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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Microfluidics for Synthetic Biology: developing technologies for study of gene
networks in single cells and large populations**

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Bioengineering

by

Ivan Alexandrovich Razinkov

Committee in charge:

Jeff Hasty, Chair
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Lev Tsimring
Kun Zhang

2013

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Chair

University of California, San Diego

2013

DEDICATION

*My family for love and support
my friends for reminding me of the good things in life
and my wife, HyeIn, for believing in me.*

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LIST OF ABBREVIATIONS

AU	arbitrary units
bp	base pair
CFP	cyan fluorescent protein
DNA	deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
eqn.	equation
fig.	figure
FL	fluorescence
FP	fluorescent protein
IPTG	isopropyl β -D-1-thiogalactopyranoside
LB	lysogeny broth
mRNA	messenger ribonucleic acid
MW	molecular weight
no.	number
OD	optical density
ODE	ordinary differential equation
PCR	polymerase chain reaction
PDMS	poly(dimethylsiloxane)
PIV	particle image velocimetry
RBITC	Rhodamine B isothiocyanate
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SD	synthetic drop-out
T μ C	Tesla microchemostat
UV	ultraviolet
CFP	cyan fluorescent protein
GFP	green fluorescent protein
YFP	yellow fluorescent protein

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Prindle, A.*, Samayoa, P.*, **Razinkov, I.**, Danino, T., Tsimring, L. and Hasty, J., 2011: A sensing array of radically coupled genetic 'biopixels'. *Nature*, 481: 39-44. (*equal contribution).

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FIELDS OF STUDY

Major Field: Bioengineering (Synthetic Biology)

Studies in Biological Dynamics

Professor Jeff Hasty and Dr. Lev S. Tsimring

ABSTRACT OF THE DISSERTATION

Microfluidics for Synthetic Biology: developing technologies for study of gene networks in single cells and large populations

by

Ivan Alexandrovich Razinkov

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2013

Jeff Hasty, Chair

Bottom-up approach in understanding and developing gene networks calls for study of cells on a single cell level. Microfluidic technology provides such necessary tools to examine single cells not only in static but even more importantly in dynamic environments. Here we present studies that focus on examining model organisms of *Escherichia coli* and *Saccharomyces cerevisiae* on single cell and colony level. First, we developed a microfluidic device for studying single cell response of Galactose network of *S. cerevisiae* in fluctuating carbon source environment. As part of the study we have developed a novel off-chip technology that allows for fast and dynamic control of extracellular environment. In addition, this microfluidic device allows for 8 independent single cell level experiments to be run simultaneously. Next, this technology was adapted to study protein expression on single-protein level in *E. coli*. Furthermore, we developed a microfluidic device to study and characterize a colony-coupled synthetic oscillator in *E. coli*. To study the diffusion characteristics of the coupling agent the scale of

the devices was increased from nominal 100,000 cells to include over 50 million cells. Lastly, we combined the ability to generate a dynamic environment with a large-scale device to study long-term population dynamics of two competing *S. cerevisiae* strains.

Chapter 1

Introduction

Systems biology, a new field of less than 10 years, focuses on description of biological networks using mathematical models, which produce experimentally testable hypotheses. Specifically, systems biology tries to look at gene networks as whole and determine the behavior of the network from the individual protein and DNA interactions. Since the onset of the sequencing age with the human genome project, it is possible to have a complete picture of all the cellular components within a system, allowing development of systems biology and other associated disciplines. The key focus of systems biology is to take complex data sets and turn them into useful descriptions of the network, leading to a top-down approach in understanding of the system. On the other hand synthetic biology tries to build up from a small number of well-understood parts to recreate a functional analog of native cellular networks. A pertinent question to both fields is how to predict the behavior of gene networks under dynamic perturbations. As life evolved in an ever-changing environment, the response of a system to a dynamic input is crucial for the survival of the organism, and understanding of the mechanisms will lead to better understanding of evolution and adaptation. Due to the interdisciplinary nature of the field, synthetic biology relies on techniques from many fields such as molecular biology, microscopy and engineering.

In the last 50 years the number of tools available for DNA manipulation has

increased tremendously. Historically, restriction digestion allowed the first meaningful DNA modification. Special enzymes, known as restriction endonucleases, are able to cut the DNA strand at very specific locations. Due to the double stranded and complementary nature of the DNA, the cuts left behind by the restriction enzymes are able to recombine with cuts made by the same protein on a different strain. Using another enzyme the DNA is then bound together to make a new, complete strand, called recombinant DNA. This technique allowed scientists to easily replace and cut out specific parts of the DNA sequence, allowing study of new DNA sequences and its effects on organism behavior. However, blindly modifying DNA leads to little useful research. In hand with the discovery of useful restriction enzymes, came the advent of rapid sequencing technology. The new method, now known as "Sanger sequencing", allowed for precise determination of the order of the nucleotides (Adenosine, Guanine, Cytosine and Thymine) within a DNA strand. With time the sequencing efficiency improved drastically, leading to the Human Genome Project which sequenced the entire human DNA. Currently, there are over 180 entirely sequenced genomes of various organisms from bacteria to mammals. Lastly, to effectively work with a DNA sequence it is crucial to have more than one copy. A technique, known as PCR (Polymerase Chain Reaction), is able to multiply a specific region of DNA. Increasing the DNA concentration improves restriction digestion and recombination reactions, allowing for more effective research. One of the most celebrated cases of application of these three technologies is in the production of recombinant insulin in yeast. The DNA sequence of the gene encoding for insulin was determined and inserted into *Saccharomyces cerevisiae* for production. Historically, the only source of insulin was an extract from sacrificed animals, such as pigs. By using unicellular organisms for production of insulin, the supply of insulin increased drastically, lowering the price and increasing availability of the life-saving substance.

By inserting special fluorescent proteins in line with the sequence of our interest, we are able to use light microscopy to determine the time and locale of protein production. Fluorescent proteins are able to absorb a specific wavelength of light and then

reemit a different wavelength of light. For example, GFP (Green Fluorescent Protein) absorbs in the blue spectrum and emits in the green part of the spectrum. So by using optical filters sets designed for individual fluorescent proteins we are able to image exclusively the protein of our interest. This technique is used in parallel to image more than one type of fluorescent protein, effectively monitoring multiple processes within the cell.

1.0.1 Microscopy Automation

Light microscopy has been extensively used by biologist for a few centuries, however the introduction of fluorescent proteins and recombinant technologies, for the first time allowed examination of protein production. In synthetic biology we employ a variety of different fluorescent markers to monitor levels of proteins in the cells. Once a specific genetic circuit has been designed and introduced into the cell, mostly *E. coli* or *S. cerevisiae*, its behavior is monitored using a microscopy setup. Depending on the function of the circuit, it might be important to look at individual cell behavior or whole populations. The number of cells in an experiment and the extracellular environment are controlled by special microfluidic chemostats, discussed in following section.

The stochastic nature of protein expression and promoter regulation, leads to increased interest in acquiring single cell data. Population statistics can always be determined from individual cell responses while allowing for unparalleled resolution. However, the total number of responses needs to be large to attain statistical significance. Through microscopy automation, it is possible to monitor large number of individual cells under various conditions for extended periods of time. Automated post-processing of data furthermore increases the throughput of our systems. During a representative experiment, the cells would be imaged in regular light (brightfield) and 2-3 fluorescent channels. To follow the response over time, the cells are imaged repetitively with a period of 30 seconds to 30 minutes. When working with faster growing strains of bacteria it is important to image as fast as possible to improve post-processing accuracy.

Automation of auto-focus, stage movement, excitation sources, filter wheels and camera exposures improves the necessary time for each, effectively allowing acquisition every few minutes. Future improvements in camera sensitivity, speed of filter changes and auto-focus techniques can potentially bring down imaging steps down to a few seconds.

1.0.2 Microfluidic Technology

Microfluidic devices are commonly used in biological applications for culturing and imaging a variety of different cell types. Due to the small scale of the architectural features in the device, with heights commonly under $50\mu\text{m}$ and lengths well below 1 mm, the flows within the device are in the laminar regime and can easily be predicted mathematically. Also, the small scale of the devices means that it is possible to use very small volumes of media and chemicals during experiments. This is advantageous for very rare or expensive chemicals. Our ability to model the devices mathematically leads to thorough computational testing using finite-element analysis techniques to determine the function of the device prior to manufacturing. This improves our ability to make usable devices with limited trial-and-error periods. Furthermore, the techniques used to manufacture master molds for these devices are decades old technologies borrowed from the electronic chip manufacturing field. The equipment and chemicals for the manufacturing process are well developed and thoroughly documented. Once a master mold has been manufactured, a silicon elastomer (PDMS) is poured over it and allowed to solidify. The silicon is then peeled off, cleaned, and bonded to a glass coverslip. Due to the inert nature of both the silicon elastomer and glass, the devices themselves do not deleteriously affect the live cells. In addition, PDMS is highly permeable to most gases allowing free gas exchange with the surrounding atmosphere of our choice such as regular, anaerobic or blood gas mixture. In this thesis we will discuss microfluidic devices for *E. coli* and *S. cerevisiae*, although it is possible to use microfluidics with mammalian cells and cyanobacteria.

Unlike a regular stirred batch culture, where cells and media are well mixed to-

gether, in our microfluidic devices the cells are held in place with media flowing around them. Cells are kept stationary by lowering the ceiling to be just smaller than the diameter of the cell. This effectively squeezes the cells, allowing small motion due to cell growth but not due to media flow. Also, only with 'immobilized' cells it is possible to switch the surrounding media within seconds. This is exactly the technique we employ for our dynamic perturbation of cells. The extracellular concentration of a chemical (inducer, inhibitor) is changed instantaneously in a variety of waveform such as steps, ramps, sinusoidal and square waves. The cellular response is then recorded with fluorescent microscopy.

1.0.3 Synthetic biology: past and future

Engineering approach to design and implementation of novel genetic circuits in living organisms has had a number of successes in the past ten years. The earliest examples of functional genetic circuits are the 'repressilator' and the 'toggle switch' (Elowitz and Leibler, 2000; Gardner et al., 2000a). The 'repressilator' is an oscillator constructed from three negative feedback components in series, where $A \dashv B \dashv C \dashv A$ ($\dashv$ is inhibition). The values of each protein (A,B,C) increase and decrease in a periodic fashion. Similarly, the 'toggle switch' is a bistable network that can change and keep a specific "state" with an outside input. Since these nominal papers, numerous other groups have produced different oscillators. A tunable synthetic oscillator has been developed for bacteria and mammalian cells (Stricker et al., 2008; Tigges et al., 2009). By varying a concentration of a specific chemical in the media it is possible to tune the period of oscillations within a certain range. In both cases individual cells have independent phases of oscillations, basically all the cells are out of tune with each other. Including a local and global coupling mechanisms into the oscillator it became possible at first, to tune a few thousand oscillators and finally up to 50 million individual oscillators (Danino et al., 2010; Prindle et al., 2012).

Although tremendous progress has been made in the field, a number of factors

have contributed to a slow down in the progress. The circuits are unpredictable and can have different behavior in different cell types. Many of the components are not compatible with other systems and cannot be simply integrated together. Random fluctuations within the cells lead to variability in circuit behavior, making them less stable. Also, the number of available promoters is limited, leading to a plateau in the complexity of circuits, Figure 1.1.

A possible solution to this complexity issue could be to harness the power of evolution itself and evolve novel circuits with desired characteristics. In the last chapter of this treatise we discuss the first step towards such a system by introducing a microfluidic platform that allows to create a dynamic environment which in turn allows a specific strain to outcompete the other strain. Since the cells within a microfluidic device can be kept in a constant logarithmic growth phase we can rapidly achieve hundreds and thousands cell division. Setting up the selective pressure of our choice we theorize that it will be possible to evolve a new strain with desired characteristics.

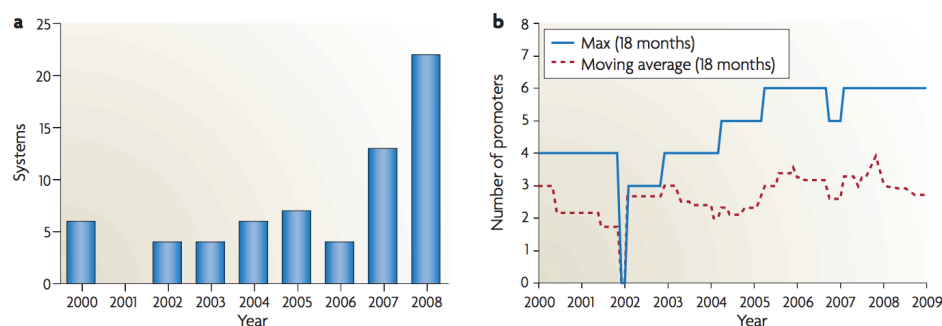


Figure 1.1: **A.** The total number of circuits published is increasing each year. **B.** The complexity of the circuits, measured as the total number of promoters used, has plateaued in recent years around 6 promoters per system (Purnick and Weiss, 2009).

Chapter 2

Microfluidics for synthetic biology

2.1 Introduction

Microfluidic technology has enjoyed considerable success and interest in recent years. Microfluidic devices have been used for everything from miniaturization of molecular biology reactions, to platforms for cell growth and analysis (Danino et al., 2010; Rowat et al., 2009; Taylor et al., 2009; Bennett et al., 2008; Hersen et al., 2008; Kurth et al., 2008; Lee et al., 2008; Cookson et al., 2005; Hong et al., 2004; Thorsen et al., 2002). A driving factor for increased use of microfluidics is the potential for more productive experiments, i.e. accomplishing the same or more using fewer resources (primarily less reagents, consumables and time). Furthermore, microfluidic devices offer the unrivaled ability to precisely control and perturb the environment of single cells while capturing their behavior using high resolution microscopy. In this report we will concentrate on how to design, build, operate and analyze data from single cells growing in the chambers of high throughput microfluidic devices. We will focus primarily on a device built to monitor the growth of *Saccharomyces cerevisiae* (yeast) in a dynamically changing environment as a case study. This device is known in our lab as the MDAW or Multiple Dial-A-Wave device.

In our lab we strongly believe in the importance of acquiring single cell trajec-

tories from our experimental runs. This requires the ability to track single cells over the course of an experiment, which generally lasts 24-72 hours. Indeed of all technologies available in molecular biology, microfluidics alone offers the ability to track the behavior of a large number of individual cells over the course of an experiment. While other technologies, such as flow cytometry, allow the acquisition of single cell data, the experimenter cannot track each individual cell in time. This leads to “snap shots” of how the population as a whole changes in time, but does not capture how individual cells progress over the course of an experiment.

The difference between the techniques can be illuminated easily if one thinks of a population of cells containing a desynchronized genetic oscillator. In this case much depends on the waveform of the oscillator. For oscillators with sinusoidal output, the population will appear bimodal with a large portion of the cells spread between the two modes. However for an oscillator with output similar to a triangle wave, the cells will be uniformly distributed between all phases of oscillation and therefore the population will have a fairly evenly distributed set of fluorescent values. Of course the behavior of a real oscillator can be somewhere between these extremes, but the point is that looking at the progression of a population as a whole does not tell you everything about its dynamics. For example in each of the cases mentioned above, other explanations are possible, such as the transient of a bistable switch, or even a genetically mixed population of cells. In contrast, using a microfluidic device to follow the temporal dynamics of single cells in such a population would allow one to easily see if any cells were oscillating.

While microfluidics is powerful, flow cytometry has the ability to capture a large amount of data quickly, much more quickly than can be done in traditional microfluidics. For this reason microfluidic and flow cytometry should be thought of as complimentary, instead of competing, technologies. We often find it useful to first characterize our genetic circuits using flow cytometry, testing as many media or inducer concentrations as possible, to look for behavior indicative of interesting dynamics. Once these conditions are determined we follow up with the more powerful, but involved microfluidic experiments.

Thus in the context of this report we will be talking about microfluidic chips designed to capture single cell data over the 1-3 days of the experiment. Unfortunately this limits the architecture of such a chip due to the difficulty of tracking cells. Regrettably cells such as yeast or especially *E. coli* have few unique features which can be used to distinguish them from their brethren. The full details of this will be discussed in a later section describing cell tracking, but suffice it to say, the only truly unique characteristic all cells possess visible by phase contrast microscopy is their position in time. As an added complexity, cells such as yeast or *E. coli* are so fast growing they can quickly fill both a trap and the camera's field of view. Once the trap is full the colony of cells will begin to move in flows resembling particulate flows (Mather et al., 2010a). These flows are due to pressure exerted by the colony on the walls of the trap. Due to this movement, phase contrast images of a colony's growth must be taken often, usually every 30 seconds to a minute, to prevent excessive movement between images.

Unfortunately, this requirement of frequent imaging imposes a physical limit to the size of the chip, usually determined by the speed of the microscope hardware. Even state of the art, fully automated microscope hardware such as the Nikon TI system, cannot autofocus, acquire phase contrast plus 3-4 fluorescent images, and then move to a new stage location in less than 4-5 seconds and sometimes as many as 7-10 seconds depending on the acquisition parameters. This limits the number of chambers and hence the number of independent experiments to at most 8-14, if the one minute interval between phase images is followed. Of course one also has to worry about overexposing cells to fluorescent excitation light, which can easily kill even the hardiest of cells rather quickly. Thus while phase contrast images are acquired every minute, we normally only capture fluorescent images every 5 minutes. Since 4 out of 5 acquisitions will not contain fluorescence capture (usually the longest step) this decreases the overall acquisition time somewhat. However, even if the phase contrast interval is lengthened the scope hardware will end up being the limiting factor in determining how large a chip can become. Of course microfluidic chips have been created with thousands of chambers (Taylor et al., 2009), however these devices cannot capture the type of single cell

trajectory data that smaller devices can, at least with current microscope technology.

The types of microfluidic experiments we will discuss here pretty much require the latest in microscope hardware for reasons mentioned above. Automation of most microscope tasks is critical, such as stage movement, phase ring and fluorescent cube changing, and shutter control. Moreover taking images every minute for days on end requires an automated focus routine, which luckily most microscope manufactures can readily provide. This also requires large amounts of hard disk space and equally important a rigorous method for space management, with backup procedures in place to prevent catastrophic data loss. Moreover the sensitivity of the camera used is extremely important. While the background fluorescence (a lower bound for the minimum detectable signal) of yeast and *E. coli* cells is easily observed using CCD cameras even a decade old, one should always use the most sensitive camera available to minimize the exposure time and hence phototoxicity caused by the fluorescent excitation lamp. The overall idea is that while older hardware may allow you to capture some data like that we discuss here, newer hardware will allow you to capture more data with a higher quality and with less damage to your cells.

2.2 The design of a microfluidic chip

To design a microchemostat chip useful for the type of experiments described in the introduction, one has to know a small amount about fluid mechanics at the microscale. We will briefly describe the physics behind microfluidics here, but the reader is directed to more complete texts if desired (Beebe et al., 2002; Nguyen and Wereley, 2002; Whitesides et al., 2001; Brody et al., 1996). Those that have not studied fluid mechanics in depth do not have to worry because making a functional microchemostat is not too difficult. The first thing to understand is how fluid flows at the microscale of a microfluidic device. From fluid mechanics we know that there are essentially two major flow regimes: laminar and turbulent flow. Laminar flows contain highly predictable, parallel flow streams resulting in fairly easy to model profiles. In contrast, turbulent flows

are unpredictable, difficult to model computationally and contain complicated flow patterns such as eddies and vortices (there is also a transition regime between these two flow types). For microchemostat devices the flow will be exclusively laminar as explained below. However to determine the flow type in an arbitrary system, the most important parameters are the type of fluid used, the dimensions of the fluid channels and the fluid's velocity in these channels. The relationship between these parameters can be expressed as the Reynolds number (Re), which is a dimensionless quantity useful for determining the dominant profile in a flow system. The Reynolds number is defined by

$$Re = \frac{\rho v D_h}{\mu} \quad (2.1)$$

Where ρ is the density of the fluid, v is the mean fluid velocity, D_h is the hydraulic diameter of the channel (a value which depends on the channels dimensions, see (Nguyen and Wereley, 2002)) and μ is the fluid's viscosity (Beebe et al., 2002). The Reynold's number represents a ratio between the inertial forces and the viscous forces of a fluid's flow. Empirically it has been determined that flows with a high Reynold's number ($Re > 10^3$), indicating the dominance of inertial forces, will be turbulent while low Reynolds number flows ($Re < 1$) will be exclusively laminar (Brody et al., 1996). Typical parameter values for microchemostats with an aqueous fluid are given in Table 2.1. Due to the low Reynolds number in these chips flow is laminar.

Table 2.1: Typical physical parameter values for microchemostat devices used in synthetic biology

parameter	variable	value	units
density of water	ρ	1×10^3	kg m^{-3}
viscosity of water (dynamic)	μ	1×10^{-3}	$\text{kg m}^{-1}\text{s}^{-1}$
hydraulic diameter	D_h	$1 \times 10^{-4} - 1 \times 10^{-6}$	m
mean fluid velocity	v	$1 \times 10^{-4} - 1 \times 10^{-6}$	m s^{-1}
Reynolds number	Re	$1 \times 10^{-2} - 1 \times 10^{-6}$	N/A

2.2.1 Mixing in microchemostat devices

A major consequence of laminar flow is that mixing will only occur due to diffusion, since bulk mixing relies on some type of turbulent flow. An important way to view the effect of diffusion in a microchemostat is to consider the diffusion length scale, which describes the one dimensional distance a molecule can be expected to travel in a given amount of time. The relationship is given as (Beebe et al., 2002):

$$d^2 = 2Dt \quad (2.2)$$

where d is the distance a molecule travels, D is the molecule's diffusion coefficient and t is the elapsed time. Since the distance traveled by a molecule is proportional to the square root of the elapsed time, diffusion will become more important at smaller length scales. For a specific example consider the Atto 655 dye, expected to diffuse $10 \mu\text{m}$ in 0.1 seconds, but taking over 1000 seconds to diffuse 1 mm. Diffusion coefficients for representative molecules often encountered in microchemostats are given in Table 2.2.

Table 2.2: Diffusion coefficients for ions and molecules commonly used in microfluidic chemostats

name	molecular weight (Da)	Diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$)	reference
Sodium ion (Na^+)	22.98	1.3×10^{-5}	(Lide, 2004)
Glucose	180.16	6.7×10^{-6}	(Lide, 2004)
Atto 655 dye	528	4.3×10^{-6}	(Dertinger et al., 2007)
Bovine albumin	67,000	5.9×10^{-7}	(Young et al., 1980)

As expected the diffusion coefficient tends to increase with increasing molecular weight and this is important to compensate for when using a tracer dye to monitor nutrient transport. For example, as can be seen in Table 2.2, one should be careful using Atto 655 dye as a surrogate for bovine albumin transport, or any high molecular weight protein, due to their order of magnitude difference in diffusion coefficients. Another important concept regarding diffusive transport in microchemostats is the Péclet number, which is another dimensionless quantity given by:

$$Pe = \frac{vL}{D} \quad (2.3)$$

Where L is the characteristic length scale, which in a microchemostat corresponds to the channel width. The Péclet number represents a ratio between advection and diffusion of a substance. Conceptually it can be thought of as the ratio of how far “downstream” a molecule is carried versus how far it diffuses across the channel in a given unit of time. In microfluidic systems reliant on diffusive mixing, knowledge of the Péclet number is critical for designing functional microchemostats. To determine the length required (Δy_m) for effective diffusive mixing of a substance the following relationship is useful (Stroock et al., 2002):

$$\Delta y_m \approx \frac{vL^2}{D} \quad (2.4)$$

$$\Delta y_m \approx PeL \quad (2.5)$$

Thus, Eq. (2.4) indicates that for two channels with equivalent Péclet numbers, the narrower one will require a shorter length for complete mixing. This statement is important because often in the design of microchemostats one wishes to carefully manage the volumetric flow rate to ensure optimal reagent use. As derived in the next section, there is often a combination of parameter values for the dimensions of a channel which result in the same resistance (and hence the same volumetric flow rate for a given pressure gradient). Often many of these parameter values will result in the same Péclet numbers as well. For example, two channels, one with a two fold greater width and a two fold smaller length will have the same resistance and equivalent Péclet numbers. However the length required for diffusive mixing will differ as described by Eq. (2.4) and this is important to consider in the design.

2.2.2 Calculating flow rates and pressure drops

While there has been some debate as to whether the general Navier-Stokes equation is applicable to the small scale of microfluidic devices, recent work has demonstrated that this is so and suggested that previously observed deviations were due to

experimental error (Bao and Harrison, 2006). As a consequence of laminar flow in a microchemostat chip, the Navier-Stokes equations reduce to a simple analog of Ohm's law. This equation is:

$$\Delta P = QR \quad (2.6)$$

where ΔP is the pressure drop across a channel, Q is the volumetric flow rate and R is the resistance of the channel. This allows the simple calculation of flow rate in a chip as a function of external pressure and channel resistance. To calculate R the dimensions of the channel have to be considered. For cylindrical channels the resistance is given by the Hagen-Poiseuille equation, equal to:

$$R = \frac{8\mu L}{\pi r^4} \quad (2.7)$$

where μ is the fluids viscosity, L is the length of the channel and r is the radius of the channel. For the rectangular channels usually encountered in microchemostats, this equation has to be modified somewhat, taking into consideration the ratio between the width of the channel and the height, known as the aspect ratio. For channels with a low aspect ratio ($w \approx h$) the equation for channel resistance is given by (Beebe et al., 2002):

$$R = \frac{12\mu L}{wh^3} \left[1 - \frac{h}{w} \left(\frac{192}{\pi^5} \sum_{n=1,3,5}^{\infty} \frac{1}{n^5} \tanh\left(\frac{n\pi w}{2h}\right) \right) \right]^{-1} \quad (2.8)$$

while Eq. (2.8) appears complicated, in practice it is not too difficult to work with if desired. Note the $\frac{1}{n^5}$ term in the infinite sum. Since this term quickly approaches zero for increasing n , only the first 5 terms need to be considered to get a reasonable approximation. However, this equation can be further reduced when using a chip with high aspect ratio channels ($w \gg h$). Usually this is the case, as typical channel heights in a yeast or *E. coli* chip will be in the range of 5-10 μm while the width will range from 60-300 μm . In this situation the bracket term in Eq. (2.8) will tend to zero and the resistance simply becomes:

$$R = \frac{12\mu L}{wh^3} \quad (2.9)$$

Using Eqs. (2.6) and (2.9) the flow rates in a microfluidic chip can be solved for in a straightforward manner, using methods similar to nodal analysis for electrical circuits. First consider a sample microfluidic chip depicted in Figure 2.1A-B, which is shown diagrammatically in stick form to make analysis easier. For each internal node labeled a-d in the figure, the flow entering must equal the flow exiting due to the conservation of mass. This is analogous to Kirchhoff's first law for electrical circuits. Thus for all nodes in the device:

$$\sum_{k=1}^n Q_k = 0 \quad (2.10)$$

where n is the number of channels joining at the node. Furthermore, note that the system will be solved once the internal pressures at the nodes are determined, since the flow rates between nodes can be found from Eq. (2.6). We will use the system described in Figure 2.1 as an example to demonstrate how to solve such a problem. The first step would be to come up with a diagram similar to Figure 2.1A, with the external ports and internal nodes clearly labeled.

Next label the current flow directions with arrows between nodes as shown in Figure 2.1B, while making sure to obey the conservation of mass. Note that you may not know the direction of flow beforehand (in fact that may be why you are doing this exercise), however this does not matter initially. As long as the conservation of mass is followed the system can be solved properly. If your initial flow direction guess is incorrect, its solution will be negative, indicating the opposite is the true direction of flow. After this step is complete, develop a system of equations describing the flow in each node. For the example system:

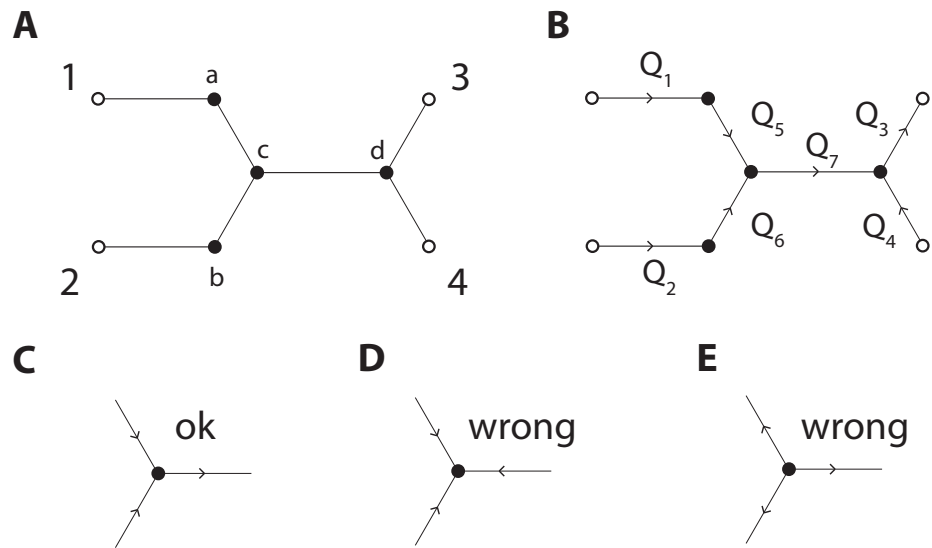


Figure 2.1: Overview of how to conceptually setup microfluidic flow problems. **A.** Stick diagram of a conceptual microfluidic device. External ports with specified pressures (open circles) are labeled 1-4. Internal junctions (whose pressures will be solved for, closed circles) are labeled a-d. **B.** Same diagram as in part A, except the port and junction numbers are removed for clarity. Volumetric flows to be solved for are given by Q_1 - Q_7 . **C-E.** Overview of the correct way to setup flow directions in a microfluidic junction, while obeying the conservation of mass. Part C has the correct setup, containing both inlets and an outlet. Part D is incorrect since there are only inlets. Part E is also incorrect since there are only outlets.

$$Q_1 = Q_5 \qquad Q_2 = Q_6 \qquad (2.11a)$$

$$Q_7 = Q_5 + Q_6 \qquad Q_3 = Q_7 + Q_4 \qquad (2.11b)$$

Next use Eq. (2.6) to substitute the pressure and resistance for the current:

$$\frac{P_1 - P_a}{R_1} = \frac{P_a - P_c}{R_5} \qquad \frac{P_2 - P_b}{R_2} = \frac{P_b - P_c}{R_6} \qquad (2.12)$$

$$\frac{P_c - P_d}{R_7} = \frac{P_a - P_c}{R_5} + \frac{P_b - P_c}{R_6} \qquad \frac{P_d - P_3}{R_3} = \frac{P_c - P_d}{R_7} + \frac{P_4 - P_d}{R_4} \qquad (2.13)$$

Since Eqs. (2.12)-(2.13) contain cumbersome fractions, it is useful to define the conductance G as the inverse of the resistance R :

$$G = \frac{1}{R} \qquad (2.14)$$

By substituting the conductance for the resistance in Eqs. (2.12)-(2.13) we get the following:

$$G_1(P_1 - P_a) = G_5(P_a - P_c) \qquad (2.15)$$

$$G_2(P_2 - P_b) = G_6(P_b - P_c) \qquad (2.16)$$

$$G_7(P_c - P_d) = G_5(P_a - P_c) + G_6(P_b - P_c) \qquad (2.17)$$

$$G_3(P_d - P_3) = G_7(P_c - P_d) + G_4(P_4 - P_d) \qquad (2.18)$$

Expanding and rearranging we get:

$$G_1P_1 = (G_1 + G_5)P_a - G_5P_c \qquad (2.19)$$

$$G_2P_2 = (G_2 + G_6)P_b - G_6P_c \qquad (2.20)$$

$$0 = G_5P_a + G_6P_b - (G_5 + G_6 + G_7)P_c + G_7P_d \qquad (2.21)$$

$$-G_3P_3 - G_4P_4 = G_7P_c - (G_3 + G_4 + G_7)P_d \qquad (2.22)$$

Or in matrix form:

$$\begin{bmatrix} G_1 P_1 \\ G_2 P_2 \\ 0 \\ -G_3 P_3 - G_4 P_4 \end{bmatrix} = \begin{bmatrix} G_1 + G_5 & 0 & -G_5 & 0 \\ 0 & G_2 + G_6 & -G_6 & 0 \\ G_5 & G_6 & -G_5 - G_6 - G_7 & G_7 \\ 0 & 0 & G_7 & -G_3 - G_4 - G_7 \end{bmatrix} \begin{bmatrix} P_a \\ P_b \\ P_c \\ P_d \end{bmatrix} \quad (2.23)$$

Equation (2.23) is a linear system which can be either solved manually or with the aid of a computer program such as Excel or Matlab. Of course the above procedure can become tedious, especially for larger microchemostat chips and a method which lends itself to automation would be preferred. To develop such a system first rearrange Eqs. (2.11) to put all currents on the LHS:

$$Q_1 - Q_5 = 0 \quad Q_2 - Q_6 = 0 \quad (2.24a)$$

$$-Q_5 - Q_6 + Q_7 = 0 \quad Q_3 - Q_4 - Q_7 = 0 \quad (2.24b)$$

Now arrange Eqs. (2.24) into matrix form:

$$\begin{bmatrix} 0 \\ 0 \\ 0 \\ 0 \end{bmatrix} = \begin{bmatrix} 1 & 0 & 0 & 0 & -1 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & -1 & 0 \\ 0 & 0 & 0 & 0 & -1 & -1 & 1 \\ 0 & 0 & 1 & -1 & 0 & 0 & -1 \end{bmatrix} \begin{bmatrix} Q_1 \\ Q_2 \\ \vdots \\ Q_7 \end{bmatrix} \quad (2.25)$$

which can be expressed as

$$0 = C\vec{q} \quad (2.26)$$

where C is an i x j matrix called the connectivity matrix for a chip with i nodes and j channels. The C matrix is unique for each chip and should be specified from a graph of the chips architecture. The $\vec{q}$ is a vector of length j representing the flows in the chip. Since $\vec{q}$ is unknown we need to use Eqs. (2.6) and (2.14) to substitute flows for pressures and conductivities:

$$\begin{bmatrix} Q_1 \\ Q_2 \\ Q_3 \\ Q_4 \\ Q_5 \\ Q_6 \\ Q_7 \end{bmatrix} = \begin{bmatrix} G_1(P_1 - P_a) \\ G_2(P_2 - P_b) \\ G_3(P_d - P_3) \\ G_4(P_4 - P_d) \\ G_5(P_a - P_c) \\ G_6(P_b - P_c) \\ G_7(P_c - P_d) \end{bmatrix} = \begin{bmatrix} G_1 P_1 \\ G_2 P_2 \\ -G_3 P_3 \\ G_4 P_4 \\ 0 \\ 0 \\ 0 \end{bmatrix} + \begin{bmatrix} -G_1 P_a \\ -G_2 P_b \\ G_3 P_d \\ -G_4 P_d \\ G_5 P_a - G_5 P_c \\ G_6 P_b - G_6 P_c \\ G_7 P_c - G_7 P_d \end{bmatrix} \quad (2.27)$$

Thus the flow vector can be split into two vectors as shown in the RHS of Eq. (2.27). The first vector contains only known values, being the external pressures and conductances of the channels connected to these ports. The second vector contains known conductances and the unknown internal node pressures which we are interested in solving for. Separating the conductances from the pressures we get:

$$\vec{q} = \begin{bmatrix} G_1 & 0 & 0 & 0 \\ 0 & G_2 & 0 & 0 \\ 0 & 0 & -G_3 & 0 \\ 0 & 0 & 0 & G_4 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \end{bmatrix} \begin{bmatrix} P_1 \\ P_2 \\ P_3 \\ P_4 \end{bmatrix} + \begin{bmatrix} -G_1 & 0 & 0 & 0 \\ 0 & -G_2 & 0 & 0 \\ 0 & 0 & 0 & G_3 \\ 0 & 0 & 0 & -G_4 \\ G_5 & 0 & -G_5 & 0 \\ 0 & G_6 & -G_6 & 0 \\ 0 & 0 & G_7 & -G_7 \end{bmatrix} \begin{bmatrix} P_a \\ P_b \\ P_c \\ P_d \end{bmatrix} \quad (2.28)$$

or:

$$\vec{q} = G\vec{s} + H\vec{p} \quad (2.29)$$

where G is a $j \times k$ matrix of j channels and k external ports containing conductance values, $\vec{s}$ is a k length vector specifying the known external port pressures, H is a $j \times l$ matrix of j channels and l internal nodes containing conductance values and $\vec{p}$ is a l length vector containing the unknown internal port pressures. Combining Eqs. (2.26) and (2.29) we get:

$$0 = C(G\vec{s} + H\vec{p}) \quad (2.30)$$

$$-CG\vec{s} = CH\vec{p} \quad (2.31)$$

$$\vec{t} = I\vec{p} \quad (2.32)$$

where $\vec{t} = -CG\vec{s}$ and $I = CH$. Note that Eq. (2.32) is the same as Eq. (2.23) and can be solved in the same ways. To solve the flow profiles for an arbitrary chip, the C, G and H matrices need to be specified, which can be done once the connectivity and channel geometries are decided upon. To automate this process our lab uses a custom matlab script, written by a former graduate student, called moca. This program has been extended to calculate how the pressure in each external port changes in time as fluid flows from the inlet ports to the outlets.

Alternatives to nodal analysis are commercial software package employing finite element techniques to solve for the flows in a more exact manner. An example of such a software package is the program Comsol, which contains an internal software package explicitly setup to solve microfluidics problems. For the design of microchemostats, this level of computation can be helpful for certain parts of the chip. For example, Comsol, unlike nodal analysis techniques, can model the diffusive transport of nutrients in complicated geometries such as cell traps or junctions. Moreover transient behavior of the chip, including how a cell chamber will respond to pressure surges, can be easily modeled in Comsol but not using nodal analysis techniques. As an additional advantage, Comsol has the ability to create models directly from Autocad files, which can save a considerable amount of time. However software programs such as Comsol are quite expensive and nodal analysis techniques are generally fine for designing basic microchemostats.

2.2.3 Designing a microchemostat chip

To design a microchemostat device one has to know a little about the overall fabrication process. The complete details will be described in the fabrication section, but we will give a brief description here. The general process is known as soft-photolithography, originally developed for the semiconductor industry. When used for microchemostats, soft-photolithography creates reusable master molds with chemicals known as photoresists. Photoresists are viscous chemicals spun on silicon wafers to very precise heights. When exposed to ultraviolet (UV) light, the photoresist crosslinks and becomes resistant to developer solvent, while the uncrosslinked photoresist remains susceptible. To make a microchemostat, a negative image of the device's features is placed between the photoresist and the UV light source. An example of such a mask is shown in Figure 2.2. When exposed, the UV light will pass through the clear sections containing the device's features, while the dark regions will prevent the background from being crosslinked. After the uncrosslinked photoresist is removed with developer, the process is repeated for the next layer. To align multiple layers, an aptly named mask aligner machine is used. This machine contains a microscopy setup so alignment patterns between the previous photoresist layer and the current mask can be viewed. Once all layers have been completed the wafer can be used to produce an almost unlimited amount of microchemostat devices.

When designing a device, the first step is to layout the architecture in a vector graphics software program such as Autocad. While it is possible to use other programs, such as Adobe Illustrator, in general Autocad is superior since it is designed for precision fabrication. Furthermore companies offering extremely high resolution mask printing generally require Autocad files. Student versions of Autocad are reasonably priced and offer more capability than is necessary for designing microchemostats. During the design stage, one needs to decide how many different channel heights will be in the device. For example the cell trap might be $3.5\ \mu\text{m}$ while the channel network is $10\ \mu\text{m}$, as is often the case for yeast chips. All features with the same height should be on

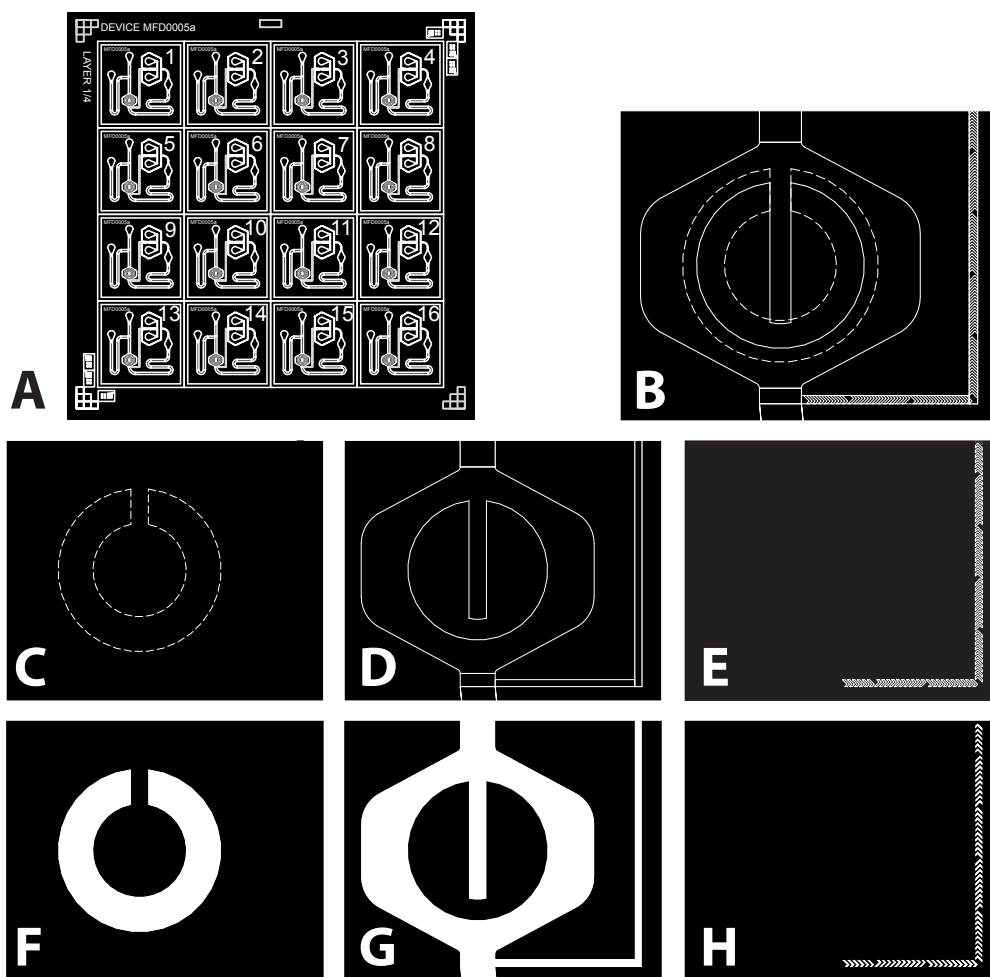


Figure 2.2: Overview of the mask design process for microchemostat devices. **A.** Overview of an Autocad file with the features of the microchemostat shown in white. Note the alignment features in the lower left and upper right corners. Each chip is individually numbered so those defective can be tracked. **B.** Close up of the cell trap region from the Autocad file shown in part A. This region contains features of three different heights, which are in different layers of the Autocad file. The cell trap will be of height $3.5\ \mu\text{m}$ and is shown with dashed lines. The central chamber will be $10\ \mu\text{m}$ and is shown with solid lines. The staggered herringbone mixers (SHM) will be of height $3\ \mu\text{m}$ above the $10\ \mu\text{m}$ mixer channel height for a total of $13\ \mu\text{m}$. Note the overlap between layers. When layers meet there should always be an overlap to compensate for small errors in mask alignment. **C-E.** Each layer from part B is shown individually, with the cell trap in part C, the cell chamber in part D and the SHM features in part E. When sent for printing the layers should be displayed individually as is shown here. **F-H.** Depiction of what the mask will look like after printing. The features of the device will be clear (white in the figure) to allow UV light to pass, while the background is black.

the same layer in the Autocad file to make work easier (see Figure 2.2).

When designing a chip with multiple layers, care must be taken to provide an accurate method for alignment during fabrication. During the alignment process one will need to look through the mask at the pattern from a previous layer and adjust the controls so the current mask will perfectly overlap. As shown in Figure 2.3 there are three degrees of freedom which need to be manipulated during the alignment process: xy translation and rotation. To make sure the wafer and mask are in perfect alignment, two locations must be viewed on the wafer to compensate for small errors in rotation. The center of the mask essentially determines the axis of rotation. The further away the two locations are from the center (and each other), the easier small errors in rotation will be to see.

In Figure 2.2A the alignment locations are in the lower left corner and upper right corner of the mask, the furthest possible from the center. The alignment features shown in Figure 2.3 are designed to have coarse and fine features to speed the alignment process and work quite well in practice. To align the patterns, one adjusts the mask aligner controls until the points of the squares meet in all locations. Note that a separate alignment pattern will be necessary for each layer other than the first, since the mask's viewing window will cross link the photoresist (and therefore remove the wafer's alignment pattern for the layer) after each alignment and exposure.

When considering a device design and alignment pattern, it is critical that the thinner layers are fabricated before the thicker ones. For example a $3.5\ \mu\text{m}$ layer should always be fabricated before a $10\ \mu\text{m}$ layer. We have found that if thicker layers are fabricated first, the later layers will spin unevenly, since the larger features from the previous layer prevent an even coating of the wafer. Furthermore, it is important not to increase the height too greatly between consecutive layers, since this limits the contrast in the mask alignment process. Recall that mask alignment occurs after spinning the current (uncrosslinked) photoresist layer, which covers all previous (crosslinked) photoresist layers. Fortunately, the wafer's alignment pattern on the previously crosslinked photoresist layer can usually be seen through the current layer. However if the height

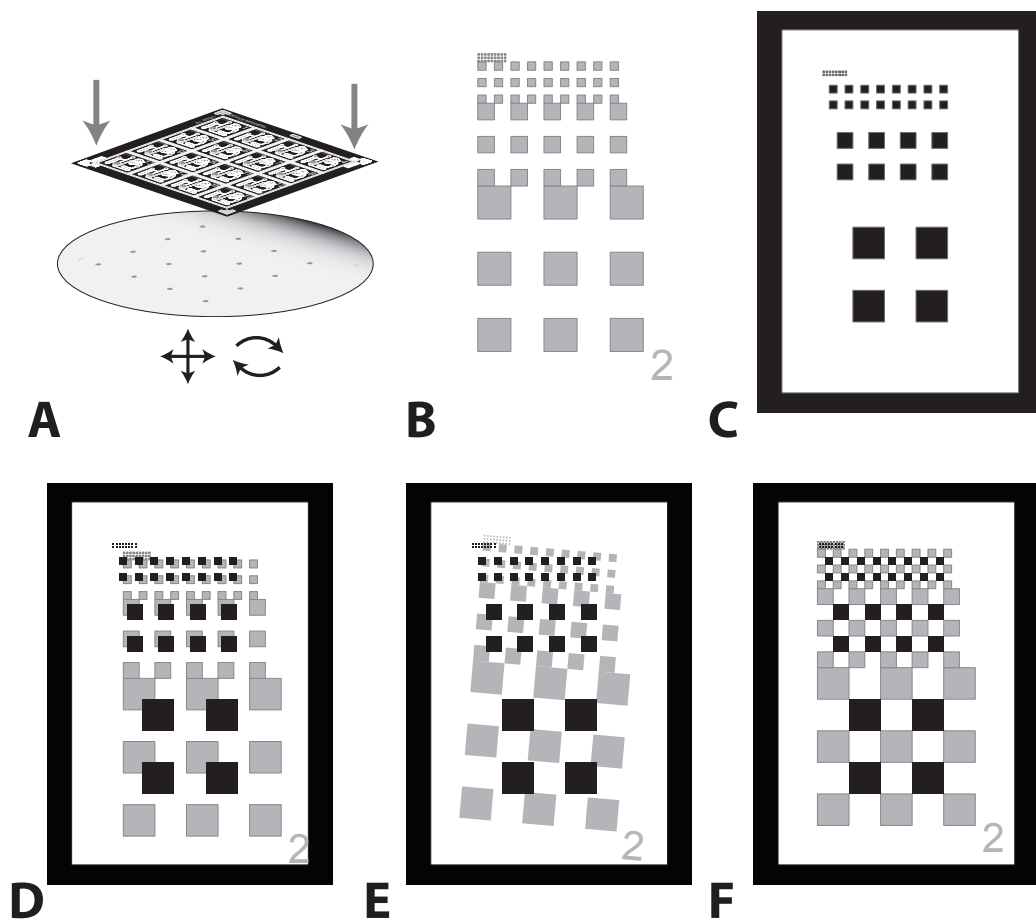


Figure 2.3: Overview of the an alignment pattern for microchemostat devices. **A.** Overview of the alignment process, with a mask shown above a wafer containing a previously deposited photoresist layer with alignment patterns. The mask aligner will have controls to compensate for both translation and rotation (bottom arrows). The arrows pointing down on the mask show the alignment pattern location. **B.** Alignment pattern present on the wafer from the previous photoresist deposition. Each layer will require a separate alignment pattern; the layer number is shown in the lower right. The pattern is composed of sets of squares whose sides are reduced by half in each iteration. **C.** Alignment pattern present on the mask. The clear window surrounding the squares allows the fabricator to view the pattern from the previous layer. The objective is to make the points of the squares from the mask and the previous layer touch. **D** Mask and wafer out of alignment by xy translation only. **E** Mask and wafer out of alignment by rotation only. **F.** Mask and wafer in perfect alignment.

ratio between the two layers is greater than about 5:1, the contrast becomes so poor that it is difficult to see the wafer's alignment pattern. In general we try and limit the height ratio to 3:1, since mask alignment is generally the most difficult and frustrating part of fabrication.

