor strain over night culture was diluted to a ratio of 1:50 into fresh LB medium + 5mM CaCl₂ + 0.2% glucose
3. The subculture was then grown at 37°C for a period of 1 -2 hours until visible growth
4. Few drops of P1 phage lysate were dropped into the subculture and grown further for 3 hours until the culture lysed
5. Chloroform was then added to the lysate and mixed thoroughly.
6. The cellular debris was centrifuged away and the lysate stored at 4°C for further use.

2.2.2 Transduction

1. 4 ml of the recipient strain MG1655 was grown overnight in LB medium
2. The over night culture was centrifuged at a speed of 3000 rpm for 5 minutes and resuspended in an equal volume of fresh LB medium + 5mM CaCl₂ + 100 mM MgSO₄)
3. Few drops of the donor P1 lysate prepared above and 200 μ L of the cells prepared in step 2 were incubated at 37°C for 30-45 minutes. The donor lysate with LB Medium and

recipient cells with LB medium were similarly treated for control experiments.

4. 200 μ L of 100 mM Na-Citrate and 1 ml of LB medium was then added and further grown for 1.5 to 2 hours.
5. The cells were centrifuged at 3000 rpm for 5 minutes and resuspended in 200 μ L of LB+ 100mM Na-Citrate and spread on selective plates and incubated at 37°C

The colonies obtained the following day were streaked for 2 times consecutively on plates containing the selective antibiotic and sodium citrate to avoid phage contamination.

The kanamycin antibiotic cassette from the FRT-Kan-FRT insert was then removed by transformation of pCP20 into the strain and selection on ampicillin at 30°C (Datsenko and Wanner, 2000). Loss of the helper plasmid pCP20 was obtained by growth at 42°C under non-selective conditions on LB agar.

All subsequent experiments involving anhydrotetracycline (aTc) induction were conducted in *E.coli* strain GN100 (F^- *ilvG rfb-50 rph-1 Δ envZ::FRT attB $_{\lambda}$::[P_{N25}-tetR lacI^q spcR]*). Lastly, the chromosomally integrated TetR/LacI expression cassette from DH5 α Z1 (Lutz and Bujard, 1997) was moved into GN101 strain by P1 $_{vir}$ transduction described as above, yielding strain GN100.

2.3 Plasmid Construction

All the plasmids used are provided in Tables 7 and 8 and the primers are provided in Table 9. The following constraints have been taking into account for the construction of the plasmids.

1. The two and three plasmid systems should have compatible origins of replication.
2. The antibiotic resistance markers should be different to enable selection for cells transformed with multiple plasmids.

The aTC sensor plasmids pGN3 and pGN12 were designed to have colE1 origin of replication and kanamycin selection marker. The positive feedback plasmids and the non- positive feedback plasmids with the Green Fluorescent Protein (GFP) reporter were designed with p15A origin of replication and chloramphenicol selection marker. The aspartate sensor plasmid was designed with pSC101 origin that was compatible with both colE1 and p15A origins of

replication and has the ampicillin selection marker.

The plasmid pPROTetE-Kan-p15A was made by swapping the ColE1 origin of pPROTet.E with the p15A origin from pZA34-luc (Lutz and Bujard, 1997) using the restriction sites XbaI and SacI and by swapping the chloramphenicol resistance gene with the kanamycin resistance gene from pZE21 using the restriction sites XhoI and SacI. The plasmid pPROTetE-Amp was made by replacing the chloramphenicol resistance gene in pPROTet.E with the ampicillin resistance gene from pZE12(Lutz and Bujard, 1997) using the restriction sites XhoI and SacI.

2.3.1 Construction of the Non-Positive Feedback Plasmid

The *luxI-GFP* transcriptional fusion was made first by PCR amplification of the *luxI* promoter using the plasmid *pluxGFPuv* (You et al., 2004) as the template with the primers KW134F. The resulting fragment was then cloned into the plasmid pPROTet. E using the restriction sites EcoRI and AatII, yielding the plasmid pGN23. The green fluorescent protein (GFP) was PCR amplified from pPROBE-*gfp* [tagless] (Miller et al., 2000) using primers GN10F and GN10R. The resulting fragment was then cloned into the EcoRI and BamHI restriction sites of the pGN23, yielding the plasmid pGN69 (Figure 6).

2.3.2 Construction of the aTc Sensor Plasmids

The LuxR* (LuxR [A221V]) expression plasmids were constructed using two rounds of PCR. In the first round, the *luxR* gene was amplified with primers KW78F1 and KW078R using pLuxRI (You et al., 2004) as the template. The resulting product was then used as a template for a second round of PCR this time using primers KW078F2 and KW078R. It was then digested with EcoRI and Sall and sub-cloned into the EcoRI and Sall restriction sites of pPROTetE-Kan-p15A. Enzymatic inverse PCR was used to introduce the Ala221Val (GCG->GTG) point mutation in the *luxR* gene with primers KW079F. The resulting PCR product was then digested with BsaI and ligated to obtain pGN3.

The *luxRA*_{Δ2-156} expression plasmid was also constructed using two rounds of PCR. The *luxR* gene was first amplified with primers KW112F and KW078R using pLuxRI2 as the template. The resulting PCR product was then amplified using primers KW078F2 and KW078R. It was then

cloned into the EcoRI and Sall cut-sites of pPROTetE-Kan-p15A, yielding pGN11. Similarly, $luxRA_{2-162}$ was made by amplifying the *luxR* gene with primers KW113F and KW078R using pLuxRI2 as the template. The resulting product was amplified again as before using primers KW078F2 and KW078R. The PCR product was then digested with EcoRI and Sall and sub-cloned into the EcoRI and Sall cut-sites of pPROTetE-Kan-p15A, yielding the $luxRA_{2-162}$ expression plasmid pGN12 (Figure 7).

2.3.3 Construction of the Positive Feedback Plasmids

The positive-feedback module was constructed using two rounds of PCR. In the first round, the primers GN09F2 and KW171R were used to amplify the $luxRA_{2-162}$ domain using pLuxRI2 [12] as the template. The resulting PCR product was then used as template for a second round of PCR this time using primers GN09F and KW171R. The resulting fragment was then digested with BamHI and NotI and sub-cloned into pGN69, yielding pGN68 (Figure 5). Similarly, pGN29 consists of a plasmid harboring the ColE1 origin of replication, chloramphenicol resistance (Cm^r), a green fluorescent protein (*gfp*) and a copy of *luxR** under the control of the *luxI* promoter P_{luxI} .

The aspartate positive feedback module was constructed first by amplifying the P_{ompC} promoter (genomic region 2310762-2310962) using primers GN03F and GN05R. The PCR product was then digested with XhoI and EcoRI and sub-cloned into the respective sites of pPROTetE-Kan-p15A, thus replacing the native $P_{LtetO-1}$ promoter with the P_{ompC} promoter. The primers GN06F2 and KW171R were used to amplify $luxRA_{2-162}$ using pGN68 as the template. The resulting PCR product was digested with Sall and NotI and then sub-cloned into the respective sites of pPROTetE-Kan-p15A, yielding pGN62.

2.3.4 Construction of the Aspartate Sensor Plasmid

The aspartate sensor module was constructed first amplifying the *taz* gene from pTJ003 (Jin and Inouye, 1993) using the primers GN13F and GN12R. The PCR product was then digested with EcoRI and SacI and cloned into the unique respective restriction sites, yielding pGN76. The ColE1 origin in pGN76 was then replaced with the pSC101 origin from the pZS24 plasmid using

the restriction sites *AvrII* and *SacI*, yielding pGN77.

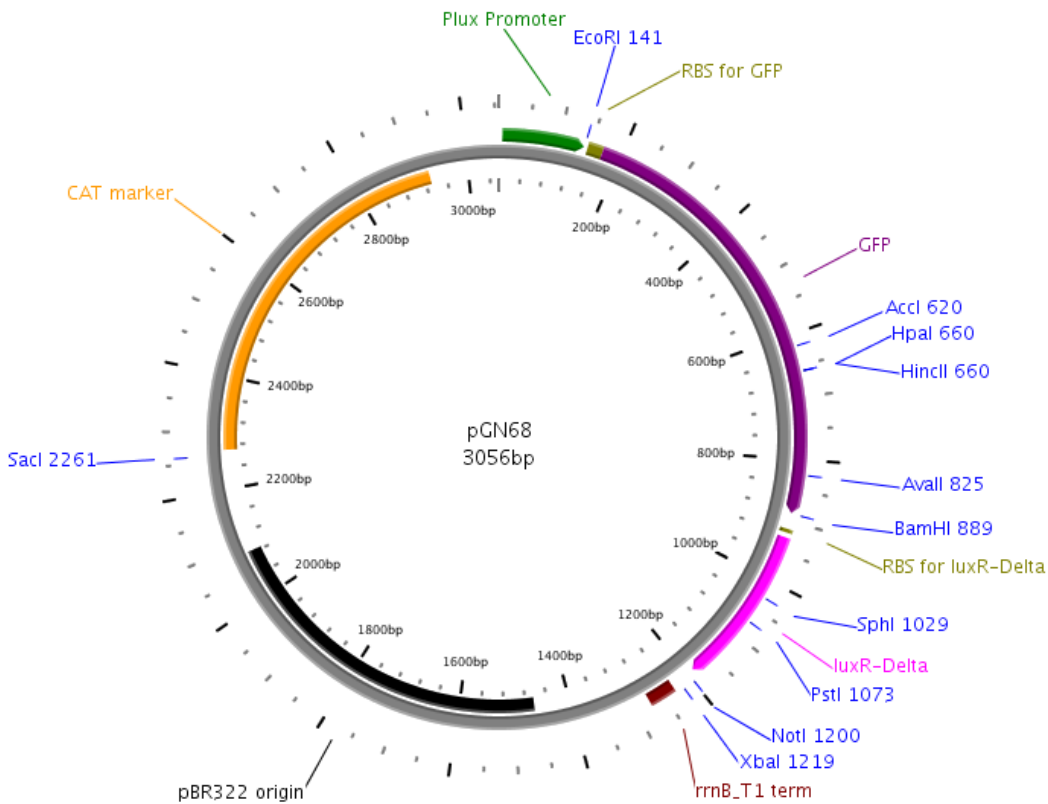


Figure 5. Positive feedback plasmid pGN68.

The positive feedback plasmid pGN68 containing the P_{lux} promoter, the GFP gene, $lux\Delta(2-168)$, ColE1 origin of replication and the chloramphenicol marker. pGN29 is similar and has $luxR^*(Ala221-Val)$ instead of $lux\Delta(2-168)$. All the plasmid diagrams (Figures 5, 6 and 7) have been made using PlasMapper(Dong et al., 2004).

