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Integrated microfluidic device for single-cell high throughput screening in dynamic gene expression analysis

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Integrated Microfluidic Device for Single-cell High Throughput Screening in Dynamic Gene Expression Analysis

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Bioengineering

by

Lawrence Kwan Yeung Hui

Committee in charge:
Professor Jeff Hasty, Chair
Professor Michael Heller
Professor Alexander Hoffmann

2008
The Thesis of Lawrence Kwan Yeung Hui is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2008
For my family,
Sherman, Josephine, and Laurel

Thank you for life, love, and laughter
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ABSTRACT OF THE THESIS

Integrated Microfluidic Device for Single-cell High Throughput Screening in Dynamic Gene Expression Analysis

by

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Master of Science in Bioengineering

University of California, San Diego, 2008

Professor Jeff Hasty, Chair

Over the past decade, interest in microfluidics has surged as applications have trended towards novel biological assays. Specifically, the ability of microfluidics to parallelize cellular studies through array-based chip designs has attracted researchers interested in investigating cellular function under a wide variety of environmental conditions. The capability of microfluidic devices to control microenvironment conditions and induce dynamic perturbation to cellular systems makes microfluidics (or “lab-on-a-chip”) an attractive platform to study gene expression dynamics.
In this project, the functionality of microfluidic technology is exploited to design and construct a device for isolation and observation of cells in high throughput. The integration of a concentration gradient with homogenous medium within each chamber was designed specifically to investigate gene regulation in *Saccharomyces cerevisiae* under various concentrations of chemical inducers. These devices were designed to sustain cells for extended periods of time with high temporal resolution to study dynamic gene expression in single cells. The device builds on previous studies by probing up to eight distinct cell cultures in parallel. The microfluidic platform was then used to study yeast cells at various levels of inducer perturbations. Further experimentation revealed the utility of a parallel gradient by producing an induction curve of the yeast response. Such high-throughput designs will prove essential to yeast systems biology research as it strives to understand the complex regulatory interactions that dictate cell function by probing vast regions of parameter space.
1

Introduction

1.1 Synthetic biology

The concept of designing genetic circuits has been around since the 1970s [1], and recent advances have spurred a renewed focus in applying quantitative analysis to the design of regulatory circuits. Extensive research in the post-genomic era is pointing towards the complete mapping of the fundamental building blocks of biology. These genes and their products are central to interactive networks which are often extremely complex. Thus, synthetic biology focuses on an approach which is to break down these elaborate networks into simpler subunits and analyzing their dynamical interactions.

Analogous to an electrical engineering approach of designing electrical circuits, the development of genetic circuits employs both mathematical and computational tools [2]. Recent advances in molecular biology and sequencing techniques allow for the construction of genetic networks in a synergistic approach to understanding universal principles in multicellular system
interactions. Moreover, systematic design of synthetic networks allows the extraction and focus of specific subsystems within natural organisms. Analysis of these simpler systems or ‘modules’ provide insight into characterizing their behavior and contributions to the underlying cellular function. This ability to isolate subsystems, such as switches and oscillators, offer the ability to expand on these basic units to create genetic ‘components’, capable of executing increasingly advanced functions [3].

The goal of such work is to synthesize increasingly complex networks that model after natural systems while maintaining the ability to extract and test each component of the system experimentally. The possibility of adding complexity to basic circuits and experimentally probing, characterizing, and optimizing these circuits lays the foundation for the development of ‘designer’ cells which can carry out pre-programmed functions in their genome [4]. Synthetic gene networks could be programmed into a cell’s genetic DNA and these cellular nano-robots could be employed for a vast number of applications including cellular biosensing, synthesizing biomaterials, initiating targeted apoptosis of tumor cells, and potential interface with bioelectronic applications [5]. The ability to control cellular behavior through the design and manipulation of gene expression regulation is a fascinating possibility but is fraught with obstacles in its path.