Once the alignment strategy is settled upon, the device features can be laid out in Autocad. For this purpose simple rectangles are usually sufficient, but arc segments can be used if more complex shapes are desired. We have found that curved sections are superior for cell containing channels, since they prevent clogging. For areas of the chip not expected to contain cells, rectangular segments meeting at sharp corners are fine. When designing channels, all features should be closed objects in Autocad, there should be no open segments. While resulting in lines across channels, these will not be printed since lines are considered to be of infinitesimal thickness by the printer and only closed regions are recognized (compare Figure 2.2D and G). Ensuring that all regions are closed in Autocad will not only make printing easier, but also facilitates importing into Comsol and Illustrator.

In general when laying out features, one must consider the tradeoff between compacting the device into as small a space as possible and maintaining usability. For example, placing two ports closer than 2 mm is not advisable since it makes it extremely difficult to plug in the port lines upon setup. Furthermore having a channel pass closer than a 1 mm to a port should also be avoided since it can be damaged if the port hole is punched incorrectly. Along these same lines, there should be at least 1 mm between a feature and the edge of the chip, so when the PDMS slab is diced into individual units no features are damaged.

In addition, when two layers are contiguous there should be some overlap between them to compensate for the small errors in alignment that inevitably occur. For example, in Figure 2.2B the cell trap layer overlaps the cell chamber layer. If the layers were designed with no overlap a small alignment error could create a gap between them resulting in a non-functional chip. Even with the alignment patterns described in Figure 2.3 and a meticulous alignment procedure, small errors will occur and can be compen-

sated for with layer overlap. When layers overlap the total height is usually a smooth transition between the height of the thicker layer alone and the sum of the heights of the overlapping layers. As shown in Figure 2.2B the cell chamber wall starts out at $10\text{ }\mu\text{m}$ and gradually increases to $\sim 14\text{ }\mu\text{m}$ in the overlapping area. This phenomenon should be remembered when modeling the flow profile of a device in Comsol for example, since a $\sim 40\%$ change in height due to overlap will have a large effect on the channel's resistance.

Another common mistake results from layers unintentionally intersecting due to small alignment errors. This can create fluidic “short circuits” and non-functional chips. The solution here is to again make sure an adequate margin is present between non-intersecting layers to compensate for fabrication problems. Most importantly, keep in mind the concept of tolerances. While the feeling for this comes from experience, always assume that some fabrication error is inevitable rather than trying to come up with the most beautiful design in Autocad. The best design will be one that can tolerate some fabrication error and still work properly, even if it is not the most “compact” design. The size of the channels is also affected by these same concepts. We have found that channel widths smaller than $60\text{ }\mu\text{m}$ should be avoided since they are prone to clogging with debris that can enter the chip (often residual PDMS). Moreover long channels should generally be $10\text{ }\mu\text{m}$ or more in height, also to prevent clogging.

Of course the ultimate limitation for microfluidic design is the resolution of the printer making your masks. This limit usually comes into play before that imposed by the UV light source or the photoresist. We use a company named CAD/Art for mask printing which has a 20,000 dpi printer. While this is normally adequate for microchemostats, higher resolution options used in the semiconductor industry are available at far greater expense. Using this process, we have been able to make features separated by as little as $13\text{ }\mu\text{m}$ *as long as they are on the same layer*. However even this is dependent on the type of photoresist used. For example the spatial resolution of a thinner photoresist, like that used to make a $10\text{ }\mu\text{m}$ layer, is generally greater than that of a thicker resist, used for making a $35\text{ }\mu\text{m}$ layer. General guidelines for recommended

channel dimensions are given in Table 2.3. Note it is certainly fine to make channels having dimensions other than those given in the table and for specialized features (like high resistance cell feeding channels) this may be necessary. For the normal fluidic “backbone” of the chip, the channel dimensions listed in Table 2.3 should be fine.

Table 2.3: General guidelines for channel dimensions in microchemostat chips, including traps and fluid channels

Channel type	organism	width range	height range
General flow network (no cells)	any	60-100 μm	10-15 μm
High flow channel (no cells)	any	300-400 μm	20-45 μm
General cell channels	<i>E. coli</i>	150-300 μm	6-15 μm
	yeast	200-300 μm	10-15 μm
	mammalian	200-300 μm	25-35 μm
Cell trap	<i>E. coli</i>	varies	1 μm
	yeast	varies	3.55 μm
	mammalian	varies	25 μm

While the general guidelines listed so far should be useful for creating a microchemostat device, as a case study we will describe our design process for an updated dial-a-wave chip. The device, called MFD005_a, was designed as an improved version of the chip described in (Bennett et al., 2008). The chip is designed to grow cells reliably in a monolayer and cope with high growth by flushing excess cells into a waste port. The chip is also designed to generate arbitrary, time varying inducer concentrations, so the cell’s response to a dynamic environment can be recorded. Often we use the chip to generate arbitrary waveforms, such as sine waves, square waves, or waves having a random period component. The waves generated by the device have high temporal accuracy and the chip is easy to use. An overview of the device is shown in Figure 2.4. The chip has five external ports, which is a reduction from eight in the Bennett chip. Reducing ports saves on consumables and eases setup, so finding the minimum number necessary to produce a working chip should always be a design goal. The chip is designed to use hydrostatic pressure and therefore no pumps are required of any kind for operation. We have found that hydrostatic pressure gives the most reliable, steady and

cost effective means of controlling the pressures in a microchemostat device. In a later section we will describe our use of linear actuators to alter the inlet hydrostatic pressure of our device and why this is advantageous compared to other means such as syringe pumps.

The role of each port of the MFD005_a chip is given in Figure 2.4. When an experiment is running, fluid will enter from ports 1 and 2 which meet at the dial-a-wave junction (Figure 2.4B). The DAW junction has two inlets and three outlets. As described in a later section the ratio of the inputs from port 1 and 2 leaving the junction to the cell chamber is determined by each ports pressure. Excess fluid is diverted through a shunt network to port 3, which is a waste port. Fluid leaving the central fork of the junction for the cell chamber travels through a long channel where it is mixed into a uniform concentration by staggered herringbone mixers (SHM). The ingenious SHM mixers (as shown in Figure 2.4C) are designed to induce a corkscrew effect in the fluid stream and increases the surface area available for mixing (Williams et al., 2008; Stroock et al., 2002). Since mixing only occurs due to diffusion in a microchemostat, as mentioned in section 2.2.1, this increase in surface area will logarithmically reduce the length of a channel necessary for uniform mixing.

Table 2.4: Role and pressures for each port in the MFD005_a device. All pressures are given in inH₂O above the height of the microscope stage

Port	Description	Contents	Run inH ₂ O	Load inH ₂ O
1	Inlet 1 for DAW	Media + inducer + tracking dye	25	25
2	Inlet 2 for DAW		25	25
3	Cell and shunt waste	dH ₂ O	5.5	5.5
4	Alternate outlet	dH ₂ O	6	17
5	Cell port	Media + cells	6	18

Even with the help of SHM features, mixing still requires a length which depends on the log of the Péclet number (Stroock et al., 2002). Thus a central question when designing our device was how long to make the mixing channel from the DAW junction to the cell port. If the channel were too short, the two inputs would not be completely mixed, resulting in a non-uniform and uneven concentration profile over the cell culture.

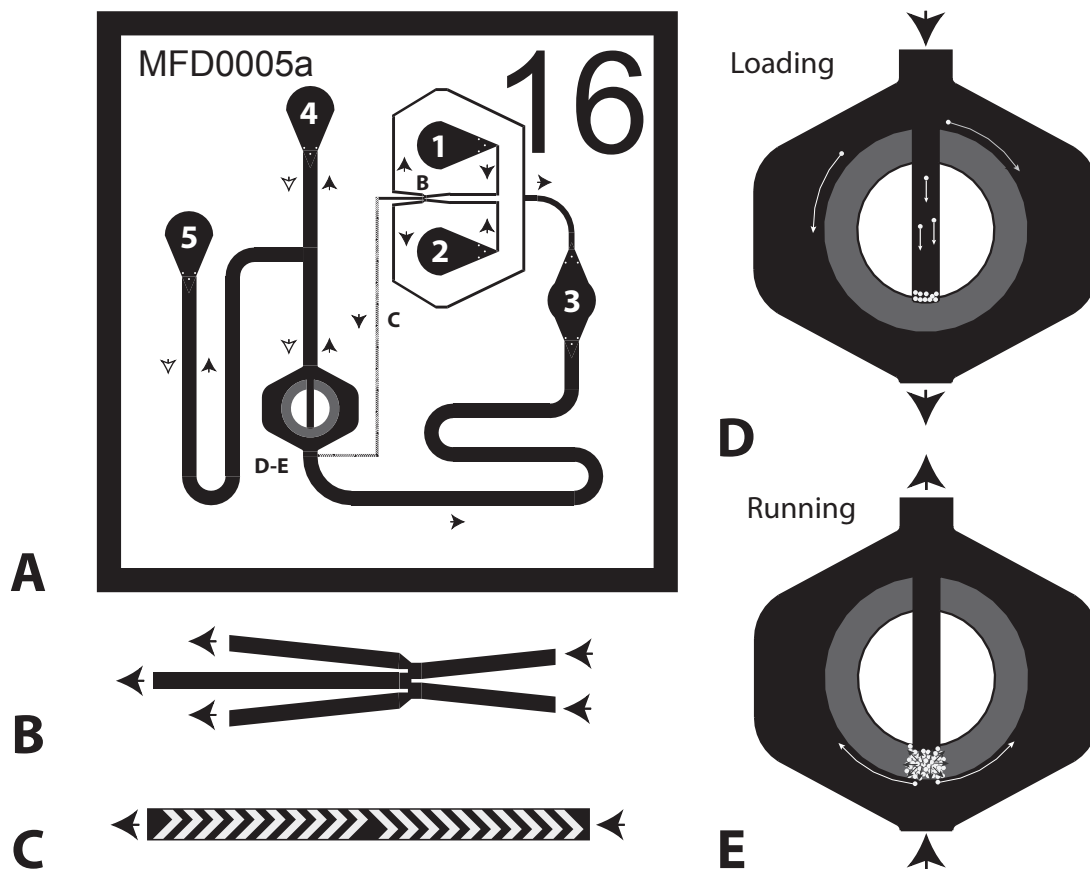


Figure 2.4: Overview of the MFD005_a chip and components. **A.** Overview of the MFD005_a chip's architecture. Flow directions in each segment during running conditions are given by black arrows, during loading conditions by white arrows. Note that only flow from ports 4, 5 and across the cell chamber changes direction during loading. Letters represent locations of the features described in other parts of the figure. External ports are numbered 1-5. Each port is described in Table IV. **B.** Depiction of the DAW junction. Flow direction is indicated by the black arrows. The two inlets on the right come from ports 1 and 2. The flow from the inlets converges in a ratio dependent on the inlet pressures of each. The middle fork of the junction leads to the cell chamber while the two outer forks lead to port 3, the cell and shunt waste port. **C.** Depiction of the staggered herringbone mixers (SHM) which reduce the channel length required for mixing. These mixers immediately follow the DAW junction and continue until just before the cell chamber. **D.** Overview of trap region of the MFD005_a chip under loading conditions. This trap is known as the yeast doughnut trap. Black region represents the cell chamber with a height of 10 μm . Gray region is the actual cell trap, with a height of 3.525 μm . White circles represent cells entering from the cell port and either passing around the trap to the cell and shunt waste (port 3), or entering the central channel and moving to the trap entry barrier. The yeast cells are slightly too large to move into the trap directly without "flicking" the cell line to assist in their entry. **E.** Cell trap upon running of an experiment. Cells begin to grow in the trap and the colony expands (black arrows). Eventually the colony fills up the gray region near where they were loaded. The growth of the cells will force some out of the trap into the outer channel where they will be efficiently carried away to the waste port (white arrows). Over the course of the experiment the cell colony will expand to fill the entire trap.

However making the channel too long is also disadvantageous since it increases the delay time for a signal to propagate the length of the channel. To find the optimum channel length we require knowledge of the flow velocities as a function of the external port pressures. This is a good example of the usefulness of the modeling techniques mentioned in section 2.2.2. Using nodal analysis or Comsol it is easy to determine the flow rates and hence the Péclet number for various substances and flow regimes. We performed just such an analysis when designing the MFD005_a device to determine the necessary channel length for efficient mixing, shown in Figure 2.5.

After mixing, fluid from ports 1 and 2 enters the cell chamber and proceeds to the outlet ports 4 and 5. Fluid also enters a diversion channel and exits at port 3. By controlling the height of port 3 relative to ports 4 and 5, one can set the ratio of fluid passing through the chamber versus exiting through the diversion channel. Modulation of this diversion ratio is important for controlling the flow velocity across the cell chamber. For example, say you wanted to minimize the flow velocity in the cell chamber why still retaining functionality of the DAW junction. Without a diversion channel you could lower the height of the input ports 1 and 2 relative to 4 and 5 and reduce the flow velocity in the cell chamber. However this would also reduce the flow velocity in the mixing channel between the DAW junction and the cell chamber. This reduction in mixing channel velocity would increase the delay time for fluid transit and negatively impact the chips function. With a diversion channel, an alternative is to maintain the height difference between ports 1-2 and 4-5 and instead lower port 3. This would increase the ratio of fluid entering the diversion channel and hence lower the fluid velocity in the cell chamber. This is another example of flow modeling's usefulness, since the diversion channel's length is critical for determining the amount of fluid diverted for a given height change.

Modeling also allowed us to solve a problem with flow reversal (backflow) in the diversion channel, which would sometimes occur over the course of an experiment in a previous version of this device. The plot in Figure 2.5C represents a time dependent solution for the flow profile in the device's diversion channel, compensating for pressure

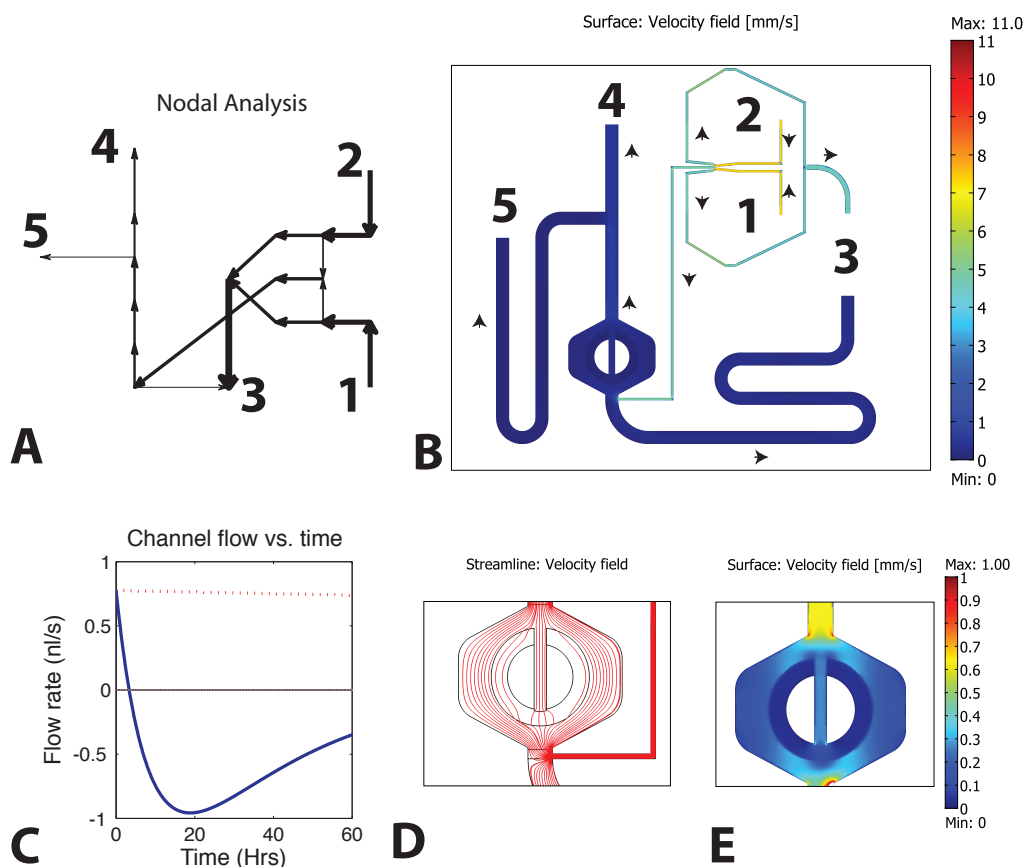


Figure 2.5: Comparison of a nodal analysis tool written in Matlab (moca) and Comsol (finite element analysis package). **A.** Graph from the moca matlab script depicting flows for the device pictured in Figure 4A. The arrow thickness and direction represent the volumetric flow in a channel section of the device. Numbers are the external ports of the device. **B.** Flow velocity through the same chip modeled using Comsol. The magnitude of the velocity is given by the channel's color, while the direction is indicated by the black arrows. Numbers are again the external ports. The MFD005_a geometry was loaded directly from Autocad, simplifying setup. **C.** Flow profile of a channel section over the course of an experiment. In previous designs we have had problems with backflow problems over the course of an experiment, as the fluid level in the external ports is altered by flow. Modeling an experiment's flow profile using nodal analysis helped to solve these problems, resulting in a redesign of the diversion channel's dimensions and using larger syringes. The blue line represents fluid flow using 1 ml syringes and the red dashed line 60 ml syringes. **D.** Streamline plot showing the path fluid particles take upon moving through the cell trap. While this plot was generated under running conditions, the streamlines are very similar for loading conditions (the direction of flow of course is opposite). Note that only about one fourth of the flow enters the central channel, most flow is directed around the trap. Hence, when loading, most cells will not enter the trapping region. **E.** Plot of the velocity field inside the trap region. Note that the velocity is lowest inside the trap itself and considerably higher in the outer channel region. This allows nutrients to be continually replenished from the outer channel into the cell trap and helps remove cells once they outgrow the trap.

changes due to fluid movement over the course of an experiment. The solid blue line represents the flow rate when small diameter syringes are used for the outlets. The fluid level in these syringes increases in height rapidly for a given volumetric flow. Under certain conditions this height increase can be large enough to change the flow velocity in the chip. When the blue line crosses the zero point of the y axis, flow reversal has occurred. The red dashed line represents the same initial setup using larger diameter 60 ml syringes. These syringes undergo far less increase in height for a given volumetric flow than the smaller syringes and therefore it takes far longer (much longer than an experiment would last) to reach a flow reversal condition. The solution was reached by redesigning the diversion channel to have a greater resistance and by using larger syringes. While this model was created using nodal analysis, it could also be done in Comsol.

2.2.4 Design of an improved DAW junction

Another opportunity for flow modeling came from designing the DAW junction. As mentioned previously, this junction is designed to combine the inputs from ports 1 and 2 of the MFD005_a device in a precise ratio depending on the input pressures. By controlling the input pressures as a function of time, one can generate precise waves of inducer concentration and hence expose cells to a fluctuating environment. To set the mixing ratio, the pressure of one input is increased and the other decreased by the *same* amount. By changing the input pressures in an opposing manner, the flow rate out of the junction remains constant and hence the downstream flow rates are not altered (this can be easily demonstrated using nodal analysis). Of course, by the conservation of mass, if the total outlet flow does not change, then the total inlet flow must not change either. Instead the ratio between the two inlet flows changes.

Initially one might think that a simple T-junction would suffice to reliably mix the two inputs streams. Indeed, when the output is derived nearly equally from both inputs (near a 50% mixing ratio), a T-junction works fine. However, as depicted in

Figure 2.6, a T-junction does not work well for skewed output ratios, when most of the output is coming from only one of the inputs. As an example of a skewed ratio, consider when 95% of the output is coming from input 1 and the other 5% is coming from input 2, with a total flow rate of 1 nanoliter / sec (nl/s). Under these conditions the input 1 flow rate will be 0.95 nl/s, while the input 2 flow rate will be only 0.05 nl/s. Going further, for a mixing ratio of 100%, the input 1 flow rate will be 1 nl/s and the input 2 flow rate will be 0 nl/s. Of course in practice, even with the most accurate system, an entirely stagnant flow is impossible to achieve. In reality this situation represents an unstable equilibrium, prone to backflow. If attempting this with a real device, either a true 100% ratio will not be achieved, or (more likely) fluid from input 1 will begin to flow into input 2. This backflow situation will result in improper mixing of the input 2 source, preventing the system from functioning properly if later switched. For example, consider if backflow had occurred for one hour and then the system was switched, from a mixing ratio of 100% to 0%. In this situation, the residual flow from input 1 would have to flow back again before fresh input 2 media could again enter the junction. Depending on the residual flow rate from input 1 to 2, this could take a considerable amount of time.

To overcome this difficulty the chip in (Bennett et al., 2008) contained a shunt network designed to direct some fluid from each input to a waste port at all mixing ratios, in addition to the junction outlet. This system prevents backflow because the inlet flow rates never approach zero, even for skewed outlet ratios. A comparison of a T-junction to the DAW junction used in the MFD005_a device is shown in Figure 2.6. While the shunt network solved the backflow problem, the response of the junction to input pressures was somewhat different than expected. Ideally the output response of the DAW junction should be linear, but we had found significant deviations from linearity with the Bennett device. These deviations made experimental setups sometimes difficult. To investigate the cause of these deviations we turned to modeling in Comsol.

We determined that diffusive transport between the input streams could cause significant deviations from an ideal response. Diffusion *at the junction* leads to transport of nutrients destined for the output into the shunts, altering the expected response. This

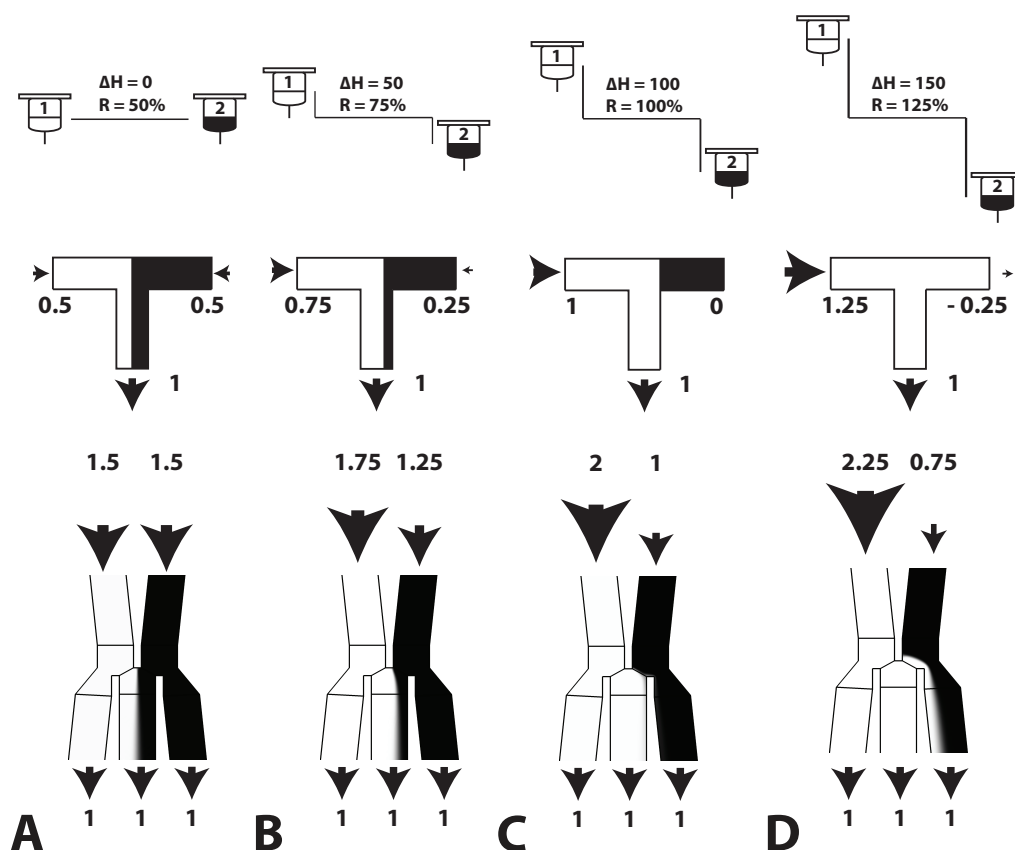


Figure 2.6: Comparison of a T-junction to our improved DAW junction for combining different source fluids in precise ratios. The figure depicts four mixing ratios from 50% to 125% and compares the performance of each junction. Note that since the system is symmetrical, flows for mixing ratios from -25% to 50% will be the reverse of those shown here. **A.** Mixing ratio of 50% ($R=50\%$), corresponding to equal flows from both reservoirs. A fluorescent dye has been added to reservoir 1, displayed in white as it would be seen under the microscope. Top portion of the figure depicts the reservoirs at equal height ($\Delta H=0$). Middle portion of the figure depicts a T-junction, each input flow is 0.5 nl/s, for a total flow of 1 nl/s. Bottom portion represents the DAW junction. Each inlet has a flow of 1.5 nl/s, for a total inlet flow of 3 nl/s. Note the smooth interface between fluid streams, as diffusion has not yet been able to cause appreciable mixing. **B.** Mixing ratio of 75%. The height of the port 1 reservoir has increased while the corresponding port 2 reservoir has decreased by an equivalent amount. Both junctions continue to perform well. Note that the flow rate in inlet 1 has increased in the exact amount it has decreased in inlet 2. **C.** Mixing ratio of 100%. The T-junction fails here as the flow rate in input 2 has dropped to zero. In practice zero flow is unattainable and will likely result in a backflow situation. Note the DAW junction continues to perform well, since all flow from input 2 is directed into a shunt. **D.** Mixing ratio of 125%. At this point backflow has occurred in the T-junction, as flow from port 1 begins to enter the input 2 source. In the DAW junction the excess flow from input 1 is directed into a shunt and flow continues from input 2. Note that the output of the junction directed to the cell chamber will be the same in both C and D (center channel). This is why the output in the cell chamber seems to plateau after increasing ΔH beyond the 100% level.

deviation was especially pronounced at skewed mixing ratios similar to what we had observed. To correct these problems we designed a new DAW junction (depicted in Figure 2.4B) to minimize the contact distance between the two fluid streams and increase the flow velocity. These changes essentially increased the flow's Péclet number in the junction to limit diffusive mixing. Moreover we altered the shunt network compared with the Bennett design so the shunt entrances would be nearly parallel to the outlet. The idea was to minimize any changes in flow direction occurring at the junction. The performance of this new junction is shown in Figure 2.7.

2.2.5 Calibration of the DAW junction

The junction is designed to be used in conjunction with linear actuators to physically move the input reservoirs up and down thereby altering their hydrostatic pressures. To map the height of the input 1 and 2 reservoirs to a mixing ratio of the DAW junction, we have come up with a simple calibration scheme. First we find two sets of reservoir heights corresponding to mixing ratios beyond 0% and 100%. Each set represents flow from one of the inputs being completely diverted into a shunt. Since the heights do not have to be exact at this step, it is relatively quick and easy to setup (unlike trying to find the exact 0% and 100% heights). Next we program the linear actuator controllers to generate a triangle input wave and begin to move the reservoirs. Generally we use an input wave with a 5 minute period. We then monitor the fluorescence near the cell chamber to record the output signal. The two signals are overlaid and any delay is removed, as shown in Figure 2.7A. In this figure the input is shown in red and the output in green. We expect the output to closely track the input signal until a plateau is reached, indicating complete diversion of a inlet into a shunt. As can be seen in the figure, this is essentially what we see, with a slight rounding at skewed mixing ratios.

Once this mapping is complete we compress the data into a single curve, as shown in Figure 2.7B. This figure depicts one of the external port pressures mapped to a mixing ratio of the DAW junction. An ideal response would be a plateau at 0% leading

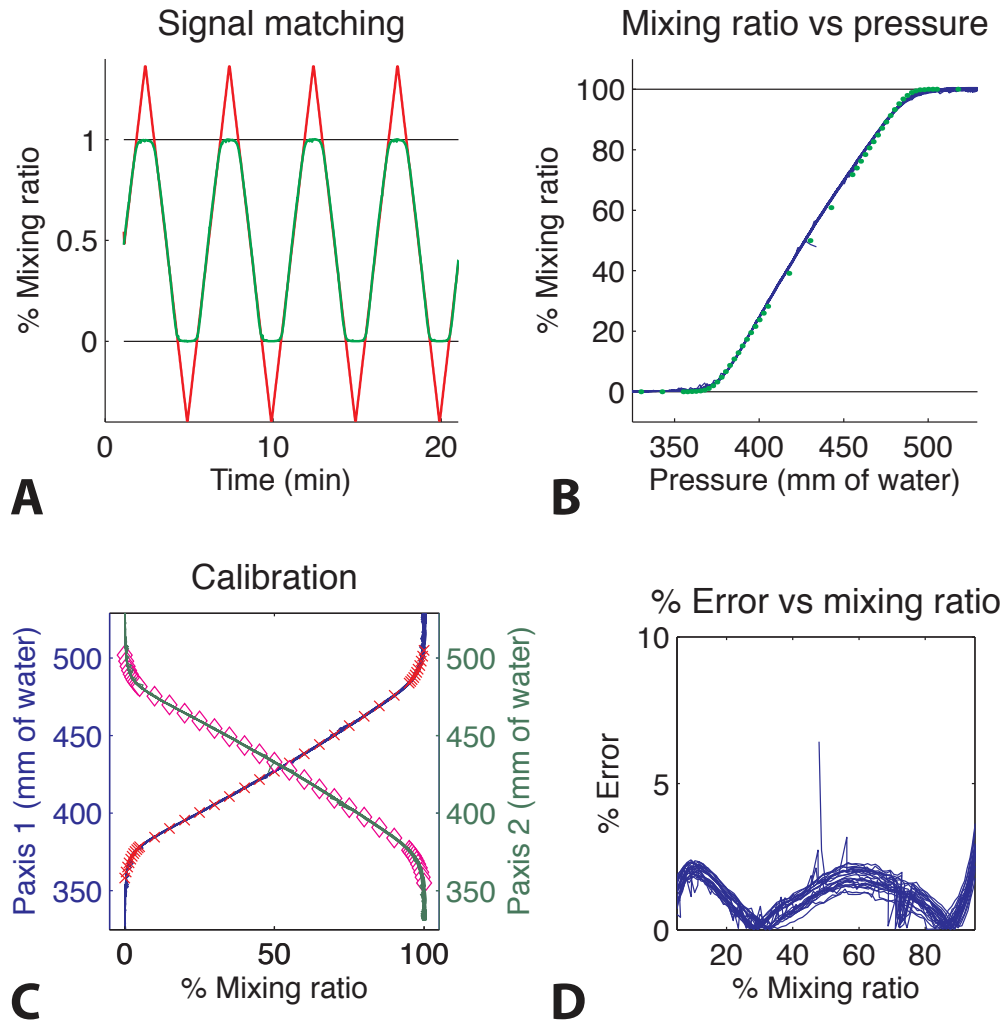


Figure 2.7: Performance of the DAW junction. **A.** Calibration signal (red line) overlaid with output signal (green line) after correction for the delay in acquisition. During calibration the system is designed to intentionally overshoot the bounds of the DAW junction. Since the starting and ending points for calibration are not critical, this makes it easier to setup as described in the text. The ideal response would be a closely tracking output signal transitioning to plateaus after the system moves beyond 0% and 100% mixing ratios. As can be seen in the figure, this is what we observe, except for a slight rounding near the plateau region. **B.** Compression of the data in part A into a single curve by mapping the input pressure directly to the output mixing ratio. Blue curve is the compressed data, while the green dots are the expected results from Comsol modeling. As can be seen in the figure, the modeling and experimental results are in excellent agreement. **C.** Completed calibration for both inputs. Red crosses and pink diamonds represent polynomial fits of inputs 1 and 2 respectively to the output mixing ratio. These fits can be used to program a linear actuator controller to generate precise inducer waves. **D.** Measure of the percent error of the *uncalibrated* output signal, which general is less than 3%.

to a linear ramp until another plateau is reached at 100%. The output of our junction (blue curve) closely approximates this, again with slight rounding near 0% and 100% mixing ratios. As an additional example of Comsol's utility, the green dots represent modeling results generated of the junction's response. As can be seen in the figure, the modeling and experimental results are in excellent agreement. In Figure 2.7C the calibration results for each input are shown. A high order polynomial fit is used for each input, which can then be programmed into the linear actuator controller. Figure 2.7D represents the percent error of the output signal as a function of mixing ratio for the *uncalibrated* system. Even without calibration the system is highly accurate, usually having an error of less than 3%.

2.2.6 Design of an improved yeast cell trap

Beyond the flow network or DAW junction, the most important part of a microchemostat chip is the cell trap. Often a successful design will hinge on a properly functioning trap. A microchemostat's cell trap should ideally be easy to load, force the cells to grow in a monolayer so they are all in the same focal plane, allow nutrients to enter the trap even when packed with cells, force cells to grow in well defined directions to assist with cell tracking and allow cells to exit the trap without clogging the device. For some cell types, specifically mammalian cells, controlling the flow rate in the trap is also extremely important. We have found that even hearty mammalian cell lines, such as 3T3 cells, can be killed by extremely low flow rates (less than 1-5 $\mu\text{m/s}$). This requires the design of highly specialized traps to prevent any flow from reaching the cells after loading. We have never encountered an issue where yeast or *E. coli* cells seem adversely affected by flow, however the flow rate can be important for intercellular communication by diffusible substances (Danino et al., 2010).

Often the goals mentioned above are difficult to achieve completely, for example a trap with high cell retention is often very difficult to load. This is the case with the T μ C chip described in (Cookson et al., 2005). To overcome these problems an improved

yeast cell trap, known as the doughnut trap, was designed. Figure 2.4D and E contain an overview of this trap. The salient feature is improved loading while retaining the ability to image cells in a monolayer. Another major issue in the trapping region is clogging of cells from excess growth. Yeast cells grown in glucose can clog a device in several hours if the microchemostat is not properly designed. As shown in Figure 2.4 the outer channel is designed with a height of $10\text{ }\mu\text{m}$. This height is large enough that no cells will be able to clog it under normal circumstances. The height of the trap is kept at $3.525\text{ }\mu\text{m}$ for yeast cells of the W303 background. Note that the height of the trap is the most critical parameter of the entire chip as will be stressed in the fabrication section. Even height differences as little as $0.1\text{ }\mu\text{m}$ can make a difference in terms of the effectiveness of the trap. If the trap is too high, yeast cells will flow right through and not be trapped at all. Even those that are trapped may not grow in a monolayer and hence a uniform focal plane will be impossible to achieve. However, if the trap is too low, then it will be impossible to get the cells into the trap. Thus, the height of the trap depends intimately on the cell type and even the cell strain. We have noticed that some larger backgrounds of yeast actually require a slightly higher trap than other common laboratory yeast strains.

Upon loading, when cells flow into the chamber containing the trap, most will actually flow around the trap to the cell and shunt waste (port 3), since this region's flow mostly goes around the trap. This is actually beneficial to the design since it allows growing cells to be quickly whisked away when they overgrow the trap, while minimizing any movements of the cells in the trap due to flow (which can make cell tracking difficult). Furthermore this difference in flow rates is primarily a consequence of the difference in the heights between the two regions. Recall Eq. (2.9) which states that resistance of a channel scales with the cube of the height. Thus while the height difference between the trap and the outer channel is only ~ 3 fold, the resistance difference will be ~ 27 fold.

Those cells entering the central channel will move to the base and become stuck at the entrance barrier. Since the trap height is slightly smaller than the diameter of a

yeast cell, the cells cannot enter the trap without some assistance from the experimenter. Once enough cells have accumulated behind the entrance barrier, the experimenter will flick the microfluidic line attached to the cell port with his index finger. This perturbation will cause a momentary pressure disturbance which will force some cells under the barrier into the trap. Once in the trap they will be efficiently held between the roof of the trap and the glass cover slip.

During the course of the experiment, cells will divide and enter exponential growth. They will quickly fill up the trap and the colony will come into contact with its walls. The pressure exerted on the trap's walls by the growing colony will generate a flow of cells, which can be modeled as a particulate flow (Mather et al., 2010a). This flow will expel some cells from the trap into the outer channel, to be carried away into ports 4 and 5 (note port 5, originally the cell port now functions as a waste port). The design of the cell trap should take cell flow into account so it can be directed in appropriate ways. For example, to track cells often it is useful to direct their movement in a regular direction to limit the difficulty of tracking. With the doughnut trap, cell flow is directed in radial directions which works fairly well. However we have been considering designing a new trap with internal baffles to limit lateral movements of the cells.

The MFD005_a device has been used successfully to generate many types of input concentration waves for numerous yeast strains and genotypes. In general the chip takes 1-2 hours to setup and can run for several days depending on the conditions. The chip is highly useful for all types of small scale experiments involving dynamic environments. However, upon building this chip we realized that most of the time during an experiment our microscope sat idle between imaging frames. To make better use of our time and resources we decided to build a parallel version of the MFD005_a device which we have named the MDAW device.

2.3 A parallel DAW device

The parallel version of our MFD005_a device was designed to have eight copies of the smaller device on a single larger device. This parallel architecture greatly increases the throughput of a run by allowing eight independent subexperiments to be conducted at a time. The utility of this design can be seen by comparing the number of ports required to carry out equivalent experiments for the progression of chip designs. With the Bennett chip, 64 ports are required to conduct eight experiments, for the MFD005_a device, 40 ports are required, while the MDAW device requires only 26 ports. Since setup time is directly proportional to the number of ports a chip contains, this reduction represents a significant savings of both time and consumables. Of course designing such a device presents its own challenges, a major one being space. Since we wanted all features to fit entirely on a single 24x40 mm coverslip, space was at even more of a premium than with the MFD005_a device.

To conserve space we compressed the features of the MFD005_a device as much as possible while retaining functionality and maintaining a margin for fabrication errors. We made the device radially symmetric in order to provide equal resistance paths to the ports shared among the subexperiments. To divide the space we separated the chip into eight circular sectors of equal area, similar to slices of a pizza. While a rectangularly shaped device would have been a better fit for the coverslip, it would have been more difficult to ensure the resistances were equal to the outlets for all subexperiments. Moreover excessive stage movement between locations during acquisition can generate bubbles in the microscopy oil. These bubbles sometimes show up after several hours into an experiment and can cause a severe loss of focus or degradation of image quality. To prevent these problems the cell chambers were placed as close to each other as possible, which essentially requires radial symmetry. As an added bonus, this lowers the amount of time for stage movement between positions.

An overview of a MDAW subexperiment is shown in Figure 2.8A. Compare this to the MFD005_a device in Figure 2.4A. Both contain a DAW junction, SHM features

and a cell trap that are essentially identical, although the length of the channel between the DAW junction and the cell chamber has been reduced slightly in order to conserve space. In fact ports 1, 2, and 5 and the channels linking them are essentially equivalent to ports A, B and C respectively in the MDAW device. The major difference is that ports 3 and 4 on MFD005_a have been consolidated in the MDAW device. In the MDAW device we call the port 3 analog the consolidated shunt port and the port 4 analog, the consolidated alternative outlet. The consolidated shunt port is connected to each subexperiment by an extensive collection network. This collection network can be seen in Figure 2.9. To create this collection network Comsol modeling was essential to ensure that the flows would be equal to their equivalents in the MFD005_a device. This modeling indicated that the height of the collection network would have to be increased to 35 μm to sufficiently lower the resistance (shown in dark blue in Figure 2.9). Moreover the shunt channels from the DAW junction now connect to the diversion channel before it reaches the consolidated shunt port, whereas in MFD005_a they both reach port 3 independently. Comsol modeling indicated that back flow from the shunt into the diversion channel could be a problem if the diversion channel was not long enough. The connection point was extended to ensure this would not happen.

It was easier to consolidate port 4 into the alternate outlet port on the MDAW device since it was in the center of the chip and each subexperiment had an independent path to the port. Thus the height of these channels could remain 10 μm . However the channel length between cell chamber and the alternate outlet port had to be reduced, which altered the resistance somewhat. Comsol modeling allowed us to determine the port pressures which led to equivalent flow. One might wonder how many ports could be shared among a device of this size. Of course if a multilayer microfluidic device were used then there would be no restriction, however we believe the time required to manufacture multilayer devices does not justify their added benefits and therefore we avoid their use if possible. For a single layer device, at most two ports can be shared among *all* subexperiments due to geometric constraints. It is possible to share additional ports between adjacent subexperiments, however, with the MDAW device this would have

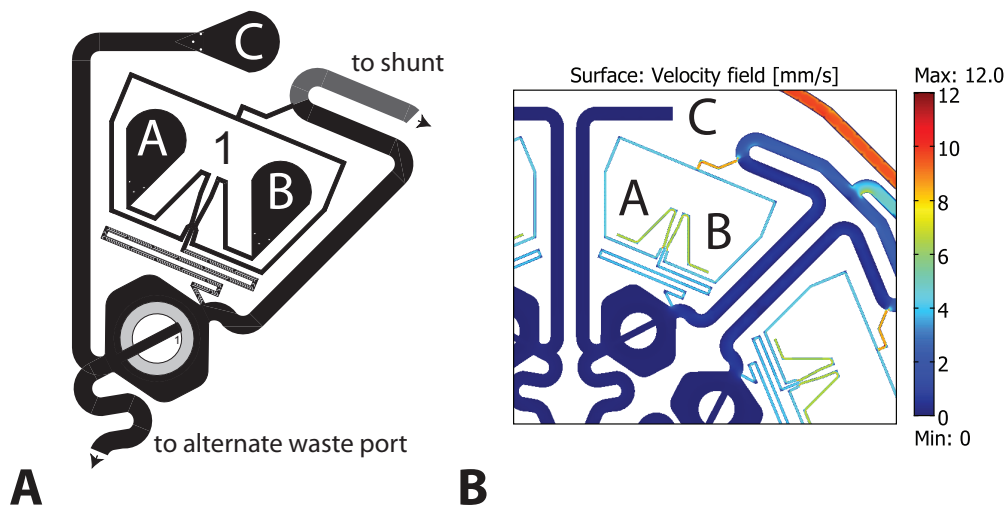


Figure 2.8: Graphic of the individual subexperiments in the MDAW microfluidic device. **A.** This is a subexperiment from the MDAW device. It is essentially a compressed version of the MFD005_a device shown in Figure 4A. The ports labeled A, B and C are equivalent to ports 1, 2 and 5 respectively in Figure 4A. The equivalents to port port 3, the cell and shunt waste and port 4 the alternate outlet port, in the MFD005_a device are shared among all eight subexperiments in this device. The arrows point to these shared ports. This port sharing reduces the number of outlets and eases the setup of such a large device. To make identification easier under the microscope, we have placed the subexperiment number above the DAW junction and near the cell trap. **B.** Closeup of a Comsol model of the MDAW device. Comsol modeling was crucial for designing the combined collection network so each subexperiment's shunt would function similar to the MFD005_a device. Since the collection network combines the output of eight subexperiments, the resistance had to be lowered so it would carry the combined flow as efficiently as that in the MFD005_a device.

meant sharing the cell ports (port C) and we wished them to remain independent. It is also possible to add y-junctions or manifolds to connect multiple outlet ports to a single reservoir. However if this is done, extra care must be taken to ensure no bubbles are introduced in the lines. This is especially a problem with small diameter y-junctions.

Even at eight subexperiments you begin to push the limit of what modern microscopes can accomplish. For example, on our current setup using the Nikon TI, the amount of time it takes to autofocus, change filter cubes, acquire a phase contrast image and 2-4 fluorescence channels and move stage positions for eight subexperiments is nearly one minute. Since phase contrast images must be taken approximately every minute for adequate cell tracking, the microscopy setup becomes limiting before the microfluidics. While laser based focus systems would offer an increase in speed, many, like the Nikon Perfect Focus System, do not work well with PDMS devices. Thus while other microfluidic devices have been produced which offer a far greater number of independent experiments, often they cannot track *individual* cells due to excessive movement between frames (Taylor et al., 2009). This prevents the acquisition of cell trajectories and the device essentially functions similar to a highly parallel flow cytometer. Thus the device chosen should reflect the type of study and data required. For generating large numbers of cell trajectories in a dynamic environment with relative ease of setup, our device works well. For generating population level data using a extremely large set of conditions the device described in (Taylor et al., 2009) would be superior.

