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Creation and observation of a library of synthetic bacterial oscillators

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ABSTRACT OF THE THESIS

Creation and observation of a library of synthetic bacterial oscillators

by

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Master of Science in Biology
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Synthetic biology utilizes computer science, physics and molecular biology to create novel gene circuits to further our knowledge of naturally occurring systems. This thesis addresses the construction and observation of a library of synthetic bacterial gene oscillators. The study furthers the characterization of known synthetically designed systems. The library is based on a change in copy number of activator and repressor units in the dual feedback oscillator network. By changing the copy number of activator or repressor units, oscillators with a wide range in periods have been created. Twelve different strains were constructed by varying the origins of replication. Three of the strains contain an integrated repressor unit, acting as a single copy in the cell. A high throughput method to observe the strains has also been created to accompany the library. A custom created microfluidic chip is used to observe the cells using fluorescence microscopy, while an automated tracking program was made to record the oscillations. The library and high throughput method has created data used to further characterize synthetic gene circuits.

Chapter 1

Introduction

1.1 Synthetic Biology and Gene Oscillators

Synthetic biology uses engineering approaches to model and design functional systems based on biological organisms. With the advent of genome mapping, biologists have created a parts list for naturally occurring molecular networks and circuits. These circuits are contained in highly complex regulatory systems within the cell. Understanding these regulatory networks has been the focus of molecular and cell biologists for decades. The inherent complexity of such systems makes it difficult to fully understand networks on a system wide scale. Systems biology takes a holistic perspective of these large complex networks to look at the overall dynamics, generally simplifying the finer details[1]. Both systems and synthetic biology have flourished in the wake of the human genome project and require an integrated approach of experimental and computational tools to evaluate complex biological systems. Synthetic biology has complemented these fields by allowing for the design and construction of simplified gene circuits; thus, increasing the understanding of detailed mechanisms that make up the networks. The construction of circuits is borrowed from engineering and uses a forward-design approach to model and accurately understand the biological circuits. The parameters for these circuits are based on established electrical engineering techniques[2]. The dynamics of these constructs allow for the characterization of the behavior in larger regulatory networks.

The difference of synthetic biology from other biology fields is its engineering approach to network design using computational and mathematical tools. It is

truly an interdisciplinary field, combining molecular biology, genetic engineering and computer science[3, 4]. Currently, synthetic biology focuses on intracellular reactions within single cellular organisms, such as *Escherichia coli.* and *Saccharomyces cerevisiae.* These model organisms are used because of our knowledge and understanding of their gene networks.

Recent synthetic biology work on fundamental systems included toggle switches and oscillators[5, 6]. The later is a ubiquitous element in circadian rhythms. Oscillators are used as clocks in nature, which allow for the determination of day and night cycles. Synchronization between various networks would not be possible without oscillators [7]. Oscillations arise from periodic changes in the concentration of proteins within a cell[8]. A whole population of cells has also been synchronized to oscillate together[9]. This state-of-the-art research represents our current knowledge in synthetic biological oscillators.

1.2 Systems Biodynamics Lab Accomplishments

In the recent past, our laboratory, the Systems Biodynamics Laboratory at University of California, San Diego, has created an engineered genetic oscillator in *E. coli.* The oscillator includes externally tunable periods that are robust and persistent[10]. Design of this oscillator circuit was based on a previous theoretical mathematical model. The oscillator was built by linking a translational activator with a translational repressor. It can be described as a dual feedback network. Each of the networks are driven by the hybrid promoter $P_{ara/lac}$. The promoter is activated by the AraC protein in the presence of arabinose and is repressed by the LacI protein in the absence of isopropyl β-D-1-thiogalactopyranoside (IPTG). The three genes within the circuit *araC, lacI* and *yemGFP* (monomeric yeast-enhanced green fluorescent protein) are under the control of the hybrid promoter. When arabionse and IPTG are present in the medium, transcription of these three circuit components will start. The positive feedback loop is driven by the increased production of AraC in the presence of arabinose. LacI is also produced by this positive feedback and as LacI acts on the promoter, it represses production thus forming the negative feedback loop. The connection of these two feedback loops is what drives the oscillatory

behavior [3, 11].

The synthetic oscillator was monitored in a custom microfluidic device, similar to the one described in this thesis. Tunable periods are achieved by varying the inducer levels, temperature and media source. The nature of the positive feedback loop accounts for the robustness and tunability of the oscillatory period. The negative feedback loop is the cause of the time delay in the circuit[11]. This previous work provides the basis of this study. Probing the dual feedback network allows further characterization and prediction of synthetic gene circuits.

1.3 Motivation

The creation of a library of oscillators allows for basic understanding of the oscillators period and amplitude. The oscillator constructed by our lab (see Section 1.2) was designed to specific parameters based on a mathematical model[11]. The ratio of activator to repressor was regulated by placing them on plasmids with specific copy numbers. The copy number refers to the number of plasmids of a certain type within a cell. When a plasmid is inserted into a cell it has a specific range in the number of copies of itself within that cell. This is regulated by the origin of replication. The different origins of replication used in the study can be found in Figure 2.2.E. Changing the ratio of activator to repressor will affect the dynamics, or even stop the oscillations. In theory, along with changing the characteristics of the oscillations, the copy number will affect the amount of noise in the system, which in turn has an effect on period and amplitude.

Noise refers to the random variability within a gene network. Gene expression consists of multiple biochemical processes, including transcription, translation and protein folding. Each process has noise generated from the varying amounts of cellular components. In gene expression, where copy numbers are typically very low. Noise can have a large impact on the amount of expression of a certain gene. This can explain many of the differences seen in clonal populations of cells[12]. Small variations in certain cellular components can also amplify the noise downstream of complex regulatory pathways. Much of this noise is produced by transcription and translation taking place at different times within a cell, despite certain cells being in identical

stages of growth. The different gene encoding sequences, as well as protein properties, will dictate much of this timing. The varying amount of transcriptional regulators, RNA polymerase and ribosomes within the cell also adds noise to the system[13, 14].

One of the objectives of the laboratory is toward the understanding of synthetic biology as a deterministic system. Understanding noise, is crucial in achieving this (theoretical) goal. A library of oscillators with different noise profiles may facilitate further characterization as mathematical models. The library of oscillators can be varied by changing the copy number for the activator and repressor units. This can be done by varying copy number on the plasmids as well as integrating certain parts to the chromosome.

The copy number of a plasmid is regulated by the origin of replication. This sequence of DNA controls the number of plasmids within a certain cell by regulating the production of the plasmid it is contained in. The origin is required for replication; otherwise a plasmid would not be able to be passed from mother to daughter cells. Three different origins of replications are used in the construction of plasmid based oscillators. They are classified into high, medium and low copy numbers. These three origins of replication are identified as colE1, p15A and pSC101* respectively. The pZ plasmid vector system, which the original synthetic gene oscillator uses, is designed around these three origins. The modular nature of the plasmid system makes changing origins an efficient process. While the plasmids are easy to work with, the origins have a range in copy numbers[15].

A tighter form of regulation for copy number is desired to reduce the amount of noise from the variation within the origin type. This could be done by taking the activator and repressor and placing them on the bacterial chromosome. This allows for one copy of the unit to exist in the cell. It is also guaranteed to remain at one because it is not extra chromosomal DNA. The repressor construct was integrated into the chromosome to achieve a single copy of the unit[16] [17]. This last variant in copy number allowed us to build a library of synthetic gene oscillators. This is the motivation for this thesis.

1.4 Contribution

This thesis is focused on the important task of creating a library of strains and on the experimental observation of this library. The mathematical modeling of this library is beyond the scope of this thesis and will be studied by another group within our lab. Specifically, the contributions of my research are:

- Successful creation of a library of twelve oscillator strains.

  Original methodology based on conventional recombinant DNA techniques (description of such techniques can be found in Appendix A) was developed to create the library. Section 2.1 describes the design and the methods used to create this library.

- Development and observation of the oscillator library with a high throughput method.

  In order to observe the library of oscillators over many cycles, new approaches were developed. A new microchemostat (general description of microfluidics and microchemostat can be found in Appendix B) allowing simultaneous dual observation of different strains. This was developed with other laboratory team members. This new approach to experimental observation was successfully executed in a timely manner. The challenges of this high throughput method, the resultant microchemostat, and the observation methodology can be found in Section 2.2.