1.2: Dynamics of synthetic biology as a multidisciplinary challenge

Evaluating dynamical interactions in synthetic gene regulatory networks and predicting outcomes is a truly multidisciplinary challenge that combines coupling tools from non-linear dynamics in physics, molecular biology, and microscopy. As explained in the section above, systems biology has flourished in the wake of the human genome project and requires an integrated approach of experimental and computational tools to evaluate complex biological systems. A primary focus of synthetic biology is the design and construction of novel genetic cir-
cuits that mimic the behavior of naturally occurring systems.

The impact of synthetic biology is dependent on the development of new methodologies and techniques for the acquisition and processing of data extracted from large samples of individual cells [6]. Single-cell measurements provide an opportunity for analyzing gene expression at the individual cell level and detail cellular phenomena such as oscillatory or nonlinear behavior in asynchronous cells. Single-cell studies have also been key to understanding the stochastic nature of gene expression [7] in live simple eukaryotic and prokaryotic cells. Recent single-cell experiments have displayed cell-to-cell variability due to noise in gene expression propagation [8], linear relationships between fluorescent reporter genes and translational efficiency [9], and examining the behavior of natural networks that contain feedback loops [10].

1.3: Current Techniques for monitoring gene expression

The post-genomic era has expedited the advancement of many techniques aimed at investigating the inner intricacies of the cell, providing light in areas such as DNA, mRNA, and protein functions. Owing to the immense complexity of biological systems, the advancement of high-throughput experimental methods is necessary in order to sufficiently generate enough data to accurately describe a system. The increasing availability of fluorescently tagged proteins [11, 12, 13] and the ease of integration with high-throughput technologies such as flow cytometry and microarrays have proven to provide a wealth of information about cellular systems. For example, flow cytometry is a technique that offers the ability to probe several parameters simultaneously at a rate of up to 10,000 cells per second. Within a few short years since the inception of high-throughput techniques, advances made in protein and gene microarrays have launched these platforms to the forefront of high throughput analysis in systems biology research [14]. In contrast, traditional microscopy methods are often limited by the length of duration of an experimental run as cells grow out of the focal plane and therefore obtain very little useable informa-
tion. Medium delivery is also an issue with traditional microscopy as cells enter a dormant stage or die in the absence of new medium.

Static cell measurements, such as those measured using flow cytometry and gene microarrays, provide useful snapshots of gene expression patterns in many individual cells with incredible precision and efficiency. However, the limitations of these techniques are exposed when monitoring the dynamics of gene expression in a single cell. Analysis of a system’s response to varying amounts of inducer and corresponding fluorescent readout is very straightforward on the flow cytometer. Cellular population characteristics can be observed using time lapse flow cytometry, but lack the means to acquire individual cell trajectories. Moreover, temporal phenomena such as oscillations or switching between metastable states in gene expression in single cells cannot be quantified by these techniques. Instead, the fluorescent readout would exhibit large variance or bimodality, and cell to cell variability is masked by population wide measurements. Since a flow cytometer provides information regarding protein levels in an individual cell at a particular time point, flow cytometry cannot be utilized to observe dynamic behavior such as oscillations of individual cells. Additionally, the flow cytometer lacks the temporal resolution necessary to determine dynamic parameters of biochemical interactions. Since the typical data acquisition period is on the scale of 1-2 minutes to extract data from a sampled state of cells, statistically relevant experimentation would require acquisition times of 5-10 minutes. With the majority of biological measurements ranging from minutes to hours, and cellular metabolic interactions occurring at time scales much faster than this at the millisecond to seconds time constants, some noise properties and other temporal characteristics are lost [15].

1.4: Microfluidic Devices

In the simplest form, microfluidic devices are networks of microscale channels that connect to transport fluids. The small-scale dimensions of these devices allow for highly controlled
cellular microenvironments and intricate perturbations. Microfluidics is the development of microscale devices for interfacing with the macroscopic world. In recent years, microfluidic devices have been used to observe cellular chemotactic response to gradients, shear stress, and other mechanical stimuli [16]. Visualization of cellular responses provided by microfluidic devices in conjunction with time-lapse microscopy offers sufficient temporal resolution to quantify short time scale interactions.