2.4 Cell tracking

For microchemostat experiments cell tracking is essential for capturing high quality data. In fact, one could argue that effective cell tracking is as important as the design of a microfluidic device itself. Like a high powered computer running an early version of DOS, even the best device is not much use if the cells cannot be tracked. Thus most articles making use of microchemostats make a reference to “custom Matlab code” used for cell tracking (Taylor et al., 2009; Bennett et al., 2008; Kurth et al.,

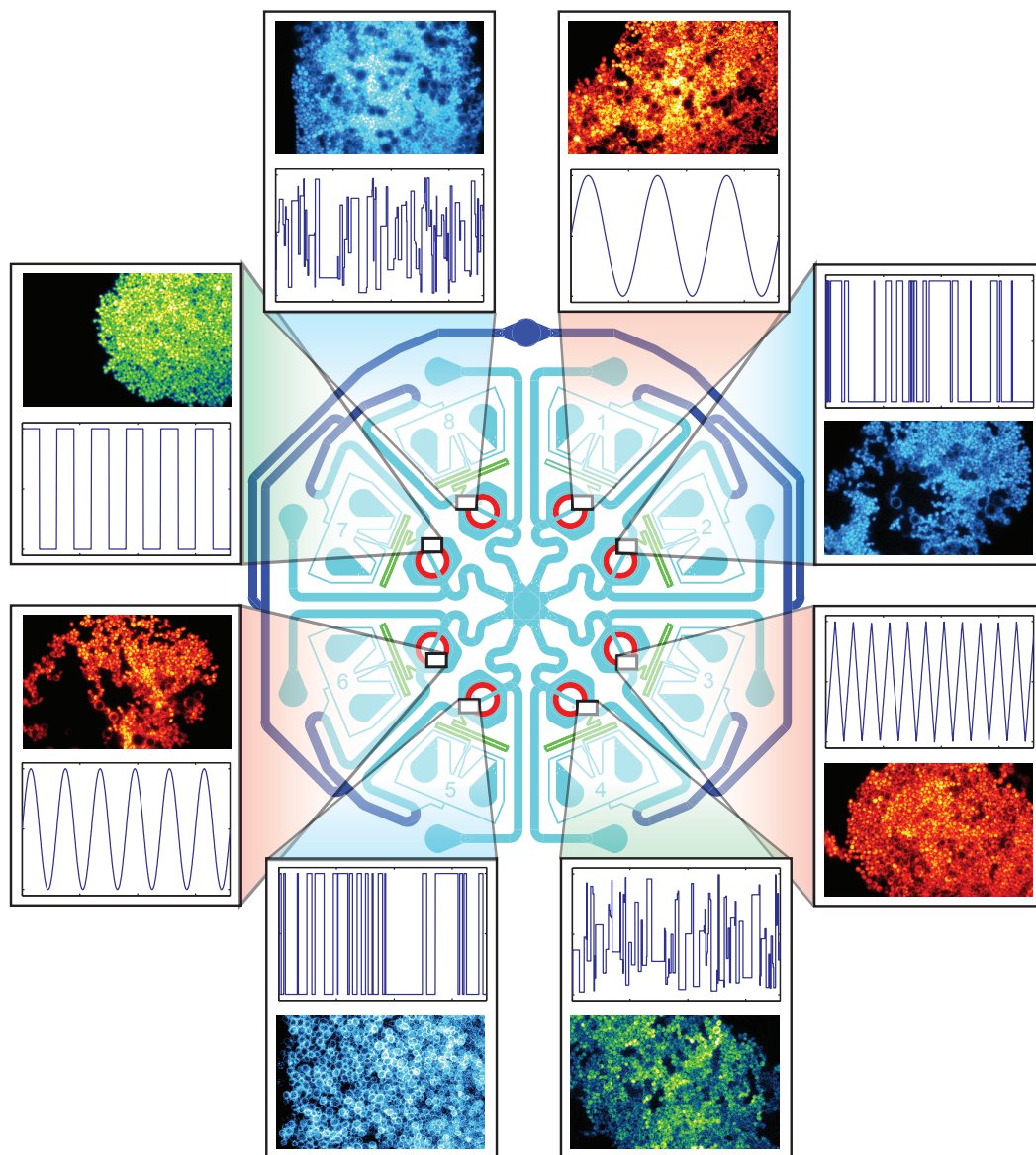


Figure 2.9: Graphic of the MDAW microchemostat device. The MDAW device has eight independent subexperiments. Each subexperiment can generate a separate inducer signal for an independent yeast strain. Examples of each are given in the breakout boxes. The system is capable of generating both periodic and pseudo random waves. The symmetry of the chip is important to ensure that all subexperiments have equal resistance outlet paths to the shared ports: the combined alternate outlet port (center) and the combined cell and shunt waste (top).

2008; Hersen et al., 2008; Lee et al., 2008). Our lab is no different and we have spent much time and effort generating a software package which works quite well but has room for improvement. There is also a program called CellTracer available free online (<http://www.stat.duke.edu/research/software/west/celltracer/>). It should be stressed that a microchemostat should be designed with cell tracking in mind from the beginning, rather than designing software to track how the cells happen to grow in the device. For example, by making the cell culture expand in defined, regular directions the cell tracking routine becomes less complex and hence works better. An excellent example of this concept is the trap described in (Rowat et al., 2009) which constrains yeast cells in essentially one dimension and makes lineage tracking quite robust.

The essential problem for tracking all types of cells, and yeast cells are no exception, is that they simply are not unique, at least as viewed under phase contrast microscopy. This can be seen in Figure 2.10 which compares different parameter values for a population of cells. Ideally each cell would occupy a unique position in some high dimensional space, corresponding to a combination of parameters, such as cell area, eccentricity and fluorescence, specific for that cell and invariant in time. This would be similar to a bar code or serial number for cells. However as seen in Figure 2.10 there is simply no combination of inherent characteristics visible under this type of imaging which can uniquely identify *all* members of the population at once. If there were, there would be no clusters of high density in the histogram. Moreover, since cells grow and divide, there is often a high amount of variability in the geometric properties between frames for the *same* cell. Unfortunately the only parameter which is unique for all cells confined to a monolayer is position. Thus it is of critical importance to keep track of cellular position during a microchemostat experiment and this explains why phase contrast images must be taken frequently. For fast growing cell types such as yeast or *E. coli*, frequent sampling is a necessity. If the cellular movement is greater than one cell diameter between frames, cell tracking becomes next to impossible.

Cell tracking software can be divided into two basic types, segmentation based methods and non-segmentation based methods (Mosig et al., 2009; Miura, 2005). Seg-

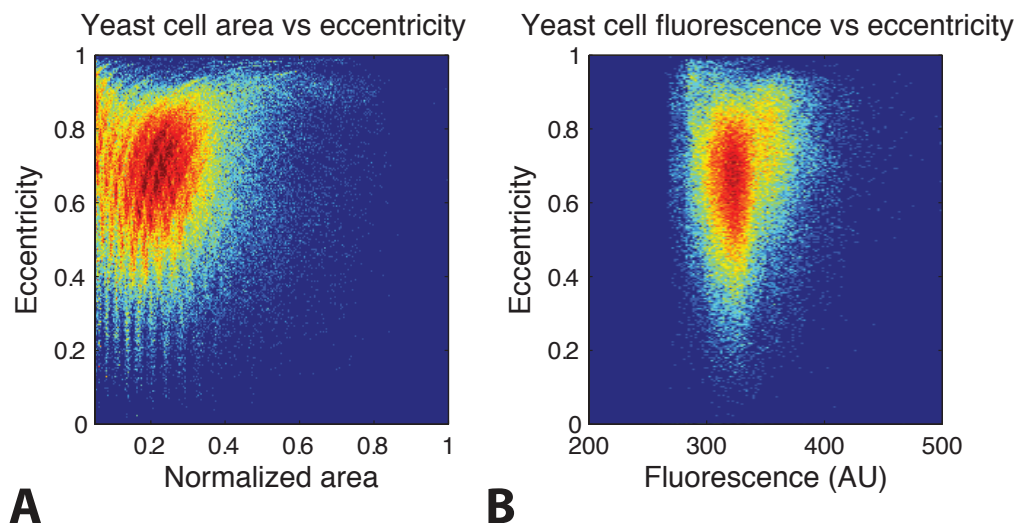


Figure 2.10: Comparison of different cell parameters for a population of yeast cells. **A.** Two dimensional histogram of yeast cell eccentricity versus area. Striations in the data are a remnant of the ellipse filter used to segment the cellular boundaries. Notice that most cells have similar values for eccentricity and area. **B.** Similar plot as part A, except here eccentricity and mean fluorescence are plotted.

mentation methods are the more common type and will be the focus of this discussion. In a segmentation method, a transmitted light image of the cell population is converted to a binary image containing only the outlines of cells. This is repeated for each image of the experiment and trajectories are formed by linking cellular objects between frames based on shared characteristics. Binary images are preferred since there are a large number of mathematical functions available for processing them. To convert a transmitted light image to a binary image the simplest method to use is a threshold. Essentially anything below the threshold is converted to black and anything above to white. Phase contrast images typically have a light halo around the boundary of cells, which provides high contrast, and thus are perfect for thresholding. A comparison of phase contrast imaging to differential interference contrast imaging, which is less suitable for thresholding, is shown in Figure 2.11.

Typically a threshold value will be chosen to retain the boundary halo while discarding all other features, thus preserving only the boundary of cells. This procedure

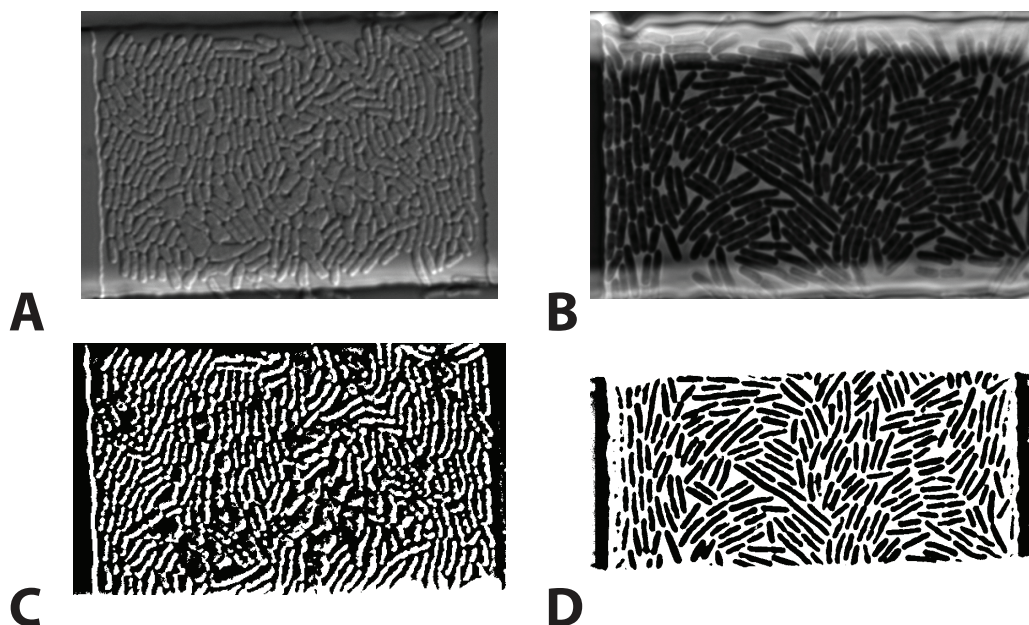


Figure 2.11: Comparison of phase contrast and differential interference contrast (DIC) imaging with regards to cell tracking. **A.** DIC image of an *E. coli* colony growing in a microchemostat device. **B** Phase contrast imaging of a similarly grown *E. coli* colony. **C.** Binary image created by thresholding the DIC image shown in part A. Notice how difficult it is to distinguish the cellular boundaries. **D.** Thresholded version of the phase contrast image in part B. Notice how much more clear the cellular boundaries are compared to C.

works fairly well assuming there are no other “phase objects” present in the cell. Unfortunately, yeast vacuoles are quite prominent under phase contrast microscopy and often are difficult to remove by thresholding alone. This necessitates later post-processing steps to remove the vacuolar artifacts to prevent errors in segmentation. Some yeast backgrounds or mutants can have especially prominent vacuoles which can be problematic. Moreover, environmental conditions, stress and aging can increase vacuole prominence. Thresholding based segmentation routines will need to cope with vacuoles and this is a downside of the technique for yeast. In spite of these issues, thresholding usually works well enough to be a reliable first step of the tracking procedure when chosen appropriately.

After thresholding the cellular boundaries are generally prominent but incomplete. Due to the aforementioned vacuole problems, often an aggressive threshold value is chosen leaving only the most prominent features of the image. While more successful in removing vacuolar artifacts, this will also remove some the cell’s boundaries. For efficient processing of binary images, the image must be composed of only completely closed objects. Thus any cells lacking completely closed boundaries will not be found by the algorithm. Even if vacuoles are not a problem, a morphological closing operation is performed to repair inevitable boundary defects. This closing operation is done using either a structuring element or the watershed algorithm. Structuring elements are small geometrical objects which can reinforce common motifs of the image. We have found them to be very useful for processing *E. coli* cells.

Implemented in ImageJ and Matlab, the watershed algorithm is good at repairing small defects in cellular boundaries. However, if even a small vacuolar remnant remains, the watershed algorithm will bisect the cell through it, causing improper segmentation. An example of how the watershed algorithm performs in segmentation is given in Figure 2.12. Note that another thresholding tradeoff comes from deciding how much to emphasize cells at the edge versus those in the interior of a microchemostat’s colony. Multiple cells in close contact reinforce their boundary halo’s, causing the signal from these areas to be greater than from isolated cells at the edge of the colony. When

choosing a threshold value that maximizes boundaries and minimizes vacuoles, often the boundaries of cells on the colony edges are removed. This can be seen in Figure 2.12. These boundary cells are consequently often dropped from the segmented image.

After segmentation the binary image is processed to extract useful data from the contained objects. To assist in this processing we fit each object to an ellipse since we have found that it generates a good approximation to a yeast cell's shape. After processing each image in a run we link cells between images to form trajectories. To accomplish this we have a scoring function which compares two cells and generates a score based on how likely they are to be the same cell. We compare the position, area, eccentricity, and orientation of each pair of cells to be scored. While we could also use the fluorescence values of the cell, we have found that this usually does not improve the score's power and often isn't possible since we take phase contrast more often than fluorescence images. The scoring of cells between frames is usually the most computationally intensive part of the entire process. To aid in the computation we only compute scores for cells in the same general location of the two images, since if the cells have moved more than one cell diameter between frames they become virtually impossible to track anyways. This greatly reduces the computational time. Images of the MFD005_a and MDAW traps generally contain ~ 1000 cells when fully packed; runs sampled every minute for 2-3 days will have thousands of phase contrast images to process. Clearly, any savings in time are important.

After scoring we remove cells which are below a threshold empirically determined to result in a poor match. To match a cell from the current frame to one from a previous frame there are several cases which need to be dealt with. These cases are depicted in Figure 2.13 The first is the easiest which is a unique match between a cell from frame n and a cell from frame $n-1$. In this case the cell from frame n is assigned to the $n-1$ cell's trajectory. An example of our algorithm's scoring output for the single match case is given in Table 2.5. The next case is when two cells match to the same trajectory. This often happens due to excessive cell movement and the cellular position becomes a less powerful discriminant. In this case the cell with the highest score will

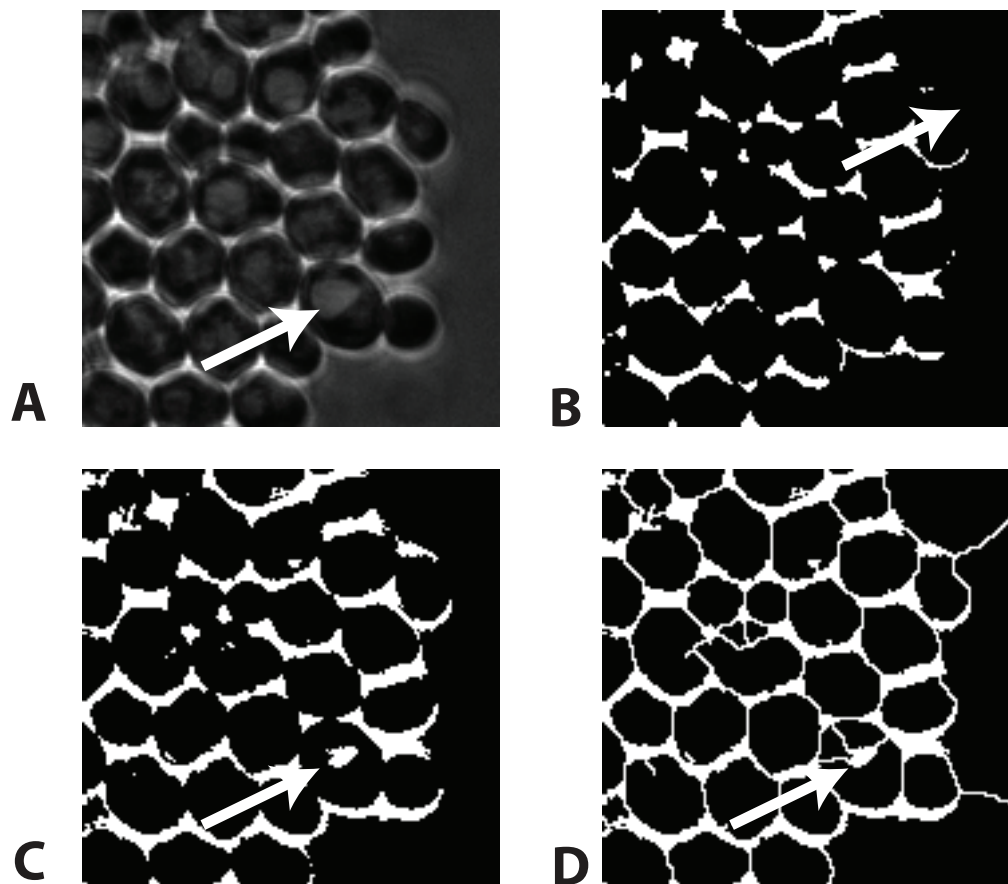


Figure 2.12: Segmentation of yeast phase contrast imagery **A.** Phase contrast image of a tightly packed yeast cell colony. The white arrow points to a cell with a prominent vacuole. **B.** Binary image created by thresholding the image from part A. The thresholding value was chosen to minimize vacuolar artifacts, but also has the effect of removing boundaries of cells on the colonies edge. The white arrow points to a cell with a deficient boundary. This cell will not be closed by the watershed algorithm and therefore will not be present in the segmented image. **C.** Binary image created by thresholding A with a less stringent cutoff value. Notice that the boundaries are thicker and well defined, but that the vacuoles are more prominent than B. White arrow points to a vacuolar artifact. **D.** Segmented image made from performing the watershed algorithm on the thresholded image from part C. Note that the vacuolar artifact has caused the segmented cell to be split into three regions. In later processing steps each of these regions will be considered cells, thus potentially causing errors in tracking.

be retained as the trajectory's match and the other cell will be moved to its next highest scoring trajectory. The third case is a skip, where a trajectory was present in frame n-2 but for whatever reason a match was not found in frame n-1. This often happens due to a segmentation error in frame n-1.

Table 2.5: Sample yeast cell tracking output. Comparison of a cell from a given trajectory and the nearest cells in the next frame. The data for the trajectory is taken from its matched cell in the previous frame. This is called the base cell. The predicted column refers to the algorithm's prediction of how the cell's properties should have changed in the current frame based on its previous behavior. This prediction is usually generated from MatPIV data of the colonies movement. While not shown here, it is also possible to predict a change in area from previous growth data. Note that a lower score is better and all scores above 1 are considered to be below the scoring threshold and thusly discarded.

Trajectory 36	Base	Predicted	Cells			
Score			0.1	2.1	2.5	2.5
Area	871.0	871.0	856.0	615.0	608.0	560.0
CentroidX	592.4	591.1	591.3	605.1	575.3	572.2
CentroidY	596.2	602.6	599.1	630.9	566.9	631.0
Eccentricity	0.5	0.5	0.5	0.5	0.8	0.5
Orientation	63.0°	63.0°	50.8°	71.7°	51.2°	-53.3°
Object	610	NaN	621	640	603	601
Fluor mean	NaN	NaN	392.6	331.2	390.3	326.4
Fluor std	NaN	NaN	603.4	316.3	538.5	277.2

If vacuoles are prominent, this type of skipping may happen often and should be corrected for. By keeping trajectories for an extra frame you can match a cell from frame n to a cell from frame n-2. The next case is the start of a trajectory. Here a new cell is formed. The last case is the removal of a trajectory, here the cell either left the field of view or died. One has to be careful that the algorithm is not too “greedy” by always finding a match for a cell in the previous frame. Cells are born and cells die, these events will happen and if an algorithm is too greedy it will end up making improper trajectories. For example, often a greedy algorithm will cause a trajectory, which should have ended due to a cell leaving the trap, to jump to an adjacent trajectory. This is sometimes worse than ending a trajectory prematurely because it can be difficult to detect unless one goes through the data very carefully. Thus to obtain long, reliable trajectories one needs

above all else good data and an algorithm which is balanced among all cases.

To reliably link cells into trajectories the number of cells uniquely matching a trajectory should be maximized. As stated earlier, the largest impediment to unique matching is movement of cells as the colony expands. This can be severe for *E. coli* or even yeast grown in rich media. In fact, sometimes it is possible to see movement of the colony due to growth in real time under high magnification. To correct for bulk movements of cells a particle image velocity (PIV) program can be invaluable. PIV programs are imaging analysis routines which are able to detect particulate flows in a sequence of images by comparing how the field of view changes in time. This is very useful for tracking bulk movements of cells and can often significantly improve the fidelity of tracking. We use a program called MatPIV, which has been conveniently implemented in Matlab, to track cell flow in our images (Sveen, 2004). Using this data we come up with a predicted position for each trajectory present in frame $n-1$ for frame n . An example of how this is useful is shown in Figure 2.14. The MatPIV generated velocity field has been used to adjust the position of the cells resulting in more robust tracking.

In principle, the change in a cell's area and eccentricity could also be predicted from previous data. These changes would be most pronounced for newer, smaller cells. However, we have not done this and it is unlikely to improve tracking appreciably. An overview of the entire procedure is given in Figure 2.14. The overall sequence of events is presented in the figure. We have done much work to improve the visualization of the trajectory data to ensure high quality. While the linking of trajectories works very well, the biggest improvements can be made in the segmentation steps of the process. Indeed, some cell tracking methods have no segmentation step at all, relying on comparison based methods for identifying cells in an image field (Miura, 2005). These methods generally rely on comparing a reference library of known cells to the current image using a cross correlation function. The cross correlation function will be maximized when the reference image matches a cell in the target image. Indeed, MatPIV works in a very similar way for tracking cell flows.

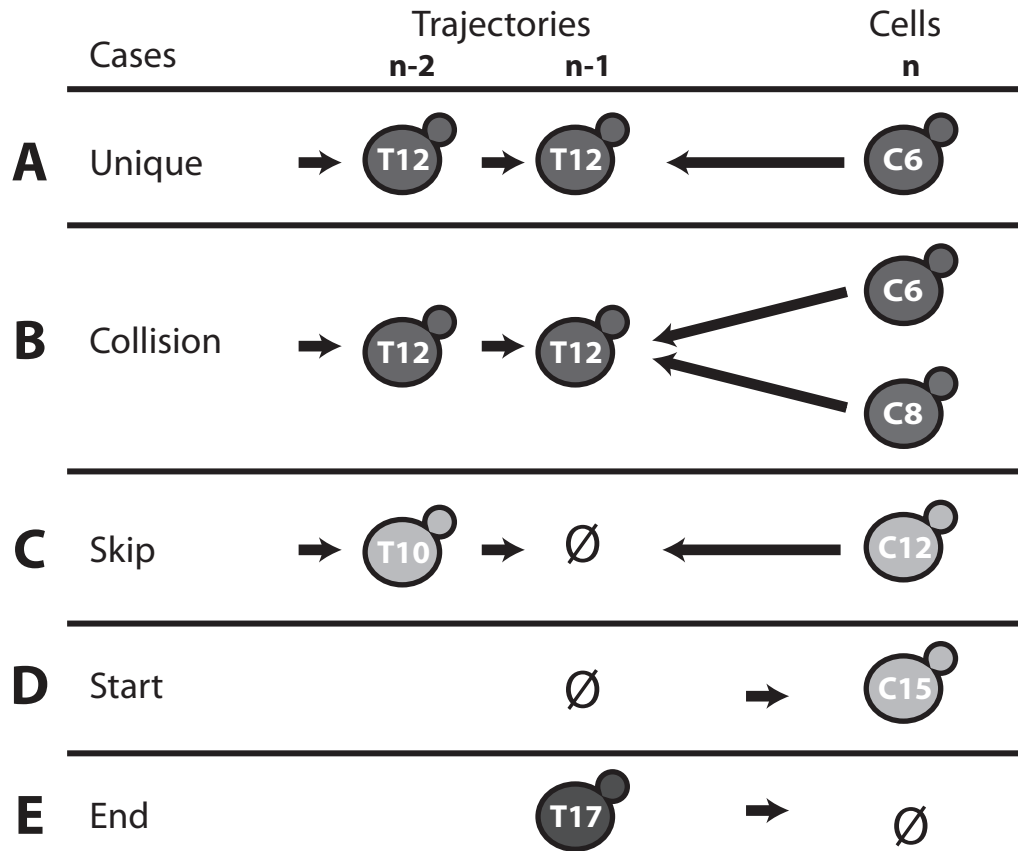


Figure 2.13: Different cases which need to be handled in cell tracking. Each case is given on the left hand side of the figure. The cells' representing trajectories present in frames n-2 and n-1 are given in the middle portion of the figure. These cells are labeled with their trajectory number (e.g. T12). Cells in the current frame (n) are shown on the right hand side of the figure. They are labeled with their cell number (e.g. C6). **A.** Unique match. A trajectory present in the previous two frames matches a single cell in the current frame. **B.** Collision. Two cells have the same trajectory as their best match. Normally the highest scoring cell is chosen as the match, however this is a symptom of poorly acquired data (cells have moved too much between frames) and will likely lead to mistakes. **C.** Frame skipping. A trajectory present in frame n-2 did not find a match in frame n-1 but does find a match in frame n. This often is caused by segmentation errors in the n-1 frame, especially vacuolar splitting of cells (see Figure 12D). If this case is handled, longer trajectories can be generated, however there is a potential for the algorithm to become overly greedy. **D.** Start of a trajectory. A cell is either born or moves into the frame. **E.** End of a trajectory. A cell either dies or moves out of the frame.

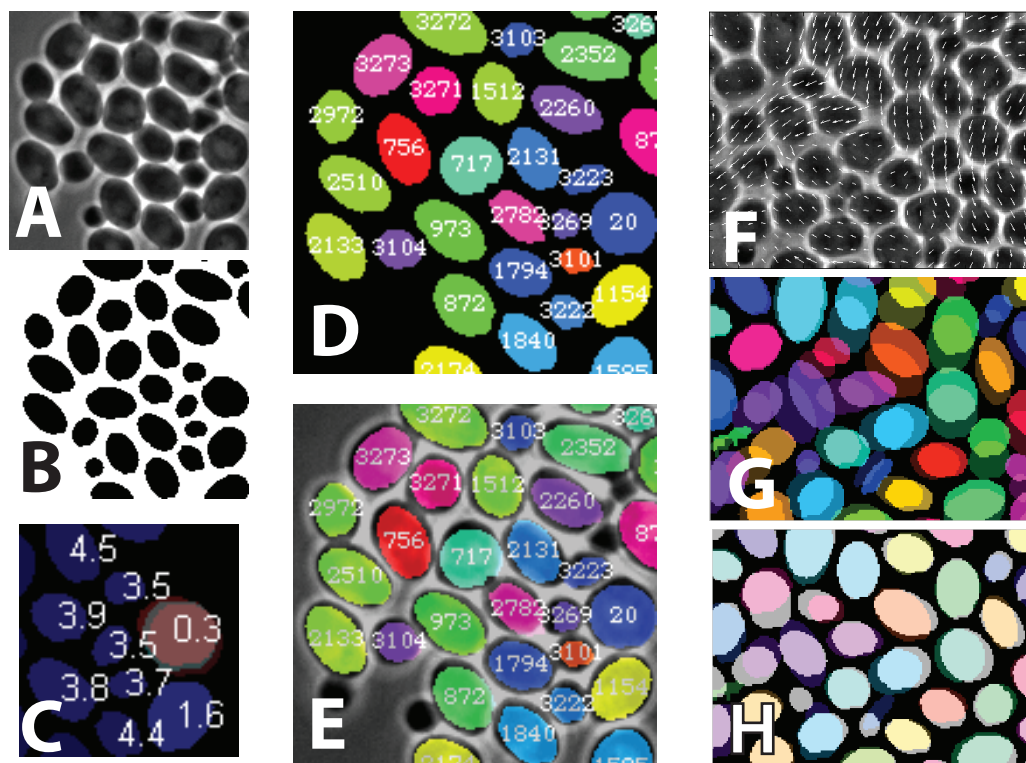


Figure 2.14: Overview of the cell tracking process **A.** Raw data: phase contrast image of yeast cells. Note the high contrast between the boundary of the cell and the exterior. **B.** Segmented image after thresholding, application of the watershed algorithm and fitting the resultant objects to ellipses. **C.** Scoring of a cell from frame n (shown in red) to trajectories present in frame $n-1$. Lower score is better. Notice that the red cell has closely overlapped with a previous trajectory and generates a better score. All other scored cells are above the scoring threshold (which is set at 1). Note that the scoring system here has generated good contrast between the ideal match and the neighbors. This is indicative of a good match. **D.** Colored image of the masks after trajectory finding is complete. Colored regions represent trajectories which are numbered. **E.** Overlay of the trajectory image from part D with the phase contrast image of part A. Note most cells were assigned trajectories except for smaller cells and cells near the exterior. **F.** Example of MatPIV processing for cell flow. White arrows indicating the cell flow velocity are overlaid with a phase contrast image of the colony. **G.** Image of cells from frame $n-1$ (opaquely colored objects) overlaid with cells from frame n (translucent objects). Notice there is an overall movement of cells towards the lower left corner of the image due to cell flow. Also note that the distance traveled here is almost one half cell diameter between frames for some cells. One can see how this movement could generate ambiguous situations for similarly shaped cells without prior knowledge of the cell flow. **H.** Same cell field as in part G except MatPIV velocity information has been applied to correct for cell flow. Notice the much better overlap compared to part G. This will lead to more reliable matches since cell position is crucial for reliable matching.

In principle, comparison methods could get around the vacuole problems mentioned above which are the bane of the segmentation approach for yeast. However, comparison methods can have problems if the cells change markedly between frames, which will happen due to growth, division and rotation. While there are ways to correct for this, in general they are computationally intensive. In fact, the whole process is much more computationally intensive than the segmentation method. We are currently working on a hybrid method which employs an initial segmentation step that is corrected using a comparison step. Since segmentation works quite well, running it initially will reduce the space to be searched by direct comparison. The subsequent comparison step will correct for any initial errors in segmentation. Fortunately, cross correlation methods lend themselves to parallel processing, and modern graphics cards can be programmed to greatly speed up computation (Owens et al., 2008). In the future we expect more use of parallel processing and comparison based methods for cell tracking. However, it should be emphasized that no matter how well designed an algorithm is, the most crucial determinants for success are the quality of the initial data and the regularity of a cell colonies movements.

2.5 DAW Hardware and software

2.5.1 Hardware

As mentioned in the chip design section of the paper, the DAW junction works by changing the relative pressures at DAW ports, while keeping the total pressure the same. Physically this can be achieved in a number of ways: by pneumatically pressurizing the syringes, using a syringe pump, or changing the hydrostatic pressures of the syringes. Our initial design relied on pneumatically pressurized syringes, but due to problems with flow control we switched to a hydrostatic system. We use two vertically mounted linear actuators to change heights of liquid filled syringes that feed into the DAW junction, Figure 2.15A. The smooth motion of the linear actuators allows for

smooth changes in mixing ratios. Linear actuators are also a better solution in case of a hardware malfunction. If the actuators break down or cannot move to a new position, they will still allow the experiment to continue, since the flow depends only on the position of the syringe. The inability to move the syringes will only result in a constant inducer level, while maintaining a steady flow. In case of a malfunction with syringe pump or pneumatically driven system the flows will change over time and might even result in flow reversal, which would most likely ruin the experiment.

The first version of DAW system had two linear actuators, which could be controlled independently, Figure 2.15A. By attaching a syringe with media to each actuator and moving them equal distances in opposite directions we were able to change the ratio of pressures at the DAW ports while keeping the total pressure constant. Since we have constrained our total pressure to be constant, the movement of one actuator has to be mirrored by the movement of other actuator in opposite direction. In essence, the second linear actuator could be replaced with a linear guide and a pulley system, as seen in Figure 2.15B. The linear guide consists of a rail and a guide block that slides along the rail. We found that the guide block does not have enough mass to keep a taught line through the pulley system. A steel block was used to weigh down the guide. Also, the length of the line between the linear actuator cart and the guide block needs to be adjustable. This can be achieved by attaching the line to guide block with a pinch mechanism operated by a screw.

The elimination of the second linear actuator proved to have a major benefit of reduced setup cost per DAW unit. However, when considering the additional parts and labor required to fabricate a pulley system the value of this benefit diminishes. Unless you intend on running a full 8 trap MDAW chip, we recommend on installing a dual linear actuator system. During installation the actuators should be securely attached to some sort of a support system. In our case we attached them to metal struts that are directly connected to the wall studs.

From Table 2.6, which lists all the required parts for a dual linear actuator system, we can see that there are 2 linear actuator controllers(RPCON) and a single commu-

nication gateway module(SIO). The SIO module is used for communication to a computer, while the RPCON's connect the actuators to the SIO. This setup seems redundant, but it allows for easy expansion. The SIO module can operate up to 16 individual linear actuators, while maintaining only a single connection to the computer. Using this system from the start will allow one to easily expand from 2 to 8 axis DAW system. Also the SIO can be wired to communicate with a computer via USB interface.

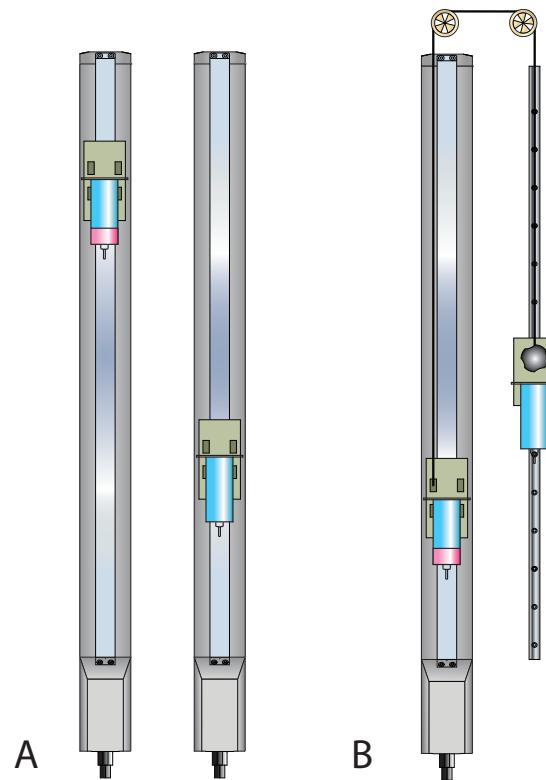


Figure 2.15: Linear actuator setup for DAW. **A.** Dual linear actuator setup. Each actuator can be move individually. One of the actuators(left) moves a media syringe with added dye. **B.** Alternate design of the DAW system using only a single linear actuator. The actuator controls the position of both syringes simultaneously. To eliminate friction in the system the following components need to be in a single plane in space: both pulleys, line attachment to actuator, and line attachment to linear guide block.

Table 2.6: Hardware required for DAW setup using commercially available linear actuators. Some manual assembly may be required.

Equipment	Qty	Part No.	Vendor
Linear actuator: fast speed, 800mm travel length	2	RCP2-SA7C-I-56P-16-800-P1-M-BE	Valin Corp.
Controller	2	RPCON-56P	Valin Corp.
Communication gateway module	1	RGW-SIO	Valin Corp.
Serial communication cable	1	CB-RCA-SIO-050	Valin Corp.
USB adapter	1	RCB-CV-USB	Valin Corp.
USB cable	1	CB-SEL-USB010	Valin Corp.
24V DC power supply	1	OMRON-S8VS-06024	Valin Corp.
AC power cable	1	70355K34	McMaster-Carr

2.5.2 Software - *iDAW*

To control the linear actuators we have created a custom software, nicknamed *iDAW*, using the National Instruments LabVIEW environment. Currently there are two major versions of the software, for the 2 and 8 actuator systems. Both versions, manuals and installation guides are freely available by request.

The graphical user interface presents the user with three main areas: actuator controls, calibration and experiment setup as seen in Figure 2.16. During a typical experiment, the actuators first have to be calibrated to the specific chip. This calibration establishes a relationship between relative positions of each actuator and the respective mixing ratios. There are two ways to calibrate the system: manually and automatically. The automatic calibration was already discussed in an earlier section. During manual calibration the actuator positions are changed to create different mixing ratios. Once all the calibration points have been acquired the software creates a calibration function. The software allows up to 11 calibration points, but we have found that a two point calibration performs very well. Also, depending on the number of points, the order of the calibration function can be increased for improved data fit.

To start the manual calibration procedure the actuators are moved together to a height that provides the desired flow to the cell trap. Since the pressures at both syringes are the same, this becomes the 50% value for the calibration. Next, the actuators are linked to move equal distances in opposite directions. The positions are adjusted until there is only media with the inducer going through the mixer, this becomes the 100%

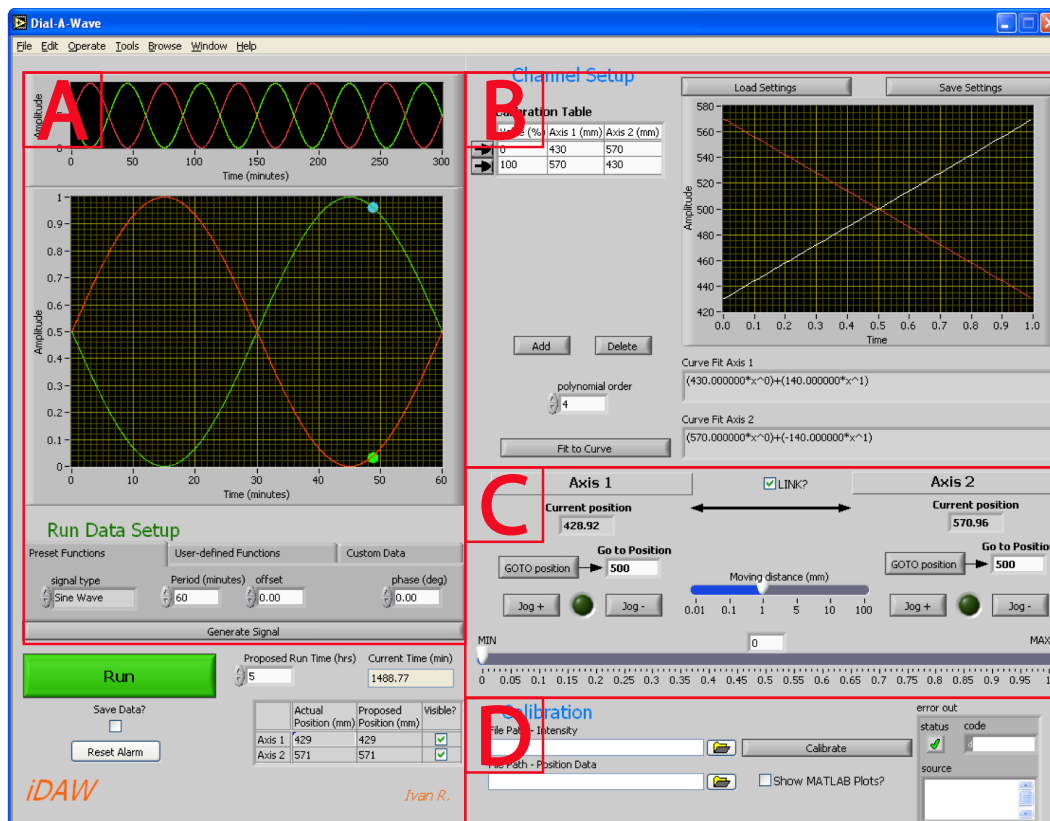


Figure 2.16: Screenshot of *iDAW* software. **A.** Experimental parameter setup allows user to setup up mixing ratios as a function of time. Most mathematical functions or an arbitrary dataset can be used as templates. **B.** Manual calibration. The table records the calibration points and the graph shows the calibration functions. In this example a two point calibration was used to create a linear calibration profile. **C.** Actuator controls allow the actuators to be moved independently or together when in "Linked" mode. **D.** Automatic calibration functions take position data from the actuators and fluorescence data from the microscope to determine the calibration profiles.

point. Similarly, the 0% point is recorded. The 0% and the 100% points are used to make a linear calibration function as can be seen on the graph in Figure 2.16B.

The experimental setup area of *iDAW* allows the user to create a profile of induction vs time. The user can choose from a number of built-in functions, such as square or sine waves, or load an arbitrary function. The software automatically adjusts the inducer values to fit between 0% and 100%. The proposed induction profile is plotted for the duration of the whole run and the individual linear actuator positions are constantly updated, Figure 2.16A. These displays eliminate errors during experimental setup and actual run-time.

2.6 Fabrication

With the design of the chip drafted and thoroughly analyzed we begin the fabrication process. An overview of fabrication is shown in Figure 2.17. The complete fabrication of a microfluidic chip can be broken down into three main phases. In the first phase we create a patterned wafer by photolithography. Next, we use this wafer to create a silicon rubber mold by a process of soft lithography. And finally the silicon is prepped and bonded to a glass coverslip to make a functional microfluidic device.

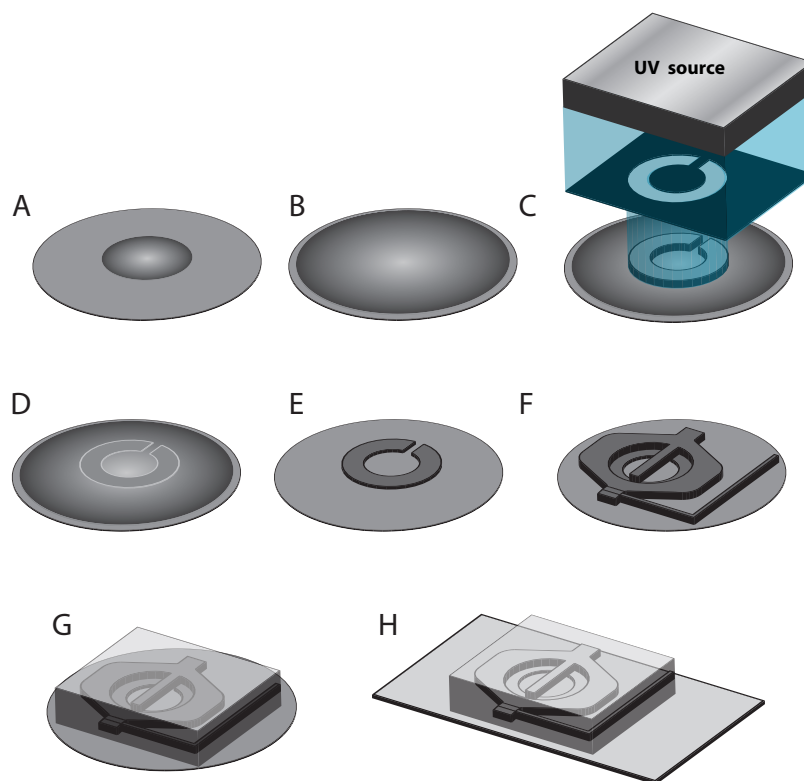


Figure 2.17: Overview of the fabrication process. Photolithography(**A-F**), soft lithography(**G**), and PDMS processing(**H**). **A.** Photoresist deposition. **B.** Spin coating: the deposited photoresist is spun at a specific speed to create a uniformly thick layer. **C.** UV exposure cross-links the photoresist creating a pattern identical to the photomask. **D.** Post-exposure baking joins the silicon wafer and the cross-linked photoresist. **E.** Developing removes the uncross-linked photoresist, revealing the features. **F.** Repeating steps A-E creates additional features. **G.** Pouring and curing PDMS over the patterned wafer creates a mold. **H.** Bonding the PDMS mold to a glass coverslip finishes a microfluidic chip.

2.7 Photolithography

Photolithography was initially developed for the semiconductor industry and later applied to a variety of fields, including microfluidics (Xia and Whitesides, 1998). The process relies on transfer of a geometrical pattern from a mask onto a photosensitive layer via light radiation. The first step involves thorough cleaning of the wafer, which will act as the foundation for all the features. It is very important to remove all debris and any chemicals from the surface of the wafer, as they will get incorporated into the final wafer design and will highly affect the adhesion properties of photoresist to the wafer. Next, we deposit a small amount of photoresist onto the wafer and spin the wafer at predetermined speed to create a photoresist film of precise height, this step is called spin-coating. The wafer is then soft-baked by gradual heating on a level hot plate, which removes solvent and enhances photoresist adhesion to the wafer. At this point the wafer is exposed to UV light through a photomask, this transfers the pattern from the mask onto the photoresist layer. We use the SU-8 2000 line of photoresist from MicroChem Corporation. SU-8 is a negative photoresist, which means that areas of the film exposed to UV radiation will form solid structures, while unexposed areas will be washed away during the developing step. The wafer is then baked again, in the post-exposure bake, to increase the level of cross-linking. And finally to complete a single photolithographic cycle, the wafer is developed by immersion in solvent which removes uncross-linked photoresist leaving only the desired pattern on the wafer. Since all of the chip designs we use require wafers with multiple heights this cycle is repeated a number of times.

Photoresist

Manufacturers, such as MicroChem, make a variety of photoresist formulations. The SU-8 line of resists alone has 3 sub-categories with a total of 18 different formulations, specific for heights ranging from 1.5 μm to 550 μm (MicroChem, 2010). We use the SU-8 2000 photoresists which have great adhesion to silicon wafers and are able to make high aspect ratio structures (del Campo and Greiner, 2007). The “negative”

denomination of a photoresist means that areas of the film exposed to UV radiation will form solid structures, while unexposed areas will be washed away during the developing step. Specically, exposure to UV radiation changes the chemistry of the resist by generating very strong acid within the film, which starts the cross-linking reaction of the SU-8 epoxy. The main difference between the various SU-8 2000 formulations is the epoxy solids content that directly relates to the viscosity of the liquid as can be seen in Table 2.7.

Table 2.7: Commercially available SU-8 2000 photoresists formulations from Microchem Corporation.

SU-8 2000	%Solids	Viscosity (cSt)
2000.5	14.3	2.49
2002	29.00	7.5
2005	45.00	45
2007	52.50	140
2010	58.00	380
2015	63.45	1250
2025	68.55	4500
2035	69.95	7000
2050	71.65	12900
2075	73.45	22000
2100	75.00	45000
2150	76.75	80000

Commonly there is a need for a non-standard formulation. It is possible to make new, less viscous, formulations by adding SU-8 thinner to the initial, more viscous, stock of photoresist. It should be noted that the manufacturer's naming scheme loosely relates to the height of the photoresist film when it is spun at 3000 rpm. Thus, for 2002 and 2005 photoresists, spin-coating at 3000 rpm would in theory produce 2 μm and 5 μm film heights, respectively. Using this information we can plot these theoretical heights against the percentage of solids for each formulation, as seen in Figure 2.18. By making a curve fit function of percent solids(s) as a function of height(h), as written in Eq. (2.33), we are able to estimate the required solids for any new formulation. For example, to make a new formulation, which would produce 3 μm height at 3000 rpm,

we use Eq. (2.33) to determine that it requires 35% solids.

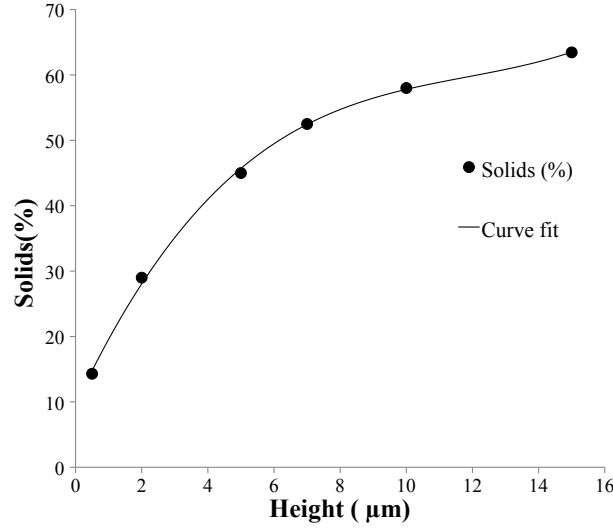


Figure 2.18: Graph of SU-8 formulation vs percent solids. Relationship between estimated height of SU-8 formulations when spun at 3000rpm and their solids content.

$$s = 0.0235h^3 - 0.834h^2 + 10.807h + 9.5781 \quad (2.33)$$

Next, the amount of thinner required for the new formulation can be calculated using the relationship described in Eqs. (2.34), where $mass_{total}$ is the desired mass of the new formulation, $mass_{thinner}$ is the required mass of thinner, $mass_{initial}$ is the required mass of original photoresist, $s_{initial}$ is the percentage of solids in the original photoresist, and s_{final} is the percentage of solids in the desired photoresist formulation.