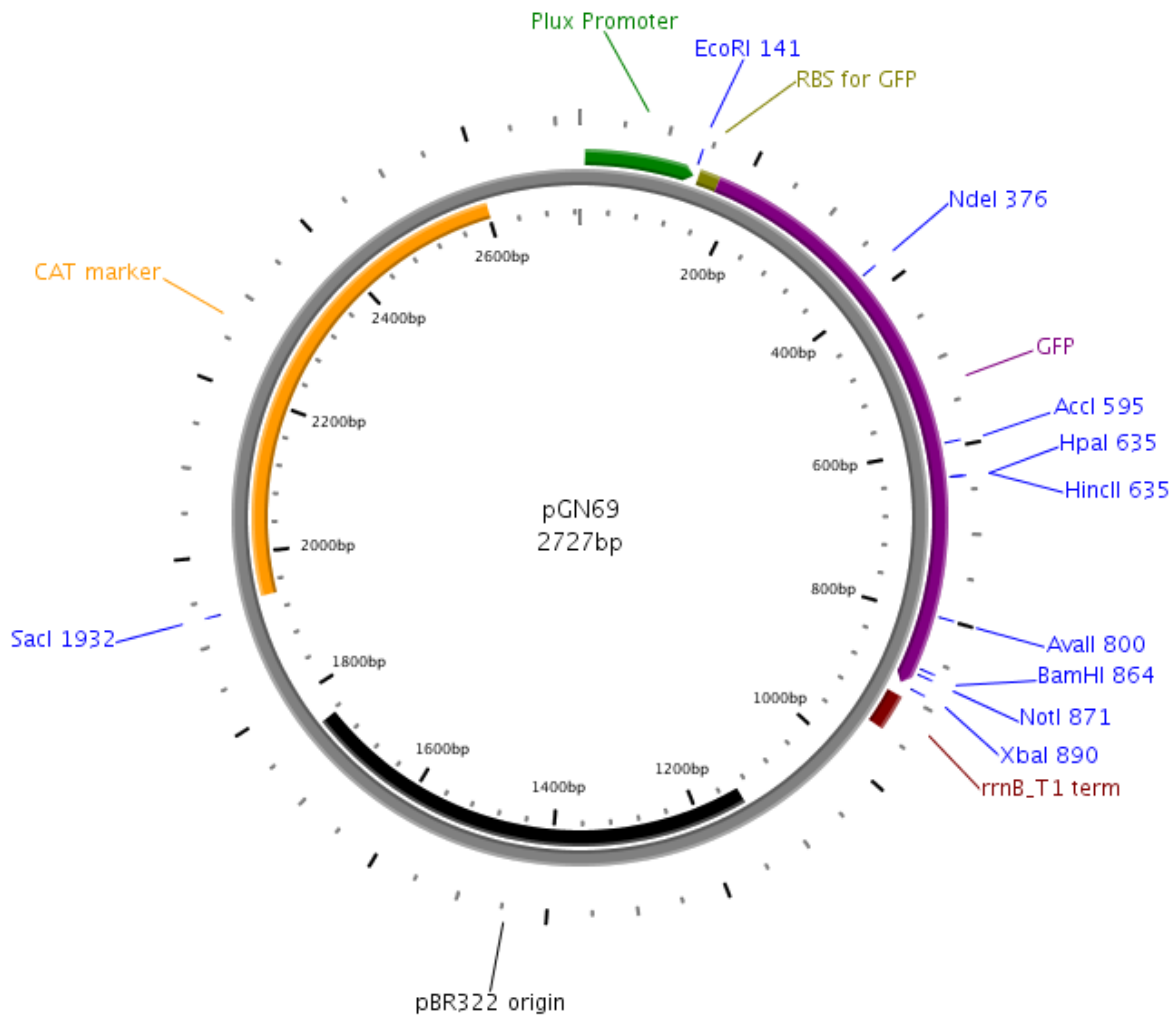


Figure 6. Non-positive feedback plasmid pGN69.

The non-positive feedback plasmid pGN69 has the P_{luxI} promoter, GFP, ColE1 origin of replication and the chloramphenicol selection marker.

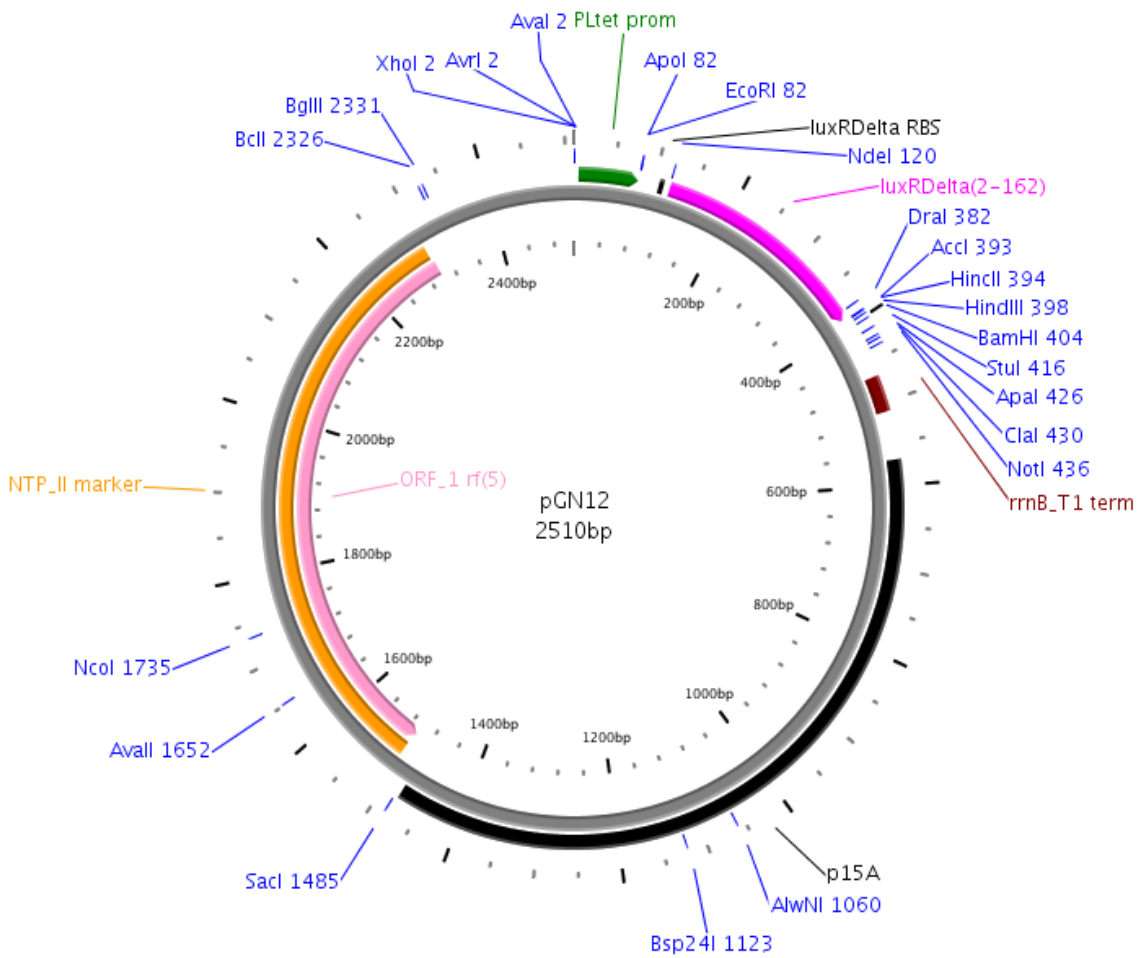


Figure 7. aTc sensor plasmid pGN12.

The aTc sensor plasmid pGN12 has the $P_{ITetO-1}$ promoter, the *luxRΔ(2-162)*, p15A origin of replication and the Kanamycin marker.

2.4 Fluorescence Assays

To measure fluorescent protein expression, cultures were first grown overnight and then subcultured to an OD₆₀₀ of 0.05 in fresh media. The cultures were first allowed to grow to an OD₆₀₀ of 0.20, at which point the inducer was added. The cultures were then grown overnight prior to taking the measurements. 100 µL of the culture was then transferred into a 96 well microplate, and the relative fluorescence and optical density at 600 nm (OD₆₀₀) were measured using a Tecan Safire2 microplate reader. The fluorescence readings, given as relative fluorescence units (RFU), were normalized with the OD₆₀₀ absorbance to account for cell density. All experiments were performed in triplicate with 95% confidence intervals reported.

2.5 Experiments for Measurement of Kinetics

Cultures were grown overnight at 37°C with vigorous shaking. The next morning, they were subcultured to an OD of 0.05 in fresh medium in triplicates and allowed to grow to an OD of 0.15. They were then induced with various levels of aTc ranging from 0 to 100ng/ml. Every hour, 100 µl of the cultures at different levels of induction conditions were transferred to a 96-well microplate. The temperature was maintained at 37°C, and fluorescence and OD readings were taken. All experiments were done in triplicate and average values with the 95% confidence intervals reported.

2.6 Single Cell Measurements

The cells were first grown overnight at 37°C in non-inducing conditions coupled with vigorous shaking. The overnight cultures were then sub-cultured to a ratio of 1:200 in fresh LB medium and grown to an OD of 0.05 at 37°C. Once an OD of 0.05 was attained, they were then induced with various concentrations of the inducer. 200 µl of the samples were collected at various time points in micro centrifuge tubes. They were then centrifuged at a speed of 3000 rpm and resuspended in 1ml of phosphate-buffered saline. In order to capture the cells in their state at the time of sampling, Chloramphenicol (34 µg/ml) added to stop all translation and stored on ice until the flow cytometry data was obtained. All the flow cytometry experiments were

performed on a BD LSR II system (BD Biosciences). FCS Express version 3 (De Novo Software) available at the University of Illinois was used for the purpose of data extraction and analysis.

Table 7. Plasmids used in this study.

Plasmid	Relevant characteristic	Reference
pTJ003	bla P _{lpp} -taz ori p15A	(Jin and M Inouye 1993)
pPROTet.E	cm P _{LtetO-1} ori ColE1	Clontech
pPROBE-GFP	kan GFP[tagless] ori p15A	(W G Miller, Leveau, and Lindow 2000)
pZE12-luc	bla P _{LlacO-1} -luc ori ColE1	(Lutz and Bujard 1997)
pZE21	kan P _{LtetO-1} ori ColE1	(Lutz and Bujard 1997)
pZS24	kan P _{lac/ara-1} ori pSC101	(Lutz and Bujard 1997)
pluxRI	cm P _{lac/ara-1} -luxR-luxI ori ColE1	(You et al. 2004)

Table 8. Plasmids constructed in this study.

pPROTetE-kan-p15A	kan P _{LtetO-1} ori p15A
pGN3	kan P _{LtetO-1} -luxR* ori p15A
pGN11	kan P _{LtetO-1} -luxR Δ_{2-156} ori p15A
pGN12	kan P _{LtetO-1} -luxR Δ_{2-162} ori p15A
pGN23	cm P _{lux} ori ColE1
pGN29	cm P _{lux} -luxR* -GFP [tagless] ori ColE1
pGN62-Kan	kan P _{OmpC} -luxR Δ_{2-162} ori p15A
pGN68	cm P _{lux} -GFP [tagless]-luxR Δ_{2-162} ori ColE1
pGN69	cm P _{lux} -GFP[tagless] ori ColE1
pPROTetE-amp	bla P _{LtetO-1} ori ColE1
pGN76	bla P _{LtetO-1} -taz ori ColE1
pGN77	bla P _{LtetO-1} -taz ori pSC101

Table 9. Primers used in this study

Primer name	Primer Sequence
KW134F	CAG ATA TCG ACG TCA GTC C
KW134R2	ATA GAA TTC TGC GTT TAT TCG ACT ATA AC
GN10F	GGG GAA TTC ATA CGT ATT TAA ATC AGG AGT GGA AAT GAG TAA AGG AGA AGA ACT T
GN10R	GGG GGA TCC TTA TTA TTT GTA TAG TTC ATC CA
KW78F1	AAC TTT ATA AGG AGG AAA AAC ATA TGA AAA ACA TAA ATG CCG AC
KW078R	ACT GTC GAC TTA ATT TTT AAA GTA TGG GC
KW078F2	TAT GAA TTC AAC TAA AGA TTA ACT TTA TAA GGA GGA AAA ACA
KW079F	ATA GGT CTC TGT GCA AAT GAA ACT CAA TAC AAC
KW079R	ATA GGT CTC TGC ACA TTG GTT AAA TGG AAA GTG A
KW113F	CTT TAT AAG GAG GAA AAA CAT ATG CCT TCT CTA GTT GAT AAT TAT C
GN09F2	AAC TAA AGA TTA ACT TTA TAA GGA GGA AAA ACA TAT GCC TTC TCT AGT TGA TAA T
KW171R	AAT AGC GGC CGC TTA TTA ATT TTT AAA GTA TGG GC
GN09F	GGG GGA TCC AAC TAA AGA TTA ACT TTA TAA GGA GGA AAA ACA T
GN03F	GGG CTC GAG GTT CCC TTG CAT TTA CAT TTT
GN05R	GGG GAA TTC TAA CTT TCA TGT TAT TAA CCC
GN06F2	GGG GTC GAC ATG CCT TCT CTA GTT GAT AA
GN13F	GGG GAA TTC TTA AAG AGG AGA AAG GTA CCC ATG ATT AAC CGT ATC C
GN12R	GGG GTC GAC TTA CCC TTC TTT TGT CGT GCC CT

Chapter 3 Results and Discussion

The gene amplifier was designed and tested in two different contexts coupled with an anhydrotetracycline (aTc) sensor and an aspartate sensor (Nistala et al., 2010). The sensory and feedback components were individually tested before integration. The construction and characterization of the positive feedback based gene amplifier is described below.