Chapter 3 is a summary of the observations showing that a library of oscillators have indeed been synthesized. These data will be crucial for the mathematic modeling task to be performed by others. Chapter 4 discusses the data collected and observations of strains. The data will need to be mathematically modeled before any conclusions can be made about the observations of this library.

Chapter 2

Materials and Methods

2.1 Strain Construction

Synthetic biology encompasses many disciplines from computer science to molecular biology. The latter, was used to create the library of oscillators by using established techniques as well as adapting newer protocols. Many of these molecular biology techniques can be found in Appendix A.

The twelve bacterial oscillator strains were constructed using conventional molecular biology techniques such as, PCR, restriction enzyme digest, ligations and agarose gel electrophoresis. Every oscillator variant was transformed or integrated into JS006 *Escherichia coli* cells which is a MG1655 *araC* and *lacI* knockout strain. This was done so the proteins AraC and LacI were produced only from the oscillator plasmids and not from the cells chromosomal genes. The first six strains were all constructed in a similar fashion, by swapping the origin of replication.

The first strain, TDCL1 was constructed using the pZ vector system. TDCL1 is the original bacterial oscillator constructed the Systems Biodynamics Lab. It was constructed by using the pZ vector system to allow for the easy swapping of components. One modular component is the use of unique restriction sites flanking the origin. The unique restriction site *AvrII* lies upstream while *SacI* flanks the end of the origin. The original activator plasmid pJS167 does not contain a unique *AvrII* site. A variation of the plasmid called pJS167NheI was created by replacing the *AvrII* site after the *araC-LAA* gene with a *NheI* restriction site. This allowed for much easier cloning of the activator plasmid variations. To swap the origins, pJS167NheI and

pZA14lacI-LAA were digested with *AvrII* and *SacI* (New England Biolabs, Ipswich, MA). This swap effectively separates the origin of replication from the rest of the plasmid vector. A gel extraction was done to isolate the origin from the vector. The pSC101* origin was obtained in a similar way from a separate plasmid. After pairing the correct origin to the correct activator or repressor vector, T4 DNA ligase (New England Biolabs,Ipswich, MA) was used to ligate the new origins into the plasmids. The ligations were then transformed into chemically competent JS006 cells. The combination of two vectors and three origins allowed for six strains with differing activator and repressor copy numbers.

Strains that were constructed follow a numbering system based on the plasmids contained in each strain. Figure 2.2.D is a table of the naming convention used to identify the strains. Figure 2.2.E lists the origin types used in the plasmids as well as the genomic copy number of an integrated construct.

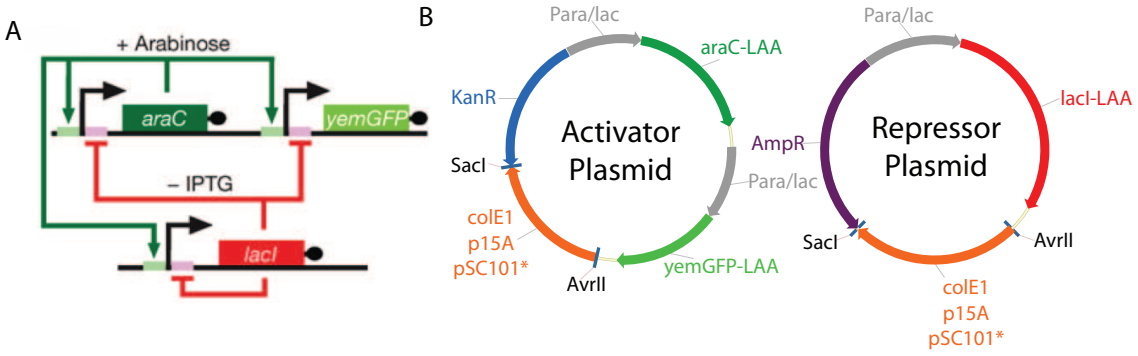


Figure 2.1: **A.** A schematic of the dual-feed back oscillator. $P_{ara/lac}$ drives the transcription of *araC-LAA* and *lacI-LAA*, forming positive and negative feedback loops. **B.** Plasmid maps of the activator and repressor plasmids used. The activator plasmid uses a Kanamyicin resistance, while the repressor plasmid uses ampicilin resistance. Origin of replication sites are flanked by an upstream *AvrII* site and a downstream *SacI* site. The activator plasmid carries the yemGFP-LAA as a reporter for the oscillator.

Single plasmid variants of the dual feedback oscillator include TDCL7, TDCL8 and TDCL9. Single plasmid strains were constructed to maintain an identical copy number of activator and repressor. Doing this on two plasmids with the same origin would not have the same effect. The variation in copy number in a single type of origin is too great to be considered identical between two plasmids. To overcome

this problem, both activator and repressor units were combined onto a single plasmid. pTDCL7 was constructed differently than 8 and 9 due to a high rate of recombination within the plasmid. This was due to the repeated promoter and terminator sequences, along with the high copy number of the plasmid. The high copy number allowed for more instances of recombination within the plasmid. The recombined plasmid always resulted in a smaller than expected size, which is less stressful to the cell′s health. Three sets of promoters and terminators per plasmid created a high number of sites for homologous recombination. This problem was unforeseen, but was eventually solved. Recombination was also seen in the construction of pTDCL8 with the p15A origin, but because of the lower copy numbers, the recombination happened less frequently, allowing for the correct length plasmid.

pTDCL8 and pTDCL9 were constructed by using PCR primers to replace the *SacI* site on the repressor plasmid with a *NheI* site. This was done by placing the sense primer right after the original *SacI* restriction site and adding the *NheI* site to the 3′ end of the PCR primer. The anti-sense primer included the *AvrII* site that is downstream of the lacI-LAA gene. The final fragment produced includes the ampR and lacI-LAA genes. These fragments were then ligated into the *AvrII* restriction site of the activator plasmid with p15A origin, creating pTDCL8. The *AvrII* and *NheI* have compatible ends, meaning the overhang on the sticky ends have complementary base pairs. The fusion of the two ends, creates a site that is not accessible to either enzyme. This keeps the *AvrII* and *SacI* sites flanking the origin unique to the plasmid. Once pTDCL8 was created the origin was swapped similar to the dual plasmid oscillator variants with the pSC101* origin, creating pTDCL9. For pTDCL7, PCR was used to create a product with $P_{ara/lac}$ and lacI-LAA with flanking *sacI* sites. The template for the PCR was pZA14lacI-LAA. This was inserted into the *sacI* site at the end of the colE1 origin. Proper orientation of the insert was checked through restriction enzyme digestion followed by agarose gel electrophoresis.

TDCL10, 11 and 12 are genomic repressor variants of the oscillator. Standard activator plasmids were used in the strains. TDCL10 uses the high copy colE1 plasmid. TDCL11 using medium copy p15A and TDCL12 with the low copy pSC101* activator. All three strains had the same single copy genomic repressor unit. To create these strains, the repressor had to be copied using PCR. Primers were designed to