To make the formulation measure out and deposit the predetermined amounts of photoresist and thinner into a clean dark glass bottle, make sure to do this in a fume hood. Drop a clean stir bar into the bottle and place on a magnetic stirrer, until it is thoroughly mixed. Due to the viscosity of photoresists removing the stir bar could be difficult, so we leave it in the bottle until the photoresist runs out.

$$mass_{thinner} = mass_{total} \left(1 - \frac{s_{final}}{s_{initial}}\right) \quad (2.34a)$$

$$mass_{initial} = mass_{total} - mass_{thinner} \quad (2.34b)$$

Finally, to complete the process it is necessary to characterize the new photoresist formulation by making a spin speed curve. This step should also be performed for any standard formulations that have not been previously characterized by your lab. To create a spin speed curve for a particular photoresist the photolithographic cycle, described later on, should be repeated 3-6 times with various spin-coating speeds. For each speed measure and record the feature heights using a surface profilometer. Plotting and curve fitting the data will produce enough data to reliably estimate spin speeds for specific heights. As mentioned earlier, the functionality of a cell trap is dependent on its height. Thus, it is critical to manufacture the exact height required by the design. The spin-curves allow us to estimate only a rough range of speeds required to achieve a height. Using this range as a starting point, we perform as many spin test as necessary to get the desired height. An example of an actual spin curve for 2003 formulation can be seen in Figure 2.19. Examining the figure it becomes evident, that our 2003 formulation produces 2.6 μm and not 3 μm height at 3000 rpm, this fact reinforces the need for photoresist characterization.

Equipment and environment

Due to sensitivity of photolithography to contamination it is usually performed in a cleanroom environment. A number of universities and research centers have shared facilities that house equipment necessary for photolithography and other dust-sensitive processes. We have made wafers in various environments from a Class 100 cleanroom to a basic HEPA filtered room with no rating. The latter type of non-cleanroom manufacturing environment is achieved by creating a dedicated fabrication space, installing HEPA filters over the air ducts and changing the ceiling panels to non-particulate releas-

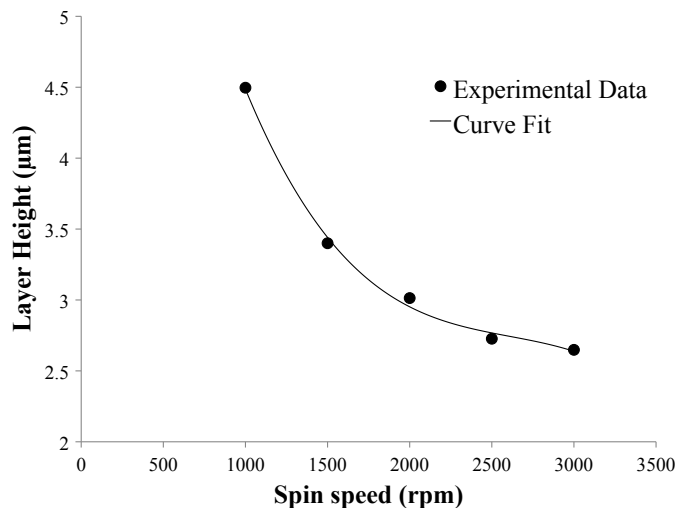


Figure 2.19: Graph of SU-8 height vs. spin speed. Actual final heights of SU8 photoresist when spun at various speeds. The nonlinear relationship between spin speed and layer height is clearly shown. Further speed increases past 3500 rpm will yield minimal drops in layer height and in this case the height will be approximately $2.5\mu\text{m}$

ing tiles. Also to prevent uncontrolled photoresist cross-linking, the lights should be fitted with UV absorbing filters. This can be easily done by placing thin filter sleeves over the fluorescent lights bulbs. Although it is beneficial to carry out the whole manufacturing process in the cleanest possible environment, in our case only photolithography is performed in the cleanroom. While soft lithography and PDMS processing are carried out in regular lab space.

It is important to point out that the chemical safety precautions are more important than the cleanliness of the facility. Some of the chemicals used in photolithography are potentially carcinogenic, labs should use a properly functioning fume hood when working with photoresists and developers at all times. A standard fume hood convects air from the environment past the user, into the hood and out a ventilation shaft. Since users are generally the largest source of particulates in a clean environment, use of a standard fume hood can increase the local concentration of particulates over the work surface in the hood, even if the surrounding environment is clean. In contrast, biosafety cabinets contain a laminar air stream between the interior and the user, preventing the

transfer of particulates into the hood. However, unless specially made and calibrated, biosafety cabinets can potentially allow chemical fumes to escape into the work area. Purpose built hoods, protecting both the user from chemical fumes and the interior from particulates, do exist but are expensive. For microchemostat fabrication we have found that a standard chemical fume hood is sufficient, however electrical engineering facilities will often contain specialty hoods. Since hot plates and spin processors are used with uncured photoresists, it is essential that they be placed in the fume hood. However, the process of soft-baking removes the solvent from photoresist, allowing one to work with a mask aligner outside of the fume hood.

Photomasks

Conventional photolithography requires expensive chrome photomasks, we use the cheaper photomasks printed on a transparency-like material as described in (Whitesides et al., 2001). As mentioned earlier, all of our masks are made by CAD/Art Services, Inc(Bandon, Oregon). They use a photographic process to print the design on a 0.007" polyester mylar sheet coated with photographic silver. Since our masks are designed in AutoCAD software, we just provide them with a *.dxf file. However, they do accept a variety of other CAD files, listed in order of preference: *.dwg, *.gds, *.cif, *.gerber and *.eps. Due to the limits of their photoplotting process, the minimum feature size is defined by a circle with 10 μm diameter. Although, it is possible to print various size masks, we usually order an 8 by 10 inch sheet. This gives us enough room to fit up to 6 individual layer masks and since most of our design require less than 6 layers we can have a whole chip printed on a single sheet. When ordering the mask, it is important to specify the polarity of the mask, considering that we are working with a negative photoresist, our masks need to have clear features on a black background. Once the masks have been printed, they are cut out and individually glued at the corners to a 3 by 3 inch glass square using clear instant adhesive. It is important to have the emulsion side of the mask facing away from the glass, since it needs to be in contact

with the photoresist later on. Also, when gluing the photomask to the glass make sure to keep the glue away from any transparent parts of the mask. For storage and transport we keep the masks in individual plastic bags, this prevents them from getting dirty and scratched.

Sample fabrication parameters

For each individual wafer we create a table with fabrication parameters, this is an effective way of condensing all of the necessary information for manufacturing the wafer. Most of the parameters, such as layer heights and number of layers, will be dictated by your design, however some of them have to be calculated after the design is done. For example, the exposure time will depend on the exposure dose required for the photoresist and on the UV lamp power. MicroChem's datasheets provide exposure energy ranges for different heights. For example, $0.4\ \mu\text{m}$ layer requires $60\text{-}80\ \text{mJ}\cdot\text{cm}^{-2}$ and $3\ \mu\text{m}$ layer requires $90\text{-}105\ \text{mJ}\cdot\text{cm}^{-2}$. Given that our mask aligner UV lamp has an effective power of $1.4\ \text{mJ}\cdot\text{cm}^{-2}$, we can calculate the exposure times using Eq. (2.35), see Table 2.8.

$$\text{Exposure Time} = \frac{\text{Exposure Dose}}{\text{Effective Power}} = \frac{\frac{\text{mJ}}{\text{cm}^2}}{\frac{\text{mW}}{\text{cm}^2}} = \frac{\frac{\text{mW}\cdot\text{s}}{\text{cm}^2}}{\frac{\text{mW}}{\text{cm}^2}} = \text{seconds} \quad (2.35)$$

Table 2.8: Calculated exposure times from the manufacturer's recommendation and experimental exposure times, actual times required for layer adhesion to substrate.

Layer height(μm)	Exposure energy($\frac{\text{mJ}}{\text{cm}^2}$)	Calculated exposure time (s)	Experimental exposure time (s)
0.4	60-80	43-57	60
3	90-105	64-75	80

With additional information from photoresist spin curves we can finalize the fabrication parameters into a table, as seen in Table 2.9 and proceed to fabrication.

Table 2.9: Sample table of wafer fabrication parameters for an *E. coli* wafer design. Layer 3 is used as a dummy layer for alignment of layer 2 and 4, no feature from this layer are present in the final design. Layer 2 is the trap layer, layer 4 is the fluidic network for media and layer 1 is for coupling channel.

Layer number	1	2	3	4
Layer height (μm)	0.4	1	3	10
SU-8 formulation	2000.5	2000.5	2002	2005
Spin speed (rpm)	3750	700	1000	660
Soft-bake at 95°C (s)	120	120	150	240
Exposure time (s)	60	60	80	100
Post-exposure bake at 95°C (s)	160	160	180	240

Photolithography: Protocol

All of the necessary equipment, supplies and chemicals for this protocols are listed in Table 2.11 at the end of this section.

2.7.1 Cleaning the wafer

Place the wafer inside the spin processor (spinner) with reflective surface facing up, this is your working surface. Try to align the center of the wafer with the center of the vacuum chuck of the spinner, this eliminates uneven rotation. If you have cleanroom paper, line the inside of the spinner with it to help with cleaning up process. Set the rotational speed to 3000 rpm and start the spinner. At this point it is recommended to turn on the mask aligner and UV source, as the lamp needs time to warm up.

2.7.2 Applying the cleaning agents

Thoroughly clean the wafer by applying chemicals in the following order: acetone, isopropanol, methanol and DI water, while gently applying pressure with a polyester swab. Make sure not to press too hard, but rather smoothly move the tip across the spinning surface of the wafer.

2.7.3 Drying the wafer

Place the clean wafer on a hot plate set at 200°C and let dry for 5 minutes. Once done with the drying cycle set the temperature to 95°C, as it will take some time to cool down. By the time your are done with step 6 your hot plate should be at the right temperature.

2.7.4 Centering the wafer on the spinner

Pick up the wafer from the hot plate with wafer tweezers and let it cool prior to positioning on the spinner chuck. Once cool, position the wafer on the chuck, making sure it is centered with respect to the chuck. To check if the wafer is centered, spin it at 500 rpm, if the wafer is centered correctly when spinning it will look like a circle. However, when off center, it will spin creating an oval shape. For best results it is recommended to center the wafer as much as possible. It is helpful to use a wafer alignment tool, although we have made a custom one, there are plenty of commercially available options.

2.7.5 Dispensing photoresist

Dispense 5-10 ml of photoresist in the center of the wafer. The total amount of photoresist depends highly on it's viscosity, with higher volumes needed for more viscous formulations. When working with photoresists make sure to never dispense directly from the main source. Constantly opening the bottle will cause solvent evaporation and build-up of dry photoresist on the mouth of the bottle. This leads to change in the viscosity of the resist and to contamination with solid particles. The best practice is to have a working 30ml amber glass bottle, which you refill from the main stock. The dark glass will limit the amount of UV entering and reacting with the photoresist. Make sure to label the bottles as all photoresist look the same.

2.7.6 Spin coating

Depending on desired layer thickness the spin speed during the second step will vary. Program the spinner for a two step cycle. Step 1: 500 rpm for 15 seconds, acceleration of 100 rpm/second; Step 2: desired spin speed for 30 seconds, acceleration of 300 rpm/second. For example, to achieve a layer thickness of 0.4 μm with SU-8 2000.5 we spin for 30 seconds at 3750 rpm; 3 μm with SU-8 2002 we spin for 30 seconds at 1000 rpm. These numbers are true for our formulations but might not be correct for your formulations, since the age of photoresist will have an effect. As mentioned earlier it is absolutely crucial to create spin-curves for each photoresist prior to final wafer fabrication.

2.7.7 Soft-baking at 95°C

Previously it was recommended to have a pre-bake step at 65°C prior to soft-baking 95°C. According to MicroChem and our own experience pre-baking step is not really necessary. We have eliminated it from our protocols and have not noticed any significant effects.

Using an infrared thermometer check the temperature of the hot plate, it should be 95°C. Place the wafer on the center of the hot plate and be careful as the wafer may sometimes slide off the hot plate. Keep the wafer on the hot plate for 1-3 minutes, depending on the layer thickness. MicroChem's material datasheets can act as a guide in selecting the baking time, however the exact time can only be determined empirically. A good way of optimizing baking time is to remove the wafer from the hot plate and let it cool. Once cool, place the wafer back on the hot plate. If the photoresist film "wrinkles" keep it on the hot plate for another 30 seconds. Repeat this process until the film no longer "wrinkles" (MicroChem, 2010).

For example for 0.4 μm layer the softbake time is 120 seconds and for 3 μm layer it is 150 seconds.

2.7.8 Alignment of photomask and UV exposure

Turn on the mask aligner UV source, if this has not been done in *Step 1*. Place the wafer on top of the vacuum chuck in the mask aligner. Turn on the vacuum, to secure the wafer on the chuck. Position the photomask in the mask holder on the aligner, with the transparency side facing the wafer, turn on the vacuum to secure the mask. During exposure the light path should be as follows: glass, printed mask, photoresist film, wafer. Make sure the z-axis of the wafer is all the way down, then move the mask into horizontal position. If the wafer is too high it can come in contact with the mask and smear the photoresist film. Move the wafer up slowly until it makes contact with the mask. Usually this creates a number of light diffraction patterns on the mask, which can be observed by looking at the mask at an angle. For alignment the best distance is usually right after the diffraction patterns appear. This distance allows for independent movement of the wafer and the mask, while keeping them close enough to each other to see the features on the wafer through the mask. For an alignment methodology see the “Special notes on alignment” section at the end of the protocol.

Once the wafer and the mask have been aligned, bring the wafer in complete contact with the mask without forcing or overextending the z-axis. Expose the wafer for a predetermined time. Move the z-axis down, lift the mask, turn off the vacuum to the wafer chuck and remove the wafer.

2.7.9 Post exposure baking (PEB) at 95°C

Bake the wafer on the 95°C hot plate for a specified time. Once again this time will depend on the thickness of the layer, with some rough estimates present by MicroChem’s datasheets. For example for 0.4 μm layer our PEB is 160 seconds and for 3 μm layer it is 180 seconds. If the exposure times are correct you should be able to see the pattern within the photoresist film within 15 seconds of baking.

2.7.10 Developing

Fill up a crystallizing dish with enough SU-8 Developer to cover the wafer. Make sure the wafer has cooled down to room temperature, before immersing it in the developer. Next, while keeping the bottom of the dish on the surface of the fume hood, move the dish in a circular fashion. This technique improves removal of uncross-linked photoresist. Continue this process for 1-2 minutes. MicroChem suggests other methods, such as ultrasonic or megasonic baths, but we have not needed them in the past.

2.7.11 Cleaning

Pick up the wafer from the dish using tweezers and rinse it with fresh SU-8 Developer, you can let the developer collect in the dish. Follow by a rinse with fresh Isopropanol and air dry using filtered air or nitrogen. At this point you should clearly see the features on the wafer. Sometimes the wafer will have white streaks, this is due to photoresist that has not been removed by development. Clean the wafer with fresh developer, rinse with fresh Isopropanol and dry.

2.7.12 Examining the wafer

Cleaning completes a single photolithographic cycle. At this point it is necessary to examine the wafer under a microscope, if the process was successful then the features will have uniform color and straight, smooth edges.

2.7.13 Measuring feature height

Using a surface profilometer measure a number of height points for each important feature. Since the height of cell traps is absolutely crucial for microfluidic chips, it is necessary to measure the height of the trap in different locations on the wafer and see that it conforms to your design specification.

2.7.14 Hard-baking at 200°C

If there are no more layers to deposit, place the wafer on 200°C hot plate for 5 minutes. If there are any cracks on the surface of the features, this step should remove them. It is beneficial to ramp up the wafer temperature to 200°C.

Special notes on alignment

As mentioned in the chip design section, the wafer is made layer-by-layer from the ground up. It is recommended to deposit the smallest height features first and gradually move in increasing order. Although the design of the chip should account for small alignment errors, this sequential approach to wafer manufacturing can result in propagation of errors from one layer to the next. Since the compounded effect of these errors can be significant, it is crucial to have the best possible alignment at each layer. Due to lack of a consistent protocol for alignment, it can be most time consuming and very frustrating step of wafer manufacturing. Here we propose a simple methodology that should let a minimally experienced person successfully align layers.

Most of the manual mask aligners use micrometers for x , y , z and θ stage movements. The micrometers are primarily used for very fine axis adjustments, but they also can be used to precisely record the position of the wafer. Also, it is easy to see that if two different alignment elements on the wafer are individually aligned to their respective alignment elements on the photomask, then the whole wafer is completely aligned to the mask. Thus, the positional data should be identical at both alignment elements. By systematically adjusting and recording the x , y , and θ positions we can find a set of values that is identical for both alignment elements.

Protocol

Photomasks presented in Figure 2.20 will be used as an example. For correct scale it should be noted that alignment elements presented in Figure 2.20A and C, are located in the center of the mask and are 80% of the width of the mask.

Using the x , y and θ micrometers on the mask aligner, find the alignment features from layer #1 and roughly position them under alignment elements of photomask for layer #2, Figure 2.20D. Adjust the magnification of the mask aligner, so that most of your field of view is covered by a single alignment element, Figure 2.20E.

Next, adjusting only the y -direction, align the top of the features to the top of the photomask alignment box. Record the position of y -direction micrometer, this is the y_1 point. Repeat this step for the bottom side of the features and record the micrometer position, this is the y_2 point. In a similar fashion obtain micrometer readings for alignment of left and right sides of the features to the alignment box, x_1 and x_2 , Figure 2.20F. Although it would seem that if the edges are aligned then position y_1 would be equal to y_2 , and x_1 equal to x_2 , however, this is rarely the case. In reality, the new photoresist layer makes the features seem somewhat distorted when viewed through the microscope. Though, assuming that the distortion is equal in all directions we can take the average of the two positions to get the actual aligned position, as seen by values in brackets in Table 2.10. Repeat the four measurements for the right side alignment element. Record all the data points into a table, as seen in Table 2.10.

In the first row of the table, the average positions for x and y are different for left and right sides. This would indicate that wafer is not aligned. Change the θ micrometer position by a small amount, in the example case we moved from 17 to 15. Repeating all the measurements it becomes evident that the left side and right sides are diverging from each other. This is probably not the right direction for θ movement. Move the θ from the initial position by the same amount in the opposite direction and repeat the measurements. In our example the θ position changed from 15 to 19. It is clear that the x - y positions are converging, but are not exactly equal yet. In the same direction, change θ position by the smallest possible step, and repeat measurements. If the positions are identical the wafer is aligned, if not, repeat θ movement and measurements. In our example, θ movement from 19 to 20 resulted in identical x - y positions for both sides, successfully terminating alignment procedure.

We have determined through experience that developing a systematic way of

placing the wafer and the photomask into the mask aligner greatly reduces the time for alignment. The wafers we use have two flat edges, so when placing the wafer into mask aligner we find a surface on the mask aligner and roughly align the edge to that surface. Same trick is applied for the photomask. This results in relatively consistent placement of wafer and photomask, thus lowering the final alignment adjustments.

For the UV exposure step, set the x and y micrometer positions to the averaged values of x and y, respectively.

Table 2.10: Sample alignment worksheet, with only 4 iterations we are able to achieve near-perfect layer alignment.

θ	Left		Right	
	$X_1, X_2, (\bar{X})$	$Y_1, Y_2, (\bar{Y})$	$X_1, X_2, (\bar{X})$	$Y_1, Y_2, (\bar{Y})$
17	15, 14 (14.5)	13, 13.5 (13.25)	16, 17 (16.5)	11, 10 (10.5)
15	14, 13 (13.5)	14, 15 (14.5)	17, 19 (18.0)	9, 10 (9.5)
19	15, 15 (15.0)	12, 12.5 (12.25)	16, 15 (15.5)	14, 12 (13)
20	15, 16 (15.5)	12, 12 (12)	16, 15 (15.5)	12, 12 (12)

2.8 Soft Lithography

Soft lithography is a microfabrication technique that relies on the use of a patterned elastomer to create structures, in our case, by cast molding. Although a number of different elastomers can be used, PDMS (polydimethylsiloxane) has become the standard choice for microfluidics. PDMS is optically transparent, permeable to biologically important gases, chemically and thermally stable, the surface can be chemically modified and it does not absorb water. The PDMS we use comes as a two part kit: silicone monomer and curing agent. Mixing the components in specific ratio creates PDMS pre-polymer that remains liquid for a few hours. The PDMS mold is prepared by pouring liquid pre-polymer over a patterned wafer, curing it at elevated temperature and removing from the wafer. Since PDMS is initially in liquid phase, it easily conforms to the geometry of the wafer. Once cured, it remains flexible and allows for easy peel-off from

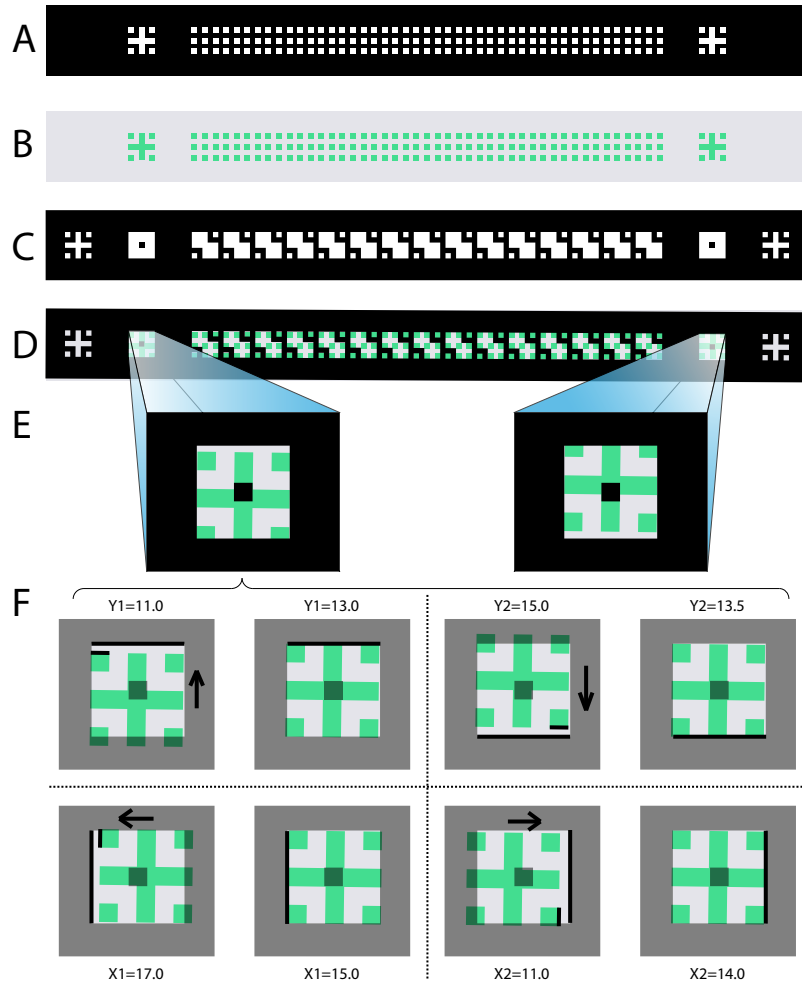


Figure 2.20: Sample layer alignment technique. **A.** Photomask of layer #1, features are created by the transparent areas of the mask. **B.** Features (green) on wafer (gray) for layer #1. **C.** Photomask of layer #2. **D.** Alignment of wafer with features from layer #1 to photomask for layer #2, as seen through the microscope of mask aligner. **E.** Close up view of alignment of the outermost left and right features. **F.** For each side (left and right), the features on the wafer are aligned to the 4 four sides of the alignment box. The mask aligner micrometer position is averaged for x (15, 14) and y (13, 13.5) directions, to provide a single xy (14.5, 13.25) position. Note that it would seem that y1 and y2 positions should be identical if the feature sides are aligned to the alignment box. In reality, due to the new photoresist layer the features from previous layer become distorted, resulting in the difference. If the xy positions from the left and the right side are identical the alignment is good, otherwise the θ position needs to be changed and the whole process repeated. The transparency of the photomask has been adjusted for demonstration purposes.

Table 2.11: Photolithography equipment, chemicals and supplies necessary for a simple microfabrication setup.

Equipment	Model No.	Manufacturer
Mask aligner	Model 200	OAI
Spin processor	WS-400BZ-NPP-Lite	Laurell Technologies Corporation
Surface profilometer	Dektak 150	Veeco
Infrared thermometer	62	Fluke
Hot plate		
Fume hood		
Chemicals and Supplies	Part No.	Supplier
SU-8 Photoresists 2000.5-2050 (500ml)	varies	MicroChem
SU-8 Developer (4L)	Y020100-4000L1PE	MicroChem
SU-8 2000 Thinner (4L)	G010100-4000L1PE	MicroChem
AlphaLite Polyester swab	18-375	Fisher Scientific
Glass bottle (amber)	41265T31	McMaster-Carr
Instant Adhesive	495045	Loctite
Borosilicate glass square, 3' X 3', 1/8" thick	8476K131	McMaster-Carr
Silicon Wafer	100MM/CZ/1-0-0/Boron/ P Type/ Resis-10-20/ Thick 500-550/Oxy 9-21/ SLBACK: ETCH ACID	WaferNet, Inc.
Wafer tray	H20-3000-01-1415	Entegris, Inc.
Wafer cover	H20-3000-02-1216	Entegris, Inc.
Wafer tweezers (125mm)	S3WF	SPI Supplies
Crystallizing Dish (740ml)	08-741E	Fisher Scientific
Wash bottles (500ml)	08-647-707	Fisher Scientific
Acetone		
DI Water	Milli-Q or better	
Isopropanol	HPLC grade	
Methanol	HPLC grade	

the wafer. Furthermore, treating the wafer with a release agent improves the peel-off process (Xia and Whitesides, 1998; Whitesides et al., 2001; Duffy et al., 1998; Sia and Whitesides, 2003). All the tools, chemicals and equipment required for soft lithography are listed in Table 2.12

Soft Lithography: Protocol

2.8.1 Aluminum holder

Cut out a 20 cm circle from aluminum foil. Place the wafer, features up, in the center of the foil. Next, carefully holding the wafer down, start to fold the foil up all the way around the perimeter. This will create 5 cm high walls around the wafer that will

hold PDMS in. Make sure that the foil is really tight against the edge of the wafer, this prevents significant leaks of PDMS under the wafer.

2.8.2 Applying release agent (for new wafers only)

It is necessary to perform this step in a fume hood following all safety precautions, as most release agents are toxic. Place the wafer into a dedicated silanizing desiccator. Using a syringe with a needle, draw up the release agent, we use (TRIDECAFLUORO-1,1,2,2-TETRAHYDROOCTYL)-1-TRICHLOROSILANE. Deposit only a single drop ($\sim 30 \mu\text{l}$) of the release agent into an open top small container inside the desiccator, see Figure 2.21. Close the lid of the desiccator and turn on the vacuum. The release agent will vaporize and evenly deposit onto the wafer. Let this reaction happen for about 15 minutes. Using too much release agent will inhibit PDMS binding to glass coverslip.

2.8.3 Preparing PDMS

In a clean weighing tray measure out, in 10:1 ratio, and mix 40 grams of silicone elastomer base with 4 grams of silicon curing agent. Continue vigorously mixing with a clean spatula. The consistency of the mixture should start to change from clear to foamy. Mix the components thoroughly for 3 minutes.

2.8.4 Degassing PDMS

Mixing introduces a lot of air bubbles into the PDMS. To degas, place the weighing tray into the dedicated desiccator and turn on the vacuum. As pressure within the desiccator drops, the trapped air bubbles will expand and PDMS might spill out of the tray. Quickly releasing the vacuum should pop a significant portion of the bubbles. Turn on the vacuum again and repeat this cycle until there are no more bubbles. Depending on the vacuum pressure this should take 10-20 minutes. Also, it is possible to degas by

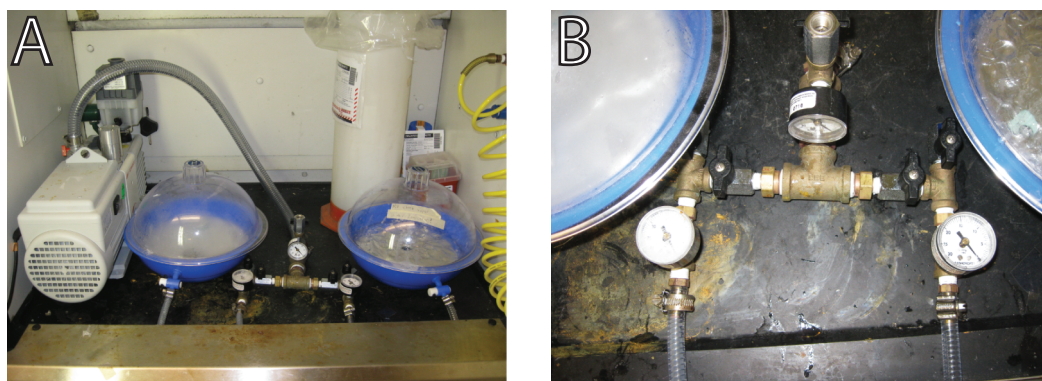


Figure 2.21: Vacuum pump and desiccators. **A.** Vacuum pump and desiccators located in the fume hood. Each desiccators for a single purpose: 1) wafer silanizing (left) and 2) PDMS degassing (right). Note the opaqueness of the silanizing desiccator, this is due to silanizing agent vapor deposition over the years. **B.** Vacuum manifold connecting the vacuum pump to the desiccators. The manifold allows for individual control of vacuum or atmospheric pressures to each desiccator. See Table XII for parts list.

pouring mixed PDMS into a 50 ml Falcon tube and centrifuging it at $\sim 2700g$ for 10 minutes.

2.8.5 Pouring PDMS

Place the wafer into the degassing desiccator and pour the PDMS over it. Since PDMS is very viscous, you might have to use a spatula to get all of it onto the wafer. This process will introduce new air bubbles into the PDMS. Repeat step 4 until there are no visible bubbles in the PDMS. Sometimes the PDMS will leak under the wafer and you will see bubbles forming around the perimeter of the wafer. You can ignore them when considering to stop degassing.

2.8.6 Curing

Place wafer in 80°C oven for 1 hour.

Table 2.12: Soft lithography equipment, chemicals and supplies. Parts necessary for casting PDMS devices from a pre-made wafer mold.

Equipment	Qty	Part No.	Vendor
Vacuum pump RV8	1	A65401906	Edwards
Vacuum pump EMF 10 exhaust mist filter	1	A46226000	Edwards
Vacuum pump oil return kit	1	A50523000	Edwards
Vacuum pump inlet connection (NW25 to 3/4" hose barb)	1	NGT908000	Edwards
Vacuum pump NW25 clamping ring	1	C10514401	Edwards
Desiccators	2	08-642-5	Fisher Scientific
Ceramic desiccator plate	2	08-642-10	Fisher Scientific
Isotemp Oven	1	506G	Fisher Scientific
Vacuum manifold parts	Qty	Part No.	Vendor
1/2" stainless steel hose clamps	5	6151K51	McMaster-Carr
1" stainless steel hose clamps	5	6151K53	McMaster-Carr
1' 3/4" ID, 1" OD wire-reinforced tubing	5	5393K45	McMaster-Carr
1' 1/4" ID, 1/2" OD wire-reinforced tubing	10	5393K31	McMaster-Carr
3/4" MPT to 3/4" barb adapter	1	5365K23	McMaster-Carr
3/4" FPT to 3/4" FPT to 1/4" FPT tee	1	4429K229	McMaster-Carr
1/4" MPT to 1/4" MPT nipple	3	9171K122	McMaster-Carr
1/4" FPT to 1/4" FPT to 1/8" FPT tee	3	4429K223	McMaster-Carr
1/4" MPT to 1/4" FPT T-handle valve	5	4912K87	McMaster-Carr
-30 in Hg vacuum gauge with 1/8" MPT at back	3	3935K21	McMaster-Carr
3/4" MPT to 1/2" MPT reducing nipple	1	9171K223	McMaster-Carr
1/2" FPT to 1/2" FPT to 1/2" FPT tee	1	4429K253	McMaster-Carr
1/2" MPT to 1/4" MPT reducing nipple	2	9171K219	McMaster-Carr
1/4" FPT to 1/4" FPT to 1/4" FPT tee	2	4429K251	McMaster-Carr
1/4" MPT to 1/4" barb adapter	2	53505K64	McMaster-Carr
PTFE thread seal tap	1	4591K12	McMaster-Carr
Chemicals and supplies	Qty	Part No.	Manufacturer
Silicone elastomer kit (TRIDECAFLUORO-1,1,2,2-TETRAHYDROOCTYL)-1-TRICHLOROSILANE		Sylgard 184 T2492	Dow Corning UCT
Aluminum foil			

2.9 PDMS processing

During the final phase of manufacturing the individual chips are cut out, cleaned and bonded to coverslips. Although the processing is performed in regular lab environment it is critical to get the chips and coverslips as clean as possible. This eliminates debris from the chip and improves the overall quality of the devices. To improve the final bond between PDMS and glass coverslip, it is recommended to complete soft lithography and PDMS processing in the same day. All the required materials and tools for this phase of manufacturing are listed in Table 2.13.

PDMS processing - Protocol

2.9.1 Removing PDMS layer

Take the wafer out of the oven and let it cool down to room temperature. Carefully peel of the foil from PDMS. Some PDMS may have gotten under the wafer. You need to remove this layer prior to peeling off the top layer of PDMS. Using a razor blade, cut the bottom layer as close to the edge of the wafer as possible. It is also possible to rub the edge of the wafer with your gloved finger. This will break the PDMS on the edge, disconnecting the bottom and top layers of PDMS. Very slowly lift up the top layer of PDMS. Allow the PDMS to lift off from the wafer by itself, this is best done by raising a part of PDMS to a small height, stopping and letting the PDMS catch up. Lift up the PDMS in 3-4 places around the perimeter of the wafer, before peeling it off completely. Wafers are very brittle, so make sure not to twist or apply excessive pressure on it, as it will easily break. For safe storage place the wafer into a labeled wafer holder.

2.9.2 Cutting PDMS

Using the dissecting scope, examine the features on the PDMS. Sometimes the angle of the light source needs to be adjusted to get enough contrast to see the microscopic features. Placing the PDMS on a dark background also improves contrast. Next, using a razor blade carefully cut out individual chips, leaving extra room around the perimeter of the chip. Try to leave at least 3mm of extra PDMS around each port, it will improve the chip's bonding and prevent port leaks.

2.9.3 Punching ports

Place the chip with feature side up and, using the dissecting scope, locate the outline of the port. Place the tip of 25 gauge leur stub within the outline and, making sure it is as vertical as possible, apply downward pressure. The PDMS should first deform and then break; sometimes a final push is required to completely break through

the PDMS on the exit. Next, carefully pick up the PDMS chip and remove the PDMS core using tweezers. Slowly pull out the puncher from the hole, while rotating it back and forth. Continue this for all ports on the chip. Sometimes the punching will tear the PDMS around the port, this is most likely due to a dull punching tip. Simply, swipe the punching tip against an abrasive surface 2-3 times and retry the punching. It is also possible to use a biopsy punch, which combines the leur stub and tweezers in a single tool, to make the holes.

2.9.4 Cleaning ports

Attach a 23 gauge leur stub to a syringe and fill it with DI water. Hold the tip of the leur stub against a port and apply pressure. A stream of water should exit from the other side of the chip. Keep the pressure for 3-5 seconds. Repeat this process of all ports on both sides of the chip.

2.9.5 Cleaning chips

Spray each chip with 70% Ethanol and gently rub using your gloved finger. Thoroughly rinse the chip with MilliQ quality water and blow dry using clean dry air. Make sure to dry both sides of the chip and all the ports. Place the dry chips in a clean Petri dish. Apply scotch tape to both sides of the chip. The next step is crucial for clean chips. Careful not to tear the PDMS, run your fingernail over the features a few times, covering the are of the whole chip. Repeat the scotch tape cleaning 3-5 times. Once done, use a fresh piece of tape to cover the chip and put the chip in the Petri dish.

2.9.6 Cleaning coverslips

Spray both sides of the coverslips with n-Heptane and gently rub the surface using your finger. To prevent the coverslip from breaking, apply pressure using your finger on both surfaces at the same time. Wipe the coverslip completely dry with a

Kimwipe. Repeat the process using Methanol. Finally, wash the coverslips with DI water and dry using clean air. Make sure the coverslips are completely free of dust, spot or streaks. If you notice something, redo the DI water wash step. Once done, place the clean coverslips into a Petri dish and cover.

2.9.7 Bonding chips to coverslips

Open the compressed O₂ valve on your tank and make sure the flow through the UVO cleaner is 0.4-0.6 scfm. Warm up the UVO cleaner, by running it for 5 minutes. Once the warm up is done, open the loading tray, there should be a faint smell of ozone. Place the chips with feature side up and coverslips onto the tray. Close the tray and run the bonder for 3 minutes. When done, open the tray and place the chip onto the coverslip using tweezers. To improve the bond, using tweezers, gently apply pressure around the perimeter of the chip. Make sure that chip and coverslip come in contact as soon as possible, as the chemistry allowing for bonding changes with time. Place bonded chips in 80°C oven overnight. If you have a lot of chips, it might be beneficial to break up the bonding step in 2 or more batches.

2.9.8 Troubleshooting

Poor chip bonding - this can be caused by a number of issues.

- 1) Too much release agent used during wafer preparation. Try lowering the amount of release agent or shortening the coating time.
- 2) Check O₂ supply to UVO bonder.
- 3) Expose the chips and coverslips exactly for 3 minutes. Make sure to bond chips and coverslips immediately after exposure to ozone.
- 4) Place a weight on top of the chips during overnight baking. Make sure not to break the coverslip.

Collapsed features:

- 1) Usually only the lowest features will collapse, but if enough pressure is applied from the top of the chip even taller features are susceptible. Lower the amount of pressure applied on the top of the chip during bonding.
- 2) Try placing the coverslip on top of the chip during bonding. This should prevent features lower than $0.5\ \mu\text{m}$ from collapsing.

Table 2.13: PDMS processing equipment, chemicals and supplies. Setup needed to clean, punch holes and bond PDMS to glass coverslips.

Equipment	Qty	Part No.	Vendor
Dissecting scope	1		
Fiber optic light source	1	Dynalite 150	A.G. Heinze, Inc.
UVO Cleaner	1	Model No. 42	Jelight Company Inc.
Flowmeter	1	FR4A37	Key Instruments
1/8 Male pipe adapter	2	5454K65	McMaster-Carr
Polyurethane tubing			
Chemicals and supplies	Qty	Part No.	Manufacturer
Leur stub (25 gauge)		75165A686	McMaster-Carr
Biopsy punch Harris Uni-Core 0.5 mm		15071	Ted Pella, Inc.
Razor blades		12-640	Fisher Scientific
10ml Disposable syringe		14-823-2A	Fisher Scientific
Cover slips No. 1 1/2, size: 24x40 mm, thickness: 0.16-0.19 mm		12-530F	Fisher Scientific
Magic Tape		810	Scotch
Kimwipes, Kimberly-Clark No. 34155		06-666A	Fisher Scientific
Compressed O ₂		Medical grade	
n-Heptane		HPLC grade	
Methanol		HPLC grade	
DI Water		Milli-Q or better	

2.10 Experimental setup for *E. coli*

Although it is possible to perform microfluidic experiments without a lot of specialized equipment, we have found that purpose-built tools, such our DAW and syringe towers, greatly increase productivity and experiment control. As mentioned in earlier sections, we use linear actuators to control the hydrostatic pressure of syringes, Figure 2.23B. However, we use special syringe towers, as shown in Figure 2.22, for controlling the height of our static syringes used for waste, cell and shunt ports. The towers are

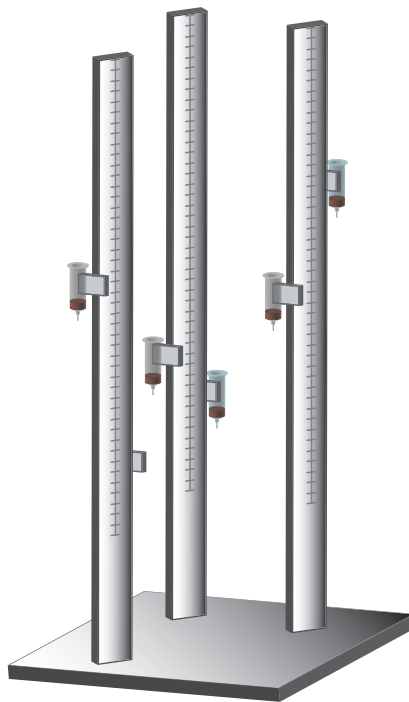


Figure 2.22: Syringe towers. Made from commercially available erector set, the towers provide support for static syringes. We use a three pillar design with six adjustable platforms, which hold 2-9 syringes each. The ticked lines in the drawing represent rulers that are used for consistent syringe placement. All the parts necessary for constructing the tower are listed in Table 2.14

equipped with rulers, allowing us to record the position of syringes for an experiment. This data is used in subsequent experiments to reliably reproduce flows within the chip. Image data acquisition is performed by a Nikon fluorescence microscope, see Table 2.16 for component list. Our complete experimental setup can be seen in Figure 2.23A, and functional mDAW chip with attached lines in Figure 2.23C.

For experiments, we modify our standard LB media, by adding 0.075% Tween 80 and filtering it through 0.22 μm filter. Addition of Tween 80 prevents the cells from sticking to chip walls without any noticeable harm to the cells. Depending on your experiment, make sure to add antibiotics and any inducers to the media.

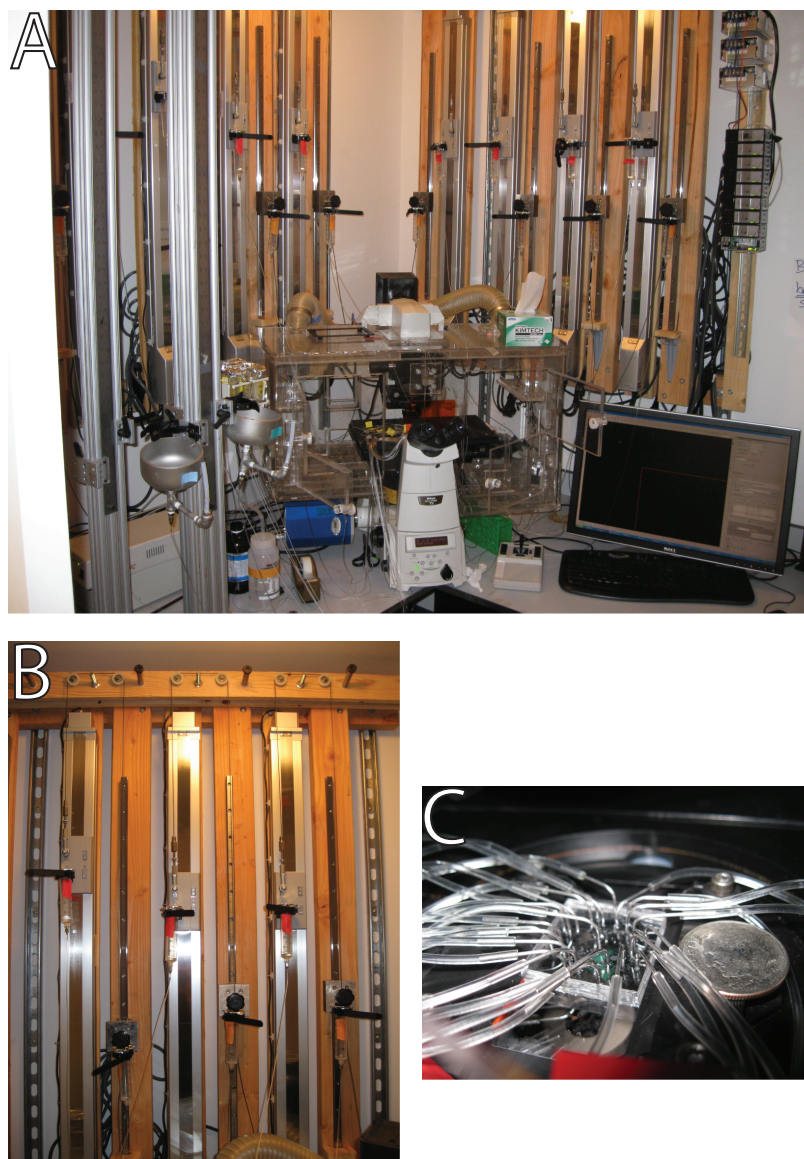


Figure 2.23: Experimental setup including microfluidics, microscope and linear actuators. **A.** The equipment setup for mDAW experiments. In the background one can see the linear actuators, it is possible to fit all 8 actuators and 8 linear guides in a compact space behind the microscope. Fluorescent microscope with environmental chamber can be seen in the foreground of the image. **B.** 3 linear actuators with linear guides and pulley systems. This is a photograph of the system described in Figure 15B. **C.** An mDAW chip with all the connection pins and lines attached. US dime coin (diameter 17.91mm) is shown for scale.

2.10.1 Overnight culture

Grow up an overnight culture of cells from -80°C stock or from a plate. Grow the cells in 3 ml of LB media with appropriate antibiotics in 37°C shaker incubator.

2.10.2 Cell growth

Dilute the overnight culture by a factor of 1:1000 into 50 ml of fresh media with appropriate antibiotics and inducers. Let the cells grow up to a culture density of OD600 0.05-1.0, we usually try for OD600 0.1. Depending on the cell type this step should take 2-3 hours. During this time perform steps 3-6.

2.10.3 Wetting the chip

Secure the chip in a chip holder, using rubber gaskets for additional contact. We have a custom chip holder, details for which can be provided by request. Basically, it securely holds a 24 x 40 mm cover slip, while allowing light access from the bottom, physical access from the top, and secure attachment to the microscope's stage. Place the chip under microscope at 4X magnification. It is important to examine the chip for dirt and collapsed channels. This is best done at lower magnification, as you can see a larger area. Make sure that there is no debris blocking the channels or the imaging areas. Collapsed traps or channels will look darker and generally resemble in shade bonded parts of the chip. If the chip looks good proceed to wetting. Wetting the chip can be done using hydrostatic or manual pressure applied through a syringe. Attach a leur stub, a microfluidic line and a connection pin to a syringe and fill with fresh media. Make sure there are no bubbles in the syringe or the line. Bubbles can be removed by flicking the syringe or the line with your finger. Carefully insert the pin into a port. The color of the channels should start to change as fluid fills them. If using hydrostatic pressure to wet, position the syringe on the towers as high as possible and let the fluid flow through the chip. If using manual pressure, make sure to apply gentle pressure as

too much pressure will lift the chip off the coverslip. As media fills the chip it will come up through the open ports and start forming droplets on the surface of the chip. Repeat this process for all the ports and until there are no more air bubbles in the chip. Media removal from the surface of the chip is best accomplished using a kimwipe.

2.10.4 Preparing syringes

Attach a sterile 23 gauge leuc stub to a clean 60 ml syringe. Take 6 feet of Tygon tubing and gently slide it over the leuc stub. Attach a connection pin to the other side of the tubing. A connection pin is basically just the metal part of a 23 gauge leuc stub. We make them by pulling out the metal tips from the plastic part of the leuc stub using pliers. The phase condensers on some microscope may come in contact with the straight connection pins. To circumvent this issue we make L-shaped pins by bending them around the shank of a 10-32 wood screw using pliers, refer to Figure 2.24 for exact instructions on making straight and L-shaped pins.