3.1 One Component Tetracycline Sensor

The positive feedback based gene amplifier was first constructed and coupled to an aTc sensor. Based on the objectives for this study, it was characterized for different properties including amplification, hypersensitivity, bistability and memory. All the experiments and the associated results are described in different subsections below.

3.1.1 Effect of the Inducer aTc on Cell Growth

Anhydrotetracycline, aTc, is an antibiotic derivative of tetracycline and was obtained from Clontech® and used as the inducer for experiments involving the $P_{\text{LtetO-1}}$ promoter. Owing to its potential cytotoxic effect, it was important to determine the toxic dosage of aTc to the cells. Strain GN100 was tested to check for the cytotoxic effects of aTc. GN100 was grown overnight in LB media and then diluted 1:100 into six test tubes containing 2 ml of LB each. They were then treated to 0, 100 and 250 ng/ml of aTc in duplicates. 500 ul of each of the cultures was transferred to a cuvette and the Optical Density (absorbance at 600 nm) was measured manually using Amersham Biosciences Ultrospec 10 Cell Density meter® every 30 minutes until the cultures reaches stationary phase (around 12 hours). The results of this experiment are shown in Figure 8. The growth of 100ng/ml aTc cultures was not significantly different from cultures lacking aTc. However, 250 ng/ml of aTc was detrimental to the growth of the cells.

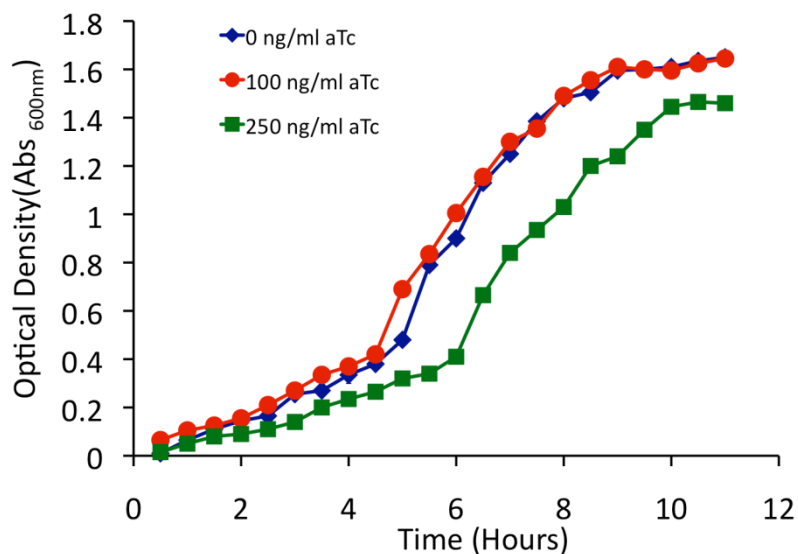


Figure 8. Effect of aTc on cell growth.

ATc has detrimental effect on the growth of cells. Strain GN100 was treated with 100 (Red circles) and 250 ng/ml aTc (Green squares) and grown until stationary phase. Optical Density (Absorbance at 600 nm) was recorded every 30 minutes. Cultures were grown in duplicates. Cultures that had no aTc (Blue diamonds) were also grown as control. It was observed that 100 ng/ml aTc did not show any significant effects on the growth as compared to the control lacking aTc. However, 250 ng/ml aTc showed significant detrimental effects on the growth of the cells.

3.1.2 Design of the Feedback Component

In order to construct a stand-alone positive feedback circuit, it is necessary to use an auto-inducer that does not interfere with native gene regulation in *E.coli*. Also, it was imperative that the activator is constitutively active and is independent of any exogenous inducer to achieve cell density independence in order to achieve. Given these constraints, the LuxR protein from *Vibrio fischeri* (Fuqua and Greenberg, 2002) was chosen.

As described in the Chapter 1, wild type *V.fischeri* displays N- (3-oxohexanoyl)-homoserine lactone (AHL)- mediated quorum sensing and cell density dependent gene regulation for the activation of its luminescence (*lux*) operon by LuxR. LuxR requires sufficient concentrations of the diffusible signal AHL, which is a product of LuxI to activate the regulatory region of the *luxICDABE* genes.

The LuxR protein is 250 amino acids in length and is composed of two functional domains. The N-terminal domain is involved in AHL-binding and the C-terminal is the DNA binding transcription regulation domain(Slock et al., 1990). The LuxR is a dimer in solution and the DNA binding domain recognizes the “lux box”, a symmetric sequence in the promoter region. The lux box “ACCTGTAGGATCGTACAGGT” is a 20-bp inverted repeat centered at -42.5 bp from the start of *luxI* transcription. AHL binds to the LuxR monomer, causing a conformational change that results in dimerization of the LuxR molecule. The LuxR dimer possesses transcriptional activity. Thus wild-type LuxR is dependent on an exogenous inducer. However, a number of structure activity relationship studies suggest that specific mutations can cause LuxR protein to become AHL independent and thus provide an autoregulatory module that is independent of cell density. These specific mutations are described below.

3.1.2.1 Point Mutations

Point mutations have been identified that result in the activation of LuxR in the absence of the autoinducer(Poellinger et al., 1995). This amino acid residue 221 enables the N-terminal domain to inhibit the activity of the C-terminal domain. The Ala221Val (Alanine to Valine) substitution renders LuxR capable of activating the expression of target genes independent of

the presence of AHL.

3.1.2.2 Deletion of N terminal Domain

Deletion of the LuxR N-terminal domain results in a derivative that is able to interact with DNA and able to activate the transcription of target genes, in the absence of AHL. Therefore, it was concluded that in the native LuxR protein, in the absence of AHL, the N-terminal portion reduces the DNA binding affinity of the C-terminal domain (Stevens et al., 1994).

3.1.2.3 Autoinducer Independence and Activity of the LuxR Feedback Components

Three constitutively active variants that are known to be autoinducer independent of LuxR were engineered to test their suitability in their use as the feedback element of the amplifier.

- 1) luxR* : It contains the Ala221Val point mutation.
- 2) LuxR Δ_{2-156} : N-terminal deletion from amino acid 2-156
- 3) LuxR Δ_{2-162} : N-terminal deletion from amino acid 2-162

The transcriptional activating strength of these luxR variants on the P_{luxI} promoter was tested in comparison to the wild type luxR in the absence of the autoinducer AHL. In order to compare the relative strengths of activation, a copy of green fluorescent protein (GFP) was placed under the P_{luxI} promoter in plasmid pGN69. The different LuxR variants LuxR*, LuxR Δ_{2-156} , LuxR Δ_{2-162} were cloned under the $P_{LTetO-1}$ promoter and are described as pGN3, pGN11, pGN12 respectively in the “Materials and Methods” chapter. They were all transformed individually with plasmid pGN69 into strain GN101, which harbors a chromosomal copy of *tetR* and then induced with anhydro Tetracycline (aTc) at 200 ng/ml to induce expression under the P_{luxI} promoter. The results are shown in Figure 9. All of the LuxR variants, including the wild-type control, were able to induce expression from the P_{luxI} promoter. It was observed that, only LuxR Δ_{2-162} was capable in of enhancing transcription relative to the wild-type control. There was no statistically significant difference between the LuxR*, LuxR Δ_{2-156} and the LuxRwt. Based on these results, LuxR Δ_{2-162} variant was chosen for use in the design of the amplifier.

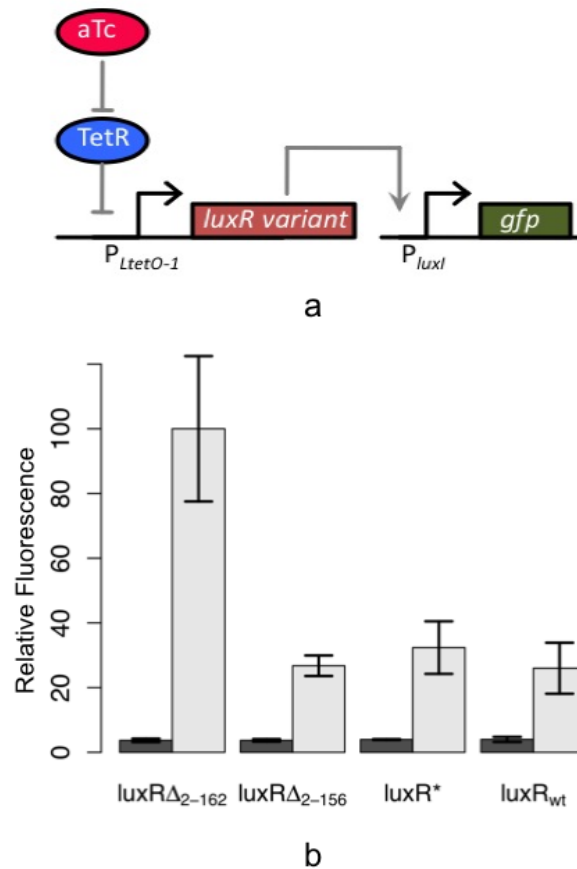


Figure 9. Comparison of constitutive luxR variants.

a. LuxR was expressed from a tetracycline-inducible promoter, $P_{LtetO-1}$, in strain GN101, which harbors a chromosomal copy of *tetR*. **b.** Activity was determined by the ability of these different variants to induce expression from the P_{luxI} promoter, using GFP as the readout, in the absence of any autoinducer. Dark bars denote the uninduced case and light bars the induced case (200 ng/mL aTc). Error bars denote 95% confidence intervals.

3.1.3 The Sensor Component

The sensor component was designed to be modular such that any inducible promoter could act as the sensor component without requiring changes to the amplifier and reporter system. The aTc inducible $P_{LTetO-1}$ promoter was chosen as the sensor component due to its popularity in the literature and availability. In order to test the response of the promoter to the inducer, green fluorescent protein (GFP) was cloned under the $P_{LTetO-1}$ promoter. The plasmid was then transformed into strain GN101 and then induced with various levels of aTc ranging from 0-100 ng/ml. The cultures were done in triplicates and the GFP expression levels were reported with the 95% confidence interval in Figure 10.

The results demonstrated that expression under the $P_{LTetO-1}$ could be regulated with various levels of aTc induction. This is an important result because it validates the design requirement that the strength of the expression of the luxR variants under the control of the $P_{LTetO-1}$ promoter should be regulatable.

The aTc signal recognition by the $P_{LTetO-1}$ promoter should be transferred to the amplifier. Therefore, a copy of LuxR transcriptional activator was cloned under the $P_{LTetO-1}$ promoter. The testing and construction of the pGN3, pGN12 and pGN12 therefore serve the following two purposes.

- 1) To grade the transcriptional activity of the various LuxR variants.
- 2) To provide an initial recognition signal to stimulate the positive feedback circuit.