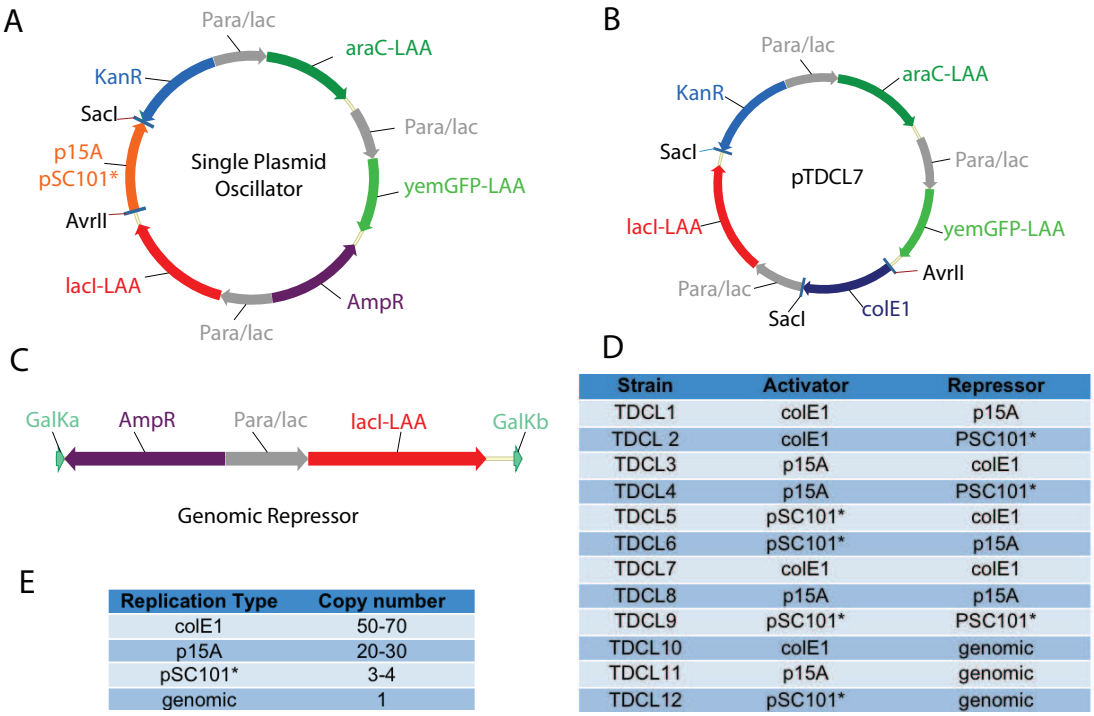
flank the AmpR and the lacI-LAA genes with 50bp GalK homology regions attached to the 3′ section of the primer. The GalK regions allowed for homologous recombination between the chromosome and the repressor construct. To facilitate this, the plasmid pKD78 was used to express the λ red recombinase. Chemically competent JS006 cells were transformed with the chloramphenicol resistant pKD78 plasmid [17]. The GalK homology sequences were obtained from Court's plasmid-based homologous recombination system[16]. The JS006 cells containing pKD78 were then made electrocompetent so the repressor unit could be added. One change from both methods was suspending the electrocompetent cells in GYT media instead of milliQ water. This was found to make the cells more competent. The repressor integration was confirmed by growing the strain on agar plates containing 2-deoxy-galactose (DOG). Cells that do not contain the GalK gene cannot properly metabolize the DOG leading to a toxic build up of 2-deoxy-galactose-1-phosphate[18].

This same method was attempted to integrate the activator of the oscillator on to the chromosome. Kanamyicin resistence, *araC-LAA* and the yemGFP-LAA were flanked by 50bp homologous ends to a few different genes within the chromosome. These genes included endA and phoR. GalK was also tried on JS006 cells without an integrated repressor. The integrations failed and this explains why an integrated activator is not included in the library. Extensive troubleshooting was used to try and solve the problem, but despite many attempts, it would not integrate.

The completed library of twelve synthetic bacterial oscillators is summarized in Figure 2.2 D. The strains were then observed using a microfluidics system along with fluorescence microscopy.

2.2 High Throughput Observation

Observing the behavior of the strains requires a quantitative method for recording the oscillations. To do this, fluorescence microscopy was used to take time-lapse images of the cells. High magnification of the cells would be needed to ensure individual tracking could be done. A specialized microfluidic device was designed in order to facilitate observation of the cells over a sustained period of time. The fluorescence microscopy working in conjunction with the microfluidics device creates a powerful



D

Strain	Activator	Repressor
TDCL1	colE1	p15A
TDCL 2	colE1	PSC101*
TDCL3	p15A	colE1
TDCL4	p15A	PSC101*
TDCL5	pSC101*	colE1
TDCL6	pSC101*	p15A
TDCL7	colE1	colE1
TDCL8	p15A	p15A
TDCL9	pSC101*	PSC101*
TDCL10	colE1	genomic
TDCL11	p15A	genomic
TDCL12	pSC101*	genomic

E

Replication Type	Copy number
colE1	50-70
p15A	20-30
pSC101*	3-4
genomic	1

Figure 2.2: Maps for oscillator variations. The single plasmid oscillator **A** contains both activator and repressor units as well as the reporter. This variant was used for the medium and low copy origins. **B.** pTDCL7 plasmid was created by inserting the lacI-LAA and its promoter into the *sacI* site of pJS167 **C.** Repressor construct for insertion into the GalK site on the genome. **D.** Table of all the oscillator variations. **E.** Table of replication copy numbers.

high throughput tool.

Nikon inverted fluorescence microscopes were used to study the various oscillators. The specific microscope setup used can be found in Stricker[11]. Instead of heated water lines to maintain temperature within the device, a plexiglass incubation unit was fitted to the microscope. A heating unit circulated air within the incubator was used to maintain a temperature of 37 °C. The thermocouple for the incubator thermal controller was placed on top of the microfluidic device to ensure that the microchemostat maintained the proper temperature. Imaging of traps was done at 100x magnification, with brightfield images being taken every 30 seconds. This high frequency of observation was required to sufficiently track the position of individual cells with the custom software. Fluorescent images were taken every three minutes to prevent photo-toxicity from frequent exposure to the light. This frequency also

prevented photobleaching the cells. Exposure times to fluorescent light were different for each strain in order to obtain the best images. The varied origins greatly differed the amount of fluorescence produced by each strain. Each strain would be tested at 1% arabinose for an appropriate exposure time. A maximum of exposure time of six seconds was used for the dimmest strains, while a minimum of exposure of 500ms was used for the brightest strains.

To study the *E. coli* under sustainable conditions at 100x magnification, a continuous-flow microfluidic chip was developed specifically to observe the varying strains of oscillators. We needed an environment that would be able to keep the cells healthy with fresh media and inducers for an extended period of time. Having a dozen strains and limited time on the microscope was another challenge. We needed a high-throughput method to study the strains. As explained in Appendix B, microfluidic microchemostats have been used to solve these problems. There are a wide variety of these types of devices to adapt to the specific needs of experiments. The original Tesla diode loop chip developed to study the original oscillator[11] would not be sufficient because of its single strain design. To overcome these challenges a new chip was devised to maximize the amount of data produced per strain.

Microfluidic devices for synthetic biology applications are made out of a polydimethylsiloxane (PDMS) casting bonded to a glass coverslide. The PDMS casting contains channels that, when bonded to the coverslide, allowed the media and cells to flow throughout the device. The coverslide allows us to image the cells without any visual obstructions.

Each chip consists of two parallel microchemostats, which share a common media source and waste port. This allowed us to study two strains at a time, reducing the time in half to run all the strains. There were multiple traps for each strain, allowing us to track multiple populations of a single strain in one overnight experiment. We found that two strains was the maximum we could study at a time, due to the physical limitations of the microscope's autofocus. Because cells were imaged every 30 seconds for the automated tracking, we could image only four positions on the chip. The fact that the fluorescent images needed to be taken every 3 minutes also added to the time constraints for tracking multiple populations.

Each microchemostat is able to maintain a single strain for an infinite amount

of time. It is only limited by the amount of media in the reservoir. The chip developed for this study consists of six ports to allow media in and out. Three of which are used in the DAW (Dial-A-Wave) section for media mixing. The other three are for cell loading and waste. Ports are supplied by a 0.020 in. diameter Tygon plastic tubing (Saint-Gobain Performance Plastics, Courbevoie, France) connected to a 60ml syringe (BD, Franklin Lakes, NJ) sans pluger, which acts as a reservoir. Adjusting the heights of each reservoir changes the hydrostatic pressure of the system, which allows us to control the flow rates within the chip. This is a very simple and reliable system that allows for quick experiment set up. Bubbles within the lines can create a hindrance in flow; and is the only problem with this system. Extra vigilance is taken when wetting the chip and inserting lines to ensure there are no bubbles in the system.

The DAW consists of the two media ports, the shunt port and the DAW mixer. This system allows for precise control over the amount of inducers delivered to the cells. The shunt acts as an overflow for mixed media, ensuring the correct ratio of desired inducers is achieved. Each media port is supplied by a reservoir of LB and 0.5mM IPTG. One of the reservoirs contains 2% arabinose, while the other one contains 0%. With the DAW, the amount of arabinose supplied to the cells can be infinitely adjusted from 0 to 2%.

The two media reservoirs are attached to independent linear actuators. These actuators are capable of adjusting the height of each reservoir by 0.1 cm. For an experiment, the actuators are calibrated for 2% arabinose and 0% arabinose in the final flows. This is done by using the tick marks in the DAW mixing chamber. As media from port 1 and 2 converge in the DAW chamber, the two flows stay laminar and do not mix. This is because of the dynamics of fluid flow in small chambers. This distinct boundary can be shifted to obtain a desired concentration of inducer. With the addition of sulforhodamine 101 fluorescent dye to one of the media reservoirs, the boundary is easily seen when excited. As the media exits the DAW mixer, it is allowed to mix thoroughly through diffusion. The length of the channel to the traps allows for this to happen. The length of the channel additionally adds resistance to the flow, making it harder for the flow to reverse from traps to DAW mixer. A computer with custom software designed in LabVIEW (National Instruments, Austin, TX) controls the heights of the actuators during an experiment. The calibration allows

the system to automatically adjust to predefined levels, allowing for exacting amounts of arabinose in the medium delivered to the cells.