Depending on the intended use for the syringe, remove the plunger and extract 100 μ l of media or dH₂O with a P200 pipetman. Insert the tip of the pipetman into the syringe and make contact with the inside of the leuc stub adapter. Slowly expel the fluid into the leuc stub adapter which should be enough to fill it, as shown in Figure 2.25C-E. Adding fluid to the leuc stub adapter in this way greatly reduces bubble formation. Tilt the syringe slightly and gently pour the rest of the media or dH₂O into the top of the syringe, letting it run down the side before it reaches the base of the syringe. This also helps in preventing bubbles. Flick the leuc stub connector to cause media to flow into the microbore tubing. If difficult bubbles are present, partially unscrew the leuc stub adapter about one half turn and then retighten. This can help release bubbles. If fluid still will not enter the microbore tubing, use the syringe plunger to force the fluid in. Note if the plunger is necessary, it usually indicates a severe bubble problem. Make extra sure all bubbles are removed before proceeding. Watch the fluid flow carefully through the microbore tubing line to the exit point at the leuc stub. Carefully look over the line to

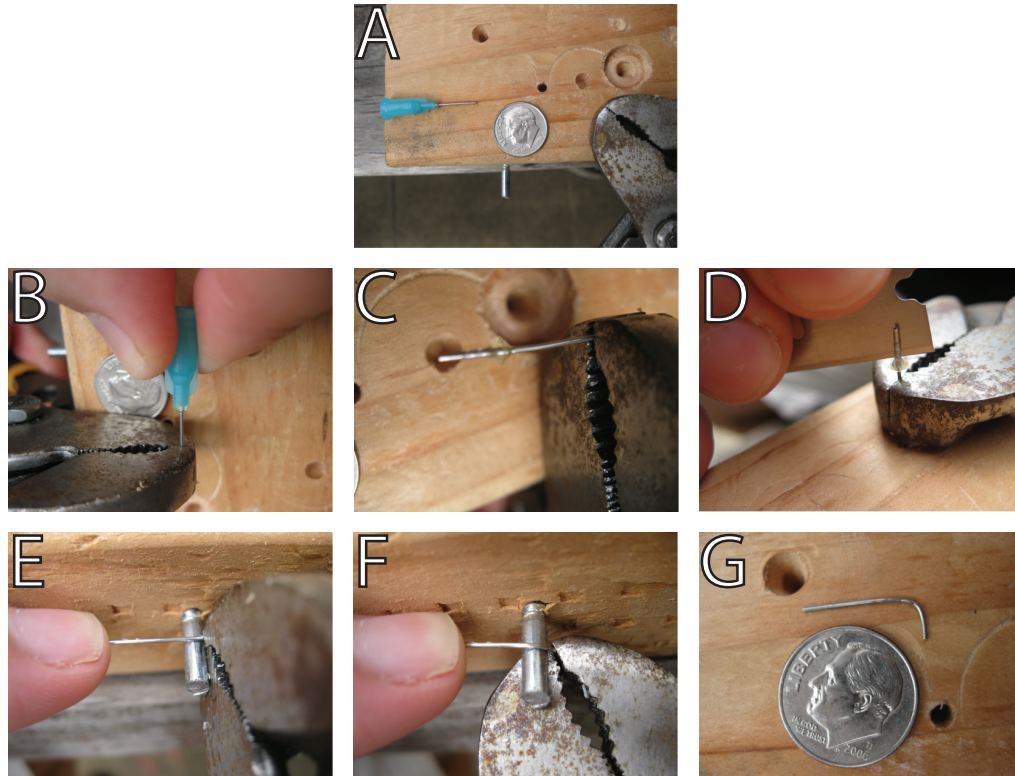


Figure 2.24: Making connection pins. **A.** A non-sterile 23 gauge luer stub, dowel pin, and pliers are used to make the connection pins. **B.** Using pliers grab onto the metal part of the luer stub, while holding the plastic part with your fingers. Pull them in opposite direction until they separate. **C.** The metal pin alone, notice all the sealant and glue on it. **D.** Using a razor blade, carefully remove all the glue from the outside of the pin. To make straight connection pins the process is finished at this point and pins just need to be cleaned in a sonicator. **E.** Holding the pin with pliers, place it over the dowel pin. **F.** While holding one of the ends of the pin with you finger, gently rotate the other end around the dowel pin. **G.** Finished L-shaped connection pin. This method preserves the inner radius of the connection pin. Simple bending it will most likely pinch the pin. US dime (diameter 17.91mm) shown for scale.

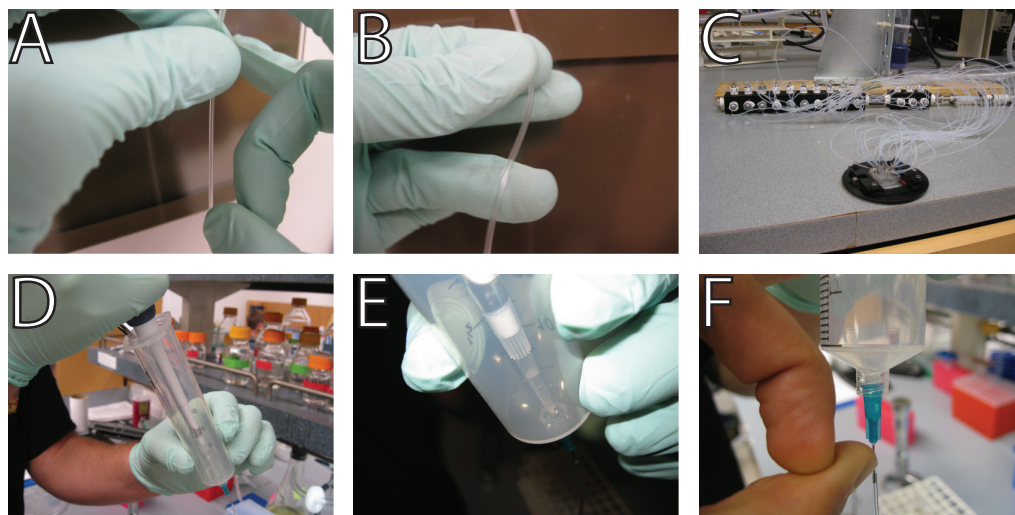


Figure 2.25: Experimental line and syringe techniques. **A.** General guide for flicking a microfluidic line. Hold the line between the thumb and index finger of one hand, while flicking the downstream the line with a finger on your other hand. In the figure, the hands are place on the line so that the left hand is closer to the syringe and the right hand is closer to the microfluidic chip. **B.** Technique for gentle agitation of fluid within the microfluidic device. Hold the line between thumb and index finger. Gently move the line back and forth using your ring finger. The ring finger is placed on the line towards the microfluidic device. **C.** The metal pin alone, notice all the sealant and glue on it. **D.** Technique for minimizing bubbles during syringe preparation. Using a P200 pipetman draw up 100 μ l of desired liquid. Remove the plunger from the syringe. Insert the pipetman into the syringe and hold both at a slight angle. **E.** Insert the pipette tip all the way into the leur stub adapter and slowly expel the liquid. **F.** Flicking the bottom of the syringe to fill the connection pin with liquid.

ensure no bubbles are present. If bubbles are present flick the lines to release them and watch them flow to the end of the microbore tubing. Cover the syringe top using a piece of foil or parafilm, while leaving a small opening to the atmosphere. Label the syringes appropriately and make sure the connection tips do not touch any surface.

2.10.5 Connecting syringes

Attach all the syringes to the sliders on the microfluidic tower, as seen in Figure 2.22. Adjust syringe heights appropriately. To prevent contamination of the media source, make sure it is always the highest positioned syringe. One by one, starting with the media, connect each syringe to the chip. Since the cells are not ready yet, connect a syringe filled with DI water to the cell port. Examine that no bubbles were introduced

into the chip during this process. If there are bubbles, see if they can slowly disappear on their own. However, it might be necessary flush the chip by disconnecting all the syringes and repeating Step 3. Once all the bubbles have been eliminated, using scotch tape secure each line to the chip holder. Tape far enough away from the chip, so that the bending of the tubing is not applying a force on the connection pin.

2.10.6 Setting up DAW software

Using the software, calibrate the syringe heights for correct mixing ratios. Create a desired profile for the syringe movement. Make sure that all the static syringes are in their "running" positions.

2.10.7 Spinning down cells

When the cells are ready, spin them down at 2700g for 10 minutes. As a backup, pour the supernatant media back into the flask and place it in incubator. Add 2-3 ml of fresh media to the pelleted cells and gently vortex them, until there are no cell clumps. Load the cells into a prepared syringe. Once again, make sure there are no bubbles.

2.10.8 Loading cells

Move all syringe to their "loading" positions. Disconnect the temporary cell syringe and plug in the actual cell syringe. At this point the flow from the media and the cell port both should be going towards the waste port. At 20X magnification you should be able to see cells flowing through the channels. Adjust the height of the cell syringe so that the cells are slowly moving past the traps. Next, securely hold the cell line between the thumb and index finger of one hand, while flicking the line with a finger on your other hand as seen in Figure 2.25A. Imagine that the pinching fingers divide the line into two parts: the syringe part and the connection pin part. The flicking should be done on the connection pin part of the line. The cells should rapidly move back and

forth within the chip, as the flicking wave propagates down the line. Adjust the flicking strength to have enough force to load the traps. Once enough traps have been loaded, adjust the syringe heights to their “running” positions. Media flow should be 20-200 $\mu\text{m/s}$.

2.10.9 Starting experiment

Allow the cells to grow in the traps for 3-5 doublings, depending on you cell type this should take 1-2 hours. Setup the microscope software for you run. Start the imaging and the *iDAW* software at the same time.

2.10.10 Checking on cells

During the experiment it might be necessary to remove stuck cells from the channels. Hold the cell line between the thumb and index finger with your middle finger further away from the syringe. Gently move the line back and forth using your ring finger and watch as the cells smoothly mirror the motion, see Figure 2.25B for a visual representation. This technique is useful for getting rid of stuck cells or controllably reducing cell density within a trap.

2.11 Methodology for setting up a MDAW microfluidic experiment

In this section we will describe how to setup a microfluidics experiment using the MDAW parallel DAW microchemostat chip since this chip presents challenges not seen for smaller chips.

Table 2.14: Experimental equipment, chemicals and supplies. Everything needed to setup and run a microfluidic experiment.

Equipment	Qty	Part No.	Vendor
Inverted fully automated microscope	1	Ti	Nikon
PDMS chip holder	1		Custom
Syringe towers			
Aluminum bread board, 12" x 12" x 1/2", 1/4-20 threaded	1	MB12	Thorlabs, Inc.
1" x 3" Extrusion 60" long	3	1030x60"	80/20, Inc.
8 Hole inside corner gusset	3	25-4138	80/20, Inc.
Slide-in T-nut	6	3382	80/20, Inc.
Double Slide-in T-nut	6	3280	80/20, Inc.
1/4-20 x 1/2" Flanged button head socket cap screw	6	3342	80/20, Inc.
1/4-20 x 1/2" Socket head cap screw	12	3062	80/20, Inc.
1/4-20 x 3/8" Socket head cap screw	6	3058	80/20, Inc.
1/4" washer - black zinc	20	3258	80/20, Inc.
Double flange linear bearing brake kit ready	8	6425	80/20, Inc.
Ratcheting L-handle	8	6850	80/20, Inc.
White UHMW Pads w/ brake hole	24	6490	80/20, Inc.
#8 x 3/8" SS standard bearing pad screw	24	3625	80/20, Inc.
48" stainless steel rule	3	2120A15	McMaster-Carr
1" adjustable strap	1 Pkg	7565K51	McMaster-Carr
Chip holder rubber gasket		7665K11	McMaster-Carr
Chemicals and supplies	Qty	Part No.	Manufacturer
Connection pins (23 gauge, ID 0.017", OD 0.025", 1/2" Long)		75165A684	McMaster-Carr
Single use, sterile leur stub (23 gauge)		14-826-19E	Fisher Scientific
Reusable leur stub (23 gauge, ID 0.017", OD 0.025", 1/2" Long)		JGM23-0.5D	Jensen Global Inc
Disposable syringe 10 ml w/ leur lock tip		14-823-2A	Fisher Scientific
Disposable syringe 30 ml w/ leur lock tip		14-829-48A	Fisher Scientific
Disposable syringe 60 ml w/ leur lock tip		13-689-8	Fisher Scientific
Tygon flexible microbore tubing (ID 0.020", OD 0.060")	1	14-170-15B	Fisher Scientific
Tween 80	1	P8074	Sigma-Aldrich
Alconox 1104	1	04-322-4	Fisher Scientific
Liquid cell media			
DI Water		Milli-Q or better	

2.11.1 Pre-experiment preparation

See Table 2.14 for the catalog numbers of supplies listed here. The steps described in this section should be performed at least one day in advance of the experiment. The microfluidic devices themselves should be prepared as described in section 2.9. Cut 26 lines using Tygon microbore tubing seven feet in length. Note the line length is dependent on the microscopy setup, there should be some slack to allow for movements of the syringe reservoirs. Obtain 26 sterile 30ml syringes. Note for the combined ports we use 4 inch stainless steel pipe caps fitted with leur stub adapters. If these pipe caps are used then only 24 30ml syringes are necessary. These pipe caps were manufactured by our university's machine shop, details can be provided by request. Due to their large

diameter, the liquid height in the pipe caps changes very little for a given volume of accumulated fluid. This is important since the combined ports receive vastly more fluid than the individual ports during an experiment. If a 30ml syringe were used instead of a pipe cap, it is conceivable that the height increase would affect flows in the chip over the course of a long experiment (2-3 days). See Figure 2.5 and the accompanying text for an example of these issues.

To clean the metal parts, sonicate 26 reusable leur stub adapters, 26 90° curved connection pins (see 2.24) and the pipe caps if they are to be used, for 60 min at 60°C in a 250 ml beaker containing 1% w/v Alconox. Sonication in 1% w/v Alconox does an excellent job of removing cell debris and residual media from small metal parts. After sonication rinse the parts in dH₂O. Flush water through the leur stub adapters and connection pins to remove residual Alconox. We use a manifold to flush all metal parts at once, see Figure 2.25C. In general we flush 3 liters of dH₂O through the entire system for rinsing. After flushing with water, air can be flushed for drying. Autoclave the leur stubs, adapters and, if using them, pipe caps for 30 minutes on a dry cycle.

Prepare the 26 (24 if using pipe caps) syringe reservoirs as described in 2.10.4. Cut 8 sections of red, orange and yellow tape. Write 1-8 on each of the colored tape sets and affix to the syringe bodies. The tape will help to identify the syringe reservoirs later in the setup. Each of the 8 subexperiments has two DAW input ports (A and B) and a cell port (C). We use the red tape to refer to A reservoirs, orange for B and yellow for C. Cut another three sets of colored tape and again write 1-8 on each set. Affix the tape near the end of the microbore tubing (just before the connection pin) for the appropriate reservoir. Labeling the end of the tubing helps to identify its connected reservoir, a necessity when many lines are nested together. Use scotch tape to affix the loose microbore tubing end to the syringe.

2.11.2 Cell Growth

Determine how many cell cultures will be needed, a maximum of 8 can be used. Inoculate the cell cultures the day before the experiment with the appropriate media and additives. In the next morning check the culture optical density at 600nm (OD600) using a spectrophotometer. Grow cells at 30°C for ~4 hours, to an OD600 of ~1.0 upon cell loading.

2.11.3 Media preparation

Prepare 4 ml of media for each of the 16 input syringes (ports A and B for each of the 8 subexperiments). Generally the media is the same in the two inputs except for the tested component. See Table 2.15 for an example of the media composition. Add dye to one of the two input reservoirs for each subexperiment to use as an inducer tracer. After the media has been prepared, add it to each of the A and B syringe reservoirs as described in section 2.10.4. Add sterile dH₂O to the shunt and alternate waste reservoirs. If 30 ml syringes are used, add 4 ml of dH₂O. If pipe caps are used add ~100 ml.

Table 2.15: Experimental medias and solutions for a sample *Saccharomyces cerevisiae* microfluidic run.

	Ingredients	Stock	Final	units	Lot	1X (μ l)	FX (ml)
1	2X SC - met media	2	1	X		2000	5
2	Methionine	50000	500	μ M		40	0.1
3	Galactose	2	0.2	% w/v		400	1
4	Raffinose	20	2	% w/v		400	1
5	ddH2O	-	-	-		1160	2.9
total (μ l):						4000	10

2.11.4 Air removal from the chip

Note that this procedure differs from that of a smaller chip (such as MFD005_a). While removal of air can also be facilitated using a vacuum, we have found this to interfere with our cell's growth under some conditions. Affix a bonded MDAW chip

onto a solid substrate, like a glass plate or a microscopy chip holder, as the one described in Step 3 of *E. coli* experimental section. Fill a 10 ml syringe with a sterile solution of 0.1% v/v Tween 80 and connect it to the central port of the chip (called the alternate waste port). Tween 80 is a surfactant that aids in clearing bubbles. It also acts as a lubricant that prevents cell clogging. Purge air from the chip by applying force to the syringe. Watch for droplets to appear at each port indicating that fluid has propagated through the chip's channel network.

We have a custom built 26 outlet manifold connected to a pressure reservoir filled with dH₂O. Each outlet of the manifold is connected to a half meter of teflon microbore tubing with a connection pin at the end. The manifold is fully autoclavable and by pressurizing all ports at once, achieves better clearance of air. Details of the manifold's construction can be provided upon request. To purge air from the manifold we pressurize it until water flows out of each connection pin. Next we connect each pin to a port on the chip, making sure a "fluidic connection" is made, i.e. there is a visible droplet of fluid above each port on the chip and fluid is leaving the connection pin of the manifold). When the manifold is fully connected it is then pressurized to 4 Psi for five minutes to flush all air from the system.

2.11.5 Connecting DAW reservoirs to the device

After the air has been purged from the system, place the chip in a microscopy holder if not done already. Secure both ends of the device with scotch tape. Place the chip above the microscope stage. Our microscope has an acrylic environmental chamber around it, whose top is about 25 cm above the stage height. We place the chip on top of this box. Attach the shunt and alternate waste port reservoirs to the syringe towers. Adjust the height of the shunt reservoir to 30 cm above the stage. Adjust the height of the alternate waste reservoir to 37.5 cm above the stage. Take the connection pin from the shunt reservoir and place it several centimeters below the reservoir's fluid level. Wait for fluid to exit the end of the microbore tubing line and then connect the leur stub to the

shunt port at the top of the device. Repeat this procedure to connect the alternate waste reservoir to the device. Connect the DAW input reservoirs for each subexperiment to each set of A and B ports on the device using the same procedure. After connecting the reservoirs attach them to the linear actuators. The linear actuators should be set so each reservoir is 60 cm above the stage height. Once all of the input reservoirs have been connected bundle the lines together with scotch tape so they do not become unwieldily.

2.11.6 Processing and loading cells

At this time remove the cell cultures from the incubator and record the final OD600 value if desired. Add Tween 80 to each cell culture to a final concentration of 0.1% v/v. Vortex on a medium setting to mix. Add each culture to the appropriate syringe reservoir as described in section 2.10.4. Be extra careful there are no bubbles in any of the cell reservoirs. While using Tween 80 helps to prevent bubbles, any that remain in the reservoir will make it extremely difficult to load cells later on. The Tween 80 will also prevent clogging of the device by excess cells.

Adjust the height of the shunt port to 11.25 cm above the height of the stage and make sure the cell reservoir holder is set to 32.5 cm above stage height. This adjustment will ensure cells flow into the shunt and not other cell ports. At this time, all cell ports will contain a bead of fluid above them since they are the outlets for the other connected reservoirs. This bead of fluid will essentially function as a small reservoir. Since the device should still be placed at 25 cm above stage height, this will be the pressure of each cell port before its reservoir is connected. Once the shunt port is lowered there will be a net flow between the fluid bead of each cell port and the shunt. When the cell reservoirs are connected their pressure will increase to 32.5 cm. If the shunt port were not lowered some flow would exit at the unconnected cell ports, possibly causing cross-contamination.

Once all cell ports have been connected, place the chip into the microscope and tape down all microbore tubing lines. Adjust the height of the cell ports to 40 cm and

observe the cells entering the system at 4X magnification. If cells are not entering from the cell ports it is usually due to residual bubbles in the cell lines. Disconnect if necessary and make sure there are no bubbles. Adjust the height of the alternate waste port to force more cells into the central region of the trap if necessary. Flick the lines for each cell port to load cells into the trapping region. Continue this procedure until an adequate number of cells have been loaded (generally 20-40 yeast cells). Once the cells have been loaded adjust the heights of all reservoirs as follows: Cell ports: 15.5 cm, combined alternate waste: 14 cm, combined shunt: 11.25 cm. All heights above stage height. The level of the DAW inputs should remain at 60 cm above stage height. If desired move to the DAW junction of each subexperiment and record the height positions for 0% and 100% mixing ratios or use the calibration procedure described in section 2.2.5.

2.11.7 Microscope setup

Record the stage locations for each of the cell traps in the 8 subexperiments in the microscopy software. Switch to a 40X or 60X objective and add microscopy oil as necessary. Update the xy positions for each trap as they will have changed slightly. Setup the microscopy software for a multiple location experiment, using appropriate exposure settings for phase contrast and any fluorescence wavelengths. Make sure the autofocus routine is properly setup. Since the MDAWchip is quite large, there will likely be a z offset between the cell traps of each subexperiment. This z offset needs to be compensated for. Moreover, due to stage drift over the course of an experiment the z offset will shift in time. Some microscope software packages cannot cope with this properly and we have written a custom macro for the NI Elements software to compensate for this changing z offset. The macro uses the median of the last five focal planes for each cell trap to calculate an updated z offset. This z offset is used as the best guess for where to start the next iteration's autofocus routine. Taking the median prevents a single poor autofocus result from causing a catastrophic loss of focus, which can happen if a bubble in oil droplet drifts into the field of view. We have had good

success with this macro, retaining focus even after almost 72 hours of an experiment. Set the linear actuator controller software for the proper input waves as described in section 2.5.2. Begin image acquisition.

2.12 Appendix

Table 2.16: Components of Nikon Ti automated fluorescence microscope used in our microfluidics experiments.

Description	Qty	Part No.
Ti-E Inverted Microscope	1	MEA53100
Ti-HUBC/A Hub Controller A	1	MEF55030
Ti-AC/A AC Adapter for HUBC/A	1	MEF51010
Ti-AC120 Power Cord 120V	1	MEF51200
USB 2.0 Cable A-B 15', Required for DS-U2 Controller	1	97050
Ti-DH Dia Pillar Illuminator 100W	1	MEE59905
D-LH/LC Precentered Lamphouse with LC	1	MBE75221
Halogen Lamp 12V 100W L.L.	3	84125
Ti-PS100W Power Supply 100-240V	1	MEF52250
Ti-100WRC 100W Lamphouse Remote Cable	1	MEF51001
Power Cord	3	79035
Filter 45mm GIF	1	MBN11200
Filter 45mm NCB11	1	MBN11710
45mm Heat Absorbing Filter	1	MBN11500
Eclipse Microscope Pad	1	92080
Eclipse Large Nylon Cover 14x26x32	1	92084
Package Lens Tissue 50 Sheets, 4X6	1	76997
CFI 10X Eyepiece F.N. 22mm	2	MAK10100
Ti-TD Eyepiece Tube D	1	MEB52320
Ti-T-B Eyepiece Base Unit	1	MEB55800
Ti-S-ER Motorized Stage With Encoders	1	MEC56100
Ti-SH Universal Holder for Motor Stage	1	MEC59110
Ti-S-C Motorized Stage Controller	1	MEF55710
Ti-S-EYOU Joystick for Motorized Stage	1	MEF55700
Ti-CT-E Motorized Condenser Turret	1	MEL51910
Ti-C-LWD LWD Lens Unit for System Condenser Turret	1	MEL56200
System LWD Ph L Annuli	1	MEH31040
TE-C LWD Ph1 Module	1	MEH41100
TE-C LWD Ph2 Module	1	MEH41200
TE-C LWD Ph3 Module	1	MEH41300
Ti-ND6-E Sextuple Motor Dic Nosepiece	1	MEP59310
CFI Plan Fluor DL 4XNA 0.13 WD 17.1mm	1	MRH20041
CFI Plan Fluor DL 10X NA 0.3 WD 16.0 mm	1	MRH20101
CFI Plan Fluor DLL 20X NA 0.5 WD2.1mm Sprg	1	MRH10201
CFI PLAN FLUOR DLL40X OIL NA 1.3/WD 0.2MM	1	MRY10018
CFI Plan APO DM 60X Oil	1	MRD31602
CFI Plan APO DM 100X Oil	1	MRD31901
50Cc Immersion Oil Each	2	MXA20234
C-FL GFP HC HISN Zero Shift	1	96362
C-FL TRITC HYQ	1	96321
C-FL YFP HC HISN Zero Shift	1	96363
C-FL CFP HC HISN Zero Shift	1	96361
C-FL Texas Red HC HISN Zero Shift	1	96365
Ti-FL Epi-Fl Illuminator for Ti-Series	1	MEE54100
Ti-FLC-E Motorized Epi-Fl Filter Turret for Ti-Series	1	MEV51110
Lumen 200 Illumination System	1	77011315
SmartShutter controller	1	77016099
35mm SmartShutter w/stand-alone housing	2	77016096
Excitation Adapter for SmartShutter	1	77016169
Transmitted Light Adapter for Ti, 35mm	1	77016168
Ti Shutter Trigger Cable for Sutter	2	MXA22088
Ti Emission Adapter	1	77016182
NIS-Elements software	1	MQS31000
NIS-Elements: Module 6D imaging	1	MQS42560
NIS-Elements: Hardware Module	1	MQS41220
C-Mount/ISO Adapter, 1X	1	MQD42000
CoolSNAP HQ2 Monochrome Camera	1	77018219
Nikon Environment-Chamber	1	77065000

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Chapter 3

A sensing array of radically coupled genetic biopixels

3.1 Introduction

While there has been significant progress in the development of engineering principles for synthetic biology, a substantial challenge is the construction of robust circuits in a noisy cellular environment. Such an environment leads to considerable inter-cellular variability in circuit behavior, which can hinder functionality at the colony level. Here, we engineer the synchronization of thousands of oscillating colony “biopixels” over centimetre length scales through the use of synergistic intercellular coupling involving quorum sensing within a colony and gas-phase redox signaling between colonies. We use this platform to construct an LCD-like macroscopic clock that can be used to sense arsenic via modulation of the oscillatory period. Given the repertoire of sensing capabilities of bacteria such as *E. coli*, the ability to coordinate their behavior over large length scales sets the stage for the construction of low cost genetic biosensors that are capable of detecting heavy metals and pathogens in the field. Synthetic biology can be broadly parsed into the “top-down” synthesis of genomes (Gibson et al., 2010) and the “bottom-up” engineering of relatively small genetic circuits (Hasty et al.,

2002; Sprinzak and Elowitz, 2005; Endy, 2005; Ellis et al., 2009; Kobayashi et al., 2004; You et al., 2004; Basu et al., 2005; Mukherji and Van Oudenaarden, 2009; Grilly et al., 2007). In the genetic circuits arena, toggle switches (Gardner et al., 2000b) and oscillators (Elowitz and Leibler, 2000) have progressed into triggers (Lu and Collins, 2007), counters (Friedland et al., 2009) and synchronized clocks (Danino et al., 2010). Sensors have arisen as a major focus in the context of biotechnology (Tamsir et al., 2010; Tabor et al., 2009; Kobayashi et al., 2004), while oscillators have provided insights into the basic-science functionality of cyclic regulatory processes (Stricker et al., 2008; Mondragon-Palomino et al., 2011; Tiggles et al., 2009). A common theme is the concurrent development of mathematical modeling that can be used for experimental design and characterization, as in physics and the engineering disciplines.

The synchronization of genetic clocks provides a particularly attractive avenue for synthetic biology applications. Oscillations permeate science and technology in a number of disciplines, with familiar examples including AC power (Westinghouse, 1887), GPS (Lewandowski et al., 1999), and lasers (Vladimirov et al., 2003). These technologies have demonstrated that operating in the frequency domain can offer significant advantages over steady-state designs in terms of information gathering and transmission. In particular, oscillatory sensors confer a number of advantages to traditional ones (Gast, 1985), since frequency is easily digitized and can be quickly updated with repeated measurements. For sensors that use optical reporters, measurements of frequency are less sensitive to experimental factors such as beam power and exposure time than intensity measurements which must be normalized and calibrated.

While the bottom-up approach to synthetic biology is increasingly benefiting from DNA synthesis technologies, the general design principles are still evolving. Within this context, a substantial challenge is the construction of robust circuits in a cellular environment that is governed by noisy processes such as random bursts of transcription and translation (Ozbudak et al., 2002; Elowitz et al., 2002; Golding et al., 2005; Blake et al., 2006; Austin et al., 2006). Such an environment leads to considerable intercellular variability in circuit behavior, which can hinder their functionality at the colony level.

An ideal design strategy for reducing variability across a cellular population would involve both strong and long-range coupling that would instantaneously synchronize the response of millions of cells. Quorum sensing typically involves strong intercellular coupling over tens of microns (Waters and Bassler, 2005; Basu et al., 2005; Danino et al., 2010), yet the relatively slow diffusion time of molecular communication through cellular media leads to signaling delays over millimetre scales. Faster communication mechanisms, such as those mediated in the gas phase, may increase the length scale for instantaneous communication, but are comparatively weak and short-lived since the vapor species more readily disperse.

3.2 Synergistic Synchronization

In order to develop a frequency modulated biosensor, we designed a gene network capable of synchronizing genetic oscillations across multiple scales (Fig. 3.1a and 3.5). We constructed an LCD-like microfluidic (Ferry et al., 2011) array that allows many separate colonies of sensing bacteria to grow and communicate rapidly by gas exchange (Fig. 3.1b and 3.13). Since previous work (Danino et al., 2010) has demonstrated that coupling through quorum sensing leads to incoherent oscillations at the millimetre scale, this mode of cellular communication is too slow for the generation of synchronized oscillations at the macroscopic scale. However, the slower quorum sensing can be used to synchronize small local colonies, provided there is a second level of design that involves faster communication for coordination between the colonies. Therefore rather than attempting to engineer a sensor from a single large-colony oscillator, we wired together thousands of small oscillating colonies, or “biopixels”, in a microfluidic array. Coupling between biopixels involves redox signaling by hydrogen peroxide (H_2O_2) and the native redox sensing machineries of *E. coli*. The two coupling mechanisms act synergistically in the sense that the stronger, yet short-range, quorum sensing is necessary to coherently synchronize the weaker, yet long-range, redox signaling. Using this method we demonstrate synchronization of approximately 2.5 million cells across a distance of

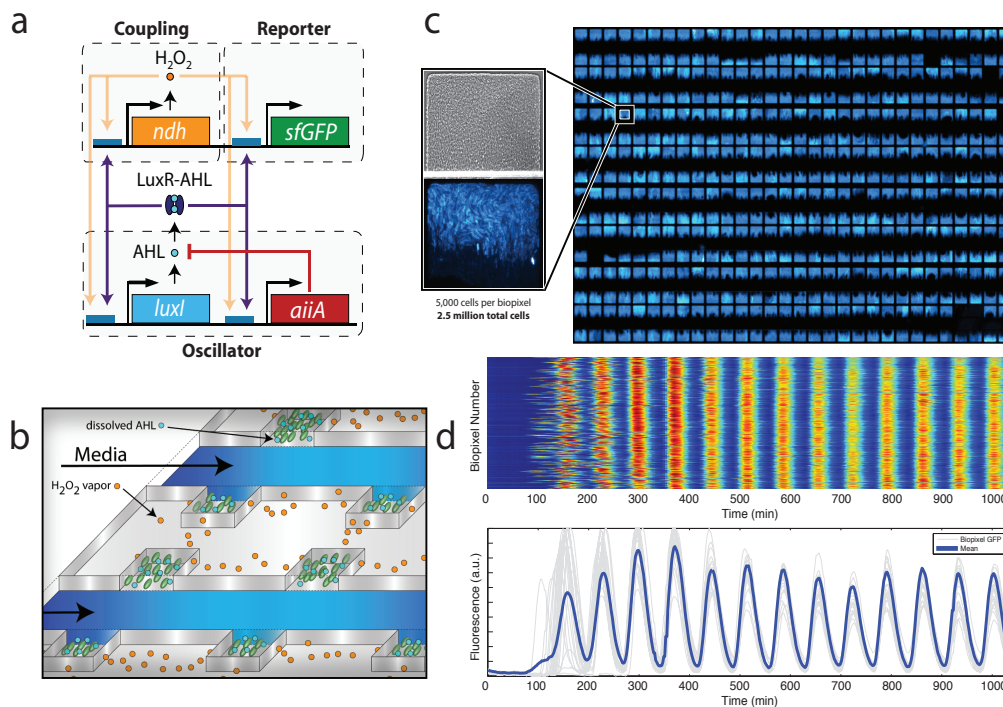


Figure 3.1: Sensing array of radically coupled genetic biopixels. (a) Network diagram. The *luxI* promoter drives expression of *luxI*, *aiiA*, *ndh*, and *sfGFP* in four identical transcription modules. The quorum-sensing genes *luxI* and *aiiA* generate synchronized oscillations within a colony via AHL. The *ndh* gene codes for NDH-2, an enzyme that generates H_2O_2 vapor which is an additional activator of the *luxI* promoter. H_2O_2 is capable of migrating between colonies and synchronizing them. (b) Conceptual design of the sensing array. AHL diffuses within colonies while H_2O_2 migrates between adjacent colonies through the PDMS. Arsenite-containing media is passed in through the parallel feeding channels. (c) Fluorescent image of an array of 500 *E. coli* biopixels containing about 2.5 million cells. Inset: brightfield and fluorescent images display a biopixel of 5,000 cells. (d) Heatmap and trajectories depicting time-lapse output of 500 individual biopixels undergoing rapid synchronization. Sampling time is 2 minutes.

5 mm, over 1,000 times the length of an individual cell (Fig. 3.1c-d). This degree of synchronization yields extremely consistent oscillations, with a temporal accuracy of about 2 minutes compared to 5 - 10 minutes for a single oscillator (Danino et al., 2010) (Fig. 3.1d).

The global synchronization mechanism is comprised of two modes of communication that work on different scales. The quorum-sensing machinery (LuxI, AiiA) uses an acyl-homoserine lactone (AHL) to mediate intracolony synchronization. In our device, the degree to which neighboring colonies are able to influence each other via AHL

diffusion is negligible owing to the high media channel flow rates. Instead, we engineered the cells to communicate via gas exchange by placing a copy of the gene coding for NADH dehydrogenase II (NDH-2) under the control of an additional *lux* promoter. NDH-2 is a membrane-bound respiratory enzyme that produces low levels of H_2O_2 and superoxide (O_2^-) (Messner and Imlay, 1999). Since H_2O_2 vapor is able to pass through the 25 μm oxygen-permeable PDMS walls that separate adjacent colonies, periodic production of NDH-2 yields periodic exchange of H_2O_2 between biopixels. When H_2O_2 enters the cell, it transiently changes its redox state, interacting with our synthetic circuit through the native aerobic response control systems, including *arcAB* which has a binding site in the *lux* promoter region (Bose et al., 2007; Georgellis et al., 2001). Under normal conditions, ArcAB is partially active so *lux* is partially repressed. In contrast, oxidizing conditions triggered by H_2O_2 inactivate ArcAB, relieving this repression. Each oscillatory burst promotes firing in neighboring colonies by relieving repression on the *lux* promoter. This constitutes an additional positive feedback that rapidly synchronizes the population (Fig. 3.6).

We investigated the effects of catalase and superoxide dismutase (SOD) to probe the nature of H_2O_2 communication. When a population of synchronized colonies was exposed to a step increase of 200 U/ml catalase, an enzyme that rapidly degrades extracellular H_2O_2 (Seaver and Imlay, 2001), synchronization was broken and colonies continued to oscillate individually (Fig. 3.7). Since the cell membrane is impermeable to catalase, this confirms that communication between colonies depends on external H_2O_2 while oscillations within a colony do not. Conversely, when we enhanced the rate of superoxide conversion to H_2O_2 by expressing *sodA* (Fridovich, 1978; McCord and Fridovich, 1969) from an additional *lux* promoter, colonies quickly fired in a spatial wave and failed to oscillate further despite no changes to growth rate or cell viability (Fig. 3.8). Since H_2O_2 is produced internal to the cell, this confirms that H_2O_2 is capable of escaping the cell and activating *lux*-controlled genes in neighboring colonies via diffusion. The apparent higher output of H_2O_2 by SOD as compared to NDH-2 is likely due to its very high catalytic efficiency (Berg et al., 2006). Finally, we observed syn-

chronization between arrays of traps even when they were fluidically isolated but held in close proximity (Fig. 3.9). These devices share no common fluid sources or channels, making communication by dissolved molecules like AHL impossible. Taken together, these results confirm that gaseous H_2O_2 is the primary mode of communication between oscillating colonies.

Based on our understanding of the mechanism for global synchronization, we expected that we could simplify the circuitry by eliminating *ndh* and achieve the same effect with intermittent bursts of high-intensity blue light. In this design, the GFP molecule acts as a photosensitizer, releasing free radicals upon exposure that produce oxygen species (ROS) including H_2O_2 (Remington, 2006). At the peak of oscillation, significant vapor-phase H_2O_2 is produced by exposing GFP-containing cells to fluorescent light. Conversely, at the trough of oscillation, cells contain almost no GFP, and therefore produce very little H_2O_2 upon fluorescing. Bursts of light thus generate bursts of H_2O_2 vapor whose concentration depends on the oscillating GFP level, just as periodic production of NDH-2 did previously. Indeed, this strategy was similarly able to synchronize our sensor array (Fig. 3.1d). Numerous controls were performed to ensure that synchronized oscillations did not occur at low fluorescence intensities (Fig. 3.10).

To probe this mode of synchronization, we investigated the effects of thiourea and the antibiotics ampicillin and kanamycin. When a synchronized population of colonies was exposed to 35 mM thiourea, a potent radical quencher (Kelner et al., 1990; Touati et al., 1995), we observed sharply decaying synchronized oscillations while growth rate and cell viability were unaffected (Fig. 3.11). This suggests that without O_2^- , oscillations cannot be produced. Next, we ran a series of experiments switching the antibiotic resistance genes on our plasmids. We noted that radical-producing antibiotics (Kohanski et al., 2010), particularly Ampicillin, significantly reduced the degree of synchronization, showing that an excess of radical species such as O_2^- also hinders communication (Fig. 3.12). Since our final constructs included a plasmid with kanamycin resistance, which was also found to produce some radicals, we used full (50 $\mu\text{g}/\text{ml}$) selection when growing up the cells but very low (5 $\mu\text{g}/\text{ml}$) selection during the ex-

perimental run. Persistence of oscillations, sequencing, and subsequent growth in full selection following the run confirmed the presence of all 3 plasmids despite this low experimental selection. Catalase and *sodA* results were identical to those with NDH-2 synchronization. These results show that fluorescence-mediated synchronization involves the production of radical species following fluorescence exposure and communication via H_2O_2 .

3.3 Sensing Array of Biopixels

With a platform for generating consistent and readily-detectable oscillations, we sought to use the circuit to engineer an arsenic-sensing macroscopic biosensor. We rewired the network to include an extra copy of the positive-feedback element, the AHL-synthase LuxI, under the control of a native arsenite-responsive promoter which is repressed by ArsR in the absence of arsenite (Fig. 3.2a). When arsenite is not present in the media, supplemental *luxI* is not transcribed and the circuit functions normally, generating baseline oscillations. However, the addition of trace amounts of arsenite relieves this repression and allows supplemental *luxI* to be produced, increasing the oscillatory amplitude and period. Tuning the level of LuxI by varying arsenite concentration results in clear changes to the oscillatory period (Fig. 3.2b). To determine the range of detection, we swept arsenite concentrations from 0 to 1 μM and measured the oscillatory period (Fig. 3.2c, top). Using statistical methods (Section: 3.7.3), we generated a sensor calibration curve (Fig. 3.2c, bottom) that depicts the maximum possible arsenite concentration present ($\alpha = 95\%$) for a given measured period. This curve is an illustration of how data generated by our array would be used to measure arsenite concentrations in an unknown sample using our device. Our system was able to reliably quantify arsenite levels as low as 0.2 μM , below the 0.5 μM WHO-recommended level for developing nations (Nordstrom, 2002).

As an alternative sensing strategy, we rewired the network to include a copy of the *luxR* gene controlled by an arsenic-responsive promoter while removing it from the

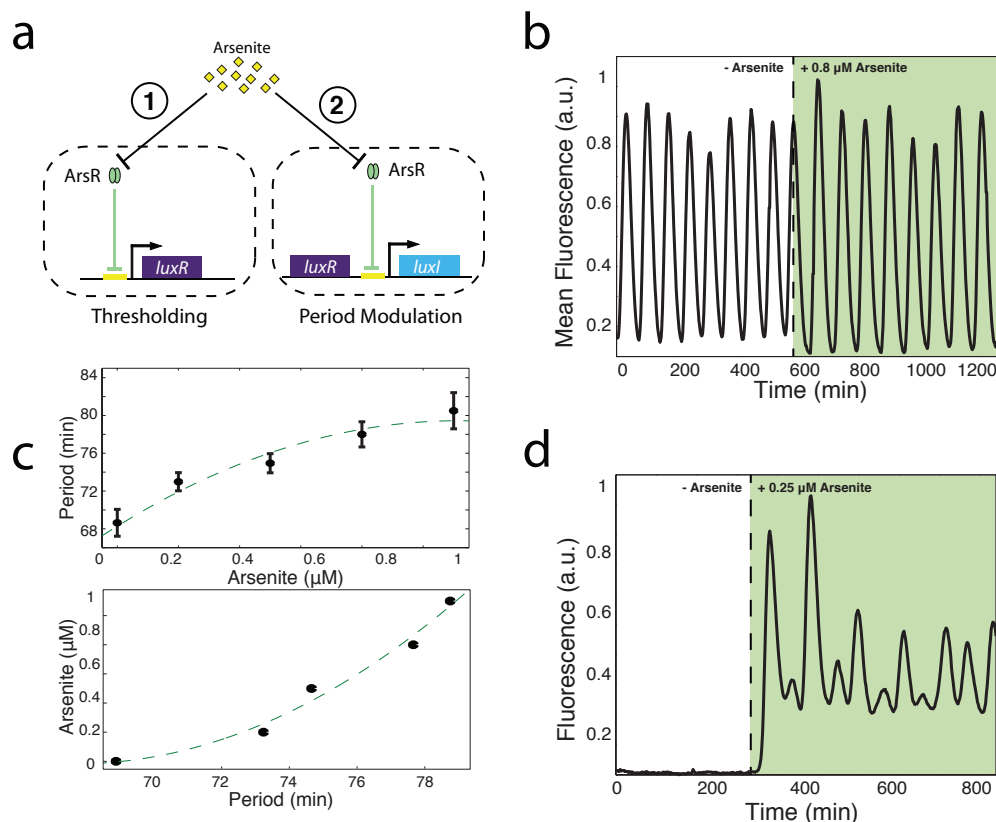


Figure 3.2: Frequency modulated genetic biosensor. (a) Network diagrams depicting two constructed sensing modules. In thresholding (1), the *luxR* gene is removed from the oscillator network and supplemented by a new copy driven by an arsenic-responsive promoter. In period modulation (2), a supplemental *luxI* gene tagged for increased degradation is driven by the arsenic-responsive promoter which affects the period of oscillation. (b) A sample period modulation sensor output following a step increase of 0.8 μM arsenite. Oscillatory period increases from 69 minutes to 79 minutes. (c) (Top) Period versus arsenite concentration for the sensor array. Error bars indicate ± 1 standard deviation averaged over 500 biopixel trajectories. Dotted line represents model-predicted curve. (Bottom) Sensor calibration curve generated from experimental data. Points indicate the maximum arsenite level with 95% certainty for a given measured period as determined statistically from experimental data. (d) Threshold output following a step increase of 0.25 μM arsenite. A dramatic shift from rest to oscillatory behavior is observed within 20 minutes following the addition of arsenite.

rest of the circuit (Fig. 3.2a). Since the LuxR-AHL complex must be present to activate the *lux* promoter (Waters and Bassler, 2005), cells produce no LuxR when the media is free of arsenite, generating no fluorescence or oscillations. The addition of arsenite stimulates the production of LuxR, restoring circuit function and producing clear, synchronized oscillations (Fig. 3.2d). This ON/OFF detection system has a threshold of $0.25 \mu\text{M}$, a detection limit that can be adjusted by changing the copy number, ribosome binding site (RBS) strength, or promoter strength of the sensing plasmid (Section: 3.7.1).

The sensing array is also capable of producing complex behaviors arising from the dynamic interaction of cellular colonies. By making modifications to the size, number, and arrangement of biopixels in the device, we are able to dramatically alter the output waveforms. For example, when we constructed a device in which trap separation distance is increased ($45 \mu\text{m}$ versus $25 \mu\text{m}$), we observed local anti-phase synchronization between neighboring colonies (Fig. 3.3d, top right). To explore this phenomenon on a larger scale, we constructed a device that contains an array of 416 traps constructed according to the specifications above. In these experiments, we observe initial global synchronization that gradually falls into local anti-phase synchronization across the array (Fig. 3.3d, middle). Phase alignment is maintained over at least 48 hours, with patches of synchronization typically 3-6 colonies in size. Alternatively, by changing dimensions such that the array contains traps of two slightly different sizes, we observe a 1:2 resonance synchronization where larger traps pulse at double the frequency of smaller traps while maintaining synchronization (Fig. 3.3d, top). Finally, when LuxR is limited as in the thresholding scheme, we observe synchronized oscillations of alternating large and small peaks in both experiment and model (Fig. 3.16). Our computational model (see Section: 3.5) captures these effects (Fig. 3.3d, bottom and Fig. 3.15, 3.16) and indicates that further array manipulation will yield new, richer dynamics that could not be produced directly by changing circuit structure .

While our sensor array is capable of performing a variety of complex functions in the laboratory, adapting this technology to a real-world device will require the elimi-

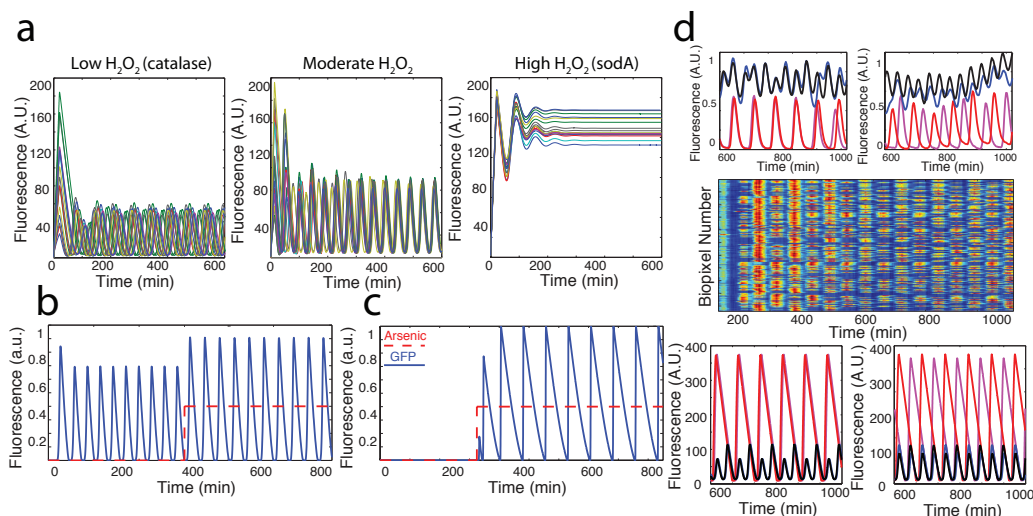


Figure 3.3: Computational modeling of radical synchronization and biosensing. (a) Time series of a population of biopixels producing varying amounts of H_2O_2 vapor. Synchronization occurs only for moderate levels while high levels lock ON and low levels oscillate asynchronously. (b) A typical time series for our period modulation sensor undergoing a step increase of arsenite. Oscillations increase in both amplitude and period. (c) A typical time series output for the thresholding sensor. Oscillations arise following the addition of arsenite. (d) Experimental and computational output depicting complex dynamic behaviors between neighboring traps. (Top 2 panels) 1:2 resonance and anti phase synchronization observed when trap size (left, black/blue = $95 \mu m$ depth and red/magenta = $85 \mu m$ depth) and separation distance (right, same colors) are modified experimentally, (Middle) Scaled-up array experimental data for increased trap separation experiments demonstrating anti phase synchronization, (Bottom) Computational model trajectories depicting 1:2 resonance and anti phase synchronization when trap size (same colors as experimental data) and separation distance are changed.

nation of the expensive and bulky microscopy equipment. However, measuring genetic oscillations in the absence of any magnification or powerful illumination will require even further increased signal. Using this mechanism of global synchronization, we were able to scale up to a 24 mm x 12 mm array that houses over 12,000 communicating biopixels (Fig. 3.4a). Synchronization is maintained across the entire array, a distance over 5,000 times the length of an individual cell, using an inexpensive LED (Fig. 3.4b,c). The signal strength generated by the large number of cells in the array (about 50 million) will allow us to adapt the device to function as a handheld sensor. In our conceptual design (Fig. 3.4d), the sensor will continuously read the oscillatory frequency using off-the-shelf electronic components costing less than \$50.