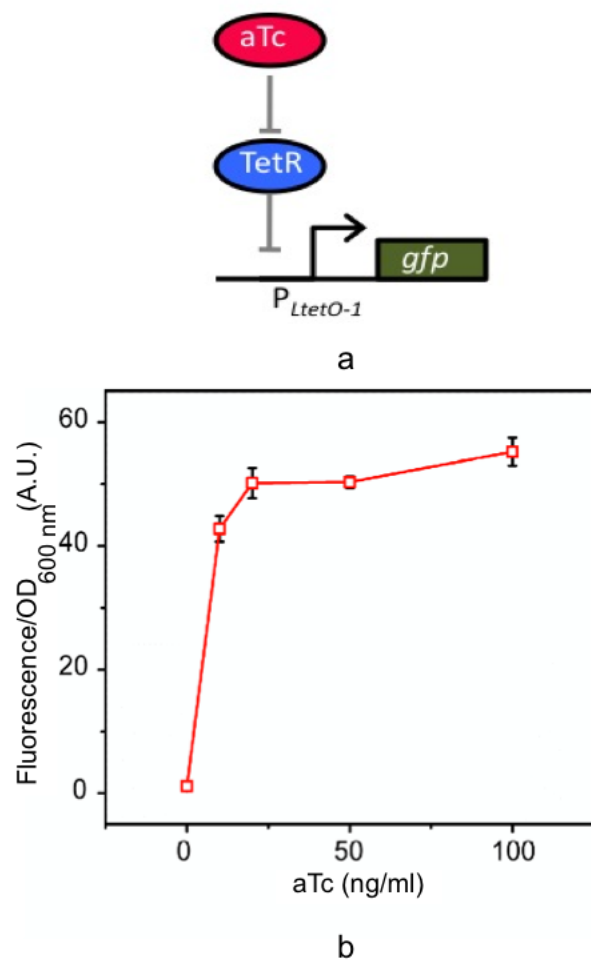


Figure 10. Activity of the sensor component.

a. GFP was expressed from a tetracycline-inducible promoter, $P_{LtetO-1}$, in strain GN101, which harbors a chromosomal copy of *tetR*. **b.** Activity was determined by inducing the $P_{LtetO-1}$ promoter with various levels of aTc ranging from 0-100 ng/ml and measuring GFP. Error bars denote 95% confidence intervals.

3.1.4 The Amplifier

In order to construct the amplifier, GFP and LuxR $_{\Delta 2-162}$ were cloned in a bicistronic configuration behind the P_{luxI} promoter on high-copy number plasmid (ColE1 origin of replication). In this arrangement, LuxR $_{\Delta 2-162}$ functions in a positive feedback loop as it can bind to the P_{luxI} promoter and activate its own transcription (Figure 11).

As a control, a non-positive feedback plasmid was also built that lacks the LuxR $_{\Delta 2-162}$. It has GFP downstream of the P_{luxI} promoter. The construction of the positive and non-positive feedback plasmids pGN68 and pGN69 respectively, is described in Chapter 2. In the positive feedback plasmid pGN68 the LuxR $_{\Delta 2-162}$ was cloned downstream of the GFP reporter to control for polar effects when results involving positive feedback were compared to those lacking it. It ensures that GFP is at the same distance from the promoter in the positive and non-positive feedback plasmids and in between the same restriction enzyme sites.

In order to induce the P_{luxI} promoter this circuit, an active variant of LuxR is needed. The most active LuxR variant, LuxR $_{\Delta 2-162}$, was used this time as the input signal through the use of pGN12. The aTc sensor produces LuxR $_{\Delta 2-162}$, which in turn activates the positive feedback plasmid pGN68. In this design, the output of the sensor is LuxR $_{\Delta 2-162}$, which in turn feeds back into the amplifier. In other words, LuxR $_{\Delta 2-162}$ is used both as the input and positive feedback signal. For the output, we used GFP as it provides a facile measure of transcriptional activity. This choice is in no way limiting, and any gene can in practice be used as the output.



Figure 11. Schematic of positive-feedback amplifier.

The basic design for the amplifier consists of GFP and LuxR $_{\Delta 2-162}$ arranged in a bicistronic configuration under the control of the P_{luxI} promoter. LuxR $_{\Delta 2-162}$ functions in a positive feedback loop as it can bind to the P_{luxI} promoter and activate its own transcription. In our design, LuxR $_{\Delta 2-162}$ is also used as the input signal to for the amplifier. LuxR $_{\Delta 2-162}$, therefore, is used both as the input and positive feedback signal. GFP provides a measure of transcriptional activity.

3.1.5 Validation of Amplifier Using a Tetracycline Sensor

For the tetracycline sensor, LuxR $_{\Delta 2-162}$ was cloned behind the TetR-regulated P_{TetO-1} promoter on a compatible, medium copy-number plasmid (p15A origin of replication) and described as pGN12. In the absence of the tetracycline analogue, anhydrotetracycline (aTc), dimeric TetR binds to the O2 operator sites within the P_{TetO-1} promoter and represses transcription. However, when TetR is bound with aTc, it no longer binds and represses the P_{TetO-1} promoter, enabling dose-dependent control of gene expression (Lutz and Bujard, 1997). Thus, the aTc-inducible promoter functions as a one-component tetracycline sensor with LuxR $_{\Delta 2-162}$ as the output. The amplifier was coupled to the one-component tetracycline sensor (Figure 12) and tested. The sensor and the amplifier plasmids were then transformed into cells (GN100) constitutively expressing a chromosomal copy of the *tetR* gene.

When this design was tested (Figure 13), it was found that the amplifier (pGN12+pGN68) increased both the sensitivity and dynamic range of the integrated circuit relative to an otherwise identical circuit lacking positive feedback (pGN12+pGN69) (Figure 12).

Sensitivity, in this context, can be described as the ability of the circuit to respond to external stimuli aTc in this case. It was found that positive feedback increased the sensitivity to aTc by roughly two orders of magnitude. In other words, equivalent levels of expression were observed in the circuit involving positive feedback at aTc concentrations roughly one hundred times less than those observed with the circuit lacking positive feedback.

Dynamic range is the ratio of expression under saturating inducing (100 ng/ml aTc) and non-inducing (0 ng/ml aTc) conditions. The positive feedback increased the dynamic range by roughly 50%.

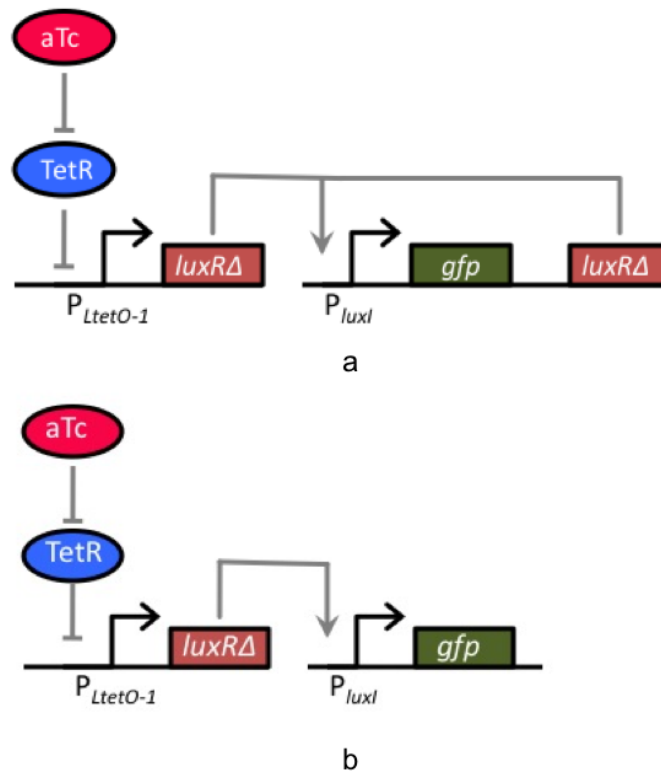


Figure 12. Schematic of tetracycline sensor coupled to the positive-feedback amplifier and the non-positive feedback control.

a. The one-component tetracycline sensor consists of a plasmid where $\text{LuxR}_{\Delta 2-162}$ has been cloned behind the TetR-regulated $P_{LtetO-1}$ promoter. In the absence of the inducer anhydrotetracycline (aTc), TetR binds to operator sites in the $P_{LtetO-1}$ promoter and represses transcription. In the presence of aTc, TetR cannot bind to the promoter, and enables dose-dependent control of $\text{LuxR}_{\Delta 2-162}$. This sensor was coupled with the positive feedback amplifier, encoded on a separate plasmid, by transforming cells (GN100) constitutively expressing a chromosomal copy of the *tetR* gene with the two plasmids respectively harboring the sensor and amplifier. **b.** The non-positive feedback plasmid lacks the $\text{LuxR}_{\Delta 2-162}$ feedback element.

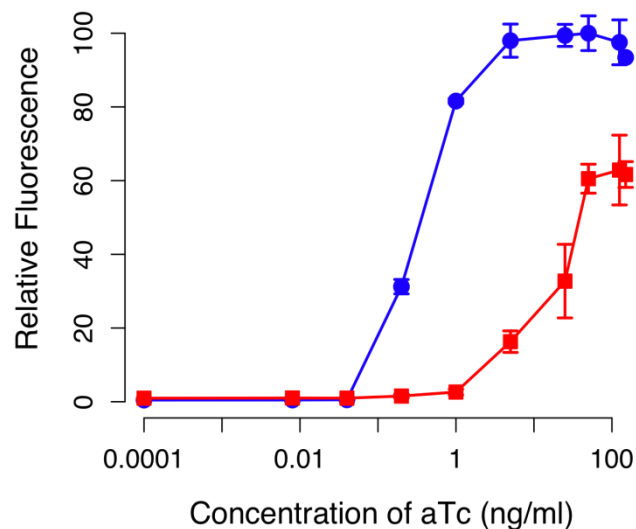


Figure 13. Comparison of tetracycline sensor with positive feedback (solid circles) and without (solid square).

Schematic of positive feedback design is shown in Figure 12. The design lacking positive feedback is otherwise identical to one with positive feedback except that only GFP is expressed from the P_{luxI} promoter. In these experiments, cells were grown overnight at the indicated concentration of aTc to an OD_{600} prior to measurements. The fluorescence values were normalized with the OD_{600} absorbance to account for cell density. Error bars denote 95% confidence intervals for the measurement average.

3.1.6 Bistability

Studies have shown that positive feedback leads to bistable behavior. Cells exhibiting bistable behavior tend to remain stably in one of two expression states. Gradient input leads to binary output (Ajo-Franklin et al., 2007). At the population level, bistability often leads to bimodality. Studies have also shown that noise can induce bimodality without the necessary presence of bistability in positive feedback loops (To and Maheshri, 2010). In bimodality the population is split into two groups where one group shows basal expression also termed as OFF state and the other group exhibits high or maximal expression also called as ON state. The end point measurement of expression does not show population level behavior. In order to probe the population level behavior of the circuit, the cells were analyzed using fluorescence activated cell sorting (FACS). The positive and non-positive integrated circuits were treated with various aTc levels ranging from 0 to 100 ng/ml and then FACS analyzed (Figure 14).

The population of cells carrying the integrated positive feedback circuit displayed basal expression and were OFF at 0 and 1 ng/ml aTc induction. When induced with higher levels of aTc at 10, 25 and 100 ng/ml all the population turned ON and displayed maximal expression. In other words, the population displayed ALL OFF or ALL ON behavior with respect to the inducer, with a threshold induction level of 10 ng/ml. It was also observed that there was a very small population of ON cells at intermediate induction levels of 10,25 and 100 ng/ml. The presence of a third set of population at an intermediate level of expression shows that the population does not exhibit bimodality. The population of cells lacking positive feedback displayed a basal expression level at 0 and 1 ng/ml aTc induction and displayed gradually increasing expression with aTc induction levels of 10,25 and 100 ng/ml aTc. In summary, the circuit with positive feedback displayed a bistable behavior but lacked bimodality at the population level.