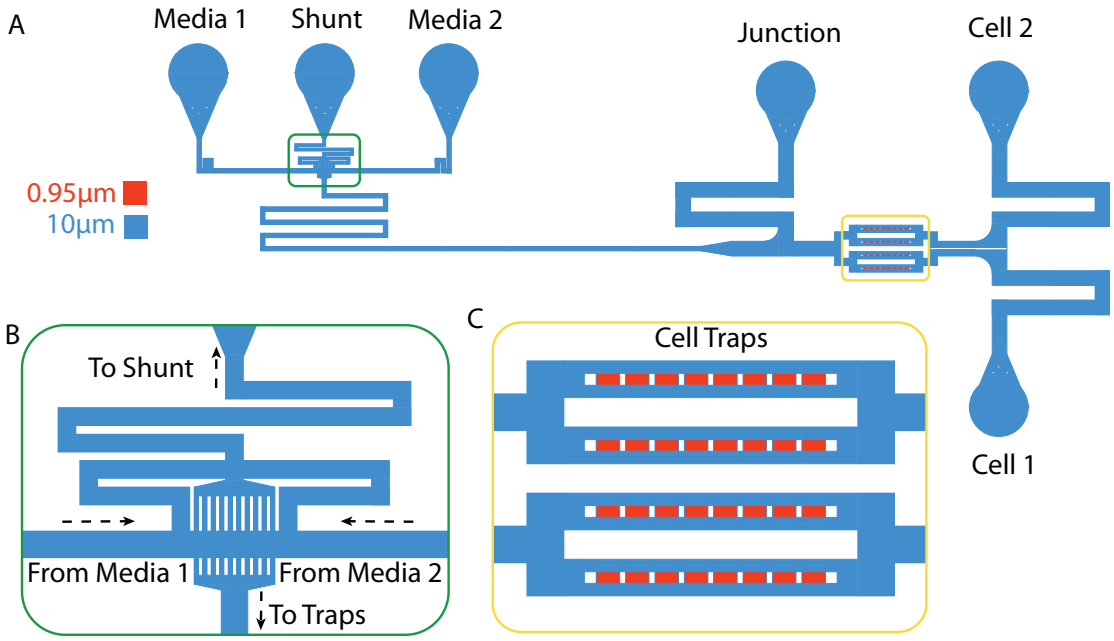


Figure 2.3: Diagram of microfluidic chip DAW6 2S. This device has traps with a height of 0.95μm and channel height of 10μm. **A** An overall view of the device. The chip has a parallel design to split to different strains between the upper and lower traps. When loading cells from the ports flow to the junction waste port. When cells have been trapped, the junction reservoir is equalized with the cell ports, allowing fresh media to flow to the junction and cell ports. **B** Enlargement of DAW mixer. Media is supplied to the DAW mixer from the media ports. Tick marks within the mixer allow for precise measurment of the proportions of media. Excess media is drained to the shunt. **C** Enlargement of cell traps. Each lane is lined with eight traps. The 0.95μm height keeps the cells stationary as they grow in the chip.

There are four rows of traps, two for each strain. Each row contains eight traps. The dimensions are 80μm long by 40μm wide and 0.95μm high. The height is 0.05μm smaller than the averaged sized *E. coli* cell's diameter, which slightly compresses the cell to hold it stationary despite the flow of media trying to carry it away. This also creates a uniform monolayer of cells, which can easily be tracked. A monolayer also keeps the cells in the same focal plane. To load the cells into the traps, the plastic lines would be flicked to create momentary high pressure to force the passing cells

into traps. During the procedure, one or two cells would enter a single trap. The top and bottom of the traps are open to the 10μm high channels, allowing media in while allowing media waste and cells out. The sides of the traps are closed off to contain the cells within the frame of view. These walls also acted as supports for the trap ceilings. Once cells were in the traps, they were allowed to grow for a couple hours to ensure they were healthy and to allow the population to fill the entire trap.

Cells orient themselves in vertical columns as the traps completely filled up. This happens naturally as the cells fill the voids and expand to the outer supports. Because of the common vertical orientation between traps, they are much easier to track with the automatic software. The common shape and orientation of the cells makes it much easier to distinguish a certain cell in the population. As the cells grow and expand they exert pressure on surrounding cells and force them out of the traps. The openings in the trap (see horizontal edges in Fig. 2.4 A) allow for the population to continually grow without physical stress. The movement of cells is tracked every 30 seconds to ensure the software can correctly follow a single cell's trajectory.

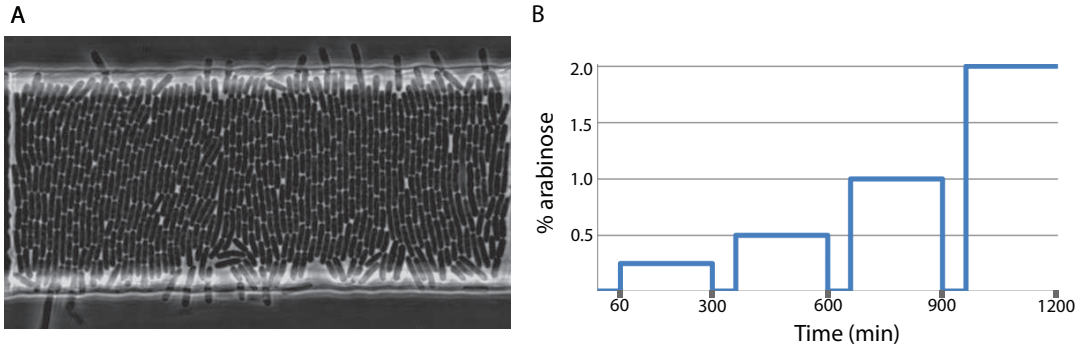


Figure 2.4: **A.** A monolayer of *E. coli* forms neat columns oriented in a vertical fashion. As cells grow, they force other cells out the top and bottom of the trap and into the channels. **B.** Arabinose inducer amounts over an experimental run. Each four hour period of induction is separated by an hour of 0% arabinose to turn off the oscillations.

For each experiment, overnights of each strain were placed in 50ml of fresh media at a 1:1000 dilution. Media consisted of LB, kanamycin (50μg/ml), ampacilin (100μg/ml), arabinose (2%), IPTG (0.5mM)and tween 20 (0.075%) as an anti-surfactant. These cultures were grown for 2 hours until the OD(Optical Density) reached 0.08. The cultures were centrifuged in 50ml conical tubes for 5 minutes at

5,000 rpm to pellet the cells. The supernatant was decanted back into the culture flasks and the cells resuspended in 3 ml of fresh media and placed in syringe reservoirs. The cells and media were allowed to drain through the Tygon line to ensure that no bubbles would enter the microfluidic device. Before the cells were loaded into the chip and media lines plugged in, the device was wetted through the shunt port (figure 2.3) by using a solution of milliQ water and 0.075% tween 20. This was done until the water and tween 20 solution pooled at each port on the chip. Wetting the chip before plugging in additional lines reduces the amount of bubbles that can form and hinder the flow of liquid. After filling each port, the media lines and junction lines were plugged in. Media was kept at a height above the other lines to prevent any contamination. This keeps the media always flowing from the DAW section of the chip to the junction. The cell ports were then plugged in so the flow would run to the junction port. As the cells flow past the traps, the lines are flicked to squeeze them into the traps. Once loaded, the cell ports would be lowered to the height of the junction port, allowing media to flow past the traps and into the cell ports, now acting as waste ports.

During an experiment, IPTG is kept constant at 0.05 mM, while the arabinose percentage is varied in steps. Each strain was first run with 1% arabinose to test whether the strain oscillates. This was done for periods lasting over 19 hours. Certain strains were then selected for more extensive testing over multiple arabinose levels. Four different arabinose levels were chosen that would reflect a diverse range in periods. These experiments contained changing arabinose at 0.25%, 0.5%, 1% and 2%. Each level of inducer was run for 4 hours at a time with one hour periods of 0% arabinose in between. The 0% hour breaks were used to turn off the cells oscillations before the next inducer level change.