There have been many examples of bacteria-based biosensors (van der Meer and Belkin, 2010; Daunert et al., 2000; Leveau and Lindow, 2002), usually involving an optical reporter driven by a single promoter. Since optical intensity readings are sensitive to imaging conditions like beam power and exposure time, measurements must typically be normalized and calibrated. Measuring period of oscillation allows us to avoid these issues since peak-to-peak time does not depend on individual peak intensity. Secondly, oscillations produced at the colony level effectively decouple the signal from the growth state of individual cells, which can also affect fluorescence intensity. By using a dynamic readout that depends on communication between biopixels, we scan and tune potential output signals by changing device parameters rather than redesigning the underlying circuit. For example, we might design a new sensing scheme in which oscillations synchronize with the addition of some toxin and shift to anti phase or resonant synchronization when critical toxin levels are present.

3.4 Scaling Up Synthetic Biology

By nesting two modes of communication we are able to expand the scale over which individual cells are coordinated and increase the complexity of their interaction. Indeed, there are many familiar examples of hierarchical systems. Airline routes are

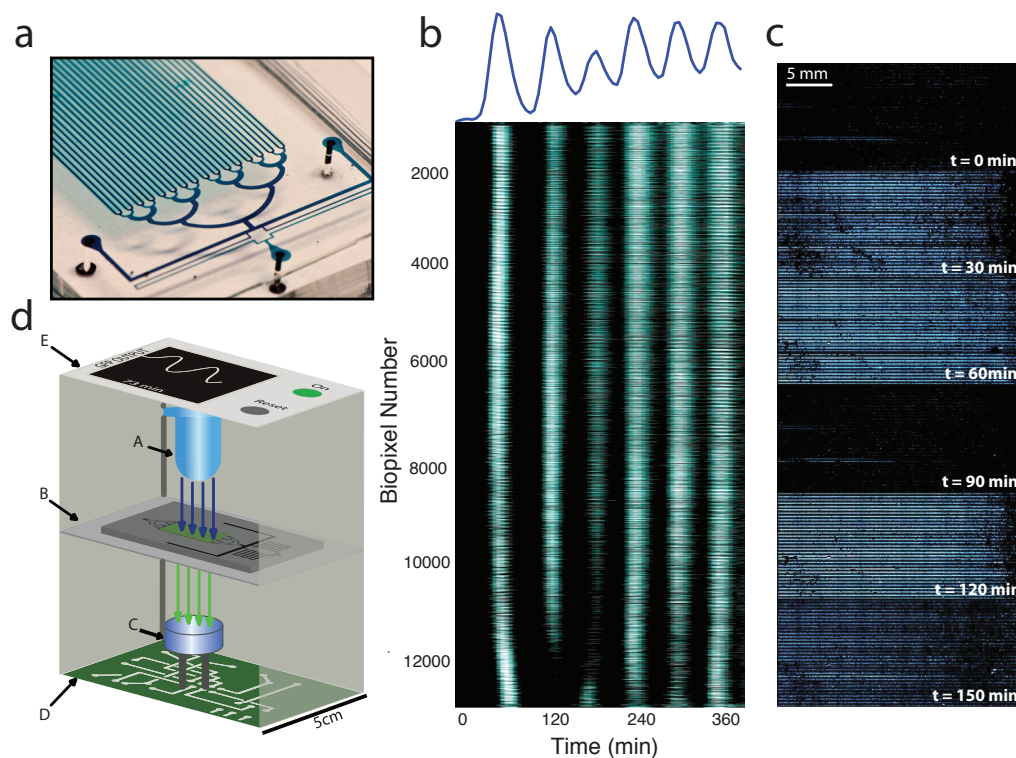


Figure 3.4: Radical synchronization on a macroscopic scale. (a) The scaled-up array is 24 mm x 12 mm and houses over 12,000 biopixels that contain approximately 50 million total cells when filled. (b) Global synchronization is maintained across the array. Heatmap of individual trajectories of all 12,224 oscillating biopixels. (c) Image series depicting global synchronization and oscillation for the macroscopic array. Each image is produced by stitching 72 fields of view imaged at 4X magnification. (d) Schematic diagram illustrating our design for a handheld device utilizing the sensing array. An LED (A) excites the array (B) and emitted light is collected by a photodetector (C), analyzed by an onboard processor (D), and displayed graphically (E).

often designed such that small airports are connected locally to larger hubs that are connected internationally. It would neither be feasible nor desirable to connect every airport together. Similarly, individual cells communicate locally by one method, generating impulses large enough to enable colonies to communicate globally by another. Nesting communication mechanisms in this way may allow us to better scale up synthetic circuits of different types, such as switches and logic gates, paving the way for the next generation of synthetic biology pursuits.

3.5 Modeling Box

Our model of the frequency modulated (FM) biosensor is based on a published model for the quorum-sensing synchronized oscillator (Danino et al., 2010). In addition to the reactions reflected in that model, we include the arsenite-induced production and degradation of LuxI and/or LuxR. From the biochemical reactions, we derived a set of delay-differential equations to be used as our model. These delayed reactions mimic the complex cascade of processes (transcription, translation, maturation, etc.) leading to formation of functional proteins. As expected, our model predicts oscillations that change frequency when changes in arsenite occur (Fig. 3.2c and 3.3b). The amplitude and period of the oscillations both depend on the concentrations of the toxin. We then modified the model to describe the LuxR-based detection system. Our model predicts a marked transition from rest to oscillations upon addition of arsenite, consistent with experimental observations (Fig. 3.3c).

The multi-scale nature of communication in our array allows us to treat colony and array-level dynamics separately, where arsenite affects the quorum-sensing machinery of a colony, producing changes to oscillatory period that propagate between biopixels in the array. To quantitatively describe the mechanisms driving synchronization at the array-level, we treat each colony as a single oscillator that acts according to degrade-and-fire kinetics (Mather et al., 2009). We also include the production of H_2O_2 and its interaction with neighboring colonies by two-dimensional diffusion. Using this

model we identified three regimes that correlate well with experimental observations (Fig. 3.3a). When the effective production of H_2O_2 is low, as with catalase, we observe unsynchronized oscillations owing to constant, mild repression of the *lux* promoter via ArcA (Fig. 3.3a, left). In contrast, when H_2O_2 production is very high, neighboring colonies rapidly fire in succession and remain on due to the permanent activation of the *lux* promoter, consistent with the SOD experiment (Fig. 3.3a, right). Finally, at intermediate H_2O_2 , we observe globally synchronized oscillations (Fig. 3.3a, middle). As colonies are moved further apart, synchronicity breaks due to slowed migration of H_2O_2 (Fig. 3.14).

3.6 Methods Summary

3.6.1 Strains and Plasmids

The plasmids were constructed using a CPEC (Quan and Tian, 2009) cloning strategy in which the origin of replication, antibiotic resistance, and circuit genes were assembled in different combinations using PCR reactions. The *ndh* and *sodA* genes were amplified directly from the native *E. coli* genome by PCR. Various arsenic-responsive promoters were tested, including a recently reported synthetic version Stocker et al. (2003), but the final design uses the native *E. coli* version. Promoter output was tuned by changing the RBS sequence and quantified using flow cytometry. All circuit components except *luxR* were tagged by PCR with a carboxy-terminal *ssrA* tag (AANDENYALAA) for fast degradation (Keiler et al., 1996).

3.6.2 Microfluidics and Microscopy

Image acquisition was performed on a Nikon Eclipse TI epifluorescent inverted microscope outfitted with fluorescence filter cubes optimized for GFP imaging and a phase-contrast based autofocus algorithm. Images were acquired using an Andor Clara cooled CCD camera or Andor DU-897 EMCCD camera, both controlled by Nikon El-

ements software. Images were acquired every 2 minutes in phase contrast and fluorescence. The cells were imaged inside a microfluidic device with an upstream switch, with the ability to mix or switch between two different media sources. A custom application written in LabVIEW (National Instruments, Austin, Texas) controlled linear actuators, to which two reservoirs of arsenite-containing and pure medium were attached. Using this algorithm, arsenite concentration was dynamically varied to probe sensor output.

The microfluidic experiments were performed as previously described (Danino et al., 2010). Briefly, 50 μ L of an overnight culture was diluted in 50mL of LB (Difco) + antibiotics the day of the experiment. When cells reached an OD₆₀₀ of 0.1, cells were spun down and resuspended in 5mL of fresh media and loaded into the device.

3.7 Supplementary Information

3.7.1 Plasmid Construction

The oscillator plasmids were constructed by modifying the constructs used in a previous study (Danino et al., 2010). The antibiotic resistance genes of pTD103AiiA was switched to chloramphenicol. The reporter protein on pTD103LuxI/GFP plasmid was switched to a recently reported superfolder green fluorescent protein, sfGFP (Pedelacq et al., 2006). The *ndh* and *sodA* genes were amplified directly from the native *E. coli* genome by PCR. Promoter output was tuned by changing the RBS sequence and quantified using flow cytometry. We initially constructed the sensing plasmid with a published synthetic background-reduced version that contains additional ArsR operator sites (Stocker et al., 2003) but failed to produce enough LuxR. To increase LuxR output, we reverted to the native promoter sequence, switched the RBS to that of pZ plasmids (Lutz and Bujard, 1997), and increased the copy number by a factor of 5 by switching to a mutated SC101 origin of replication. All circuit components except LuxR were tagged by PCR with a carboxy-terminal *ssrA* tag (AANDENYALAA) for fast degradation (Keiler et al., 1996). Modular pieces (resistance genes, promoters, origins, and

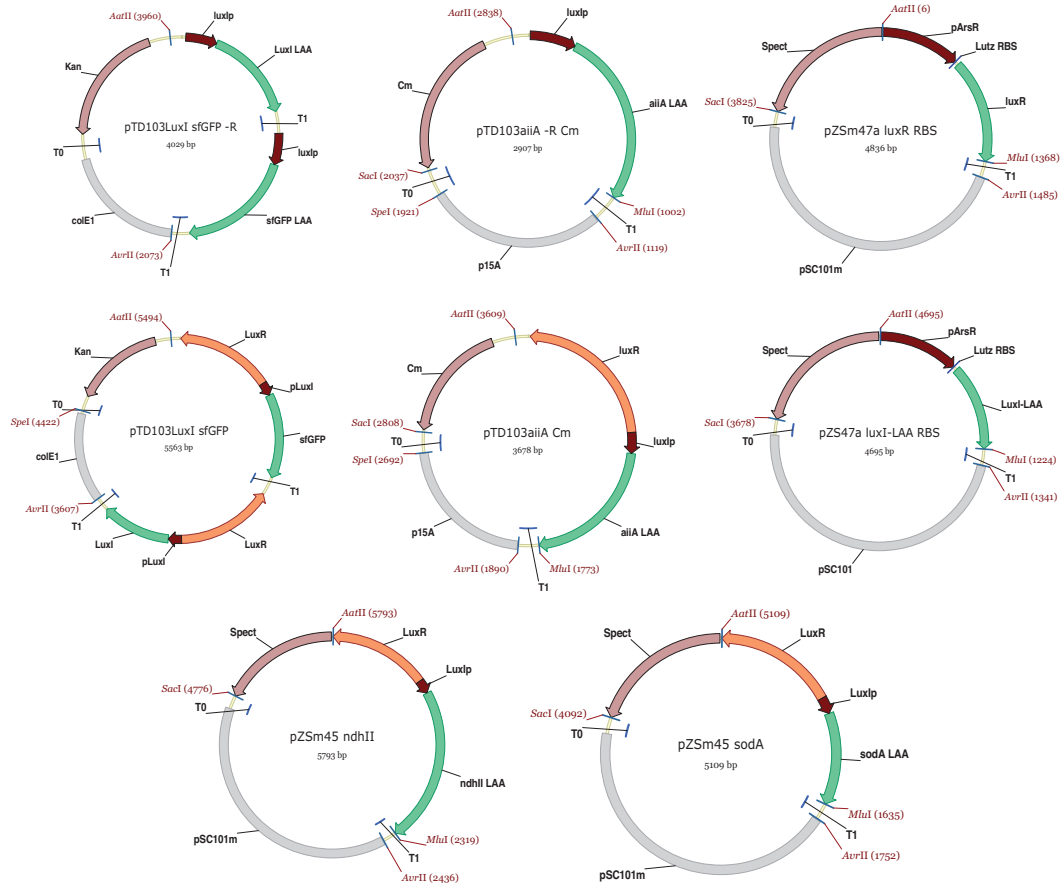


Figure 3.5: Plasmids used in this study. Top row is the thresholding sensor: 2 oscillator plasmids with luxR genes removed and a plasmid containing pArs::luxR. Middle row is the period modulator: 2 oscillator plasmids and a plasmid containing pArs::luxI-laa. Bottom row contains 2 plasmids used to study H_2O_2 production and synchronization: pLux::ndh and pLux::sodA. NDH-2 synchronization strain is the oscillator plasmids with pZSm45 ndhII.

ORFs) were assembled using a PCR-based cloning scheme named CPEC (Quan and Tian, 2009).

3.7.2 Additional Experimental Results

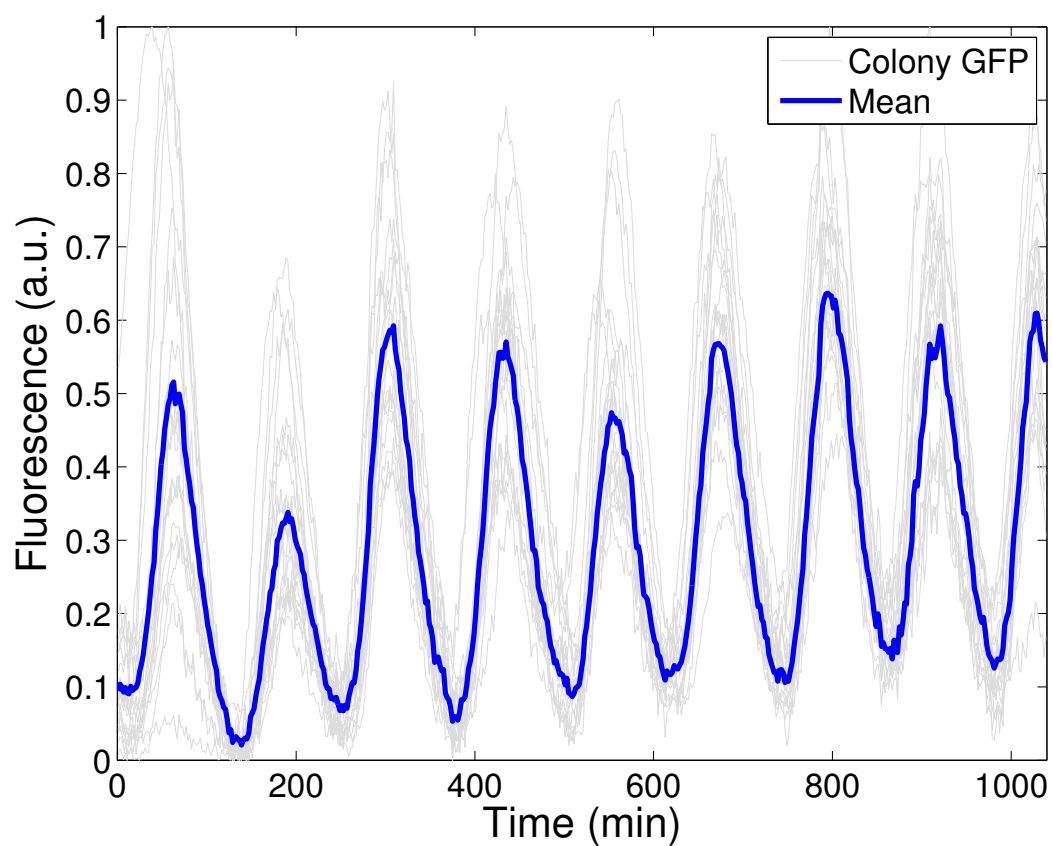


Figure 3.6: Biopixels with NDH-2 engineered synchronization observed at ultra-low fluorescence (4X, 20ms exposure, 3% power) using an EMCCD camera to ensure no fluorescence interaction. Synchronized oscillations are maintained across the array for the length of the experiment (14 hours).

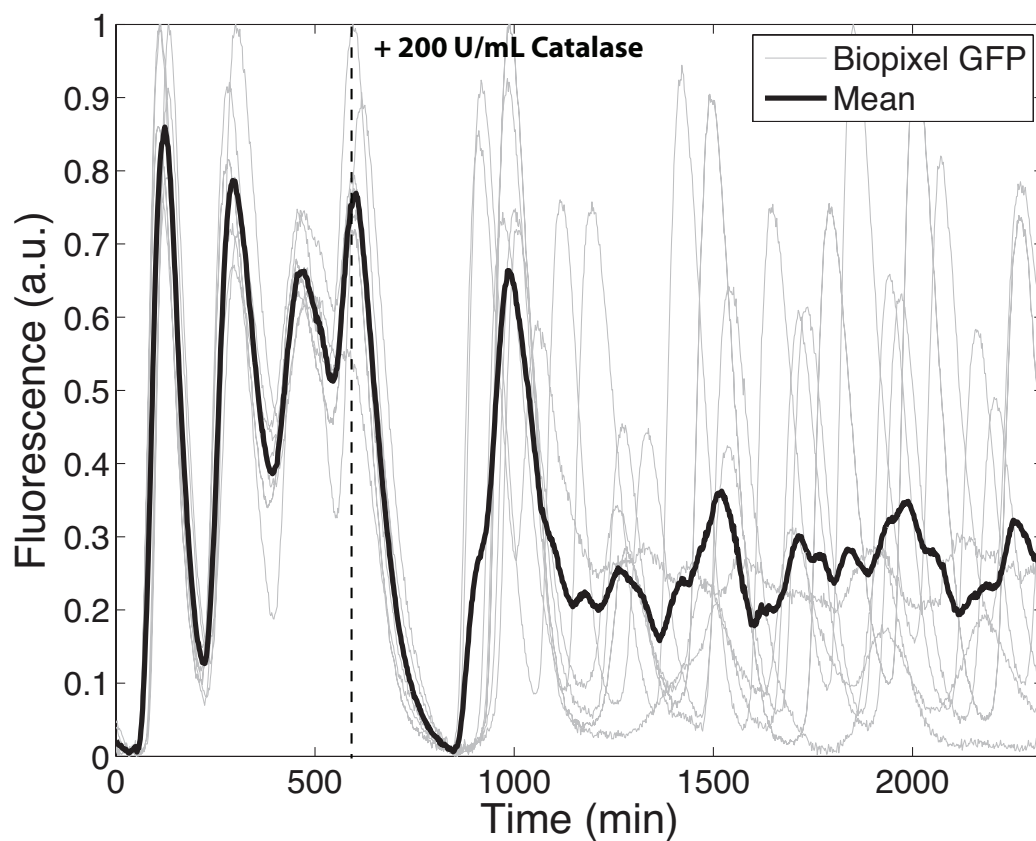


Figure 3.7: Catalase degrades external H_2O_2 and prevents communication between colonies. When a synchronized population of biopixels was exposed to a step increase of 200 U/ml catalase, synchronization was broken and biopixels continued to oscillate individually. Since catalase can't cross the cell membrane, this shows that synchronization between colonies depends on H_2O_2 but oscillations with a colony do not.

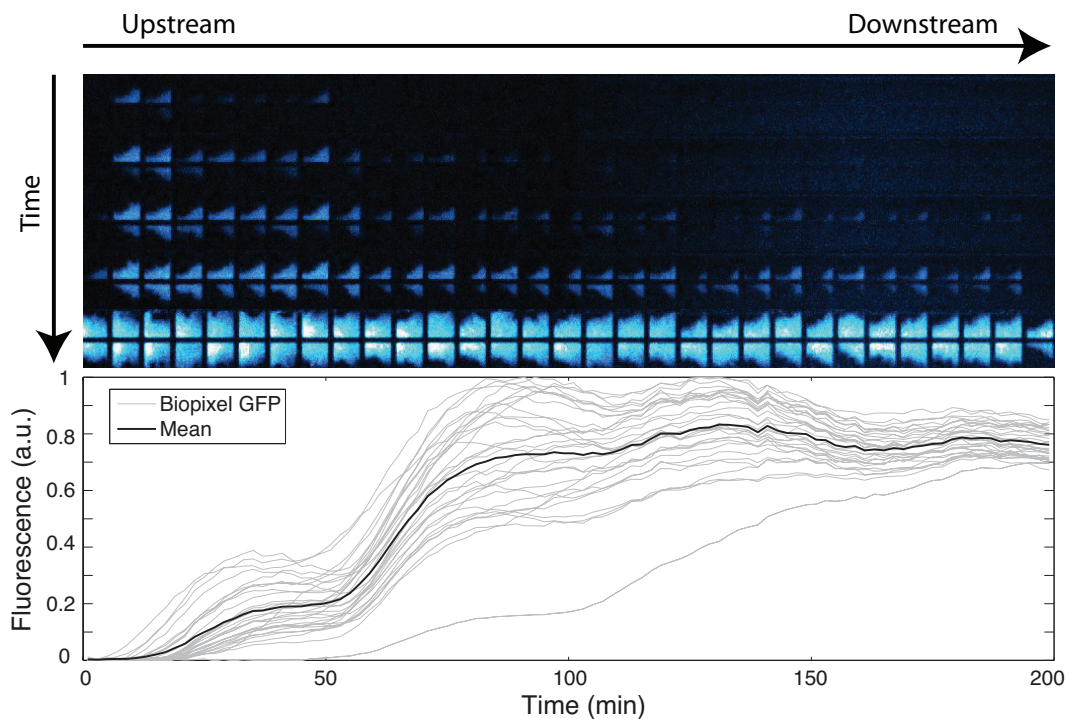


Figure 3.8: SodA produces H_2O_2 internal to the cell, permanently switching the cellular redox state (oxidizing) thereby activating lux-controlled genes. Biopixels rapidly fire and lock on in a spatial wave, far earlier than is typical for colonies of this size. The propagation of ON biopixels suggests that colonies are capable of activating those nearby via migrating H_2O_2 species.

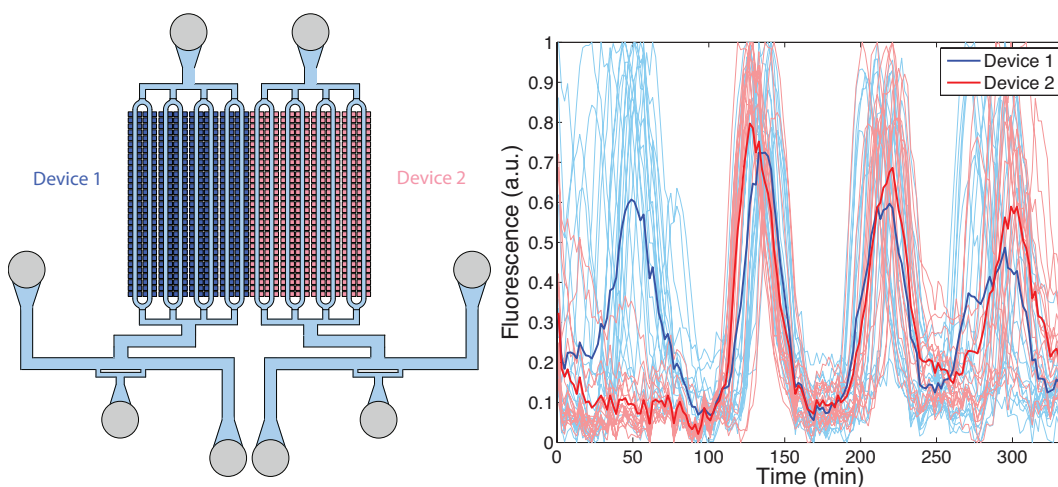


Figure 3.9: Synchronized oscillations occur across 2 fluidically isolated devices held in close proximity. In this experiment, the devices were started at different times yet become synchronized. Since these devices share no common fluid sources or sinks, this confirms that synchronization is mediated by vapor species.

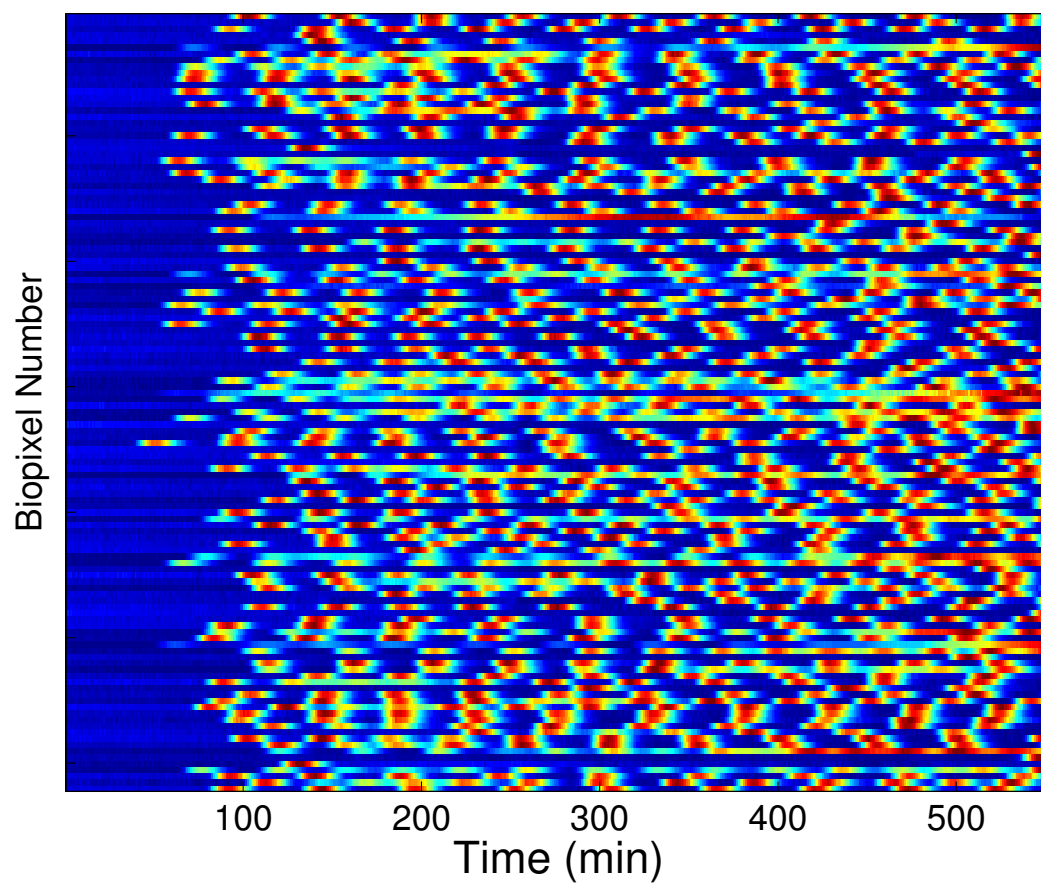


Figure 3.10: Heatmap of trajectories extracted from low fluorescence intensity control when NDH-2 plasmid is not present. Biopixels oscillate individually but fail to synchronize.

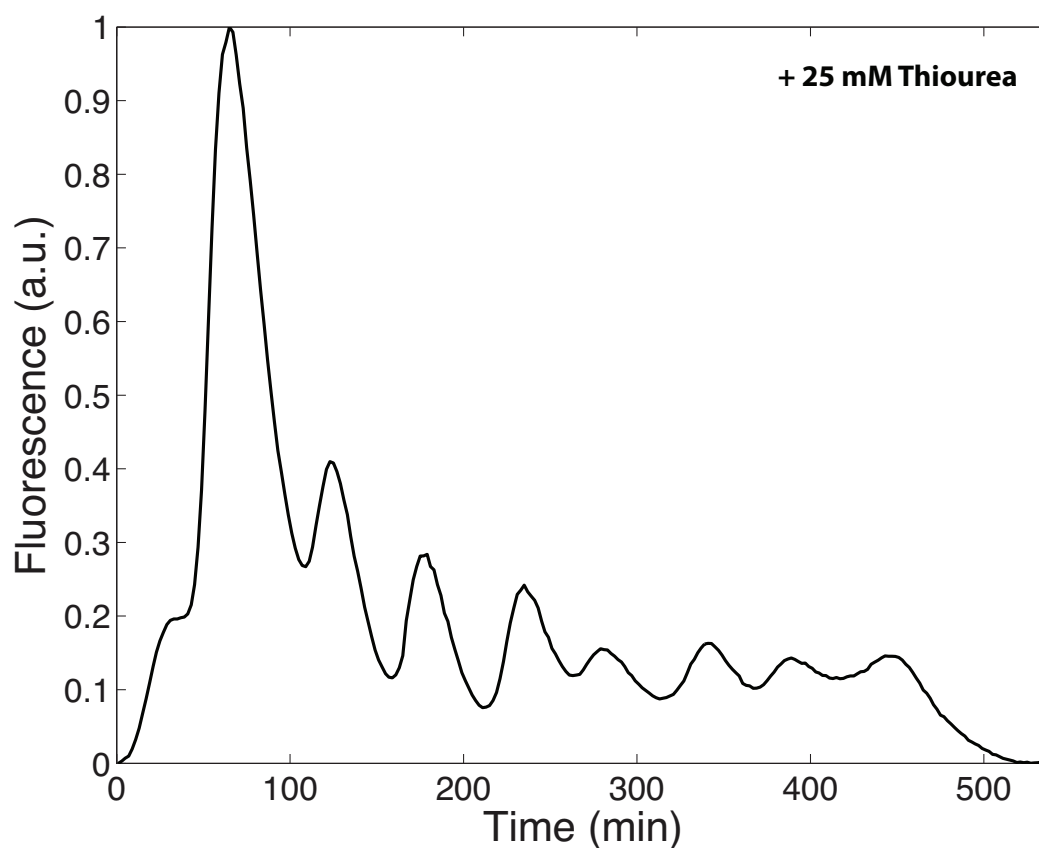


Figure 3.11: The introduction thiourea, a potent radical quencher, produces decaying synchronized oscillations across a population of biopixels. Because radical species are precursors for H_2O_2 , eliminating them lowers the production of H_2O_2 and therefore dampens the oscillations. Colonies are still able to synchronize because, while thiourea eliminates radicals within cells, it does not prevent H_2O_2 from diffusing between cells.

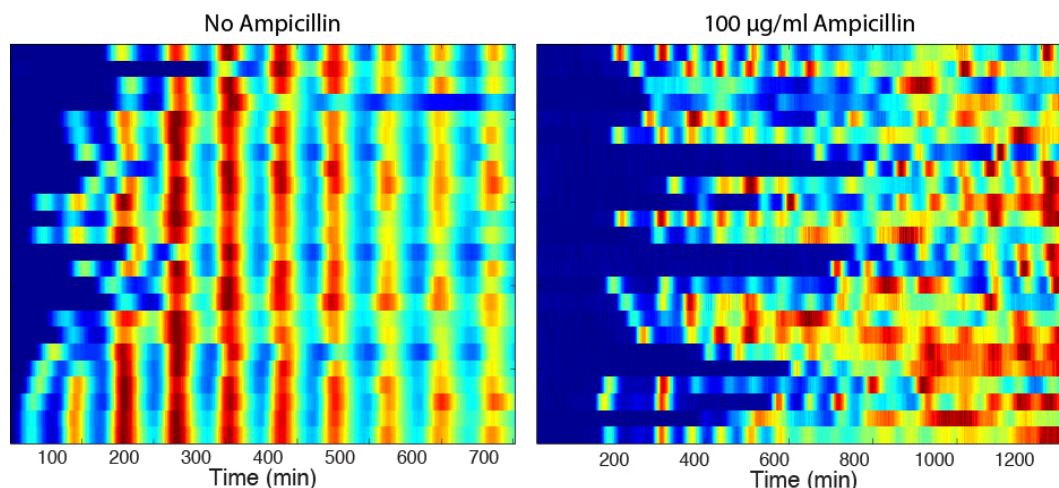


Figure 3.12: Synchronization is prevented when 100 $\mu\text{g/ml}$ Ampicillin is used in the media. The constructs, strains, and experimental conditions are otherwise identical.

3.7.3 Data Analysis

Fluorescence data was obtained by importing fluorescent images into ImageJ and subtracting cell signal from background signal. Oscillatory period was taken to be the average of peak-to-peak and trough-to-trough distance, calculated using a MATLAB script. The data represented in Fig. 3.1d and 3.2b-d were collected by stitching 4 images taken at 4X magnification. The mean trajectory in Fig. 3.1d was found by averaging 373 individual biopixel trajectories, of which 20 are shown. Biopixel trajectories were extracted from image series using a MATLAB script, where a bright field image of the corresponding array was used to generate a mask. The data shown in Fig. 3.2c was measured over 4 separate experiments using 10-30 oscillatory periods per data point.

Sensor calibration curve (Fig. 3.2c, bottom) was generated using a series of 2-population ttests comparing the experimental datasets to randomly generated new sample sets. The mean of generated sets was decremented until the test failed with $\alpha = 95\%$, indicating the lowest period that could be associated with that arsenite concentration. We repeated this process for each arsenite level and fit the points with a quadratic since we expected it to take the inverse shape of the period vs. arsenite measurements.

3.7.4 Microscopy and Microfluidics

We used a microscopy system similar to our recent studies (Danino et al., 2010), with the addition of a high-sensitivity Andor DU-897 EMCCD camera. Fluorescent images were taken at 4X every 30 seconds using the EMCCD camera (20ms exposure, 97% attenuation) or 2 minutes (2s exposure, 90% attenuation) using a standard CCD camera to prevent photobleaching or phototoxicity.

In each device, *E. coli* cells are loaded from the cell port while keeping the media port at sufficiently higher pressure than the waste port below to prevent contamination (Suppl. Fig 8). Cells were loaded into the cell traps by manually applying pressure pulses to the lines to induce a momentary flow change. The flow was then reversed and allowed for cells to receive fresh media with 0.075% Tween which prevented cells from adhering to the main channels and waste ports.

To measure fluid flow rate before each experiment, we measured the streak length of fluorescent beads ($1.0\ \mu\text{m}$) upon 100 ms exposure to fluorescent light. We averaged at least 1,000 data points for each.

We constructed several microfluidic devices over the course of the study. The trap dimensions were always $100\ \mu\text{m} \times 85\ \mu\text{m} \times 1.65\ \mu\text{m}$ high, which we previously found to be optimal for oscillator function, except when size was varied to study dynamic interactions. Spacing between traps was $25\ \mu\text{m}$, except in devices designed to study the effects of increasing separation distance between traps. For sensor array devices, we constructed 500 and 12,000 trap arrays as well as a tandem device which holds two 150 trap arrays in close proximity ($25\ \mu\text{m}$) without sharing fluid sources or sinks.

3.7.5 Modeling

To model the dynamics of the quorum-sensing oscillator, we used our previously described model for intracellular concentrations of LuxI (I), AiiA (A), internal AHL

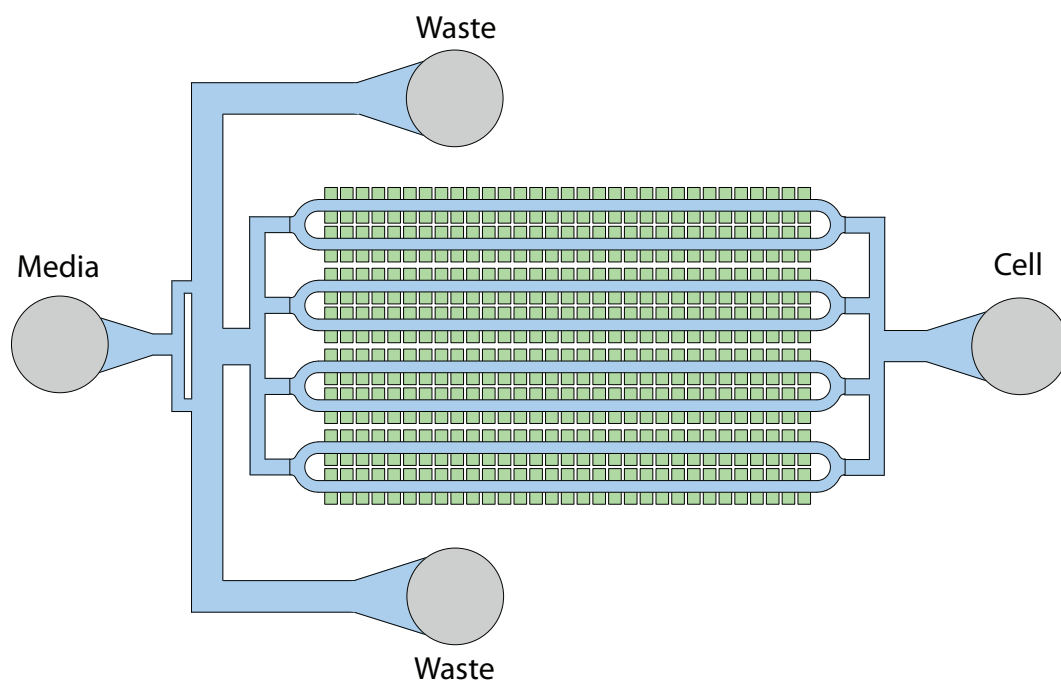


Figure 3.13: Primary microfluidic device used for this study. Media containing variable arsenite concentration is fed through the cell port, flowing past the biopixel array into the cell and waste ports. During loading, pressure is increased at the cell port and decreased at the waste ports to reverse the flow, allowing cells to pass by the trapping regions. Other microfluidic devices used have the same layout with trap number, separation, and size varied.

(H_i), and external AHL (H_e) (Danino et al., 2010),

$$\frac{\partial A}{\partial t} = C_A[1 - (d/d_0)^4] G(\alpha, \tau) - \frac{\gamma_A A}{1 + f(A + I)} \quad (3.1)$$

$$\frac{\partial I}{\partial t} = C_I[1 - (d/d_0)^4] G(\alpha, \tau) - \frac{\gamma_I I}{1 + f(A + I)} \quad (3.2)$$

$$\frac{\partial H_i}{\partial t} = \frac{bI}{1 + kI} - \frac{\gamma_H A H_i}{1 + gA} + D(H_e - H_i) \quad (3.3)$$

$$\frac{\partial H_e}{\partial t} = -\frac{d}{1-d} D(H_e - H_i) - \mu H_e + D_1 \frac{\partial^2 H_e}{\partial x^2} \quad (3.4)$$

In the original model, the concentration of the constitutively produced LuxR protein R was assumed constant. In the ON/OFF threshold arsenic biosensor circuit, LuxR production is induced by arsenic, which we model by the equation

$$\dot{R} = \frac{\alpha_c A}{(A_0 + A)} - \gamma_R R \quad (3.5)$$

in which the LuxR expression from the arsenic promoter follows a standard saturating function of the arsenic concentration A . Accordingly, we modified the Hill function for Lux promoter to include the explicit dependence on R :

$$G(\alpha, \tau) = \frac{\delta + \alpha(R_\tau H_\tau)^2}{1 + k_1(R_\tau H_\tau)^2} \quad (3.6)$$

For modeling the period-modulating sensor, we modified the equation for LuxI (Eq. 3.2) to include additional production from the arsenic promoter,

$$\dot{I} = C_I[1 - (d/d_0)^4] G(\alpha, \tau) + \frac{\alpha_c A}{(A_0 + A)} - \frac{\gamma_I I}{(1 + f(A + I))} \quad (3.7)$$

The following additional parameters were used for the biosensor simulations: $\alpha_c = 50$, $A_0 = 2$, $\gamma_R = .1$.

Arsenic levels were swept across the dynamic range of the arsenic promoter to produce the curve in Fig. 3.2c. The period for each arsenic level was calculated from the peak-to-peak average of 15 oscillatory periods.

To model the spatial synchronization of oscillating colonies across a microfluidic array, we generalized a simplified “degrade-and-fire” model (Mather et al., 2009). The

delay-differential equation

$$\dot{X}_{i,j} = \frac{\alpha(1 + \nu P_{i,j,\tau_2})}{(1 + \frac{X_{i,j,\tau_1}}{C_0})^2} - \frac{\gamma X_{i,j}}{k + X_{i,j}} \quad (3.8)$$

describes oscillations of individual biopixel $\{i, j\}$ as a combined effect of production and delayed autorepression (first term in the r.h.s.) of the colony-averaged LuxI concentration $X_{i,j}$ and its enzymatic degradation by ClpXP (second term). Unlike (Mather et al., 2009), the first (production) term in Eq. 3.8 describes both delayed auto-repression of LuxI and its delayed activation by H_2O_2 proportional to its local concentration $P_{i,j}$. Subscripts τ_1 and τ_2 indicate the delayed concentrations, $X_{i,j,\tau_1}(t) = X_{i,j}(t - \tau_1)$ and $P_{i,j,\tau_2}(t) = P_{i,j}(t - \tau_2)$. The dynamics of $P_{i,j}$ is described by the equation

$$\dot{P}_{i,j} = \mu + \alpha_p X_{i,j} - \gamma_p P_{i,j} + \hat{S}\{P_{i,j}\} \quad (3.9)$$

where the first three terms describe the basal and induced production and degradation of H_2O_2 . The last term models the spatial coupling of neighboring biopixels via the H_2O_2 exchange. For a square $N \times N$ array of traps, we used the following discrete diffusion form of the spatial operator,

$$\hat{S}\{P_{i,j}\} = D\Delta^{-2}[P_{i-1,j} + P_{i+1,j} + P_{i,j-1} + P_{i,j+1} - 4P_{i,j}] \quad (3.10)$$

Each colony is affected by the H_2O_2 produced in four neighboring colonies, two in each dimension of the array, separated by the equal distance Δ . We used the boundary condition $P_{i,j} = 0$ for the edges of the array $i, j = 0, N + 1$. This represents the infinite external sink of H_2O_2 diffusing out of the microfluidic chip. The diffusion operator above can be generalized if the row spacing differs from the column spacing, or for other spatial arrangements of colonies within the biosensor.

We introduced variability among different traps by randomizing oscillator parameters for individual traps in each simulation. Specifically, LuxI (X) activation and degradation parameters ($p = \{\alpha, \gamma\}$) of each of the oscillators in the array were varied around their nominal values (p_0) as $p = p_0 + \delta$ where δ is a random number uniformly distributed between -0.25 and 0.25 . We used the following dimensionless parameters

for most of our simulations: $\alpha_0 = 8.25$, $\gamma_0 = 5.75$, $\nu = 1$, $\tau_1 = 10$, $\tau_2 = 20$, $C_0 = 6$, $k = 10$, $\mu = 20$, $\alpha_p = 1$, $\gamma_p = 10$, $D = 7$, $\Delta = 1$.

For the characterization of various regimes of array synchronization, 16 colonies were modeled in the 4×4 array. Scaling up the simulation with larger numbers of colonies produced equivalent results. Overproduction of H_2O_2 by expressing *sodA* was captured by increasing α_p 20-fold. This is consistent with expression from a pSC101m plasmid with a copy number of 20-30. Depletion of external H_2O_2 by catalase was modeled by increasing H_2O_2 degradation (γ_p) and decreasing H_2O_2 diffusion, D . In Fig. 3.13 we show the variance of the concentrations $X_{i,j}$ within the array averaged over time and parameter variations. This plot demonstrates that the synchronicity among the biopixels decreases with increase of spacing among them, and for $\Delta > 5$ is completely lost.

Increasing the trap spacing Δ 2-fold while simultaneously decreasing k 4-fold allowed us to reproduce the more complex waveforms observed experimentally in our arrays. Note that changing k models the change of the trap depth. As the size of the trap decreases, the flow of media is able to more rapidly sweep away AHL and increase the effective degradation for the colony. Simulating smaller and more sparse trap sizes recovered antiphase behavior for neighboring biopixels (Fig. 3.14). We also simulated the arrays with traps of two different sizes in different rows and recovered the experimental 2:1 biopixel resonance or 2:1 + antiphase behavior depending on the trap spacing (Fig. 3d, bottom).

The model was also able to capture the alternating large and small amplitude oscillations observed in the ON/OFF biosensor (Fig. 3.15). This behavior was seen when C_0 was increased 2-fold, capturing the decreased level of LuxR in ON/OFF experiments where it was the limiting factor for oscillations.

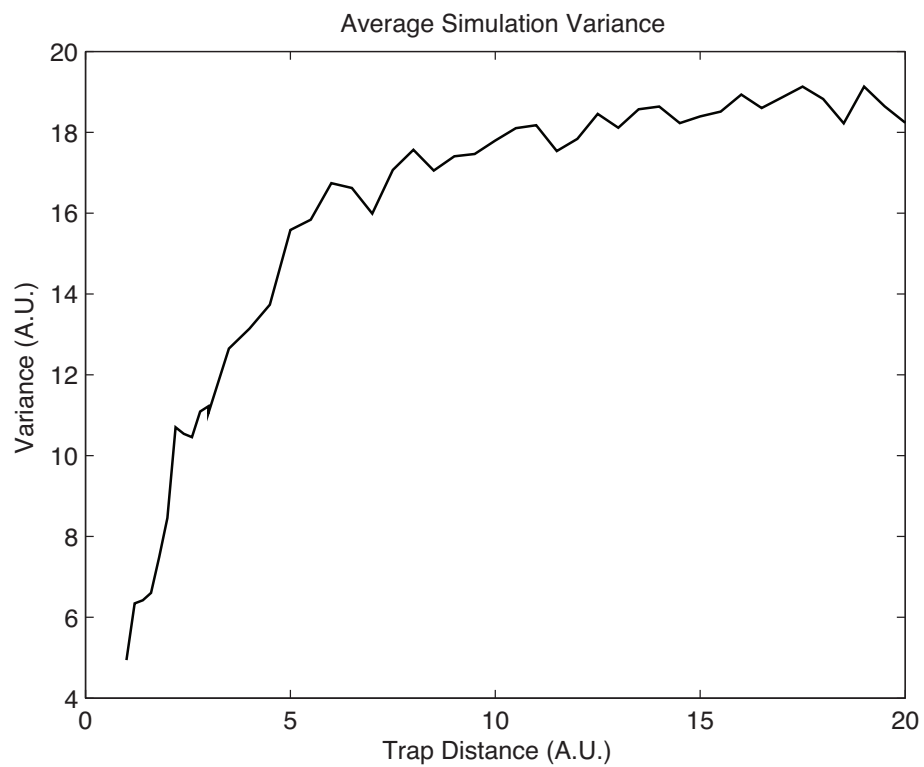


Figure 3.14: Computational results depicting biopixel synchronicity as a function of trap separation distance. As biopixels are moved farther apart, the entropy increases due to decreased effective migration of H_2O_2 between colonies.