As explained in the previous section, there is a need for new technologies to have the capability of analyzing high-throughput whole cell experimentation. These new technologies must be able to probe a wide spectrum of highly controlled environmental conditions and maintain individual cellular scale resolution. The ability to isolate and manipulate local cellular environments makes microfluidics a suitable platform for large-scale determination of dynamic gene expression properties, protein expression, and population dynamics [17]. Microfluidic devices overcome limitations that hinder current instrumentations via miniaturization, increased fluid flow, low-cost of production, technological integration, and significantly reduced sample requirements [18].

Microfluidic technology also offers the potential for significant advancement in research capabilities in a diverse range of biological applications such as high-throughput drug screening, genetic analysis [19], biomolecular separations [20], and polymerase chain reactions [21]. The following sections detail some of the basic background regarding the design and manufacturing of microfluidic devices.

1.4.1: Modeling on-chip pressures and flows

The fundamental physics that govern fluid flow changes drastically as dimensions are reduced to the micrometer scale. Small length scales quickly bring other physical phenomena to the forefront that are less familiar on the macroscale [22]. This section will take a look at the
physics of the microscale and how manipulation of fluid flow allows for certain devices to be possible.

The velocity field of a Newtonian fluid follows the Navier-Stokes equation,

$$ \rho \left( \frac{\partial \mathbf{u}}{\partial t} + \mathbf{u} \cdot \nabla \mathbf{u} \right) = - \nabla p + \eta \nabla^2 \mathbf{u} + \mathbf{f}, $$

(1.1)

and the conservation of mass gives rise to the incompressible fluid flow condition given by,

$$ \nabla \cdot \mathbf{u} = 0. $$

(1.2)

Now Reynolds number \((Re)\) is a dimensionless number that compares the ratio of inertial forces \((f_i)\) and viscous forces \((f_v)\),

$$ Re \equiv \frac{f_i}{f_v} = \frac{\rho U_0 L_0}{\eta}, $$

(1.3)

where \(\rho\) stands for the fluid density, \(U_0\) is the characteristic velocity of the fluid, \(\eta\) is the fluid viscosity and \(L_0\) is a typical length scale. The standard transition to turbulent flow through a straight circular pipe typically involves \(Re\) values between 2000 and 3000 [22]. However, at length scales pertinent to microfluidic fluid flow (~100 μm), \(Re\) is \(<< 1\). Therefore, such low values for \(Re\) affirm that the resulting fluid flow lies in the laminar flow regime and that viscous forces dominate fluid flow and inertial forces become negligible. As a result, all viscous terms drop from equation 1.1 and only linear terms remain,

$$ \rho \frac{\partial \mathbf{u}}{\partial t} = - \nabla p + \eta \nabla^2 \mathbf{u}. $$

(1.4)

Under steady state conditions, eq. 1.4 further simplifies to,

$$ \nabla p = \eta \nabla^2 \mathbf{u}. $$

(1.5)

Solving eq. 1.5 for fluid flow through a cylindrical pipe of radius \(r\) and length \(L\) gives the fol-
following solution,
\[ \Delta P = Q \cdot \left( \frac{8\mu L}{\pi r^4} \right). \] (1.6)

where the resistance of a cylindrical pipe is
\[ R = \frac{8\mu L}{\pi r^4}. \] (1.7)

However, channels built on microdevices typically have rectangular cross-sections and solving for the resistance gives [23],
\[ R = \frac{12\mu L}{wh^3} \left[ 1 - \frac{h}{w} \left( \frac{192}{\pi^5} \sum_{n=1,3,5}^{\infty} \frac{1}{n^5} \tanh \left( \frac{n\pi w}{2h} \right) \right)^{-1} \right], \] (1.8)

here \( w \) is the channel width and \( h \) is the channel height. Geometries with high aspect ratios in which \( w \gg h \) lead to fluidic resistance of the rectangular channel,
\[ R = \frac{12\mu L}{wh^3}. \] (1.9)