This high throughput method was able to combine multiple experiments into an a single overnight procedure. The ability to run two different strains in multiple traps has quadrupled the amount of data produced from one Tesla microchemostat experiment. It does this by first doubling the amount of strains that can be observed as well as doubling the experiments for each strain. With the added ability to vary inducers at four separate levels, the amount of data is quadrupled again. The DAW section of the chip allows for a seamless change in inducer level without the need to

plug in new media reservoirs. The automated actuator system and software adds to the convenience of the system. Allowing the experiments to run overnight optimizes the amount of time the microscope is free for other types of work. The success of this high throughput method allowed for the quick data acquisition of multiple oscillator strains.

Chapter 3

Data

The microfluidics device and fluorescence microscopy were used in combination to obtain the data from each strain. As described in the previous chapter, each chip is capable of sustaining two different strains. Experiments consisted of two different strains with similar fluorescence levels. This was done to optimize exposure times between the strains for the best possible data.

Strains were run at a constant inducer level of 1% arabinose and 0.5 mM IPTG to see if they would oscillate before running at multiple arabinose inducer levels. Strains such as TDCL9, single plasmid oscillator on low copy pSC101* origin, proved too difficult to detect on the scope. This difficulty stemmed from several sources. First the strain was extremely dim even during 2% arabinose, which is when oscillations for other strains are their brightest. Secondly, the limitations of the scope camera could not pick up any detectable form of fluorescence, even with high binning set on the microscope software. Thirdly, autofluorescence was observed at exposures of six seconds or more of fluorescent light exposed to the cells. These factors along with the possibility that the strain does not oscillate, made it impossible to determine whether there were sustained oscillations. For other strains, exposure to fluorescent light had to be limited to prevent the cells from becoming sick and dying, while images had to be taken every three minutes to track the oscillation periods. Along with TDCL9, TDCL12 was also too dim to determine whether it oscillated.

Periods vary from 33 to 52 min for the different strains when induced with 1% arabinose. Many of the strains oscillate with consistent distinct periods. While most strains could be imaged easily under these conditions, the two genomic repressor

strains, TDCL10 and 11, were particularly difficult due to their dim nature. They were bright enough to determine distinct periods at 1%, but at lower concentrations they were much too dim. These two strains were not included in the multiple arabinose level experiments because of this difficulty. Once strains were determined to have detectable sustained oscillations, certain strains were chosen to test the robustness of the oscillations. This was done by exposing the strains to varying percentages of inducers. The strains chosen were TDCL1,2,3,4,7 and 8. Figure 3.1.B outlines the period curves over different amounts of arabinose inducer. Each strain follows a trend of increasing period with increasing arabinose.

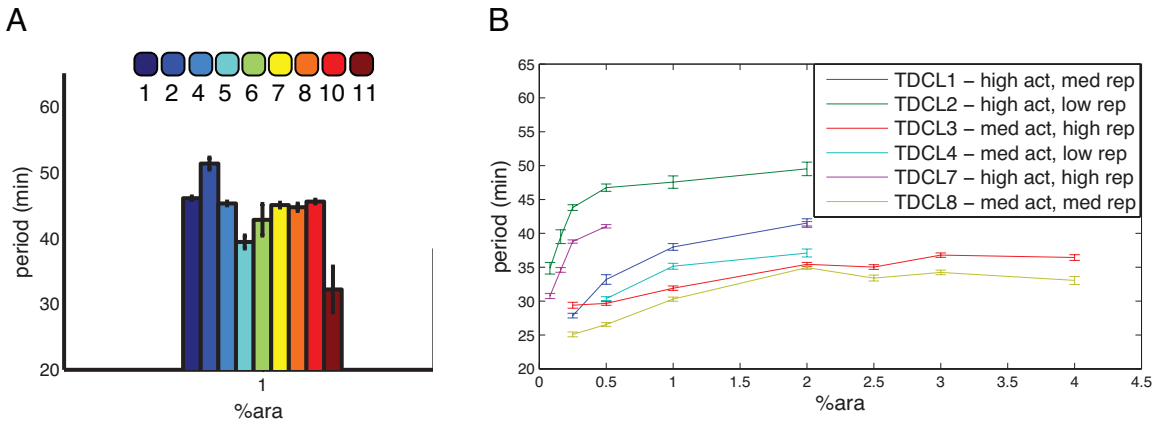


Figure 3.1: Mean periods for various inducer levels. **A.** Mean period for strains at 1% arabinose. Numbers correspond to strain number. **B.** Strains run at multiple arabinose concentrations TDCL2 and TDCL7 have additional points at 0.08% and 0.16%. TDCL3 and TDCL8 have additional points at 2.5%, 3%, and 4%.

To expand our knowledge of the periods, the inducer levels were varied beyond the range of 0.25% to 2%. The range dropped to 0.08% and 0.16% for two different strains. TDCL2 and TDCL7 were the two brightest strains, due to their high copy activators. These two were chosen, because there was a trend of low inducer level and low amplitude. TDCL2 recorded periods of approximately 35 minutes for 0.08% arabinose and 40 minutes for 0.016%. TDCL7, run at identical conditions had approximately 31 minute periods at 0.08% arabinose and 35 minute periods at 0.16% arabionse. Both strains show a continuation of an increasing period with increasing inducer levels. It should be noted while this is one of the brightest strains, the amount

of fluorescence recorded at these two arabinose levels was very low. It was this observation, which led us to the conclusion that 0.25% arabinose would be the base level of inducer for the different strains. This level allowed for a detectable amount of fluorescence from all strains.

The range was also expanded above to 2.5%, 3% and finally 4% arabinose. Strains TDCL3 and TDCL8 were used to test the arabinose range from 2.5% to 4%. As seen in the Figure 3.1.B, the periods varied by a little beyond the 2% level. TDCL3 and TDCL8 changed approximately three minutes in period. This change can be attributed to noise in the system and the fact that fluorescent images were taken every three minutes. Without a faster sampling of cell fluoresces, determining the period more accurately will be much more difficult. Because of the much lower change of period when compared to the 0.25% to 2% range, other strains were not explored beyond the 2% arabinose level.

Periods for a single strain seem to vary about 10 minutes between 0.25% and 2% arabinose. The increase in period does not follow a linear trend for increasing inducer. In general, the trend appears to be closer to an inverse logarithmic scale. This trend can be clearly seen in the strains TDCL3 and TDCL8 as the inducer levels are pushed from 2.5% to 4%. It is interesting to note that the strains with high copy activators (TDCL1, TDCL2 and TDCL7) in general have longer periods than all of the strains constructed with medium copy activators (TDCL3, TDCL4 and TDCL8). This is true for arabinose levels above 0.5%

Experiments were then tracked with a custom automated tracking program written by Tal Danino and Jangir Selimkhanov using MATLAB (Mathworks, Inc., Natick, MA)[unpublished work]. Images from experiments were loaded into the program and were individually tracked for each inducer level. The automated tracking code works by segmenting images and then tracking the centroids of cells in subsequent frames. The cell that has the closest centroid is deemed the same cell in the previous frame. In addition, J. Selimkhanov [unpublished work] wrote a common image processing method (region of highest overlap) as a secondary check in the tracking code method. From there, a trough finding algorithm was developed to extract periods and amplitudes for tracked cells.