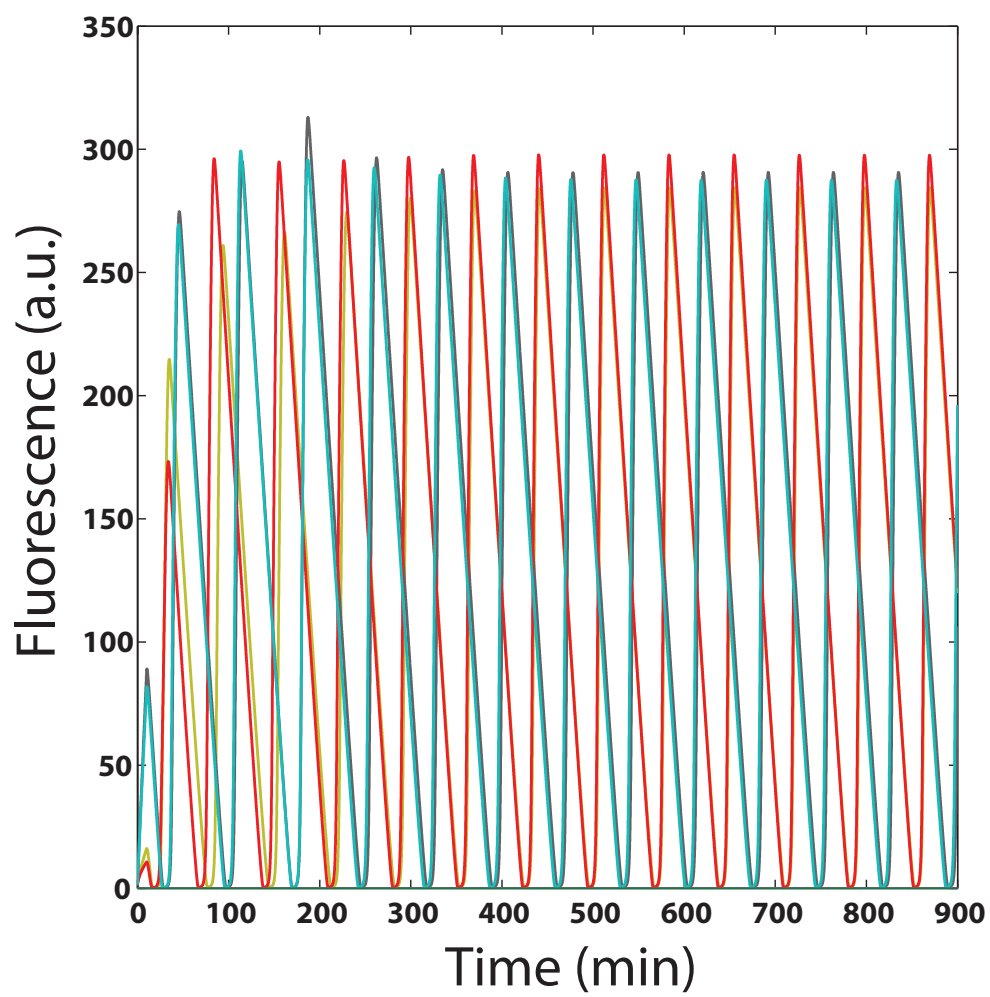


Figure 3.15: Antiphase behavior of 4 neighboring biopixels having equal trap sizes and spacing $\Delta = 3$.

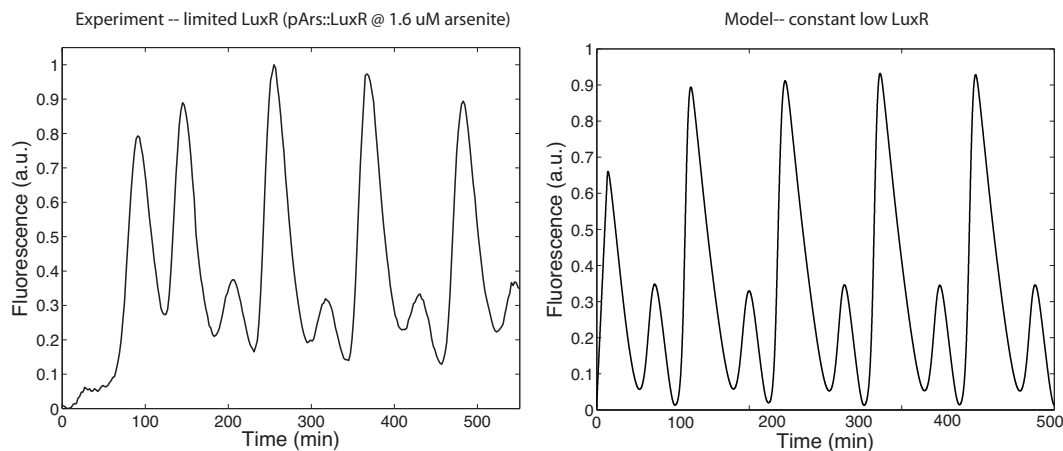


Figure 3.16: Oscillations of alternating large and small amplitude when LuxR is limited in experiments and simulations. The alternating oscillations vanish when LuxR is restored to its normal level in the model. Experimentally, we were unable to build a system in which LuxR is tunable between big/small and normal amplitude regimes. This is probably due to the small dynamic range of arsenite promoter-driven output of LuxR compared to the level produced by 3 constitutively expressed copies in the original circuit.

3.8 Acknowledgements

Chapter 3 contains material originally published as Prindle, A.*, Samayoa, P.*, **Razinkov, I.**, Danino, T., Tsimring, L., and Hasty, J., 2012: A sensing array of radically coupled genetic 'biopixels'. *Nature*, 481: 39-44. (*equal contribution). Copyright permission to republish here was granted by Nature Publishing Group.

Chapter 4

Hi-throughput gene expression at the level of single proteins using a microfluidic turbidostat and automated cell tracking

4.1 Introduction

Using time-lapsed phase-contrast and fluorescence microscopy, it is possible to monitor live bacterial cells and simultaneously quantify the expression of their highly expressed genes as the activity of introduced fluorescence reporters (Elowitz et al., 2002). However, for many of its native protein species, a bacterial cell expresses only a few copies per generation (Xie et al., 2008). In order to study processes involving these proteins, fluorescence microscopy methods sufficiently sensitive to resolve individual molecules have been developed. For instance, Yu et al. reported in 2006 of the use of a fast maturing yellow fluorescent protein variant, Venus (Nagai et al., 2002), fused to a membrane tag, Tsr, to profile the absolute expression of the *lacZ* gene, in live *Escherichia coli* cells in its repressed state (Yu et al., 2006). The Tsr domain immobilizes

the fluorophore at the membrane so that it appears stationary for periods of 50-100 milliseconds and can be detected as a diffraction-limited spot. However, tethering to the membrane will disable molecules that rely on intracellular mobility for their function. For this reason method for counting expression events for cytoplasmic proteins have been limiting. A possible solution is suggested by the single molecule tracking experiments performed in the Xie lab, in which stroboscopic illumination pulses were used to image the transcription factor LacI-Venus non-specifically bound to DNA in live *E. coli* cells (Elf et al., 2007). This suggests that short excitation pulses could be used also to profile the synthesis of cytoplasmic low copy number transcription factors or other proteins binding to relatively immobile intracellular targets.

Single-protein counting experiments *in vivo* reveal that isogenic cells under seemingly identical experimental conditions display considerable diversity in expression as previously described (Taniguchi et al., 2010). In order to confidently draw conclusions on the nature of this diversity it is necessary to sample a sufficient number of cells. Several microfluidic devices have been reported to substantially increase experimental throughput by harnessing the reproduction of bacterial cells to continuously regenerate the sample and also allowing imaging of many replicate colonies in parallel (Danino et al., 2010; Wang et al., 2010). However, the sheer size of image data sets that can be generated in this fashion overwhelms manual analysis efforts and consequently several initiatives of automation have been undertaken (Sliusarenko et al., 2011; Young et al., 2012). In this study we report of a method combining microfluidics, single-molecule fluorescence microscopy and automated image analysis, enabling the study of the expression and super-resolution localization of low copy number transcription factors throughout thousands of bacterial life spans per experiment. To illustrate the performance of the method we quantify the dynamics of synthesis and intracellular localization of the lactose repressor by monitoring LacI-Venus expressed from its native promoter in live *E. coli* cells. We compare these observation with those obtained under identical conditions for cells expressing the reporter construct Tsr-Venus from the lactose permease gene, *lacY*, of the lactose operon.

4.2 Materials and methods

4.2.1 Design, fabrication and use of the microfluidic device

The chip design was inspired by Mather et al. (Mather et al., 2010b). The features of the microfluidic chip used in this study were designed in three layers using AutoCAD. The layers correspond to structures of different step heights of the mold and ultimately to the different depths of the structures of the finished microfluidic device (described under Mold Fabrication and Chip Fabrication). The device contains four structural motifs; ports, channels, a chamber and traps (Fig. 4.1a, top right). The chamber houses three evenly spaced rows, each containing 17 traps (Fig. 4.1a, top right). Each trap is $40 \times 40 \times 0.9 \mu\text{m}$ (Fig. 4.1a, top left), which is bounded by two opposite walls and two open sides connecting the trap to the $10 \mu\text{m}$ deep surrounding. This geometry restricts the cells to form a monolayer colony in the focal plane while imaging. Cells close to the openings are released as the colony expands (Fig. 4.1a, top left). The microfluidic device is connected to media reservoirs and imaged using an inverted microscope (Fig. 4.1a, bottom). The master mold was fabricated using standard UV-soft lithography techniques. Three masks for microfabrication were printed in chrome. Custom formulations of SU8 Photoresist (MicroChem) were deposited on clean polished silicon wafers (University Wafer) using a spin coater. The wafers were then aligned to the mask and exposed using a mask aligner (Suss MA6). This process was repeated to deposit layers of step heights 0.9, 2.7 and $10 \mu\text{m}$ per wafer. The first layer corresponds to the trap depth of the microfluidic device; the intermediate layer enables the alignment of the first and third layer, which corresponds to the channels and ports. Each layer of the molds was measured using a stylus profilometer and inspected under a microscope before applying the next.

A master cast of the mold was made from polydimethylsiloxane, PDMS (Sylgard 184, Dow Corning), using the master mold. Bubbles were removed by vacuum desiccation. The cast was cured at 80°C for 30 minutes. One master cast contained 12

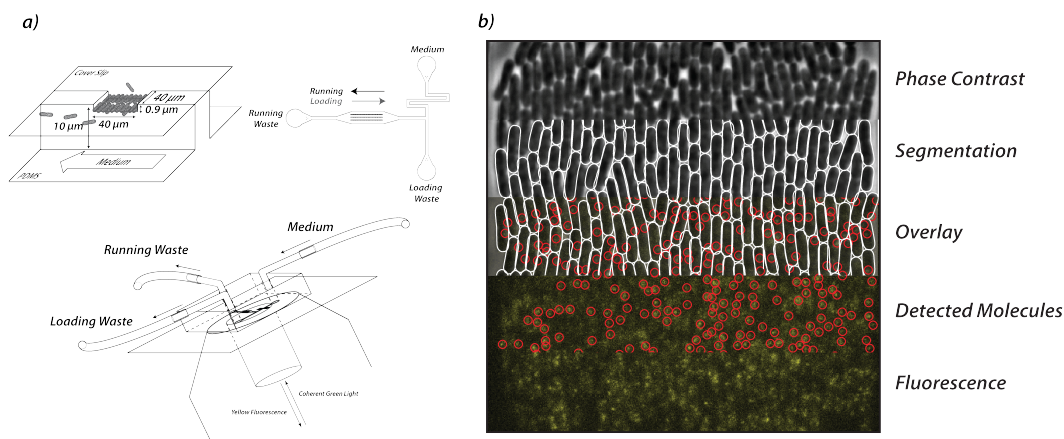


Figure 4.1: The experimental set-up and data processing. (a) The microfluidic device has three ports designated for medium, running waste and loading waste. The chamber houses three rows, each containing 17 traps. The direction of the flow through the chamber is alternated between the loading and running phase of the experiment. The cells are introduced from the running waste and are caught in the traps. (b) Each trap is a $40 \times 40 \times 0.9 \mu\text{m}$ compartment which is bounded by two rigid walls and two openings. Cells that reach the openings are released from the traps into the 10 mm deep surrounding. (c) The device is connected to reservoirs at the ports and imaged using an inverted microscope. The various parts of the microfluidic chip are not drawn in scale. (d) Data processing: cells are detected and segmented from the phase-contrast image (top). Molecules are detected within the fluorescence images (bottom). The coordinates from the detected molecules and cells are used to map molecules to cells (middle).

identical chip structures, which could be excised and used individually. When fabricating each device, port holes (0.5 mm diameter) were punched out of the device cast. Debris was removed from the cast by vortexing in Ethanol. The chip cast was bonded to a coverslip (40 mm diameter, 200 μ m thick, thermo-Scientific) after Oxygen/UV-plasma treatment (UVO-cleaner 42-220, Jelight Co.) for 5 minutes at 0.5 bar Oxygen pressure. The bond was stabilized by incubating at 80°C for 10 minutes. Just prior to loading and running the device, the ports were treated with a high frequency generator (model BA 20 D, Electro-Technic Products Inc.) and the device was flooded with de-ionized water. Gravity flow was used to control the direction and the magnitude of the flow inside the microfluidic device. The pressure gradients between the different ports of the device were established by differences in elevation relative to the sample of the connected reservoirs. During loading, the seeding culture was introduced into the device through the running waste port. The cells were caught in the traps by introducing pressure waves into the tubing. Once all traps were sufficiently occupied (10-100 cells per trap), the direction of the flow in the chamber was reversed, exchanging the seeding culture with fresh medium (Fig. 4.1a top right). The cells were allowed to acclimatize and grow until the traps were fully occupied ($\approx$ 4 hours) before imaging. The temperature of the sample was maintained at 37°C using a custom-fitted incubator hood (OKO-lab).

4.2.2 Strains and medium

Two bacterial strains, SX701 and JE116, based on *E. coli* strain BW25993 (Datzenko and Wanner, 2000), were used in this study. In strain SX701, the lactose permease gene, *lacY*, was replaced with the *tsr-venus* construct (Choi et al., 2008). Strain JE116 is based on strain JE12 (Elf et al., 2007), in which the *lacI* gene was modified to encode a C-terminal fusion of LacI and Venus. The auxiliary lactose operator site, *O3*, was replaced with the main operator sequence, *O1*, to increase auto-repression by LacI threefold. Further, in strain JE116 the downstream sequence, including the native *O1*, *O2* binding sites as well as parts of the *lacZ* gene was removed, leaving only one specific

binding site sequence for LacI-Venus molecules per chromosome copy (Hammar et al., 2012).

Cells were grown in M9 minimal medium, with 0.4% Glucose, either with or without supplemented amino acids (RPMI1640 (R7131), Sigma-Aldrich). An overnight culture was diluted 200 times in 40 ml fresh medium and incubated for 3-5 hours (6-8 hours for cells grown without amino acids) at 37°C and shaking at 225 rpm. During this incubation the microfluidic device was prepared. Cells were harvested into a seeding culture by centrifugation at 5000×rcf for 2.5 minutes and the pellet resuspended in 50-100 μ l fresh medium. In order to prevent the cells from sticking to the surfaces of the microfluidic device a surfactant, Pluronic F108 (prod. Number 542342, Sigma-Aldrich), was added to all medium to a final concentration of 0.85g per liter.

4.2.3 Microscopy and imaging

Imaging was performed using an inverted microscope (TI Eclipse, Nikon) fitted with a high numerical aperture oil objective (APO TIRF 100× / N.A 1.49, Nikon) and external phase contrast to minimize loss of fluorescence signal. The phase contrast channel and the fluorescence channels were imaged using separate cameras, a model CFW-1312M (Scion Corporation) and a Ixon EM plus (Andor Technologies) respectively. Focus was maintained by the Perfect-Focusing-System of the microscope. The light source for fluorescence excitation was an Argon ion laser (Innova 300, Coherent Inc.) dialed to 514 nm for excitation of YFP-reporters in the sample. For fluorescence imaging, a slower shutter (LS6Z2, Uniblitz) was used for strain SX701 (Tsr-Venus) and a fast shutter (LS2Z2, Uniblitz) was used for strain JE116 (lacI-Venus). The fast shutter was controlled using a signal generator (AFG3021B, Tektronix) which was triggered by the Ixon camera, exposing the sample for 1 millisecond. A 2x magnification lens was used in the fluorescence emission path to distribute the point spread function ideally on the 16 μ m pixels of the Ixon camera. Image acquisition was performed using RITAcquire, an in house GUI-based plugin for micromanager (v1.3.4.7, www.micro-

manager.org). The RITAcquire package; containing the plugin, installation instructions and a description of the functions, will be distributed at request. In each experiment three positions (traps) were subjected to the following acquisition program in parallel: every thirty seconds (every frame), a phase contrast image (125 ms exposure) was taken for all positions. Every three minutes (1/6 frames) for all positions, in addition to the phase contrast image, two fluorescence images (50 milliseconds exposure for SX701 and 1 ms exposure for JE116) were taken in rapid succession, followed by a bright field image (100 ms exposure) of the fluorescence channel, i.e. using the white-light lamp of the microscope as illumination source. This programming cycle was repeated for 1001 frames (8.3 hours). Fluorescence images were acquired in tandem to account for the effects of bleaching on molecular counting (see under 4.2.6). The bright field images were acquired to allow alignment of phase contrast and fluorescence images for each frame. Our automatic method for cropping the phase images and aligning them to the fluorescent images is described in the supplementary methods.

4.2.4 Cell segmentation and tracking

For segmenting and tracking individual cells in the microfluidic device, we have modified and further developed existing MATLAB software suite, named MicrobeTracker (Sliusarenko et al., 2011). MicrobeTracker uses the position of cells in the previous frame as an initial guess and applies an active contour model (Kass et al., 1988) to fit each cell with a sub-pixel resolution boundary. In order to accurately track mobile cells over several generations, three supervised algorithms (Bishop, 2006) were implemented in MicrobeTracker: a cell pole tracker and two separate error detectors. The cell pole tracker is used to help the active contour model find the cell poles correctly for moving cells; otherwise this will lead to error propagation in the subsequent frames. The first error detector identifies errors made by the cell pole tracker. This is usually the result of an occasional large displacement of the cell between frames. This activates the cell tracker, which attempts to correct the segmentation of the erroneous

cell. The accuracy of the cell tracker is in turn monitored by a second error detector. Any cells histories triggering this detector are terminated. In addition, a novel division function was added to MicrobeTracker in order to more accurately detect cell divisions. Each supervised algorithm was constructed by first identifying features that efficiently discriminate between two classes, for instance, true or false cell division. In the second step, training data was extracted manually from the image sets for creating training examples for the algorithm in order to achieve accurate classification. A linear classifier (Bishop, 2006) was used in all supervised algorithms. The algorithms, cell tracker and the classification method are described in detail in the supplementary methods. To increase the computational speed, parts of MicrobeTracker were rewritten to allow parallel computing using MATLABs Parallel Computing Toolbox (PCT).

4.2.5 Single molecule detection, localization

Fluorescent particles in the sample were detected as diffraction limited spots in the fluorescence micrographs according to the method described in Ronneberger et al. (1998), in which the normalized cross-correlation between the fluorescence image and an idealized optical point spread function (a symmetric bi-variate Gaussian function) is calculated. The standard deviation (s.d.) for this function is obtained experimentally by imaging and the signatures of immobilized highly fluorescent beads (data not shown). The image resulting from the correlation is transformed using the Fisher transform. A Fisher transformed Gaussian function with s.d. corresponding to the point spread function is fitted to the Fisher transformed correlation image using the Levenberg-Marquardt method (More, 1978) implemented in MATLAB, and the obtained parameters are used to localize each molecule with super-resolution accuracy and estimate the localization error.

4.2.6 Maximum likelihood Estimate of Synthesis

For gene expression studies, we want to estimate how many molecules have been newly synthesized between two fluorescence images given that there is a chance that some of the fluorophores present in the previous frame have not been bleached. We formulate this as a maximum-likelihood problem where there are M molecules observed in frame $i - 1$ and N molecules observed in frame i . The number of molecules surviving bleaching, m , can be calculated by maximizing the probability $p(m|M, N, p, \lambda) = \text{Bin}(m, M, 1 - p) \cdot \text{Po}(N - m, \lambda)$, where Bin is the binomial distribution and Po is the Poisson distribution. The maximum-likelihood estimate of the number of new synthesized molecules is $n_{max} = N - m_{max}$, where m_{max} maximizes $p(m|M, N, p, \lambda)$. The parameter p is the bleaching probability per fluorophore per frame and is assumed to be constant. λ is the number of molecules synthesized between two frames.

In the special case of cell division between frame $i - 1$ and i , where $N1$ molecules are found in one daughter cell and $N2$ in the other, the most likely number of newly synthesized molecules n_{max} are calculated for both cells based on $N = N1 + N2$. Given n_{max} the most likely number of newly synthesized molecules in daughter cell 1 is the $n1$ that maximizes $\binom{N1}{n1} \binom{N2}{n_{max} - n1}$ because this gives the number of possible combinations of picking $n1$ molecules from $N1$ and $n - n1$ from $N2$.

4.3 Results

4.3.1 Throughput

Currently, one experiment returns approximately 3000 complete cell histories from three traps imaged in parallel. The total time of expenditure is 36 h. The manual effort of a single operator amounts to 3 h, of which roughly 80 percent is spent prior to image acquisition. The manual work effort to acquire and analyze the images constitutes less than 2 percent of the total time required to complete these processes (Figure 4.2a).

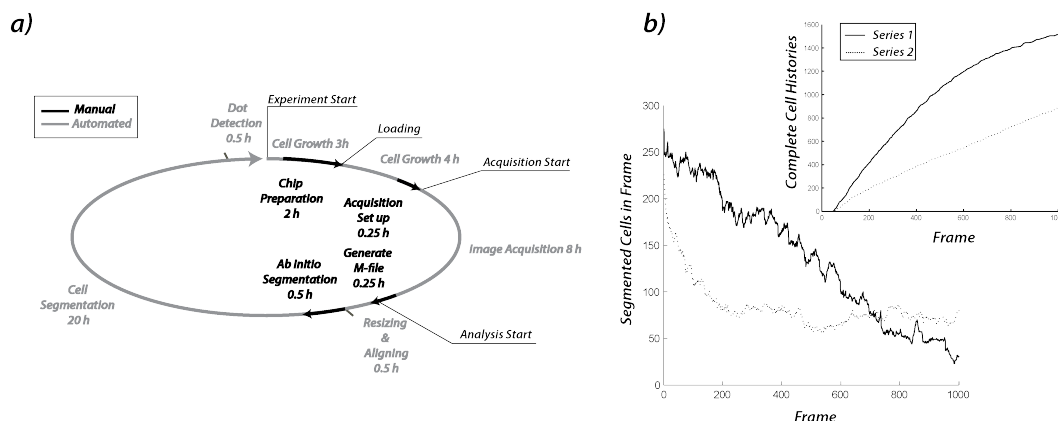


Figure 4.2: Throughput of the method. (a) Time distribution for an experiment. The minimum time expenditure for an experiment is around 36 h. Currently, the method generates around 3000 complete cell generations per experiment. The protocol contains mostly automated steps, i.e. they require no attention from the operator. The manual time expenditure accounts for less than 10% of the total time and less than 2% of the analysis time. (b) The number of segmented cells remaining at different frames during the analysis of two different time-series. Owing to large movements in the cell colony, the loss of correctly segmented cells varies between series. The nature of the decay is observed to depend on the pattern in which the cells grow in the trap, for which no sufficiently accurate prediction model has yet been found. (c) The integrated numbers of cells acquired from cell division to cell division, i.e. the number of complete cell histories, for the two series in (b).

Several overlapping experiments can be performed to use the alternating availability of the microscope and the computational framework to further improve throughput. The number of cell histories acquired from an image series is determined in the segmentation process. The cells sometimes make large displacements between two frames. When the cell tracker fails to track the cell, the cell history is terminated. Therefore, the number of cells that the program keeps track of decreases over time. The rate of decay varies considerably between image series, even when acquired under seemingly identical conditions (Figure 4.2b). Only the set of cell histories that completely cover the time from division-to-division enter the analysis (Figure 4.2c).

4.3.2 Morphology and growth in microfluidic device

The generation time defines the growth rate of exponentially growing cells and is often used as an indicator of the health or fitness. We compare cells grown with and

without amino acids in the medium (Figure 4.3a, red and blue, respectively) and observe average generation times of 26.4 ± 7.2 and 46.8 ± 17.0 min, respectively. Further, we observe an exponential growth of the cell length over the cell cycle (Figure 4.3b). In contrast to previous reports (Mather et al., 2010b), we observe no obvious dependencies of the growth rate on the position the cell occupied in the trap (Figure 4.3c). This uniformity also holds for morphology and bacterial age, i.e. the number of divisions during which the oldest pole of a cell has been observed. We find that the generation times of mother and daughter cells are weakly correlated ($r = 0.27 \pm 0.02$ with amino acids, $r = 0.07 \pm 0.05$ without amino acids; Figure 4.3d). The relation between the length at birth and the generation time of a cell history displays a correlation (Figure 4.3e), indicating that comparatively longer newborns complete their cell division faster. Although this holds qualitatively for cells grown both with and without supplemented amino acids (red and blue), it is less pronounced for cells grown without amino acids. Also, cells grown with amino acids vary more in length at birth than in generation time and the opposite is observed for cells grown without supplemented amino acids. The correlation for cells with amino acids is $r = -0.43 \pm 0.02$ and without amino acids $r = -0.28 \pm 0.04$. No significant differences in growth or morphology between strains SX701 and JE116 are observed.

4.3.3 Localization of transcription factors during the cell cycle

In figure 4.4, we compare the intracellular localization of the reporter constructs, Tsr-Venus (Figure 4.4a) and LacI-Venus (Figure 4.4b), over the cell cycle. A localization distribution function (Figure 4.4, left) is constructed by mapping the detected molecules to their position along the major axis of the cell (x-axis) at the time in the cell cycle they were detected (y-axis) and smoothed using a Gaussian filter. To increase synchronicity, only observations occurring in cells with generation times between 25 and 32 min and terminal lengths of $4-7 \mu\text{m}$ are included (780 for SX701 and 1176 for JE116). In the right of figure 4.4, we visualize the detected molecules of each construct as bi-

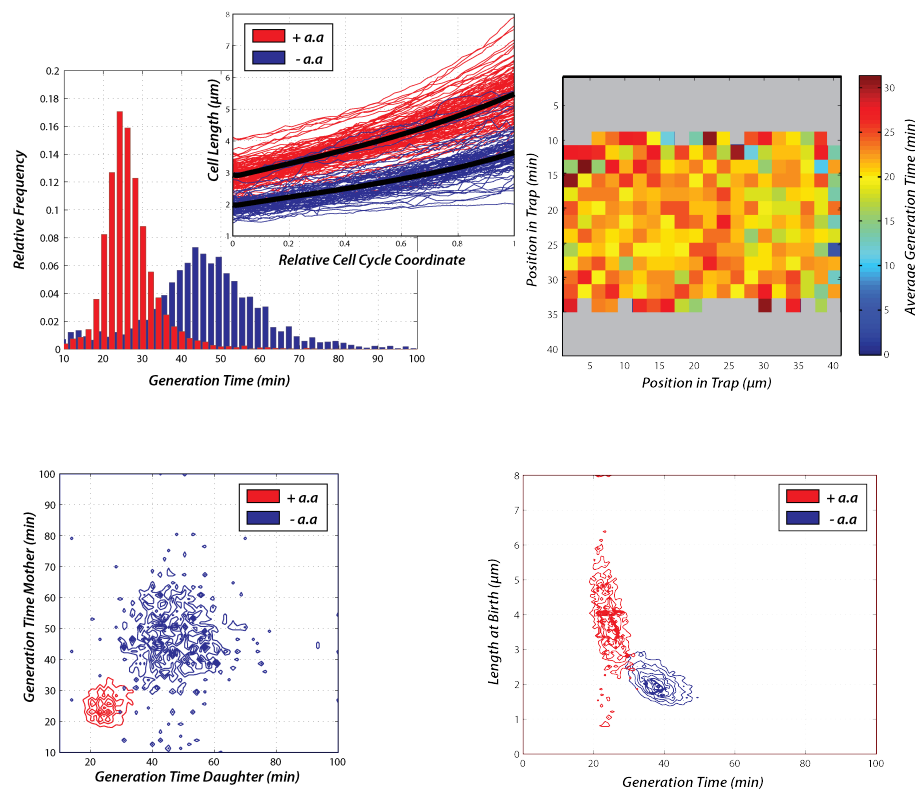


Figure 4.3: Morphology and growth of cells in the microfluidic device. (a) The distribution of generation times of cells grown with (red, $n = 6755$) and without (blue, $n = 2298$) supplemented amino acids. (b) Cell length as a function of relative cell cycle coordinates, i.e. time from birth normalized by the generation time, for randomly selected cells with (red, $n = 6755$) and without (blue, $n = 2298$) supplemented amino acids. (c) Growth rate as indicated by average generation time over the geometry of the trap for cells growing with supplemented amino acids ($n = 6755$). The figure is oriented so that the outlets of the trap are on top and bottom (Figure 4.1b). (d) Joint distribution of generation times for daughter and mother cells with (red, $n = 6755$) and without (blue, $n = 2298$) supplemented amino acids. (e) Joint distribution of generation time and cell length at birth for cells with (red, $n = 6755$) and without (blue, $n = 2298$) supplemented amino acids.

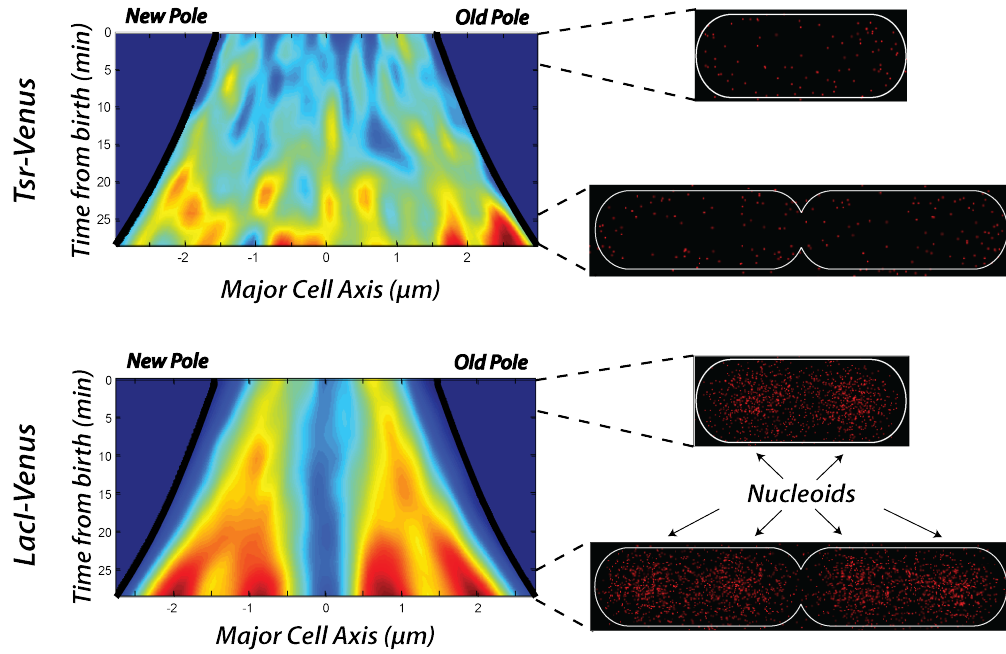


Figure 4.4: Intracellular localization of molecules over the cell cycle. On the left are the observed molecule densities projected onto the major axis of the cell as a function of time from birth to division for (a) Tsr-Venus and (b) LacI-Venus. The units are the average number of molecules per minute and μm . The black line at the edge indicates boundary of cell at the apex of the cell poles and expands as the cell grows. On the right is shown the localization of individual (a) Tsr-Venus and (b) LacI-Venus molecules with super-resolution accuracy for early and late stages of the cell cycle.

variate symmetric Gaussian functions to create a PALM style super-resolution plot of the intracellular distribution. We do not observe the typical polar localization that may be expected for Tsr (Figure 4.4a). This is most likely because the protein is inserted at random positions in the membrane and bleaches before reaching the Tsr clusters in the polar regions (Nagai et al., 2002). For LacI-Venus molecules (Figure 4.4b), we observe a tendency to cluster at positions corresponding to the nucleoids of chromosomal DNA. The number of nucleoids doubles from two, early in the cell history, to four in the later stages, which is consistent with expectations for our growth conditions.

4.3.4 Synthesis dynamics of an auto-repressed transcription factor throughout the cell cycle

Figure 4.5 shows lineage trees of cell histories stemming from a single ancestral root of strain SX701 (Figure 4.5a) and JE116 (Figure 4.5b) with bars corresponding to the number of Tsr-Venus and LacI-Venus molecules at the times they were synthesized. The trees are pruned as cells are lost from the segmentation and/or from the trap. For Tsr-Venus expressed from the *lacY* gene, we observe 1.5 ± 0.1 molecules per expression event and 1.7 ± 0.1 events per cell cycle. For LacI-Venus, 2.2 ± 0.05 molecules per expression event and 2.5 ± 0.04 events per cell cycle are observed. The average expression rates of Tsr-Venus and of LacI-Venus molecules over the cell cycle are shown in Figure 4.5c,d. Both show relatively large statistical errors, especially Tsr-Venus. The cell histories with generation time 25-32 min and terminal length $4-7\mu\text{m}$ are used. For strain JE116, 1418 complete cell histories and 7910 LacI-Venus molecules are observed. For strain SX701, 780 cell histories are retained from the experiment and 1176 Tsr-Venus molecules. The combination of fewer cell histories and lower expression levels leads to larger statistical uncertainty in determining the expression rate of Tsr-Venus from the *lacY* gene. However, our results indicate a greater expression rate of LacI-Venus at the beginning of the cell cycle.

4.4 Discussion

In this study we report on a method combining microfluidics, time-lapsed single-molecule microscopy and automated image analysis capable of monitoring the growth and absolute number of gene expression events throughout approximately 3000 complete individual *E. coli* life-spans per experiment. Further, we demonstrate that it is possible to use a functional transcription factor, LacI-Venus, nonspecifically interacting with DNA, to retrieve information on both expression dynamics and super-resolution localization dynamics throughout the cell cycle. We show that the microfluidic chip

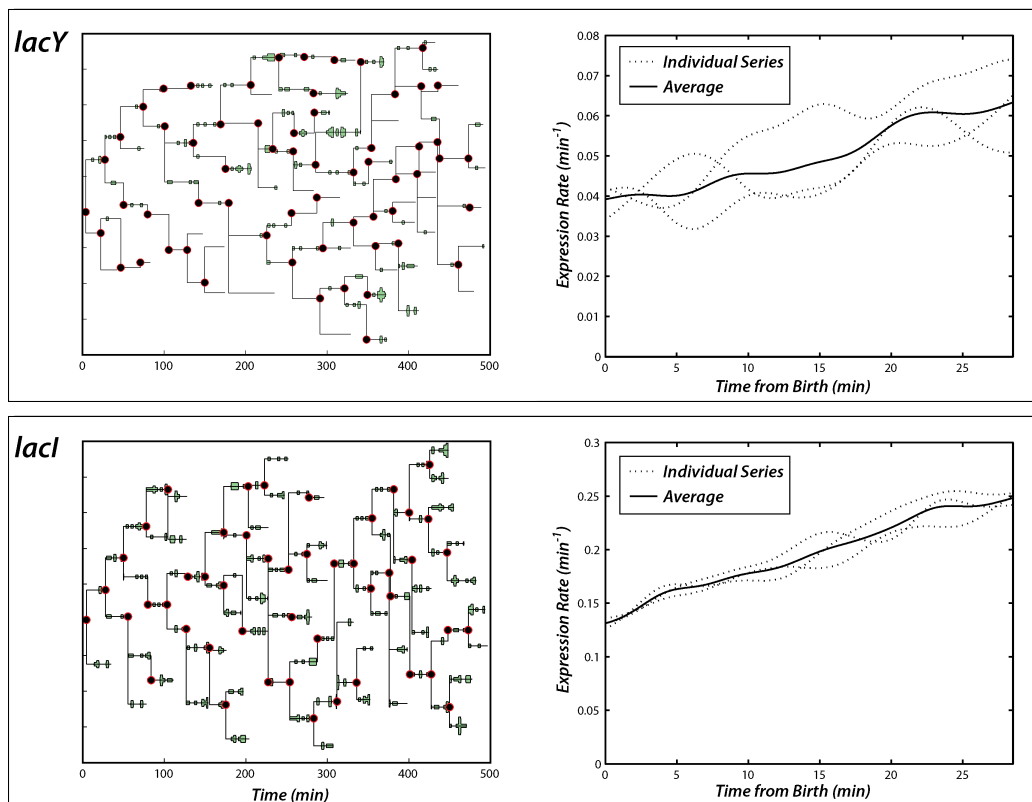


Figure 4.5: The rate of gene expression over the cell cycle. (a,b) Representative lineage trees for strains (a) SX701 and (b) JE116 stemming from one ancestral root. The absolute number of newly synthesized molecules expressed from the *lacY* and *lacI* gene are indicated as grey bars at the time they are detected. (c,d) The average expression rates from the (c) *lacY* and (d) *lacI* genes over the cell cycle. Solid lines show the average of all three series for each construct. The average of the individual series is shown as dotted lines as an indication of the uncertainty in determining the mean.

provides a beneficial and stable environment for exponentially growing *E. coli* cells and a high degree of control and reproducibility. We observe a significant variability in generation times of individual cells. However, we find that generation time is relatively memory-less from generation to generation. More interestingly, cells living in richer media vary more in length at birth than in generation time and that the opposite is true for cells living in poorer media. The underlying causes for this size-generation time uncertainty relation and for which range of conditions it holds are presently unclear. LacI-Venus molecules localize onto the nucleoids in the cell. It appears that non-specifically interacting transcription factors are uniformly distributed over the DNA. As expected, we find that LacI-Venus is more highly expressed than Tsr-Venus from the *lacY* gene position. Our result for the latter is consistent with the findings of Yu et al. (2006) in the number of gene expression events from the *lacZ* gene position during the cell cycle. However, we observe fewer Tsr-Venus molecules per expression event (1.7 ± 0.1 instead of 4.2 ± 0.5). Given that *lacZ* and *lacY* are transcribed to a polycistronic mRNA, we conclude that the translation rate at the *lacY* position is two to three fold lower than that of the *lacZ* position. The average rate of LacI-Venus expression is slightly higher in the beginning of the cell cycle. We propose that this may be due to partition inequalities at cell division, in which disfavoured cells replenish their transcription factor pools. The experiments confirm the highly variable nature of *in vivo* single-molecule observations (Figure 4.5). We estimate that to obtain a 5 percent accuracy of the mean expression rate per minute for all points in the cell cycle would require to a total of 4000 and 16 000 complete cell histories of JE116 (lacI-Venus) and SX701 (Δ lacY::Tsr-Venus), respectively. Sufficient observations could therefore be obtained with three additional experiments for JE116 and fifteen additional experiments for SX701. The Mather design can potentially sustain a population of bacterial cells in a state of exponential growth indefinitely. Many biological phenomena, such as the development of antibiotic resistance, occur in a small subpopulation of all cells and on longer time-scales than the current longevity of an experiment using our method. Further increasing the throughput and the longevity of the method to enable the study of such phenomena represents a formidable

image analysis challenge. However, to our advantage is that the performance of supervised algorithms improves and can be made more advanced as more training data accumulate. We are confident that more advanced algorithms can be implemented to increase both accuracy and speed, which would make it possible to acquire an arbitrary number of cell histories from a single experiment.

4.5 Acknowledgements

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Chapter 5

Measuring competitive fitness in dynamic environments.

5.1 Introduction

The theory of evolution asserts that genotypes that convey optimal fitness in a given environment will be selected for through a competition for resources among individuals with various genetic sequences. The ability to predict the selective advantage of a particular allele has been exploited throughout human history in various contexts from farms to laboratories, where a specific environment is created to artificially select for desired traits. However, competitive fitness has been less well explored for cases where the selective advantage of an adaptive trait is strongly dependent on environmental dynamics. While it is easy to accept that polymorphisms that affect gene expression dynamics would influence fitness in fluctuating environments, experiments to measure actual fitness advantages in dynamic situations have been technically challenging. The goal of the present study was to invent an experimental platform for the long-term monitoring of mixed population dynamics in fluctuating environments and to use this technology to measure the selective advantage conveyed by a genetic mechanism that enhances a cell's ability to respond to environmental changes.

The ideal system for conducting dynamic evolution experiments would allow a mixed population of cells to be co-cultured in a precisely controlled environment over long periods of time while granting the periodic collection of culture samples. Mixed populations can be cultured long-term in fermentors or bioreactors, but the environmental dynamics cannot be precisely controlled in these devices and the cost of replacing the medium once in every generation time can quickly become prohibitive. Microfluidic chemostats are a potentially suitable alternative because the environment can be controlled dynamically, while the small volume of the growth chamber dramatically reduces the cost of media consumption over long experiments. However, most microfluidic devices are not designed to hold enough cells for evolution experiments and do not allow the culture to be collected. We sought to combine the best features of large-scale fermentors and microfluidic chemostats to create a device that could be used to measure population dynamics. The resulting microfluidic device has a cell growth chamber large enough to maintain a log-phase culture of $\sim 10^6$ yeast cells for at least 500 generations. In addition, samples of culture can be collected from the effluent line for real-time monitoring of population dynamics using any of several genetic or biochemical assays. Alternatively, the monolayer growth chamber allows the experiment to be monitored by fluorescence time-lapse microscopy. The design of the media ports was based on our dial-a-wave (DAW) chip and can be used to generate media fluctuations of practically any frequency or wave form, providing exquisite control over environmental dynamics. We named this device the evoDAW (evolution dial-a-wave) chip and tested it by monitoring the population dynamics of a mixed culture of two isogenic yeast strains that differ in their ability to respond to changes in carbon source.

Glucose is the preferred carbon source of yeast cells, though they are capable of growth on many types of sugar. Yeast metabolic networks contain several mechanisms to ensure that secondary carbon sources are not consumed in the presence of glucose. The most well studied of these networks is that of galactose metabolism, which is inactivated by transcriptional and post-transcriptional means in response to glucose addition. Cells growing on galactose respond to glucose by rapidly degrading specific gene tran-

scripts encoding enzymes for galactose metabolism and entering the cell division cycle. The ability of galactose-induced cells to respond to glucose has been attributed to the spatial co-regulation of transcripts for galactose network genes and the cell cycle regulator, cyclin 3 (Cln3p). The physical proximity of the transcripts creates competition for translational resources when galactose is the sole carbon source. When glucose is re-introduced, the *GAL* gene transcripts are specifically degraded and the competition is relieved, inducing the translation of *CLN3* messages, which leads to cell cycle entry. The spatial regulation of *GAL1* and *CLN3* is only detected in cells that have recently experienced growth in both glucose and galactose. This is thought to be a mechanism that allows the cell to anticipate the return of glucose to the growth medium when glucose availability has been transient.

As part of a previous study, we created a mutant version of the *GAL1* transcript that is stabilized in the presence of glucose. Strains expressing this allele of *GAL1* (called ST for stable) degrade *GAL1* messages inefficiently in response to glucose addition, and consequently the translation of *CLN3* transcripts is delayed in response to glucose relative to WT cells. When growing in a constant environment containing either galactose or glucose, WT and ST cells have identical growth rates. However, the stabilization of *GAL1* mRNA causes ST cells to have a transient cell cycle defect immediately following the addition of glucose to a galactose culture; although, these cells eventually attain wild type cell cycle dynamics. We began the current study by developing a computational model to predict whether the transient difference *GAL1* mRNA levels would convey a measurable selective advantage to WT cells over ST cells growing in conditions where the sugar source alternates continuously between glucose and galactose. We then used the microfluidic evoDAW chip to experimentally validate the predictions put forth by the model. Our experimental results support the model's prediction that the WT genotype does convey a selective advantage to cells growing in dynamic conditions. Interestingly, the strength of the adaptive advantage conveyed by glucose-sensitive *GAL1* mRNA decay depended on the total number of times the sugar source switched from galactose to glucose, not on the period of media oscillations. Our

results underscore the importance of dynamic processes in the evolution of species.

5.2 Methods

5.2.1 Experimental

Microfluidic EvoDAW devices were assembled as previously described (Ferry and Razinkov (2011)). Overnight cultures of WT and ST were grown in 25 ml of 1%galactose/1%glucose synthetic complete media (Baumgartner et al., 2011). The devices were loaded with an equal mixture of WT and ST. During the experimental run, two different media were attached to the microfluidic devices: media 1 - 0.5%galactose synthetic complete, media 2 - 0.5%galactose/0.25%glucose synthetic complete. The devices were incubated at 30°C in a custom-fabricated acrylic enclosure. The media syringes were placed on automatic DAW tracks with different periods of square wave oscillations, as previously described (Ferry and Razinkov, 2011). The exiting media and cells were collected from the waste port of the device into a 14 ml falcon tube. The devices were periodically monitored for cell growth and full occupancy using an inverted (Nikon Diaphot) light microscope equipped with 4x (Nikon, N.A. 0.1) objective.

5.2.2 Data collection and Analysis

Samples were collected every 24 hr, serially diluted 1:1000 and plated on -Leu and -Trp plates to select for WT and ST, respectively. Once colonies formed, the plates were imaged and recorded using UVP Biodoc-It Imaging System. The number of colonies in each image was counted using ImageJ. The ratio of WT cells was calculated as the number of WT colonies divided by the sum of WT and ST colonies $R = WT/(WT + ST)$. Multiple repeats were performed for each media switching period. The data for each experiment was normalized to a starting ratio of 0.5, then the average and standard error were calculated. Linear fit lines were calculated in Excel using a linear regression algorithm with y-intercept set to 0.5.