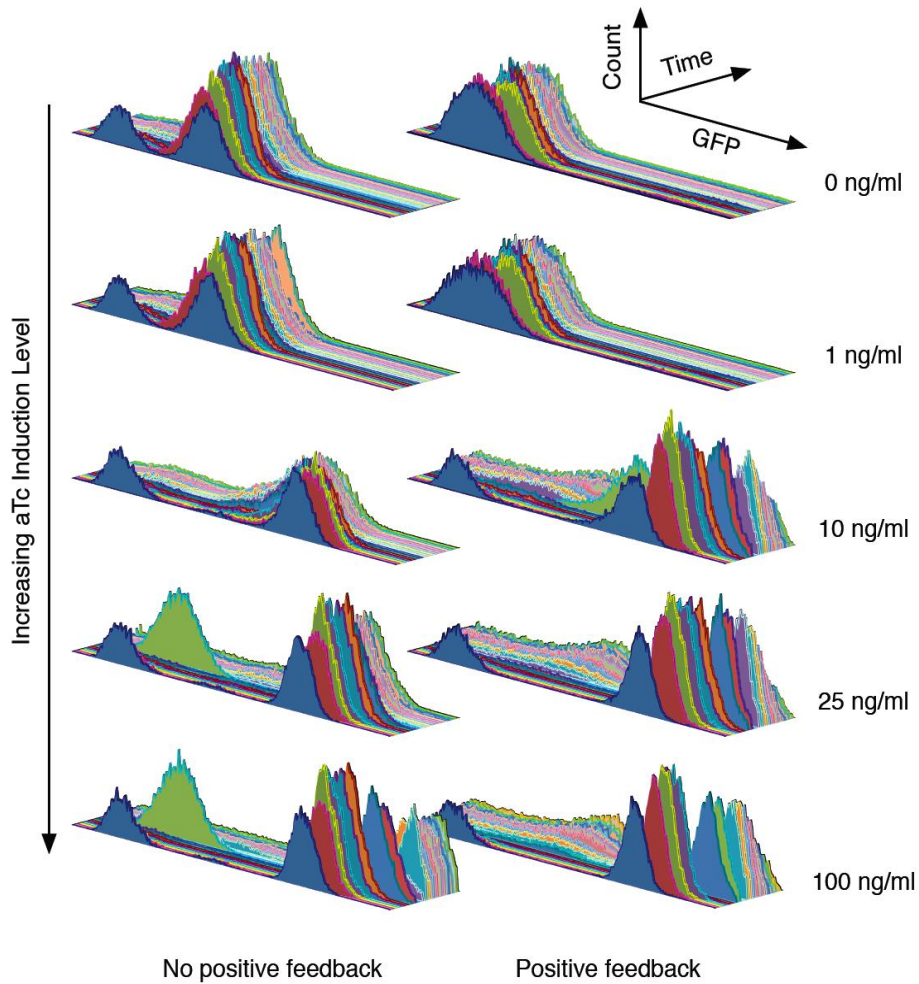


Figure 14. Bistability of the cell population due to positive feedback.

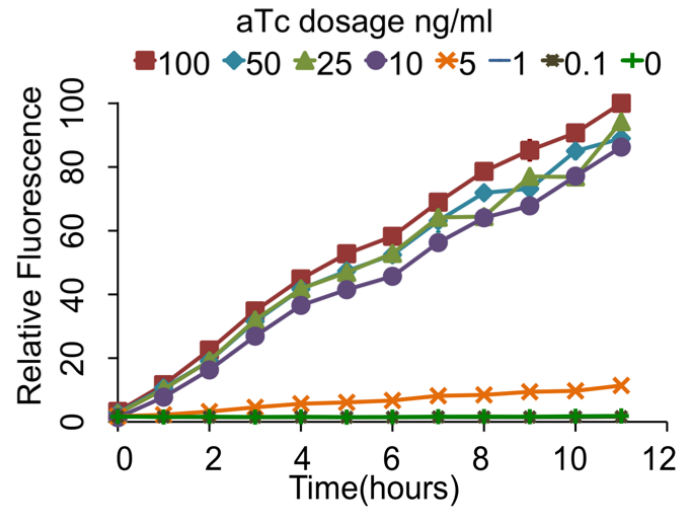
Cells carrying the positive feedback integrated circuit display ALL OFF or ALL ON (at maximal expression) behavior in response to gradient levels of aTc induction as compared to cells lacking positive feedback. Cells lacking positive feedback display gradient expression in response to increasing levels of aTc induction.

3.1.7 Kinetics

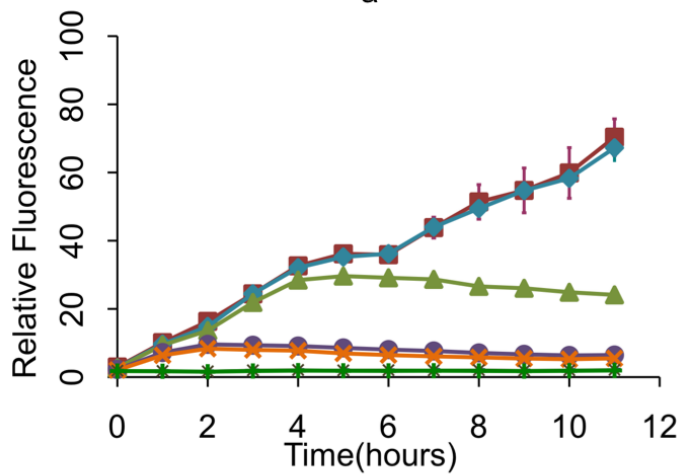
Kinetic experiments were also performed to observe the response over a twelve-hour interval to varying concentrations of aTc (Figure 15). Consistent with the end-point measurements, it was found that the design involving positive feedback was more sensitive to aTc and had a wider dynamic range of expression levels.

Particular attention should be paid to the expression at 25 ng/ml (green triangles) between both the circuits. The positive feedback circuit shows higher final expression relative to the circuit lacking positive feedback, which levels off. In case of applications that need higher final expression with the lowest possible level of induction, 25 ng/ml would represent an optimum level of induction.

The threshold induction level between positive and non-positive feedback circuits described in Figure 13 is different from the threshold value observed in the kinetics. This difference might have arisen from the difference in culture volumes. The expression levels in Figure 13 are end-point measurements of 2 ml cultures whereas the kinetics are based on hourly readings of 200 μ l cultures, leading to differences in threshold values as well as kinetics. Collectively, these results demonstrate that the genetic amplifier is capable of both increasing the sensitivity and dynamic range of this one-component tetracycline sensor.



a



b

Figure 15. Kinetic analysis of tetracycline sensor with positive feedback (a) and without (b).

In these experiments, cells were grown for 12 hours at varying levels of aTc induction with measurements taken every hour. The fluorescence values were normalized with the OD_{600} absorbance to account for cell density. The scale for both sets of experiments is the same. Error bars denote 95% confidence intervals for the measurement average.

3.1.8 Memory and hysteresis

Many studies have shown that positive feedback can lead to hysteresis. Hysteresis is history dependent behavior. It is easier to maintain the system in one state than to toggle the system between two states. Hysteresis is also called memory-like behavior because the expression of cells harboring positive feedback circuits at a particular level of induction depends on the previous level of induction. In other words, the expression of cells at the current induction level would be different if the current induction level was attained through concentration or dilution of the inducer.

In order to display memory like behavior, the cells harboring the positive feedback circuit should remember their previous exposures to aTc. In order to show hysteresis like behavior, the system requires a larger dosage of aTc to switch from low to high state than from a high to low state. In effect, the system should follow a different path when the induction is decreased to the same level that the system had experienced before. The cells with the amplifier circuit were tested to verify the presence or absence of memory like behavior.

The cells containing positive feedback and non-positive feedback plasmids were grown overnight without induction. The cells were then diluted 1:100 into 2 ml of LB containing the appropriate antibiotics and grown for 2 hours without induction. They were then induced with various concentrations of aTc ranging from 0-100 ng/ml and grown for 8 hours. They were then diluted 1:100 into fresh LB media containing antibiotics and lacking the aTc inducer. Samples were stored at 0, 1, 2, 8, 12, 14 and 20 hours and analyzed using flow cytometry as described in the Materials and Methods section.

In Figure 16.a, the purple data represents hour 8 (fourth level on time axis) when all the samples were diluted into media lacking aTc. If there was memory like behavior then the behavior of the system should be path dependent. The expression levels would be different for the same level of induction based on if the previous level of induction was higher or lower than the current level of induction. The cells harboring no positive feedback displayed basal levels of expression after the dilution as seen in 12, 14 and 20 hours for all aTc levels. The cells harboring positive feedback circuit however displayed higher GFP expression even after a 100 times

dilution as seen in the 12, 14 and 20 hours data for 10 and 100ng/ml aTc induction. After 1:100 dilution, the carried over aTc would be 0.1 and 1 ng/ml respectively. This data was then compared expression from 1 ng/ml aTc induction after 8 hours. The expression at 14 hours (6 hours after dilution) is similar to expression at 8 hours (before dilution). These results have been summarized in Figure 16.b.

These results indicate the positive feedback loop involving $\text{LuxR}_{\Delta 2-162}$ is able to amplify the response to an inducer but is incapable of sustaining the response in the absence of inducer. Based on the properties of LuxR, specifically the role of AHL in stabilizing LuxR, the reason the circuit does not sustain activation is likely due to the protein dimer being degraded too quickly. In other words, it can be hypothesized that $\text{LuxR}_{\Delta 2-162}$ dimer is being degraded at a rate greater than it is being produced by positive feedback alone (No specific experiments have been done to make this measurement). More specifically, positive feedback alone is unable to sustain the expression of $\text{LuxR}_{\Delta 2-162}$ in the absence of some exogenous source, in our case the one-component sensor. That said, the positive feedback is still strong enough to amplify the response when an external input signal is present.

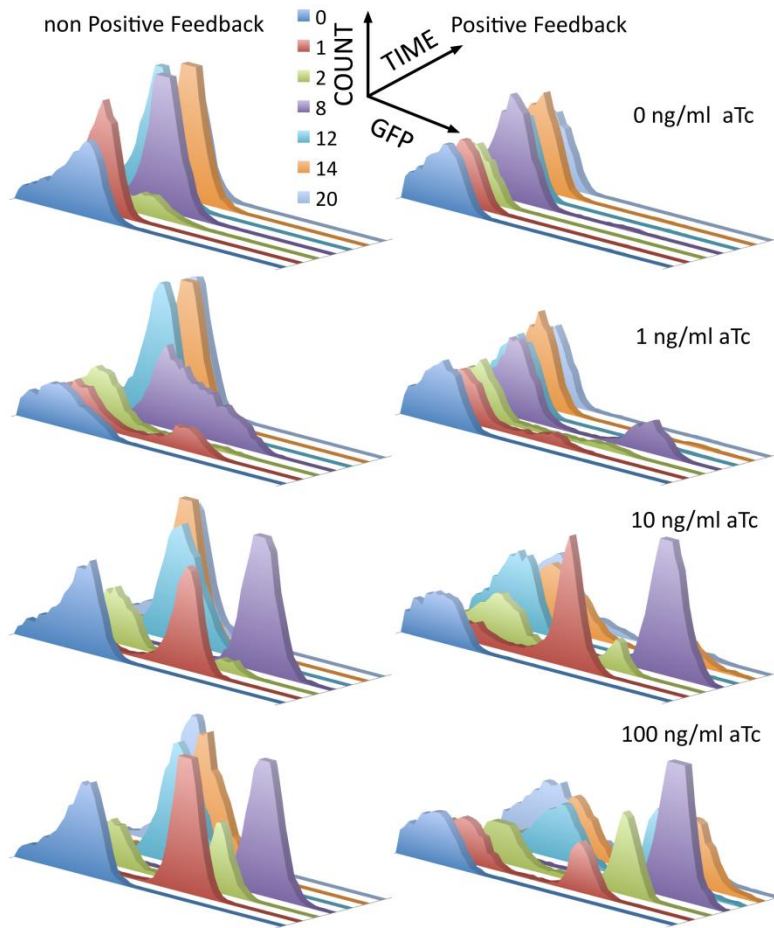


Figure 16.a. Test for hysteresis and memory.