Figure 3.2 shows the data generated from tracking TDCL8 over a 4 hour span

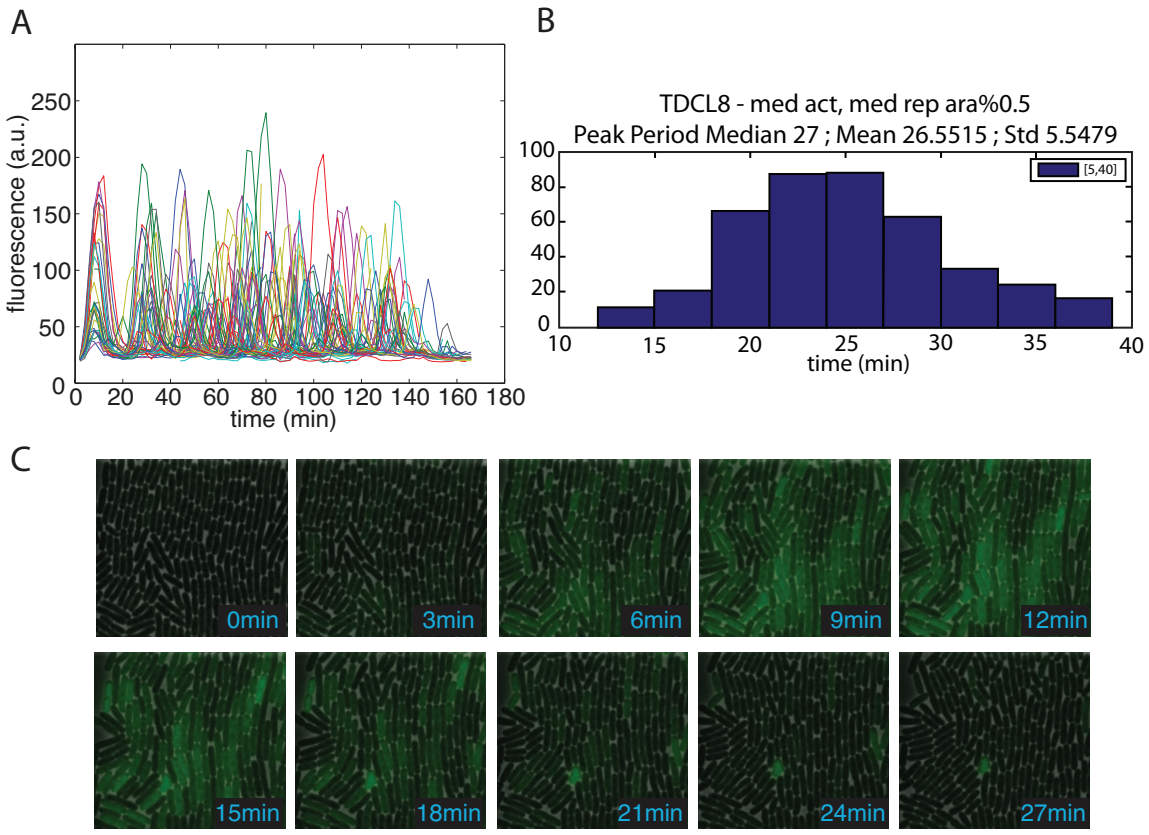


Figure 3.2: **A.** Multiple tracked trajectories of individual cells. **B.** Period data for TDCL8 over a 4 hour span with 0.5% arabinose. **C.** A single oscillation of TDCL8 induced with 0.5% arabinose.

with 0.5% arabinose. The trajectories of individual cells are tracked and plotted to show their distinct oscillations, as seen in Figure 3.2.A. The graph starts at 0 min with the induction of arabinose. There is a distinct first period, where all tracked cells are synchronized. The cells quickly lose synchronization because of the noise in the oscillator. Period median and mean is extracted from the trajectory data and can be seen in part B of Figure 3.2. There is a wide range of periods for each individual strain. Much of this can be attributed to the noise of the oscillator. We hope to discover more about these results with the tuning of mathematical models. A frame by frame set of images during the first induction can be seen in Figure 3.2.C. These are composite images of the brightfield and fluorescence views. They are combined so the individual cells can be seen along with the GFP produced. One thing to note about the single plasmid strains is the fact that there are no overly bright cells within

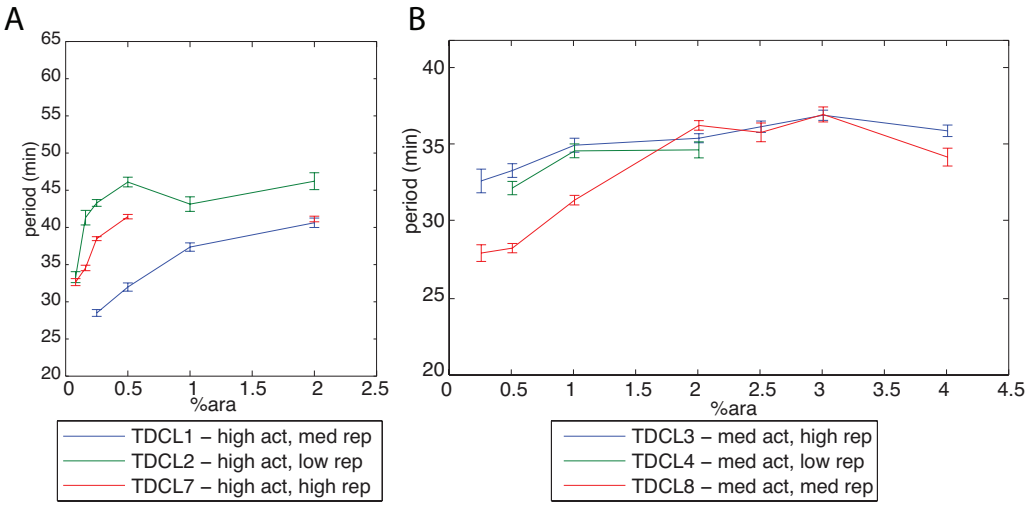


Figure 3.3: Mean periods classified by activator type. **A.** . Strains with high copy activator colE1. **B.** Strains with medium copy activator p15A.

the population. Dual plasmid oscillator strains exhibit cells that do not oscillate but do produce GFP when induced with arabinose. Instead of the cells creating peaks and troughs in fluorescence, they continue to stay bright. There are a couple possibilities for these types of cells; the most likely possibility is explained in the results section of this thesis. In section C of the Figure, the cells can be seen over their first initial oscillation. The entire population can be seen in synchronization as well. All of the cells, with the exception of one, return to zero fluorescence at the end of the first period. The time can be seen in the bottom right of each frame. As seen in the trajectories graph, not all cells share the same period. Cells start to reach the trough of the oscillation around the 21 minute mark, with all cells being off after 27 minutes of induction. The dual plasmid oscillator strains usually contain three to five bright cells per trap and typically do not look like the oscillations of TDCL8. They seem to be much noisier in their oscillations.

The strains classified by the activator copy number are displayed in Figure 3.3. Each strain has an increase in period as the inducer level rises. Part A of the figure displays the strains with high activator. TDCL1 has periods ranging from 27 minutes to approximately 36 minutes. It is also the strain with the fastest period among the strains with high copy activator. TDCL2 is the slowest of the strains and also contains a decrease in period of approximately 3 minutes from 0.5% to 1%

arabinose. The strain did increase in period from 1% to 2% arabinose, although it exhibited similar periods at 0.5%. TDCL7 exhibited similar behavior as its period slightly decreases from 0.5% to 2% arabinose. Data for TDCL7 at 1% is missing because of an inconsistent period. The standard deviation for the data was much too high to include in the results. Without that data point, the period does decrease by approximately 2 minutes and is similar to the period of TDCL1. Part B of Figure 3.3 displays the strains with medium activator. TDCL8 is the fastest of the strain with medium copy activators. Its periods range from 27 to 36 minutes from 0.25% to 2% arabinose. While it is generally the fastest strain among medium copy activator oscillators, it has the slowest period of the strains at 2% inducer. At levels greater than 2% the periods seem to settle around 35 minutes, fluctuating by 2 to 3 minutes in either direction. It shares this pattern with TDCL3, the slowest of the strains. While it is the slowest, it has similar periods to TDCL4, only differing by 2 minutes. The faster strain, TDCL4 uses a low copy repressor; while the slower strain uses a high copy repressor. They both vary in periods from 32 minutes to 35 minutes. Unlike TDCL8, there is much less variation in the period over the range of induces. These oscillators share much more similar periods than the strains with high copy activator.

Chapter 4

Results

Observations made from experimental data will be discussed in this section, but much of it is speculative, as the mathematical models have not been applied to the data. The varying copy numbers and oscillator types seem to have a strong correlation to period duration. All dual plasmid oscillators exhibited bright cells during the experiments. As explained in Chapter 3, these cells continue to express GFP after being induced by arabinose. These cells can be quite numerous in certain traps and often grow slower than other cells. They usually do not divide. Single plasmid oscillator strains do not exhibit these bright cells during experiments. One probable explanation for this is the possible loss of the repressor plasmid in dual plasmid strains. Many of the characteristics of the bright cells suggest that this is the case. The continued expression of GFP would make sense, as there is no *lacI-LAA* being produced by the repressor plasmid. This would allow for unrestricted production because of the activator plasmids positive feedback design.