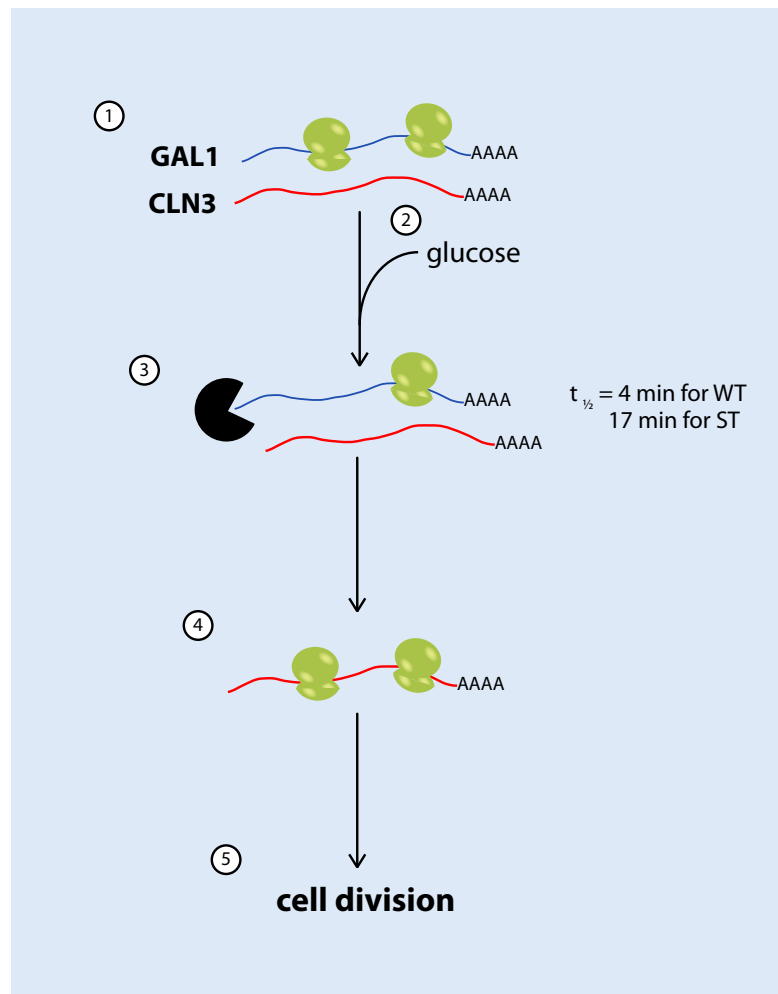


Figure 5.1: Translational competition mechanism regulating response of galactose-induced culture to glucose addition. (1) When cells are in galactose medium lacking glucose, GAL1 transcripts are highly expressed and spatially sequestered with less-abundant CLN3 transcripts. The GAL1 transcripts have a higher translational efficiency, so CLN3 translation is kept low and the length of G1 phase is long. (2) Glucose is introduced into the medium triggering catabolite repression that includes the transcriptional silencing of GAL genes, and (3) the rapid degradation of GAL transcripts. The half life of GAL1 transcripts in the presence of glucose is determined by sequences in the 5'UTR and is ~ 4 min in the WT strain and ~ 17 min in the ST strain. (4) The degradation of GAL1 transcripts relieves the competition for translation components, leading to an increase in Cln3p synthesis. (5) Cells enter S-phase as the result of Cln3p-induced gene expression. The longer half life of GAL1 transcripts in ST cells causes a delay in the cell cycle response to glucose.

5.3 Results

5.3.1 Modeling dynamics of mixed WT and ST populations

It has been shown that WT colonies grow better than ST colonies when the sugar source oscillates between galactose and glucose. The reason for this is not obvious because the differences in cell cycle dynamics between the two strains is slight and exists for only a few minutes following the transition from galactose to glucose. For most of the time, the two strains have identical growth rates. To predict whether the transient cell cycle difference provided by glucose-sensitive GAL1 mRNA could contribute significantly to population dynamics, we turned to mathematical modeling. We created a stochastic model of cellular growth and division consisting of a modified version of Gillespie's algorithm that takes into account various phases of the cell cycle (Figure 5.2, see Supplementary Information for details). In yeast, the restriction point beyond which a cell is committed to entering the cell cycle is called START. While in G1 phase, each cell spontaneously begins the cell cycle (i.e. passes START) with rate r_{START} , which depends on the current carbon source and the time since the last switch. Simply put, r_{START} will switch between two values, r_{gal} and r_{glu} , for galactose and glucose environments, respectively. However, as the carbon source is changed from galactose to glucose, the switch happens according to the function

$$r_{START} = r_{glu} - (r_{glu} - r_{gal})\exp(-\lambda t^*) \quad (5.1)$$

where λ is the decay rate of GAL1 mRNA and t^* is the time since the addition of glucose. Once past START, a dividing cell enters the S/G2/M phases. We chose to model these phases with a variable delay of average length t_c , which also depends on the carbon source. Since the decay rate of GAL1 mRNA differs between the WT and ST strains, so too does the rate at which r_{START} relaxes after the addition of glucose. In our simulations, this difference causes the relative fraction of cells in G1 phase to decline faster in WT cells than in ST cells, similar to what was observed by flow cytometry (Baumgartner et al., 2011). Interestingly, even though the half-lives of GAL1

mRNA while in glucose are short in both strains (≈ 4 min for WT and ≈ 17 min for mutant), the relative difference in the G1 fraction persists for much longer after the introduction of glucose. Figure 5.2 shows the time evolution of the population fraction, $R(t) = n_{WT}/(n_{WT} + n_{ST})$, of a mixed population containing n_{WT} WT cells and n_{ST} ST cells in several dynamic environments, obtained from stochastic simulations. The symbols represent the averaging of 100 trials in which the media was regularly and periodically switched from galactose to glucose, and back, such that each medium was in the environment for an equal amount of time. In each simulation, the WT strain eventually takes over the entire population at a rate that depends on the period of the cycling. In particular, one can show that if the population undergoes m switches from galactose to glucose, and if they remain in either environment sufficiently long, then the population fraction of the WT strain after the m^{th} switch, R_m , is given by

$$R_m = \frac{1}{1 + \frac{1-R_0}{R_0} \exp(-m\sigma\tau_{eff})} \quad (5.2)$$

where R_0 is the population fraction of the WT cells before the first switch, τ_{eff} is the effective delay in growth rate change caused by slowly decaying GAL1 mRNA, and σ is the difference in growth rate between glucose and galactose environments (see Supplementary Information for details). The solid lines shown in Fig. 5.2 are Eq. (2) simultaneously fit to all three trials, with $\tau_{eff} = 10.5$ min and $m = t/T$, where T is the period of environmental cycling. Note that the rate of increase of the relative population fraction depends primarily on the total number of switches from glucose to galactose. If we plot each of the relative population fractions obtained from the three trials shown Figure 5.2 against the total number of switches, we find that they collapse onto a single curve defined by Equation 2 (see Figure 5.2).

5.3.2 Experimental Results

To test the predictions made by our computational model, we developed a novel microfluidic device capable of long-term culturing in a dynamic environment. Previous

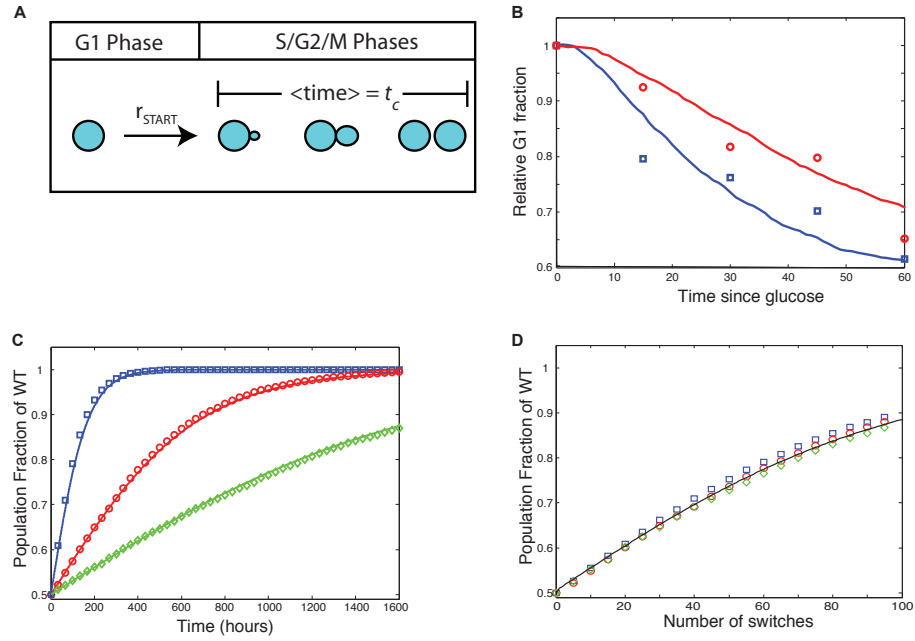


Figure 5.2: Modeling transcript decay-dependent growth. **A.** Cellular growth in the stochastic model consists of two phases. Cells leave G1 phase according to the rate r_{START} , and then enter a delayed growth and division cycle (S/G2/M phases) that takes, on average, a time t_c . **B.** Relative fraction of cells in G1 phase for WT (blue) and ST cells (red) immediately after the introduction of glucose. Solid lines are the results of a single stochastic simulation and symbols are the experimental results obtained from flow cytometry. **C.** Population fraction of WT in a mixed population of WT and ST cells in a dynamic environment. Symbols represent the average population fraction of 100 stochastic trials for an environmental cycling period of 100 min (squares), 400 min (circles) and 1000 min (diamonds). The solid line is the analytical prediction, Eq. (2), fit to all curves simultaneously with $\tau_{eff}=10.5$ min. **D.** The same three trials plotted against the total number of switches from galactose to glucose.

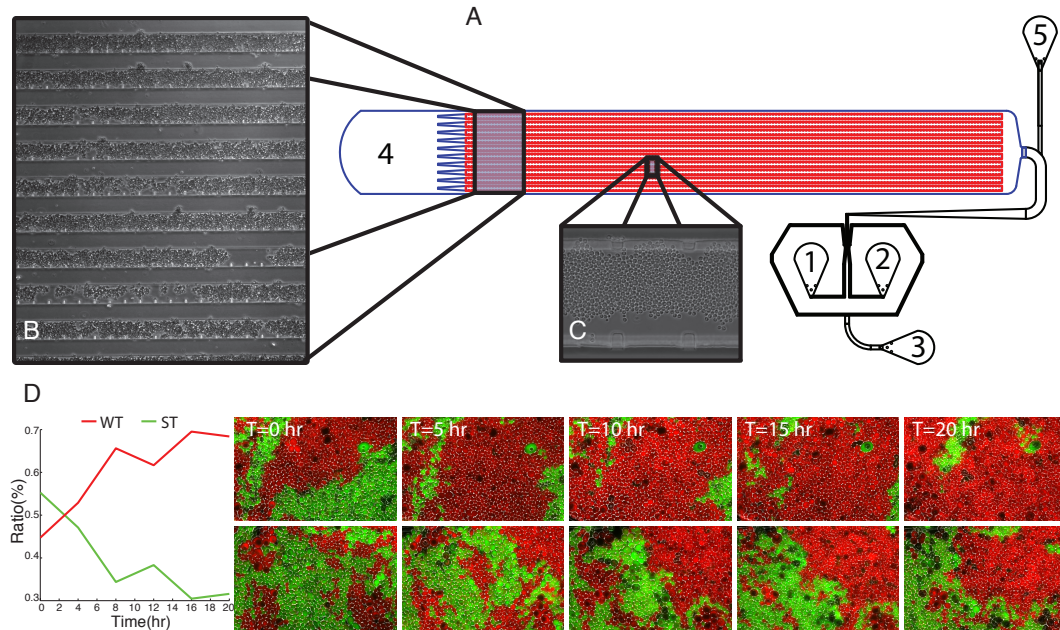


Figure 5.3: Overview of the EvoDAW microfluidic chip design for evolutionary experiments. **A.** These 3 ports comprise the Dial-A-Wave (DAW) network of channels for continuous media switching. Port 1 and 2 connect to two different media syringes, while port 3 acts as an overflow shunt for media not going to the cell chamber. Media is supplied to the cells from the DAW region and exits, along with daughter cells, through port 4. During loading the cells enter the device through port 4 and exit through temporary port 5. Once the cells have been loaded port 5 is permanently filled in with silicon elastomer. **B.** 4x magnification of an actual device with trapped *S. cerevisiae*. **C.** 20x magnification. Cells fill most of the trap and maintain a uniform morphology throughout the experiment. **D.** Time-lapse fluorescent microscopy images of a mixed culture of WT (red) and ST (green) cells grown in the microfluidic device. The cells were exposed to a square-wave signal of 0.25% glucose over 0.5% galactose background with 8 hour period. Plot of ratio change as the experiment progressed. Each glucose period produced an increase in the ratio of WT cells. Time between frames is in hours. The upper and lower sets of images, while from different devices, exhibit the same take over by the WT strain.

microchemostat designs, based on a mixed culture bioreactor, have the significant drawback of being unable to produce instantaneous changes in the growth medium (Zhang et al., 2006). We designed our device based on a flow-through bioreactor. The height of the trapping region is on the order of the diameter of a single yeast cell. Although previous studies have successfully used a height of $3.5\ \mu\text{m}$, we found that lowering the ceiling to $3.25\ \mu\text{m}$ resulted in a more consistent loading and growth (Ferry and Razinkov, 2011). Because the cells were immobilized in the device, the surrounding media could be changed as fast as necessary without the risk of dilution. This allowed us to achieve medium switching within a 1 sec interval. Due to the limited size of the traps the population experiences logarithmic growth only during the initial loading phase of the experiments. Any subsequent changes in population are due to loss of a cell from the trap. The height of the media channels next to the traps was an important factor in long-term viability of the device. The final design had $50\ \mu\text{m}$ channels and we found that lower values increased the probability of clogging. The DAW channel network height was kept at $10\ \mu\text{m}$ to increase fluid resistance in the device.

Our computational model predicted that the final ratio of WT cells in a mixed population depends on the total number of glucose switches but not on the frequency of the switches. Wild-type cells experience an advantage in growth during the first division in a glucose-rich environment. To test these predictions, we performed a series of experiments where a mixed population was exposed to square wave oscillations of galactose and glucose with various periods. The shortest period length was determined by the generation time ($\approx 2\text{hr}$) of a yeast cell and set at 4 hr (2 hr galactose, 2 hr glucose). Since cells have galactose memory for around 6 division cycles, the total time spent in glucose was limited to under 9 hr (Brickner et al., 2007). We did not intend to study the effects of galactose memory loss, so we chose our longest period to be 10 hr (5 hr galactose, 5 hr glucose).

To control for any effects in long-term growth in either of the carbon sources, we measured the WT:ST ratio of cultures experiencing growth in only galactose or only glucose. The change in the population composition was minimal, with the greatest

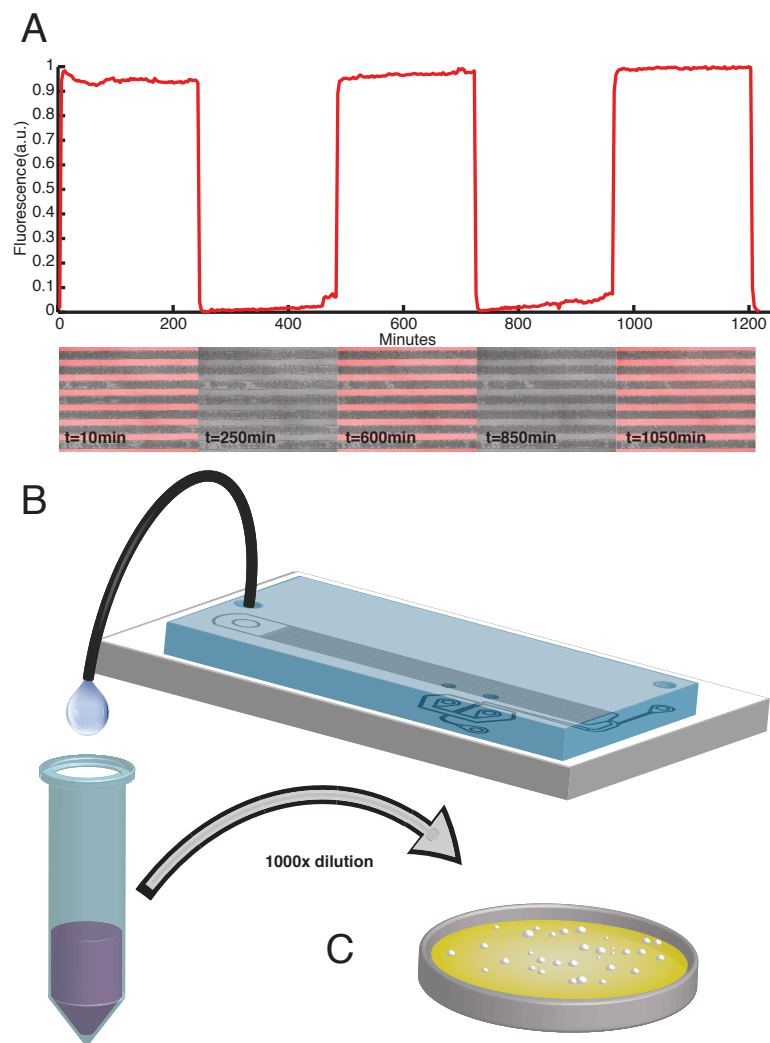


Figure 5.4: Overview of the experimental procedure. **A.** Square wave of 0.25% glucose with different periods pulsed over a background of 0.5% galactose synthetic complete media. Tracer dye was added to the glucose media during testing and experiments to confirm proper function of the device. Pressure changes for media switching were accomplished off-chip using a custom built device described in Supplementary info. Cell growth and function of media switching was confirmed using fluorescent imaging. Sample images show that traps remain full throughout the entire experiment. **B.** Waste from the device, carrying population samples, was collected in a tube over the period of 24 hours. **C.** The samples were serially diluted 1:1000 and plated on -Trp(ST) and -Leu(WT) plates. Colonies were visible after 48 hour incubation at 30°C. Plates were imaged and the total number of colonies on each plate was determined.

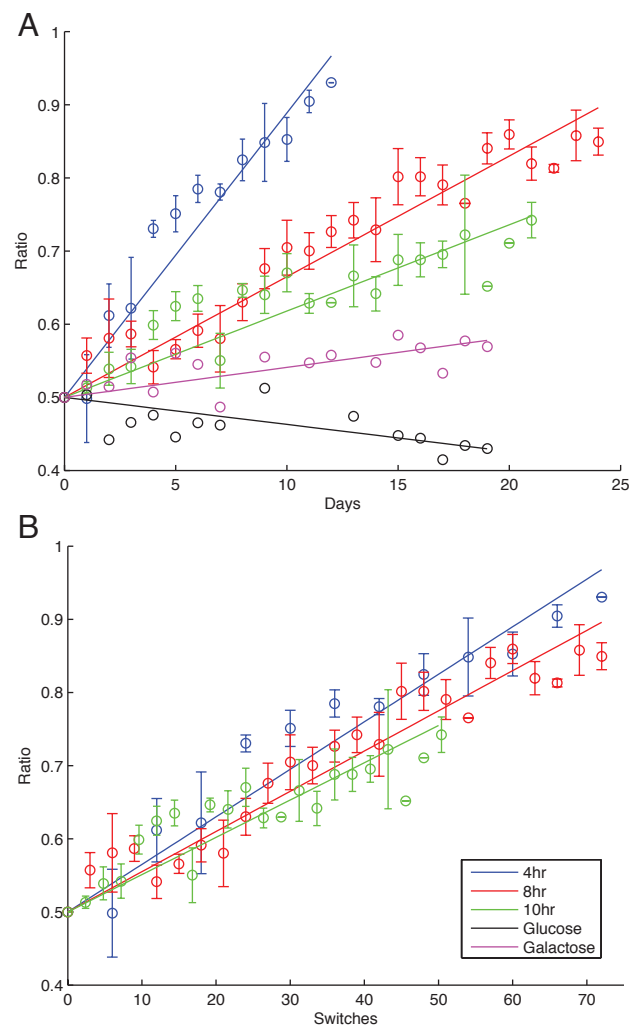


Figure 5.5: Experimental results of driving WT and ST mixed populations with different periods of galactose/glucose. **A.** A mixed population of WT and ST cells were grown in constant 0.5% galactose background with square wave of 0.25% glucose pulsed at different periods. Samples were collected at the exit port of the microfluidic device and plated on auxotrophic plates. The ratio of WT to total number of cells for each experiment was scaled to start at a common value of 50%. Actual starting ratios were with 10% of the common value. Glucose and galactose data point represent experiments in constant mediums. **B.** The ratios were plotted vs calculated number of switches for each period. Error bars represent standard error.

change occurring in the glucose media of -7% over 19 days. The constant galactose environment produced a slight increase of 5% over the same 19 days (Fig. 5.5A). In contrast, dynamic conditions with a 10 hr period lead to a 22% change over 19 days. Therefore, the large changes in population ratios observed in dynamic conditions are due to media switching and not to a competitive difference between the strains in either medium.

For all periods of sugar switching, the WT strain gradually took over the population of the culture. The rate of change was dependent on the frequency of media switching (Fig. 5.5B). To achieve comparable number of switches between different driving frequencies, the total length of the experiments varied from 12 d ($T=4$ hr) to 25 d ($T=8$ hr). The 4 hr period resulted in the fastest rate of population change, with WT > 90% within 12 d. We found that doubling the period of driving nearly doubled the time required to reach population take over by WT, with 8 h period taking 24 d to complete. Increasing the period even further resulted in an even slower rate of takeover, with 10 h driving achieving WT > 75% in 22 d. Consistent with our model predictions, plotting the population ratio as a function of number of switches, the different periods fall on the same line (Fig. 5.5B). We determined that the total number of switches required to reach WT > 85% is 60 for 4 and 8 h periods. Due to the length of the experiments the 10 h period did not reach WT > 75%, however they were on track with other periods. We performed statistical analysis on the slopes obtained from linear fits of the population ratios versus time for each period and found that they were all statistically different (z-test with $p = 0.05$). However, when we performed the same statistical test of the slopes of the population ratios versus the number of switches, we found that they were not statistically different.

5.4 Discussion

Cells growing in a constant environment, such as a laboratory culture, are free to dedicate all of their resources to growth and division and often reach their maximal

growth rate. However, in the natural world, cells rarely experience static conditions. Whether they exist as a single cell or as part of a large multicellular organism, natural-living cells must cope with frequent changes to their surroundings. To survive in a dynamic environment, cells are equipped with gene networks that allow growth to continue in spite of changing conditions. But this flexibility comes at a price, and cells experiencing environmental fluctuations usually do not attain their fastest growth rate. In light of this, it is likely that there are genetic mechanisms that exist because they have been selected for in natural environments, though they appear to have little competitive advantage in laboratory experiments. In this study, we measured the adaptive advantage of one such mechanism that allows cells to respond rapidly to changes in glucose availability. As illustrated in Figure 1, cells quickly shorten the time spent between cell divisions when glucose is introduced into the environment. Interestingly, this mechanism is only active in cells that have experienced glucose in their recent past. Cells that have only been previously grown in galactose do not respond quickly to glucose, but have shorter cell division times in galactose than those with glucose experience. Therefore, in a dynamic environment, cells trade slower growth when glucose is absent for the ability to rapidly respond to its return. This can be thought of as a history-dependent form of bet-hedging, whereby a cell anticipates the presence of its favorite carbon source for a while after its removal, but the anticipation fades over time if glucose does not return. Similarly, mechanisms involving chromatin organization and the transcription of *GAL* genes exist to provide cells with galactose memory.

Glucose-sensitive transcripts have been described as part of several glucose-repressed pathways, suggesting that this may be a conserved mechanism for regulating the response to transient glucose availability in general, not just in the presence of galactose. This further suggests that glucose memory conveys a significant selective advantage to cells growing in dynamic environments. Consistently, we have observed that ST colonies do not grow well, relative to their WT counterparts, when galactose and glucose alternate continuously in the environment. However, whether the competitive translation mechanism is sufficient to explain the advantage of the WT genotype

is less clear, as the resulting difference in cell division dynamics between WT and ST is small and fleeting. Our computational model was designed to specifically answer this question. In the model, the cell doubling time for any given cell is dependent on the level of GAL1 mRNA. Therefore, differences in growth rate between WT and ST are directly related to the half-lives of GAL1 transcripts in the two strains, which differ in the presence of glucose. Our computational simulations, as well as our experimental results, confirm that glucose-mediated degradation of GAL1 transcripts provides a sufficient growth advantage to be selected for in dynamic environments. These results are important in that they illustrate the selective power of environmental fluctuations on seemingly modest phenotypic differences in cellular response dynamics.

A general interest in how life evolved has been at the center of biological study for more than a century. Maps of the evolutionary trajectories of species that are currently inhabiting the planet, and hypotheses of how others have become extinct are always rooted in speculation. To meet the challenges that go along with retroactively interpreting evolutionary mechanisms, researchers set up forward evolution experiments using microbes to measure the competitive fitness of particular genotypes in predetermined environments. Combined with the power of genomics technology, this method for determining the fitness associated with particular genetic variations is potentially revolutionary. However, genome-wide measurements of competitive fitness using the yeast deletion collection have yielded only moderate results that have called into question long held theories explaining natural selection. Particularly bewildering has been the discovery that only $\sim 20\%$ of genes are essential and most gene deletions have little to no effect on competitive fitness. How, then, is genetic integrity maintained if the majority of the genome is dispensable? The answer could be that the fitness contribution of individual genes is dependent on interactions with a dynamic environment that yeast cells evolved in. As we show in this study, the relationship between genes and fitness is potentially highly nuanced, incorporating changing conditions in the present as well as information about past growth environments and the anticipation of future events.

We invented our evoDAW device to measure fitness advantages that are strongly

dependent on environmental dynamics. To test the device, we set up a competition experiment between two strains with a predicted fitness difference in environments that were known to favor the growth of one strain over the other. The resulting population dynamics that we measured were in good agreement with theoretical predictions for each environment, and were highly reproducible relative to competition experiments carried out in batch cultures. The reproducibility of our results may be attributed to the multiple cell traps (10 total) that each device contains. Any variation due to uneven loading of the two strains is likely to have been normalized because each device essentially contains 10 individual populations. We tracked the evolution of the population by selecting for prototrophic markers that differed between the two strains. However, the amount of cells collected each day ($\sim 10^7$) is sufficient for use in many cell and molecular assays, such as immunoblotting, flow cytometry, and qPCR. If more material were desired, for use in genomics techniques for example, the collected cells could be amplified through growth in rich medium. In this study, experiments were observed for ~ 500 generations or ~ 30 days, but there is no theoretical limit to how long these cultures can be maintained. Finally, the dimensions of the growth chambers can easily be altered to accommodate microbes other than yeast or animal cell cultures. The evoDAW chip provides a powerful platform to measure the fitness contributions of individual genes in dynamic conditions that more accurately reflect the environments in which cells evolve.

5.5 Supplementary Information

5.5.1 Microfluidics Device

The microfluidic device used in this study is an adapted version of the design described in Baumgartner et al. (Baumgartner et al., 2011). The new design implemented a much larger trapping region for approximately 10^6 *S. cerevisiae* cells. To increase the viability of the device for long-term experiment (>20 days) a number of key features were added to the design. Specifically, the fluidic mixing region (DAW) was connected

to the cell trap region through only a single fluidic channel. This reduced the complexity of the fluidic network during the experiments. All the fluid leaving the DAW region, exited through the sample collection port clearing all the channels between the traps of free moving cells. Effective removal of non-trapped cells was crucial for long-term viability of the device. During loading the cells entered the device from sample/waste collection port and flowed towards the DAW region. To prevent contamination of the media ports, an auxiliary port was used to divert the flow away. The cells were grown for 24 hr in galactose-only media. Once the cells have filled the trap regions, the auxiliary port (Fig. 5.3A, Port 5) of EvoDAW was filled in with fast curing silicon (Sylgard 170, Dow Corning) using a 3 ml syringe, tygon tubing, and 23 gauge metal pin. After silicon curing, the cell loading syringe was removed, leaving a small piece (5-10 cm) of tubing connected to the device for sample collection into a 14 ml falcon tube. Due to the high viscosity of the uncured material, it was possible to watch silicon slowly move through the channel as slight pressure was applied on the syringe. By reducing the height and width of the auxiliary channel, thus increasing the resistance, the overflow of the silicon into the trap region was prevented. The initial sample was collected after additional 24 hr (48 hr since loading) in constant galactose-only media. Media containing glucose was labeled with fluorescent dye, sulforhodamine 101 (0.01 mg/mL), to visualize media switching at the sample collection port. Neither media contained a selective marker. To prevent contamination reusable metal pins were autoclaved, only new sterile syringes and tubing was used for each experiment and medias were filtered through 0.22 μ m filter.

Since the experiments called for only bimodal switching of medias, the presence of staggered herringbone mixers (SHM) was not required. Furthermore, the channel from DAW region to the trap region was designed to have a gradual change in width for improved flow through the trap region.

The design was simulated using a finite-element analysis software (COMSOL Multiphysics 4.0) using the Laminar Flow and Transport of Diluted Species physics simulations. Due to the large number of cells present in the device it was important to determine the glucose uptake by the cells. If we consider an trap long enough, then due

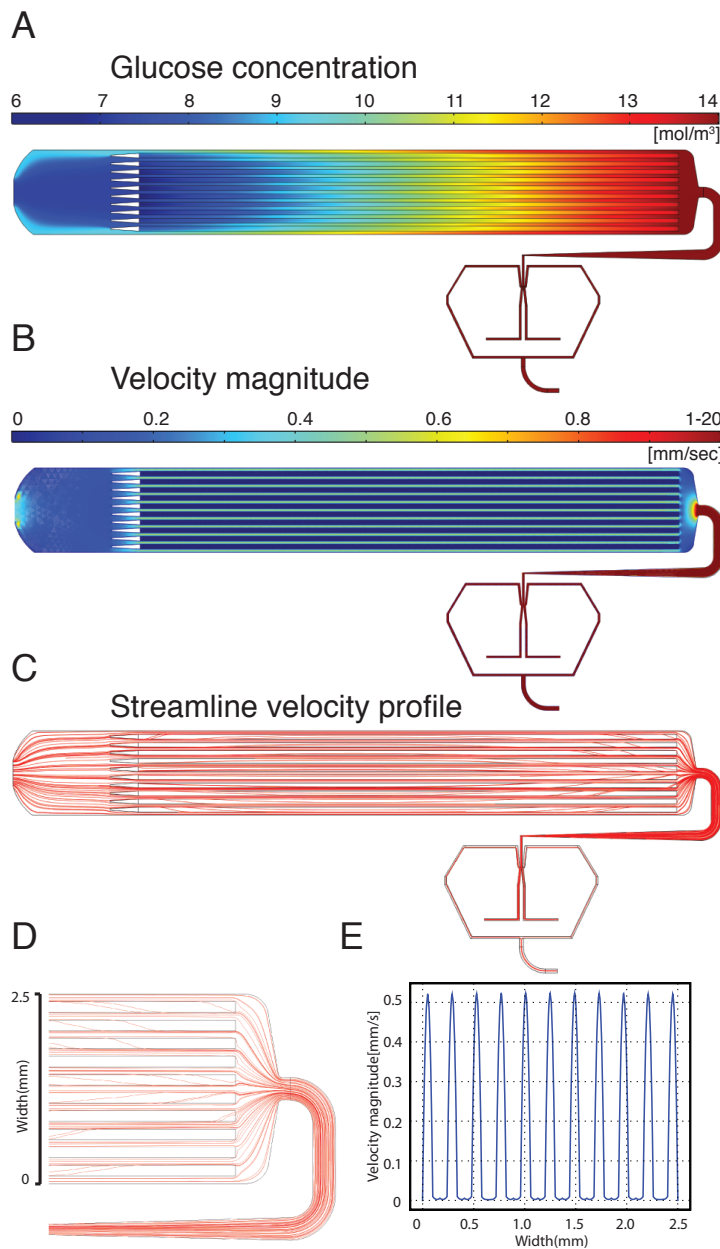


Figure 5.6: Finite element analysis of device design. **A.** Glucose consumption by *S. cerevisiae* was determined for the entire geometry. The initial media concentration of 0.25% w/v glucose corresponds to $14 \text{ mol} \cdot \text{m}^{-3}$. **B.** Velocity profile in the device. Note regarding scale: values of velocity of $1\text{-}20 \text{ mm} \cdot \text{s}^{-1}$ are all grouped together in the same color (red). The velocity is constant in the entire trap region of the chip, exposing all the cells to similar conditions. **C.** Streamline profile of the device. Since the curvature of the streamlines is related to pressure changes perpendicular to the streamline, we can easily visualize areas of large and sudden pressure changes within the device. As expected the major pressure gradients are present at the junction of trap and DAW region (**D**), while the trap region remains even. **E.** Cross-section of the trap region. Magnitude of the velocities in the traps is only a fraction of the velocity in the channels.

to cell uptake there would be a point where all glucose is gone from the medium. Our simulation has shown that cells along the whole length of the trap see the presence of glucose (Fig. 5.6A). However the concentration at the end of the chip drops to half of the incoming media. Considering 0.25% w/v glucose at the start, this would result in 0.125%w/v glucose at exit, although a significant change, this concentration still fully represses the galactose network(Bennett et al., 2008). Glucose consumption was derived from glucose uptake of $r_{cell} = 5.4 [mmol \cdot g^{-1} \cdot hr^{-1}]$ and cell density of $\rho_{cell} = 1.130 [g \cdot mL^{-1}]$ to be $r_{population} = r_{cell} \cdot \rho_{cell} = 1.695 [mol \cdot m^{-3} \cdot s^{-1}]$ (van Dijken et al., 1993; Bryan et al., 2010). Due to the difference in heights of the trapping region and the media channels, the volumetric consumption rate was scaled by the factor of the ratio of the channel height to trap height(≈ 16) to $0.1059[mol \cdot m^{-3} \cdot s^{-1}]$.

Fluid dynamics significantly contributed to proper function of this design. Uneven flow velocities could potentially create a growth advantage to colony of cells. The low flow spot could more easily accumulate just a single strain of cells and form a fast growing colony that could potential push out other colonies from the trap. To make sure our design eliminates low flow spots we examined the velocity magnitude and streamline profiles of the entire device. The highest velocities were around the DAW region, $18[mm \cdot s^{-1}]$, while the flow velocity dropped significantly to $\approx 0.1 - 0.2[mm \cdot s^{-1}]$ in the trap region and remained constant along the entire length of the chip(Fig. 5.6B). This effect was due to convective deceleration, the flow velocity drops when the fluid exits a smaller diameter channel into a larger one. Furthermore, it was important that all parts of the chip have the same volumetric flow rate. By placing a 100 virtual beads in our DAW region and following their trajectories through the device the simulation showed an even distribution of streamlines throughout the trap region(Fig. 5.6C and D). Lastly, it was important that cells leave the trap only due to pressure from their neighboring cells and not from fluid pressure. We examined the flow profiles in and around the traps. The peak velocity in the channel was $0.52 mm \cdot s^{-1}$, while the highest velocity in the middle of the trap was ≈ 100 times less at $5 \mu m \cdot s^{-1}$ (Fig. 5.6E).

Due to the large number of long-term experiment requiring linear actuator setup

from Ferry and Razinkov(Ferry and Razinkov, 2011), we developed a cheaper, more reliable version. By using of-the-shelf stepper motors and controllers, we were able to reduce the price of a single setup to approximately \$500. A new, more reliable software support package was written in JAVA and tested to run for over 60 days. We made the software and the design files available on our wiki page: <http://dialawave.wikispaces.com>

5.5.2 Modeling

Section A - Derivation of Equation (2) of the main text

Assume that there are two strains, each of which grows exponentially with rate γ_{gal} while in galactose-rich media and γ_{glu} while in glucose-rich media, with $\gamma_{gal} < \gamma_{glu}$. Upon a switch from galactose to glucose at time t_s , the wild-type strain will immediately begin growing at the faster rate. If we assume constant log-phase growth, we can write the differential equation for the wild-type strain as

$$\dot{n}_{wt} = \begin{cases} \gamma_{gal}n_{wt} & t < t_s \\ \gamma_{glu}n_{wt} & t \geq t_s \end{cases} \quad (S1)$$

where $n_{wt}(t)$ is the number of wild-type cells at time t , and the over-dot represents differentiation with respect to time. The ST strain with the randomized 5' UTR inefficiently transitions from growth on galactose to growth on glucose. To model this, we assume that the switch between the two growth rates after the introduction of glucose is delayed by a time $\tau > 0$. Therefore, the dynamics of the randomized strain during one switch from a galactose-rich medium to a glucose-rich medium can be written as Equation (S2):

$$\dot{n}_{st} = \begin{cases} \gamma_{gal}n_{st} & t < t_s + \tau \\ \gamma_{glu}n_{st} & t \geq t_s + \tau \end{cases} \quad (S2)$$

where $n_{st}(t)$ is the number of cells with a stabilized GAL1 transcript at time t . Solving Eqs. (S1) and (S2) gives us for time $t > t_s + \tau$

$$n_{wt}(t) = n_{wt,0} \exp\{\gamma_{gal}(t_s - t_0) + \gamma_{glu}(t - t_s)\} \quad (S3)$$

$$n_{st}(t) = n_{st,0} \exp\{\gamma_{gal}(t_s - t_0 + \tau) + \gamma_{glu}(t - t_s - \tau)\} \quad (S4)$$

where $n_{wt,0}$ and $n_{st,0}$ are the numbers of WT and ST cells at time $t_0 < t_s$, respectively.

Define the R to be the population fraction of the WT strain in a mixed population of both types of cells, i.e.

$$R \equiv \frac{n_{wt}}{n_{wt} + n_{st}} \quad (S5)$$

From Eqs. (S3) and (S4) one can show that the population fraction after one complete switch, R_1 , is

$$R_1 = \frac{1}{1 + \frac{1-R_0}{R_0} \exp(-\delta\tau)} \quad (S6)$$

from which follows

$$\frac{1 - R_1}{R_1} = \frac{1 - R_0}{R_0} \exp(-\delta\tau) \quad (S7)$$

where $\delta = \gamma_{glu} - \gamma_{gal}$. Note that the population fraction only changes during the interval $(t_s, t_s + \tau)$, so that only the initial population fraction, R_0 , and the final population fraction, R_1 , need to be calculated. Next, assume that after one full switch from galactose to glucose, the mixed population is switched back to a galactose environment for a long enough time to return the growth rates of both strains to γ_{gal} . Because the randomized 5' UTR of the ST strain only affects the galactose to glucose switch, and not the reverse, there is no change in the population fraction. If the population is then again switched to glucose for a second time, the population fraction becomes

$$R_2 = \frac{1}{1 + \frac{1-R_0}{R_0} \exp(-2\delta\tau)} \quad (S8)$$

which follows from Eq. (S7). In general, after n switches from galactose to glucose we have

$$\frac{1 - R_n}{R_n} = \frac{1 - R_0}{R_0} \exp(-n\delta\tau) \quad (S9)$$

giving us

$$R_n = \frac{1}{1 + \frac{1-R_0}{R_0} \exp(-n\delta\tau)} \quad (\text{S10})$$

which is Eq. (2) of the main text.

Section B - Numerical simulation of cellular growth and division

To simulate the competitive growth of WT and ST cells, we implemented a modified version of Gillespie's algorithm. Populations of both cell types were partitioned into two groups, those in G1 phase and those in S/G2/M phases. While in G1 phase, cells spontaneously pass through START into S/G2/M, with a rate, $r_{START,i}$ that depends on both the carbon source (galactose or glucose) and the strain ($i = 1$ for WT and $i = 2$ for ST). While in galactose, this rate is given by , $r_{START,i} = r_{gal}$ for both strains. In glucose the rate is given by

$$r_{START,i} = r_{glu} - (r_{glu} - r_{gal}) \exp(-\lambda_i t^*) \quad (\text{S11})$$

where λ_i is the GAL1 mRNA degradation rate in the i^{th} strain, and t^* is the time since the last switch to glucose.

Once a cell has passed START and entered into the S/G2/M phases, it is assigned a random delay time, τ_c representing the time from the beginning of START to the completion and division of the daughter cell. This time is given by

$$\tau_c = t_{glu/gal} + \eta \quad (\text{S12})$$

where η is a uniformly distributed random number on $(-\sigma, \sigma)$, and $t_{glu/gal}$ is the mean delay time that depends on the current environmental carbon source. Numerically, this is achieved by removing one cell from the G1 population and adding the randomly chosen delay time into an ordered stack, representing all cells of the same strain that are currently in the S/G2/M phase. The first number in the stack then gives the time at which the next division for that strain will occur. Separate stacks are kept for each strain.

Because reactions in this simulation contain 1) reactions that do not have exponentially distributed waiting times and 2) time-dependent rates, we implemented a version of Gillespie's algorithm that incorporates both dynamical delay and time-dependent rates. First, at each incremental step a randomly chosen time step for the next reaction, τ_{exp} , is calculated from all exponentially distributed reactions, here being the spontaneous entrances into START of cells in G1 phase for both strains. Next, this randomly chosen time is compared with the time until the next delayed reaction (i.e. the minimum of all values in the two stacks), τ_{stacks} , and with a minimum step size, dt , determined by the time scales of all time-dependent rates. Here, we have $dt \ll 1/\lambda_i \forall i$. Depending on which of the three times above is the *least*, different outcomes occur according to Table 5.1.

Table 5.1: Different time step used during the modified Gillespie simulation of the competition model.

minimum	outcome
τ_{exp}	The associated G1 phase cell type begins to bud. The number of G1 phase cells of that cell type is decremented by one, and a random delay time is added to the associated S/G2/M phase stack. Time is incremented by τ_{exp} .
τ_{stacks}	The exponential reaction is thrown out. The delayed reaction time is pulled from the associated stack and the number of cells in G1 phase of that cell type is incremented by two. Time is incremented by τ_{stacks} .
dt	The exponential reaction is thrown out. No reaction occurs. Time is incremented by dt .

Note that, because some of the rates in the process are time-dependent and some reactions are delayed, the entire process is non-Markovian. Setting a minimum time, in essence, does two things. First, it assumes that the entire process is locally Markovian on time scales of order dt . Second, it approximates the true continuously differentiable rate functions with piece-wise constant functions of step sizes no larger than dt .

The total population size of the two competing strains was controlled in our stochastic simulations, as well. To do this, we implemented a chemostat-like environ-

ment that simulates constant exponential growth in a population size-limited culture. Simply, the maximum population size was set to some constant, N_{max} . Upon cellular division, i.e. exit of a cell from the S/G2/M phase, if the new population size was greater than N_{max} , a random cell, chosen from all cell types and phases, including G1 and S/G2/M phases and both cell types, was removed from the simulation. The values of the constants used in our simulations are given in Table 5.2.

Table 5.2: Value of constants used during our simulation, based on experimental data from literature.

Constant	Value	Units	Description
r_{glu}	$\ln(2)/20$	min^{-1}	Rate constant for cells exiting G1 phase in the long-time limit in a glucose-rich environment.
r_{gal}	$\ln(2)/40$	min^{-1}	Rate constant for cells exiting G1 phase in a galactose-rich environment.
λ_1	$\ln(2)/4$	min^{-1}	Half life of GAL1 mRNA in wild-type cells in a glucose-rich environment.
λ_2	$\ln(2)/17$	min^{-1}	Half life of GAL1 mRNA in mutant cells in a glucose-rich environment.
t_{glu}	70	min	Mean time for cells to pass through S/G2/M phases in a glucose-rich environment.
t_{gal}	80	min	Mean time for cells to pass through S/G2/M phases in a galactose-rich environment.
σ	10	min	Variance of time for cells to pass through S/G2/M phases.
N_{max}	20,000	unitless (# cells)	Maximum number of cells allowed in the simulation.
dt	0.1	min	Minimum time step.

5.6 Acknowledgements

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Chapter 6

Summary

The field of biology has been revolutionized by DNA sequencing technology, which opened many fields of research that focus on figuring out how these different sequences produce the top-level behavior that we can observe in humans, plants and animals. It has been known for some time now that specific sequence translate into various proteins, which in turn interact with other proteins or even more importantly with DNA itself. Through various methodologies the connections of various proteins have been mapped out, producing a network of interactions. The next step is to probe the network in a dynamic fashion to determine not only the overall behavior of the network, but more importantly the underlying mechanism of these interactions. One approach to deciphering this hairball of interactions is to construct simple gene network synthetically and study their behavior on a single cell level. Alternatively, we could deconstruct an native network while examining its behavior in a dynamic environment. Here, we have presented studies that examine behavior of simple single cell model organisms with native and synthetic network under dynamic environments.

In Chapter 2, we developed a novel microfluidic chemostat for study of single cell gene perturbations in *S. cerevisiae* (Ferry*, Razinkov and Hasty, 2011, *equal contribution). We first created a microfluidic switch that can effectively on-chip mix two different streams of fluid. By changing the hydrostatic pressure of fluid reservoirs lo-

cated off-chip we were able to change the ratio of two fluids going into a single inlet. Downstream of the inlet the fluid with two fractions was mixed by folding the fluid on to itself using 'chaotic' mixers. Fluid reservoirs were attached to linear actuators, which with help of a custom software package were controlled to move in a predetermined waveform. Combining the mixing technology with a trap design for a monolayer of *S. cerevisiae*, we created a microchemostat that allowed us to track single-cell response to a dynamic environment. Furthermore, scaling this technology up we were able to create a device that ran 8 independent dynamic experiments. By tagging fluorescent proteins to genes of interest we used fluorescent microscopy to determine protein abundance. Using this technology we probed the Galactose network in *S. cerevisiae*, determined individual cell response using an automated cell tracking algorithm. Our results indicate the importance of microfluidics for gene network studies, by providing insight into behavior of individual cells over the course of a whole generation.

In Chapter 3, we constructed and characterized a novel synthetic circuit for a coupled oscillator (Prindle*, Samayoa*, Razinkov, Danino, Tsimring, and Hasty, 2012, *equal contribution). We built on previous experiments of a coupled oscillator, to including a circuit component that would allow synchronization at a larger scale. Using microfluidic devices, we were able to create 'biopixels' - 100 μm by 100 μm traps filled with *E. coli*. Individual cell oscillations were synchronized within the trap through a freely diffusing coupling molecule. To achieve synchronization between individual 'biopixels' the circuit was altered to create a gas molecule that could freely diffuse between adjacent 'biopixels', effectively synchronizing their oscillations. Gas synchronization was characterized by increase the total number of 'biopixels' 100-fold. Also, the synthetic oscillator was tested as a bio-sensor for detection of various chemicals. We found that it can easily detect arsenic at concentration below currently available chemical tests. Our results suggest that this technology can potentially be used for detection of low levels of chemical in and out of a laboratory setting.

In Chapter 4, we applied microfluidic, automated microscopy and cell tracking software to study low level protein expression in *E. coli*. Using microfluidic devices, we

were able to constrain bacterial cells to a monolayer, that grew in a predictable fashion. By creating a trap with two open side parallel to the flow of the media around the trap, the cells spontaneously organized into a well structured, brick-line, formation. This led to improved cell tracking algorithm, allowing more data to be collect per experimental run. By fusing a fluorescent protein to a low level produced protein, the production of the protein was recorded as fluorescent signal. The location of the produced protein was determined very accurately using a series of algorithms. Time-lapse microscopy of the cells allowed us to capture the dynamics of protein production from 'birth' to 'death' of the cell. Automation of microscopy and image analysis, allowed us to gain meaningful information about the localization of protein production.

In Chapter 5 we used microfluidic devices to track population dynamics of two different *S. cerevisiae* strains (Razinkov, Baumgartner, Bennett, Tsimring, and Hasty, 2013). Wild-type yeast cells respond very fast to a change in external carbon source. By mutating a portion of gene responsible for galactose utilization, we were able to increase the response time 4-fold when external carbon source switched from galactose to glucose. Small-scale study in a microfluidic device, mentioned in chapter 2, showed that the wild-type are able to divide sooner upon switch to glucose. This in turn pushed other cells out of the trap, effectively allowing the wild-type to outcompete the mutant strain. However, the growth rates of both strains were identical in static conditions. To test the effects of switching on a co-culture population we developed a large-scale microfluidic device that would allow dynamic stimulation for over 30 days. By switching the carbon sources with different frequencies we were able to determine that wild-type overtake of the population is dependent only on the number of switches and not the time spent in the device.

The consistent theme in these studies is the perturbation and study of genetic network in dynamic environments, while allowing long-term data collection. Microfluidics are at the core of these technological and scientific breakthroughs, allowing us to generate dynamic environments while simultaneously recording time-lapse microscopy images for single-cell analysis. The ease of design and production of microfluidic de-

vices allowed us to study cell behavior on multiple levels, from single-cell to tens of millions. Effectively allowing us to gain understanding of gene networks in single cells and using that knowledge to predict and test behaviors on larger scales. With advances in synthetic biology and microfluidic technology we gain the necessary knowledge for developing real-world solutions for biosensors, medicine and environment remediation.

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