Cells containing positive feedback and cells lacking it were induced with various levels of aTc and then diluted 1:100 into media lacking it after 8 hours of growth (expression show in purple). The cells lacking positive feedback displayed basal levels of expression after dilution for all aTc levels. The cells carrying the positive feedback circuit at 10 and 100ng/ml induction levels, however, displayed higher GFP expression after dilution with an effective aTc concentration of 0.1 and 1 ng/ml aTc respectively.

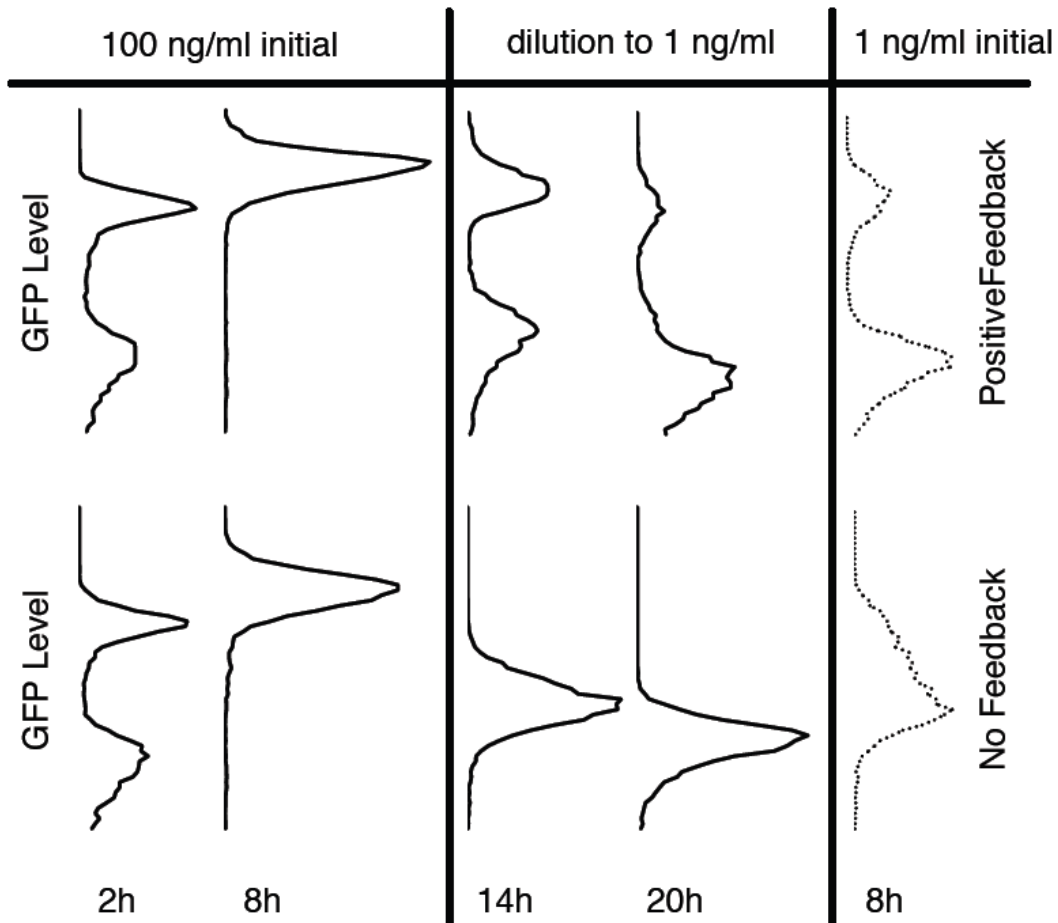


Figure 16.b. Summary of test for hysteresis and memory.

When the expression of positive feedback cells from an original induction of 1 ng/ml aTc after 8 hours was compared to the expression of cells diluted from original induction of 100 to 1 ng/ml at 14 hours (6 hours after dilution), they were strikingly similar. The cells with positive feedback, therefore, do not display hysteresis and also do not remember their previous state of higher expression at 100ng/ml. In other words, the positive feedback in the current scenario does not endow memory.

3.2 Two Component Aspartate Sensor

One of the design objectives was to test the amplifier in various contexts, in order to demonstrate its modularity. The amplifier was coupled to a two-component aspartate sensor (Figure 19) and tested for amplification and sensitivity.

The hybrid Tar-EnvZ (Taz) sensor kinase (Jin and Inouye, 1993) was used as the sensor component in place of the one-component aTc sensor. This chimeric, transmembrane sensor kinase controls the levels of phosphorylated OmpR (OmpR-P), which in turn activates the expression from the P_{ompC} promoter. When the Taz sensor kinase is bound with aspartate, it increases the levels of OmpR-P, leading to increased expression from the P_{ompC} promoter. In addition to amino acids, EnvZ chimeras have been constructed to sense other inputs such as sugars and light (Dattelbaum et al., 2005; Levskaya et al., 2005).

3.2.1 Testing the Aspartate Sensor

Two different genomic regions of varying lengths of the OmpC promoter ranging from 2310762-2310962 and 2310721-2311221 containing the operator sites were chosen to and tested for response to aspartate. The reporter green fluorescent protein was cloned behind these two promoter regions and tested with varying concentrations of the aspartate inducer as shown in Figure 17. The promoter with genomic region 2310762-2310962 responded better to aspartate and resulted in higher expression. Therefore this region was chosen for the cloning of the plasmid pGN77.

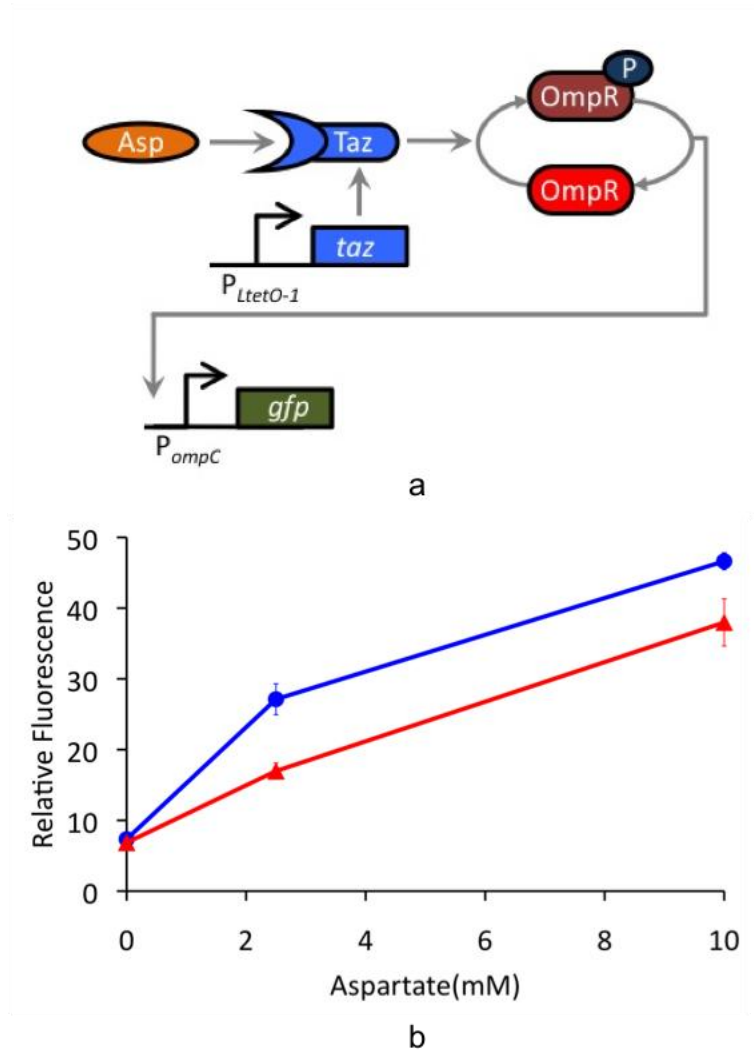


Figure 17. Testing the aspartate sensor.

a. The reporter green fluorescent protein had been cloned behind different lengths of the OmpC promoter. The Taz sensor kinase, when bound with aspartate, increases the levels of OmpR-P and activates the expression from the P_{OmpC} promoter. **b.** The blue circles show the GFP expression under P_{OmpC} from genomic region 2310762-2310962 and the red triangles show the GFP expression under P_{OmpC} from genomic region 2310721-2311221. The promoter with genomic region 2310762-2310962 demonstrated higher expression and had been used for cloning the plasmid pGN77.

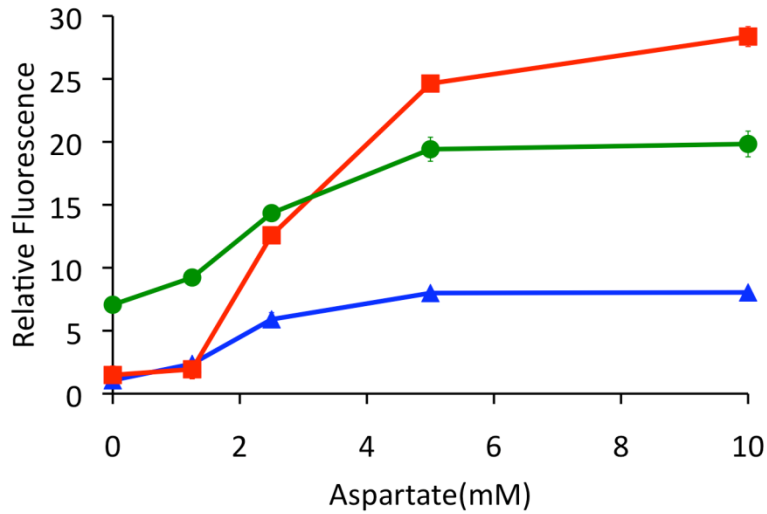


Figure 18. Polar effects of the fluorescence gene.

The position of the reporter protein relative to its promoter affects the expression. In these experiments, the red squares show the expression of the positive feedback circuit in which, $LuxR_{\Delta 2-162}$ is in between the P_{luxI} promoter and the reporter green fluorescent protein. The green circles show the expression of the integrated circuit lacking the positive feedback in which the green fluorescent protein is directly behind the P_{luxI} promoter. The blue diamonds show the expression of the control circuit carrying the wild type $luxR$. It can be seen that having reporter directly behind the promoter resulted in a basal “leaky expression” even in the absence of induction. Therefore, the design was improved to have the reporter at the same distance from the promoter in the positive and non-positive feedback plasmids.

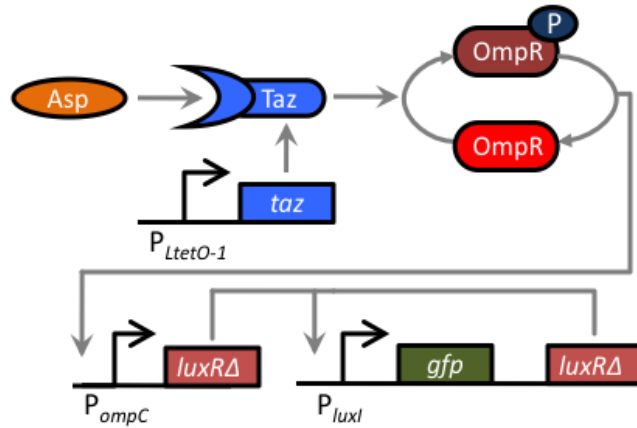


Figure 19. Schematic of aspartate sensor coupled to the positive-feedback amplifier.