The slow growth and inability to divide is most likely explained by the loss of ampicilin resistance. Ampicillin is a beta-lactam antibiotic, which inhibits the production of cell wall and binary fission. The cells are able to perform normal cellular functions, which explains the continued transcription and translation of the GFP gene. The single plasmid oscillator is able to avoid this loss of repressor, mainly from the fact that it is contained on the same plasmid as the activator. The ampicillin resistance is also adjacent to the *lacI-LAA* gene, preventing homologous recombination and loss of the repressor. This recombination event has been seen in the construction of the oscillator, but not during experiments. Overall the single plasmid oscillator is a much

more consistent in terms of the cell population.

The two strains TDCL3 and TDCL8 were run beyond the standard 2% arabinose inducer level to explore the dynamics of the oscillator. TDCL3 is a version of the dual plasmid oscillator, while TDCL8 represents a single plasmid oscillator. Both of the strains have the same relative brightness, which is why they are run in the same experiment. As explained in Section 3, inducer levels of 2.5%, 3% and 4% were added on top of the normal range. Figure 3.1.B illustrates the oscillator periods observed from these three new inducer levels. There is much less variation, as the periods seem to stay within a 4 minute window, ranging from 31 minutes to 35 minutes for both strains. The relative steadiness in periods could possibly be explained by the fact that the oscillator has reached a saturation point.

The positive feedback loop functions when arabinose and the AraC protein form a complex to induce its own hybrid promoter. Any level above 2% arabinose could possibly be saturating the system under the specified experimental conditions. At this level, the amount of free AraC protein is equal to zero; or the amount of arabinose/AraC protein complex is equal to the number of bindable promoters. Either of these situations would create a limit for the amount of AraC produced. This could effectively limit the period to around 33 minutes. The leveling off of period due to over saturation of activator is only speculative. The mathematical model could produce a different explanation for the consistency of the period after 2% arabinose.

Variations in average period can be seen between runs with a single level of 1% arabinose and runs with 1% arabinose in varying inducer levels. This difference could possibly be an effect from memory within the cell. Between different inducer levels, there is an hour run of 0% arabinose to stop the oscillations. This single hour might not be enough time to fully degrade all the AraC present in the cell, as the enzyme *ClpXP* must degrade all proteins marked with the *LAA* tag. While there is no arabinose present, there is constant IPTG in the media. Research indicates that there is possible crosstalk between IPTG and the *araC* portion of the hybrid promoter [19]. The levels of IPTG in the media are low enough that this crosstalk effect should not take place, but given the low copy numbers of some plasmids, it could be a possible effect.

One observation that can be made is that in this regime of inducers to the

number of activators/repressors is a strong determinant of period. For instance, at 1% arabinose the period increases from 28, 37, 47 minutes for ratios of activator/repressor of 1, 2.5 and 17 respectively (TDCL8 then TDCL1, TDCL2). Sufficient modeling will be used to account for the behavior that causes this change. In addition to this observation the behavior of oscillators with common activator plasmids should be further explored. As seen in figure 3.3, the above pattern of increasing period with increasing activator/repressor ratio does not occur. When grouped by high activator copy number, the strains seem to follow the pattern of increasing periods for activator/repressor ratios of 2.5, 1 and 17. When grouped by medium activator copy number, the strains follow a pattern of increasing periods for activator/repressor ratios of 1, 7 and 0.5.

The library of oscillators was successfully created, and observed using the microscopy and microfluidics high throughput method. While some work does remain that can help further characterize the oscillators, such as the integration of the activator, this work provides a basis for a much more detailed model of synthetic gene oscillators. The data generated from this study will be used to develop a model to account for the behavior and variability seen in these strains.

Appendix A

Recombinant DNA Techniques

The central dogma of molecular biology is the relationship of deoxyribonucleic acid (DNA) to ribonucleic acid (RNA) and finally to functional proteins. This underlying flow of processes is what allows molecular and synthetic biologists to manipulate as well as create novel genetic circuits. The transition from DNA to RNA is known as transcription. DNA can be thought of as a blueprint for a protein. This blueprint is highly valuable and the cell goes through many precautions to ensure that it does not change. These changes can lead to mutations in the genome. The most direct way the cell protects the DNA blueprint is to make a photocopy. This copy is created with RNA and is known as messenger RNA (mRNA). Once the mRNA is transcribed from the DNA, it can then be translated into a protein. Proteins are responsible for every function in the cell. Molecular biology exploits this process by splicing in new or different sections of DNA to create novel cellular functions.

Several tools used to accomplish this include restriction enzyme digests, expression cloning, polymerase chain reaction (PCR) and gel electrophoresis. Restriction enzyme digestion is a method that is highly efficient at cutting DNA at specific locations known as restriction sites. This is a powerful tool for excising specific genes, promoters and origins of replication. Restriction enzymes are found naturally in bacteria and archaea. They have been isolated and purified for use in cloning. Each enzyme has a specific recognition site within the DNA. (The enzymes used in this study all have a 6 base pair site.) Depending on the enzyme, the cut will either create a blunt end or a sticky end. Figure A.1 illustrates that a blunt end can be very useful as it can be ligated back to any other blunt ended DNA molecule. The sticky end

can only be ligated to another sticky end with compatible base pairs.

Ligations are another important aspect of modern molecular biology. They serve the opposite function of a restriction enzyme. Once the cut vector and the gene of interest are combined, the ligase will connect the ends of the gene with the vector. The DNA ligase will join the phosphate backbone of DNA between the $3'$ and $5'$ groups through a covalent bond. Ligase is a naturally occurring enzyme typically used in DNA repair and replication.

Expression cloning consists of work with plasmids. Plasmids are naturally occurring extra chromosomal molecules of DNA. They are usually circular and are found mostly in bacteria. They are able to self replicate, by using the cells machinery. Plasmids provide a natural way for gene transfer among a population of cells. In expression cloning, plasmids are used to introduce synthetic constructs into host bacteria, this is known as a vector. These constructs contain the desired genes to be expressed by the cell. All vectors contain an origin of replication, multicloning site (MCS) and a selectable marker. The origin of replication has already been explained in Section 1.3. The MCS is a section of the plasmid vector, which has multiple unique restriction sites adjacent to each other. This is convenient for splicing in the genes to be expressed. The plasmid is first cut open at one of these sites with a restriction enzyme, and the gene containing the matching site is ligated in. A MCS allows for the quick insertion of genes into a vector. The selectable marker is a gene on the vector, which encodes a protein for antibiotic resistance. This acts as an indicator that the vector is inside the bacterial cell. Without the plasmid vector, the cell would die from the antibiotics present in the medium. By adding the resistance selectable marker, the cells are able to survive and the genes of interest on the vector are guaranteed in the cell. (See Figure A.1)

Once plasmid vectors are constructed, they must be transferred into the cells, this is known as a transformation. Prior to a transformation the cells must first be made competent, this means that the cells posses the ability to uptake foreign DNA. This can be done by two different ways. The first is to treat the cells in a special media, which makes them chemically competent. The vector is then added and the cells are able to absorb the DNA through holes in the membrane created by the media. The cells are then heat shocked to close the holes. The second is to wash them with

water and then shock them with electricity to induce the uptake of the vector. This is known as electrocompetency and is much easier and faster, although the chance for an error is much greater. (Both of these methods were implemented during this study.)

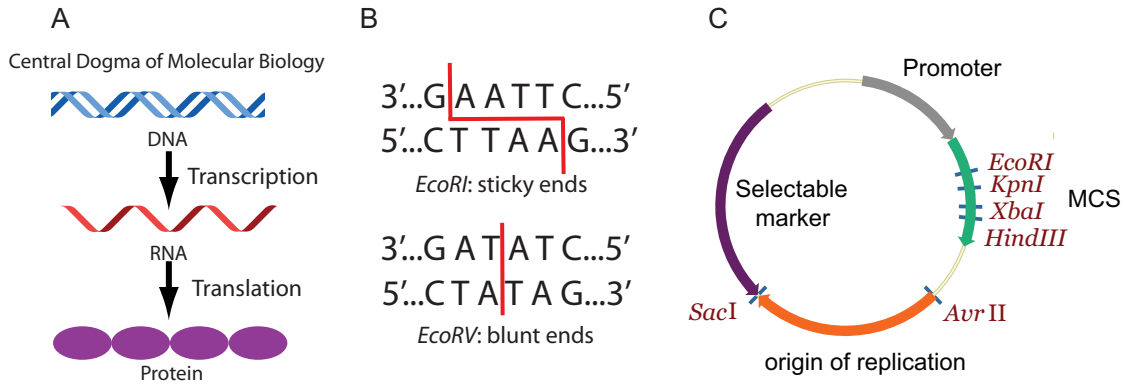


Figure A.1: **A.** An illustration of the central dogma of molecular biology. DNA which contains the genes for proteins to be produced is maintained by the cell. When a certain protein is needed, transcription occurs, where RNA polymerase creates an single stranded mRNA copy of the gene from the double stranded DNA. This mRNA transcript is then translated into a protein by ribosomes. The protein is constructed from amino acids and once translation is completed, the amino acid chain folds into a functional protein. **B.** Two different types of restriction enzyme cuts at 6bp restriction sites. The *EcoRI* site, when cut by the enzyme *EcoRI*, creates a staggered cut with nucleotide overhangs on the 3′ end of each strand. *EcoRV* enzyme creates a blunt when it cuts the restriction site. This type of site can be ligated to any blunt end. *C.* A map of a typical double stranded DNA plasmid vector. The plasmid contains a selectable marker , origin of replication and MCS. This one also happens to contain a promoter for and gene inserted in the MCS. The MCS contains four unique restriction sites. The plasmid also has unique restriction sites flanking the origin of replication.

PCR is a very powerful tool in molecular biology for quick and efficient cloning. PCR is used to amplify pieces of DNA. It can be used instead of restriction enzymes to copy a specific gene for cloning. The most useful part about this process is that it allows for the addition of flanking sequences, such as new restriction sites or even the addition of new genes. Developed in 1983[20], PCR has become a routine method for cloning.

A PCR reaction contains a few basic components; a template, primers, deoxynucleoside triphostates (dNTPs) and a heat stable DNA polymerase. The tem-

plate is the piece of DNA that will be copied and amplified. The primers are used to mark the boundaries of the piece to be synthesized. The dNTPs are the building blocks of DNA and are used to form the new base pairs. The DNA polymerase is what synthesizes the new DNA molecule. It must be heat stable so it does not denature during the change in temperature.

PCR has three main steps; denaturation of template DNA, annealing of primers and elongation of product. When a template piece of DNA is placed in a PCR reaction, it must first be denatured from its double stranded structure to single stranded. Denatureation of DNA occurs at 96 °C. This allows the primers to bind to the DNA template. The primers mark the outer edges of the PCR product. If a gene is to be copied by PCR from a genome, the sense primer is placed at the start of the gene; and the anti-sense primer is placed at the end. The product that will be produced is a copy of what lies between these two primers. When adding new restriction sites or linker DNA sections to PCR products, one just has to add the new sections to the ends of the primers. These new sections will be incorporated in the final product.

During the annealing phase of the PCR cycle, the temperature is lowered to the optimal temperature for the primer. This temperature depends on the base pairs that make up the region that binds to the DNA template.

The third step in a PCR reaction is elongation. The temperature is brought up to 72 °C, at which the DNA polymerase is most efficient. These three steps are then repeated up to 32 times. The PCR product will undergo exponential replication. [See Figure A.2] PCR is a very robust and fast way to clone a desired gene. It makes for an invaluable tool in molecular biology.

Gel electrophoresis has become an invaluable tool for quick verification of constructed plasmids and PCR fragments. Gel electrophoresis uses an agarose gel matrix and electrical current to separate DNA fragments by size. This can then be visualized using ultraviolet light.

An electrophoresis apparatus consists of a box filled with conductive buffer and an electrical supply. The agarose gel is situated in the box and submerged by the buffer. The gel contains small wells that are used to hold the DNA. Once the electrical current is applied, the DNA is allowed to run through the gel matrix. The principle behind the separation comes from the negatively charged backbone structure

of DNA. This backbone contains a single phosphate group in every nucleotide, giving the DNA molecule an overall negative charge. The electrical charge is applied with the negatively cathode is closest to the wells; while the positively charged anode lies at the opposite side of the gel. DNA to be separated is mixed with a loading buffer, which contains a dye. The dye illuminates when excited by ultraviolet light. DNA is also mixed with glycerol, which allows the DNA to sink to the bottom of the agarose wells. As the DNA runs through the gel, the fragments that are larger in size will run slower, while smaller fragments of DNA run faster. A DNA ladder of fragments is run in an adjacent well as a standard. This allows for the user to compare their samples to the known lengths of the DNA in the ladder. Gel electrophoresis is a very quick and efficient way to verify PCR products, as well as constructed plasmids.

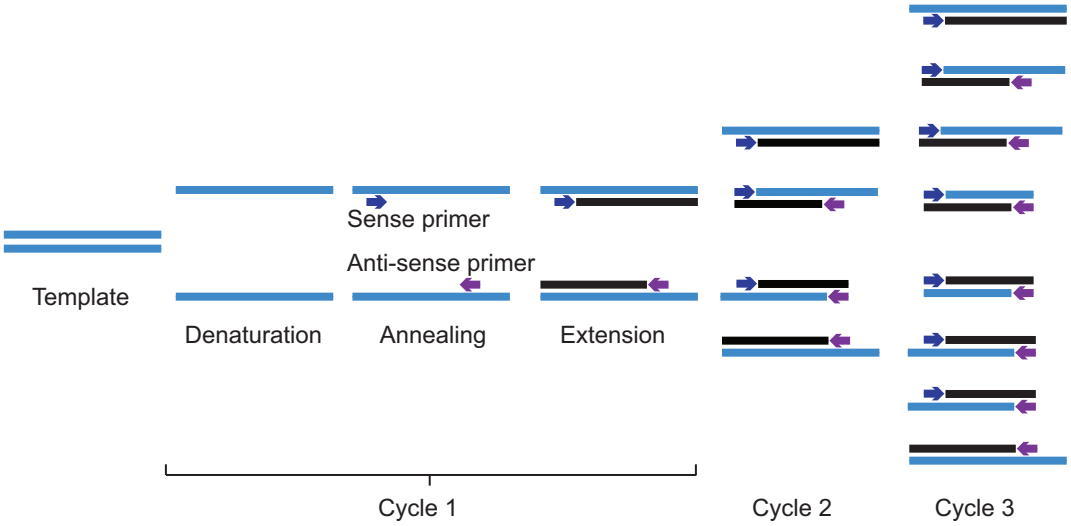


Figure A.2: PCR amplification illustration. During each cycle, there are three steps; denaturation of double stranded DNA, annealing of primers and elongation of product. The sense primer is illustrated as the right facing arrow, while the anti-sense primer is left facing arroww. During extension new DNA is synthesized (black) from dNTPs. Newly synthesized DNA is represented in black, while DNA from previous cycles is the blue. Over the 30 cycles, the short product is produced at an exponential rate.

Appendix B

Mircrofluidics

Microfluidics is a nascent multidisciplinary field that contributes greatly to biotechnology. This integrated field of engineering, physics, chemistry and biology encompasses the manipulation of liquids on a small scale, usually on the order of microliters to nanoliters. Using microfluidics to create novel devices for synthetic biology has become increasingly popular. So far, other fields of research have also discovered innumerable uses for this technology. Applications include genetic analysis[21], polymerase chain reactions (PCR)[22] and two-dimensional electrophoresis[23]. These devices are able to overcome conventional hurdles through its miniaturized environment, lower cost, reduced sample size and integration with other technologies.

Microfluidic devices allow for highly controlled experimental conditions on a small scale. These devices consist of channels and chambers ranging from under one micrometer up to hundreds of micrometers. There are unlimited number of possibilities to the design and implementation of microfluidic devices. These properties, along with the short fabrication time, make it ideal for constructing a microchemostat[24, 25, 26]. A chemostat is a bioreactor which allows for the constant addition of fresh growth medium and the constant removal of used medium and cell waste. The rate of fluid entering and leaving the reactor is equal in order to ensure the volume of the culture does not change. These properties are ideal for keeping a culture of cells in constant logarithmic growth. This environment allows for continuous growth of cell culture for an indefinite amount of time, only limited to the amount of available of fresh medium.

Microfluidics allows for a chemostat to be miniaturized on a small scale. Inte-

grating the microchemostat into a microscope creates a very powerful tool to observe bacteria over many hours[27]. Along with time-lapse fluorescence microscopy, which has become the preferred method for synthetic biologists[28], the microchemostat is a powerful tool to analyze sustained oscillations.

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