The two-component sensor consists of the Taz sensor kinase and the OmpR response regulator. Taz controls the level of phosphorylated OmpR (OmpR-P), which in turn activates the expression from the P_{ompC} promoter. When the Taz sensor kinase is bound with aspartate, it increases the levels of OmpR-P, leading to increased expression from the P_{ompC} promoter. The Taz sensor kinase has been cloned behind the constitutive $P_{LtetO-1}$ promoter on one plasmid (the cells used in these experiments do not possess TetR). On a second plasmid, $LuxR_{\Delta 2-162}$ has been cloned behind the P_{ompC} promoter, resulting in the expression of $LuxR_{\Delta 2-162}$ being aspartate dependent. The third plasmid harbors the positive feedback amplifier. The sensor was coupled to the amplifier by transforming the three plasmids into a $\Delta envZ$ null mutant (GN101).

3.2.2 Validation of the Amplifier Using an Aspartate Sensor

In order to couple the two-component aspartate sensor with the genetic amplifier, LuxR $_{\Delta 2-162}$ was cloned behind P $_{ompC}$ promoter on a compatible, medium copy-number plasmid (p15A origin of replication). In order to introduce the Taz sensor kinase into *E.coli*, the taz gene was cloned behind the constitutive P $_{LtetO-1}$ promoter on a compatible, low copy-number plasmid (pSC101 origin of replication). These experiments were performed in cells lacking a chromosomal copy of the *tetR* gene, so the P $_{LtetO-1}$ promoter in this background is constitutive.

A design was tested where the positive feedback plasmid and the non-positive feedback plasmid differed in the position of the reporter relative to the promoter (Figure 18). The position of the reporter protein relative to its promoter affects the expression. The presence of reporter protein directly behind the promoter resulted in a basal “leaky expression” even in the absence of aspartate induction. Therefore the design was then been improved to have the reporter at the same distance from the promoter in the positive and non-positive feedback plasmids.

The three plasmids including the aspartate sensor (pGN77), the luxR input plasmid (pGN62-Kan) and the positive feedback plasmid were transformed a $\Delta envZ$ null mutant (GN101) to achieve the integrated circuit (Figure 19). As a control, the circuit lacking positive feedback was achieved by transforming the plasmids pGN77, pGN62-Kan and pGN69 into strain GN101.

Similar to observations with the one-component tetracycline receptor, it was found that the amplifier increased both the range and sensitivity when coupled to the two-component aspartate sensor as compared to an otherwise identical circuit lacking positive feedback (Figure 20). However, when compared to the one component tetracycline sensor, only a minor increase in sensitivity was observed but the amplification response was retained. In particular, the amplifier increased the dynamic range by roughly an order of magnitude whereas the sensitivity increased by approximately a factor of two. While these results demonstrate that the amplifier is modular as it can readily be applied to different sensor systems, they also demonstrate that the performance of the amplifier is context dependent.

3.3 Results Summary

In summary, in the context of a one-component sensor, an increase in the sensitivity was primarily observed whereas in the two-component sensor, an increase in the amplification. It should be noted that only weak activation, roughly two-fold, was observed in response to aspartate in the absence of positive feedback. This level of activation is less than what has been previously observed in other studies using Taz, where the degree of activation is greater than ten fold (Michalodimitrakis et al., 2005; Yoshida et al., 2007). However, unlike the present work, these studies measured the expression directly from the P_{ompC} promoter. In the present work, the expression was measured from a downstream promoter, P_{luxI} . Thus, there is an additional stage between the sensor and reporter in this design. Likely, expression of LuxR $_{\Delta 2-162}$ from the P_{ompC} promoter is not sufficiently strong to activate the P_{luxI} promoter without further amplification. However, addition of amplification by including positive feedback results in robust expression.

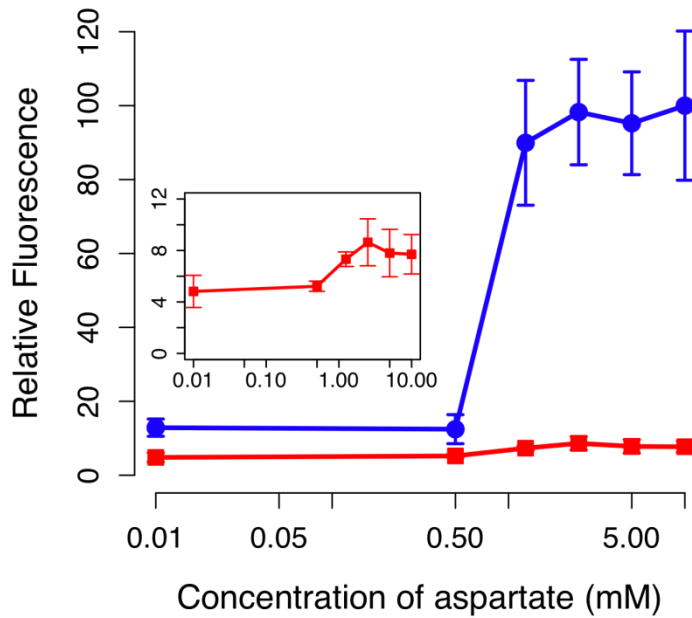


Figure 20. Comparison of sensor output in the presence (solid circles) and absence (solid squares) of the positive feedback amplifier.

Schematic of positive feedback design is shown in Figure 19. The design lacking positive feedback is otherwise identical to one with positive feedback except that only GFP is expressed from the P_{luxI} promoter. In these experiments, cells were grown overnight at the indicated concentration of aspartate prior to measurements. The fluorescence values were normalized with the OD_{600} absorbance to account for cell density. Inset figure shows the magnification of the response for the design lacking positive feedback. Error bars denote 95% confidence intervals for measured averages.

Chapter 4 Mathematical Model of the Positive Feedback

The positive feedback has been extensively modeled using differential equation (Ferrell et al., 2009; Becskei et al., 2001; Keller, 1995; Maeda and Sano, 2006a) and stochastic simulation (Pfeuty and Kaneko, 2009) based approaches. There are a number of models with varying degrees of complexity that capture the various dynamic and steady state properties of a positive feedback circuit.

4.1 Rate of Synthesis and Decay of Autoregulatory Transcription

Factors

Autoregulatory transcription factors can have multiple steady states in the cells causing the genetic circuits encoding them to have multiple steady states. The positive feedback model was derived with respect to transcription factor and the active transcription activation sites as demonstrated by Keller (Keller, 1995).

The rate of synthesis of a transcription factor is proportional to the rate of transcription of the gene encoding that factor. The rate of synthesis is the sum of products between the frequency of each possible configuration of promoter occupancy and the rate of synthesis resulting from that configuration.

Let us assume that the lux promoter has been known to have 2 active sites to which luxR activator binds with affinities K_{A1} and K_{A2} . The possible configurations of the promoter occupancy have been given below along with their relative frequencies and rate of transcription factor synthesis.

[A] is the cellular level of the activator.

S_{XA} is the activator dependent rate of synthesis of transcription factor X

S_{XB} is the activator independent rate of synthesis of transcription factor X also known as basal expression.

The relative frequency for each case scenario is given below for no occupancy, single occupancy and double occupancy of the promoter sites by the transcription factor.

Relative frequency	Rate of synthesis of TF X
1	S_{XB}
$K_{A1}[A]$	$0.5S_{XA}+S_{XB}$
$K_{A2}[A]$	$0.5S_{XA}+S_{XB}$
$K_{A1}K_{A2}[A][A]$	$S_{XA}+S_{XB}$

Rate of synthesis of the transcription factor X is then

$$\left(\frac{((K_{A1} + K_{A2})[A] + K_{A1}K_{A2}[A]^2)}{1 + (K_{A1} + K_{A2})[A] + K_{A1}K_{A2}[A]^2} \right) S_{XA} + S_{XB} \quad (1)$$

The rate of decay can be assumed to be exponential. Considering the case where the transcription factor X can homodimerize with dimerization constant K_{X2} to form X2 and heterodimerize with constant K_{XY} to form XY. The monomeric, homodimeric and heterodimeric species will each decay with respective decay constants d_x , d_{X2} , d_{XY} .

The overall decay rate of the transcription factor X will then be

$$d_x[X] + 2d_{X2}[X2] + d_{XY}[XY] = d_x[X] \left(1 + \delta_x \frac{[X]}{[X]_0} + \delta_{XY} \frac{[Y]}{[Y]_0} \right)$$

where

$$\delta_x = \left(2 \frac{d_{X2}}{d_x} \right) K_{X2} [X]_0 \text{ and } \delta_{XY} = \left(\frac{d_{XY}}{d_x} \right) K_{XY} [Y]_0 \quad (2)$$

At steady state, the rate of synthesis is equal to the rate of decay. If S_{X0} is the rate of synthesis of transcription factor X which produces $[X]_0$ steady state cellular levels of monomer X in the presence of $[Y]_0$ steady state cellular levels of monomer Y, then

$$S_{X0} - d_x[X]_0(1 + \delta_x + \delta_{XY}) = 0 \quad (3)$$

Solving this equation for d_x and inserting it into the decay rate equation gives the rate of decay

$$S_{X0} \left(\frac{[X]}{[X]_0} \right) \frac{(1 + \delta_x \frac{[X]}{[X]_0} + \delta_{XY} \frac{[Y]}{[Y]_0})}{1 + \delta_x + \delta_{XY}} \quad (4)$$

Let us assume that the transcription factor x only homodimerizes, then the rate of decay will be

$$S_{X0} \left(\frac{[X]}{[X]_0} \right) \left(\frac{1 + \delta_x \frac{[X]}{[X]_0}}{1 + \delta_x} \right) \quad \text{since} \quad \delta_{XY} = 0 \quad (5)$$

At steady state

$$\text{Rate of synthesis of TF} = \text{Production-Degradation} = 0 \quad (6)$$

$$[X'] = \left(\frac{0.5(K_1 + K_2)[X] + cK_1K_2[X]^2}{1 + (K_1 + K_2)[X] + cK_1K_2[X]^2} \right) S_{XA} + S_{XB} - S_{X0} \frac{[X]}{[X]_0} = 0 \quad (7)$$

At steady state the production rate of the transcription factor must equal the degradation rate. Equation 7 can be empirically captured by the Hill input function as done by Becskei and colleagues (Becskei et al., 2001). Hill input for an activator is a curve that rises from zero and approaches maximal saturated level.

$$\frac{dx}{dt} = \frac{sx^n}{d^n + x^n} - kx \quad (8)$$

x is the expression level of the gene amplified by the positive feedback, luxR transcription factor TF in the present case, t is time. Only the existent positive feedback from the transcription factor has been considered in this case. The variable s is a lumped parameter that takes into consideration various parameters like rate of mRNA production through transcription and protein synthesis through translation. The variable d is the dissociation constant between the activator luxR and lux box, its cognate promoter. d can be accounted for in s for the sake of simplicity. (8) then becomes

$$\frac{dx}{dt} = \frac{sx^n}{1+x^n} - kx \quad (9)$$

The variable n is the co-operativity factor capturing the dimerization of luxR and is equal to 2 in the current study and k is the degradation rate of the protein. The various behaviors of the positive feedback can be explained through the parameters s and k. The activation is done by the self-sustaining positive feedback captured through first term in equation 1. The inactivation is assumed to be unregulated; its rate is proportional to the amount of x.

At steady state,

$$\frac{d}{dx} \left(\frac{sx^n}{d^n + x^n} \right) = \frac{d}{dx} (kx) \quad (10)$$

$$snd^n x^{n-1} = k(d^n + x^n)^2 \quad (11)$$

When synthesis = degradation

$$\frac{sx^n}{d^n + x^n} = kx \quad (12)$$

From 11 and 12

$$s = kdn(n-1)^{\left(\frac{1}{n}-1\right)} \quad (13)$$

When the transcription factor is a dimer n=2, therefore

$$s = 2kd \quad (14)$$

or

$$d = \frac{s}{2k}$$

Bistability is therefore attained when the conditions described in 14 are met. The gain of the system can therefore be manipulated by tuning the value of s or d. The gain also depends on

the unregulated degradation constant k , which is directly proportional to the amount of the output.

4.2 Model Using Ordinary Differential Equation

Equation 1 has been modeled using `ode15s` in MATLAB. Various initial conditions are given to simulate the effect of the presence of luxR transcription factor at different levels. The final steady state expression was determined at 4 different times. The solutions to a first order differential equation model of the positive feedback shows the population to divide between all 'ON' and all 'OFF' based on the strength of the transcriptional activator and its degradation rate. For example, the steady state expression was determined at a constant degradation rate of $k=0.2$, with different binding strengths of the transcription factor s ($s = [0.395, 0.4, 0.5, 2, 10]$). When $s=0.395$ all the population is turned OFF. When $s=0.4$, the population is split into no expression "OFF" and some expression "ON". When $s=10$ all the population is ON.

At a constant degradation rate k , the ratio of "OFF" to "ON" population can be manipulated by varying the strength of s . The final steady state expression level or in other terms "amplification due to positive feedback" can also be manipulated by manipulating s . As long as the transcription factor is stable and the degradation rate is not high, it is possible to manipulate the system behavior from gradient to switch like behavior and at the same time obtaining higher amplification and steady state expression levels. The parameter k was varied while s was kept constant and vice versa and then the ratio of ON cells to total number of cells ($ON/ON+OFF$) was calculated in order to understand relation between the synthesis of TF and its degradation. Figure 21 shows the synthesis-degradation phase space where the ratio ($ON/ON+OFF$) is plotted with relation to s and k . The ratio ($ON/ON+OFF$) is high when s is high and k is low.

The model of positive feedback captured by the ordinary differential equation using the Hill Input Function shows that the ratio of number of ON cells to OFF cells can be controlled. This also informs us that the Hill input function is an efficient way of representing positive feedback.

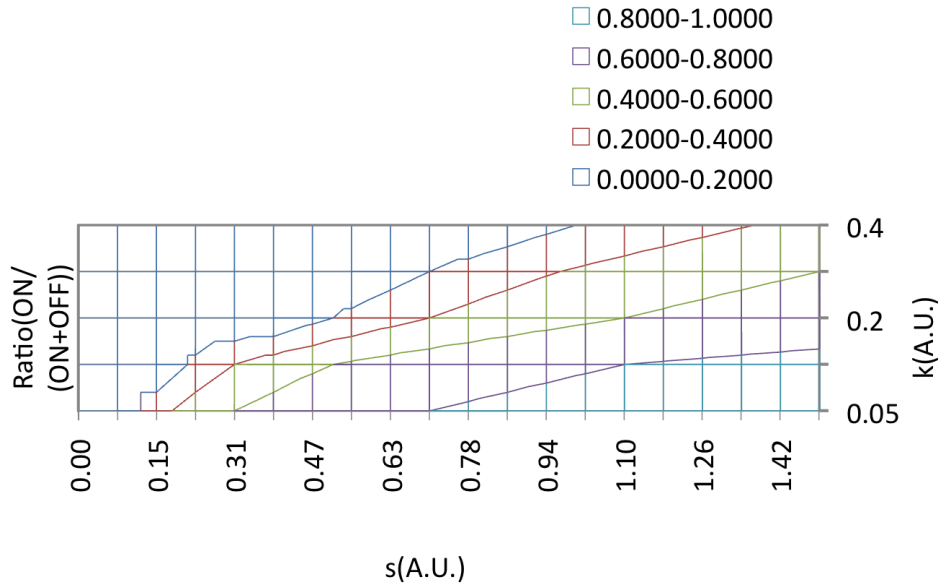


Figure 21. The ratio of ON cells to the whole population (ON/ON+OFF) can be controlled by manipulating k and p.

The ratio of ON cells to total number of cells (ON/ON+OFF) was calculated in order to understand relation between the synthesis of TF and its degradation. Both s and k can be manipulated to change the ratio of ON cells to the OFF cells. The ratio (ON/ON+OFF) is high when s is high and k is low.

The response at the single cell resolution was modeled using a stochastic approach. Noise was introduced through a random number generator to the initial conditions using the same Hill function.

4.3 Model Using Stochastic Differential Equation

A stochastic differential equation method was developed for the positive feedback circuit as described by Higham (Higham., 2001). Using a stochastic differential equation approach, the system response at a single cell resolution when all the cells start from the same state (same initial conditions) and the strength of the positive feedback is changed from strong to feeble was observed. To track the heterogeneity in the system the time points in between are monitored. The final GFP expression is plotted as a histogram. A Monte Carlo approach was used where random variables are simulated with a random number generator and the expected values are approximated by the computed averages. The random number generator randn

from MATLAB was used.

When the positive feedback is stronger ($p=2$) than degradation ($k=1$) the system can be switch like or gradient (Figure 22). If T_{\max} is the total time, the histograms show the output (GFP) and the number of cells at three different values of T_{\max} , 20, 100 and greater than 600. Therefore, the stochastic differential equation model of the positive feedback circuit showed that strong transcription factor binding strength resulted in stronger feedback activation and caused the system to move from gradient to switch like behavior.

When the positive feedback strength is feeble ($p=1$ and $k=0.9$) the system did not exhibit gradient to switch like behavior (Figure 23). If T_{\max} is the total time, the histograms show the output (GFP) and the number of cells at three different values of T_{\max} , 20, 100 and greater than 600. The stochastic differential equation model of the positive feedback circuit, therefore demonstrated that weak transcription factor binding strength couldn't cause the system to move from gradient to switch like behavior.

Transcription factor binding affinity can therefore cause the positive feedback system to change between switch-like and gradient behaviors. The histograms shown in Figure 22 and Figure 23 look similar to the experimental data shown in Figure 14. The stochastic differential equation model, therefore, sufficiently captures the behavior of the positive feedback where the population of cells can be modulated from gradient to switch like behavior by manipulating the strength of the positive feedback and the degradation rate.

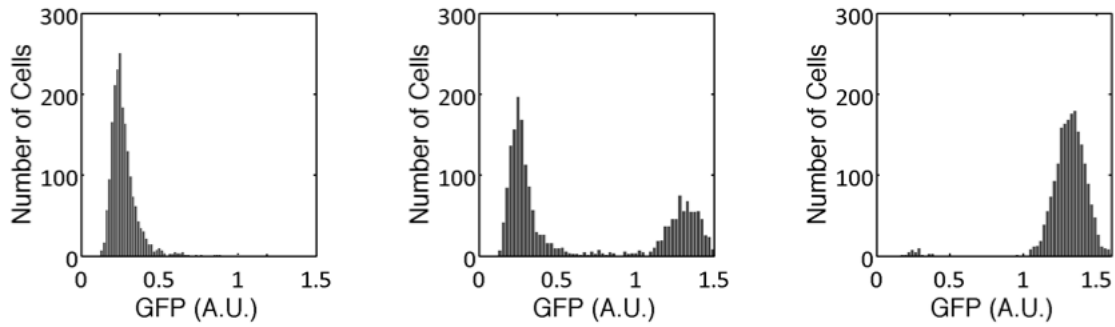


Figure 22. SDE model of the positive feedback circuit with strong feedback activation.

The histograms show the output (GFP) and the number of cells at three different values of T_{max} , 20, 100 and greater than 600. The stochastic differential equation model of the positive feedback circuit demonstrates that strong transcription factor binding strength ($p=2$ and $k=1$) resulted in stronger feedback activation and caused the system to move from gradient to switch like behavior.

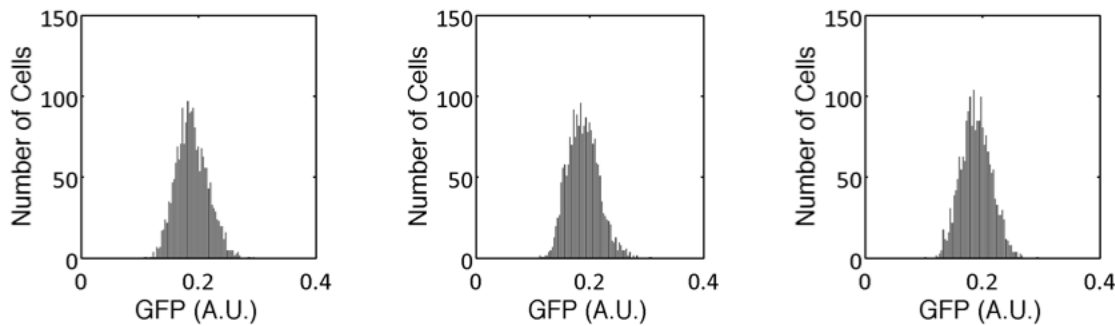


Figure 23. SDE model of the positive feedback circuit with feeble feedback strength.

The histograms show the output (GFP) and the number of cells at three different values of T_{max} , 20, 100 and greater than 600. The stochastic differential equation model of the positive feedback circuit demonstrates that weak transcription factor binding strength and relatively high degradation ($p=1$ and $k=0.9$) couldn't cause the system to move from gradient to switch like behavior.

Chapter 5 Conclusions

In this work, a modular genetic amplifier based on a constitutively active variant of LuxR was successfully developed. The use of autoinducer independent LuxR for construction of positive feedback element is truly unique to this study and has not been employed in any other studies before. This approach obviates the cell density dependence of the gene regulatory circuit.

The positive feedback amplifier was coupled to a one-component tetracycline sensor and a two-component aspartate sensor and tested. In accordance with the design objectives, the amplifier was able to increase the dynamic range and sensitivity of the integrated circuit. The amplifier can most likely be coupled to any cell-based sensor where the output involves the transcription of a gene. The design objective of modularity was also achieved through the positive feedback component. The feedback component was not manipulated and was successfully reused in entirety in two different scenarios. The amplifier has also met the other criterion of displaying bistability and hypersensitivity.

In addition to sensing applications, the amplifier can also be used to create devices of greater complexity in function. One intriguing application concerns impedance matching. Impedance mismatch occurs when the output range of one sub-circuit does not match the input range of another sub-circuit to which it is connected. To effectively link these two sub-circuits, the respective output and input ranges should match one another. As positive feedback can significantly alter the response of a sub-circuit, it can be used as an 'impedance matching' device by coupling two different sub-circuits together that have disparate requirements for signal levels to operate correctly.

A primary goal of synthetic biology is to design modular components with defined behavior that can be reused in diverse applications. The ideal component should have predictable behavior regardless of the context in which it is applied. This is a significant challenge.

While the hypothesis that amplification would be observed due to positive feedback was right, a different response was observed when the amplifier was coupled to the two different

sensors. For instance, the tetracycline sensor showed a major increase in sensitivity but only moderate increase in the dynamic response. The aspartate sensor, however, showed a major increase in the dynamic response but only a moderate increase in sensitivity. The amplifier also increased background expression in the case of the aspartate sensor but not in the case of the tetracycline sensor. The origins of these differences are unknown, but may arise due to variations, for example, in plasmid copy number, promoter strengths, and the metabolic burden imposed by each circuit. While further engineering can be used to control for these individual factors, their effects are often non-trivial to isolate and quantify.

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