Note that the resistance of the microchannels relates to the first power the width and length dimensions while the height is a higher power. The given solution given by eq. 1.6 is analogous to Ohm’s law for linear electric circuits,
\[ V = I \times R. \] (1.10)

Therefore, the analogous nature of fluid flow through a system with linear circuits allows for similar analysis of the system. Using principles of nodal analysis with reservoirs acting as power supplies and channels as wires, flow rates were calculated using Kirchoff’s current law. The whole process of modeling on-chip pressures and flows can be automated, thus allowing more complex systems to be simplified and solved in an efficient process. Throughout the design process, the geometry of fluidic chambers and channels were kept as simple as possible.
so advanced computation of flow modeling was unnecessary.

1.4.2: Device Fabrication

Initial development of microfluidic devices began with etching microchannels onto glass substrates. These devices were time consuming in fabrication and restrictive in design. More recently, the adaptation of microfabrication techniques from the semi-conductor industry have also brought over the use of silicon based devices. However, alternative fabrication materials and techniques such as hydrogels, plastics, and elastomers are quickly gaining popularity due to increased efficiency and reduced production costs. Another alternative technique is soft lithography, which utilizes rapid prototyping techniques to produce replica moldings of polydimethylsiloxane (PDMS) against a photolithographically produced master mold.

In particular, soft lithography is an alternative to silicon-based micromachining that retains the advantages of rapid prototyping, ease of fabrication, and increased compatibility to biological surface changes [24]. The use of UV established photolithography procedures allows for an increased flexibility in design. Also, there is a simple method of bonding PDMS components by plasma oxidation to seal microfluidic channels to glass substrates. The benefit of such a technique is its speed in production. From photomask concept design to finished product, the whole process can be quickly accomplished in a matter of 2-3 days.

More advanced polymer-based devices may take longer, including incorporation of monolithic valves, pumps, and other three dimensional structures [24]. These chips require the fabrication and assembly of multilayered polymers. Another alternative to polymer-based three dimensional devices is the use of laser-cut Mylar laminar sheets [25]. This technique stacks multiple layers of high transparency laminates cut by a laser to produce channels and mixers.

This work uses well established PDMS techniques along with soft-lithography technology and replicate molding to develop microfluidic devices. The fabrication process can be di-
vided into three phases: photolithography, replica molding, and processing. Photomasks are designed in vector based drawing programs (e.g. AutoCAD, Autodesk Inc. or Adobe Illustrator, Adobe Sys.) and printed on high resolution phototransparencies. A more detailed procedure is outline in Chapter 2, Section 2.

1.4.3: Driving and Controlling Fluid Flow

Microfluidic applications require methods for pumping fluids and a fluid control. The most typical means of mechanically driven fluid flow is the use of pumps which apply a constant force on the plunger of a syringe. These high precision pumps are quite expensive and offer the ability to reverse flow, as well as the drawing and dispensing of fluid simultaneously. Lower end vacuum pumps suffer from extraneous noise in the form of pulsatile pumping and introduce variations into the microenvironment.
The most basic method of driving fluid flow in microfluidic devices is the use of hydrostatic pressure on connected fluid filled syringes [26]. Syringes may either be free-standing reservoirs using gravity feeding or it may be driven using vacuum pumps. Using gravity feeding, reservoirs can be manually adjusted and the net pressure is the differential height between the relevant reservoirs. However, one drawback is the inability to precisely control fluid switching. Vacuum pumps offer higher pressures to attain flow rates in the devices that may be impractical to attain in laboratory settings with just gravity feeding. They are also advantageous over mechanical pumps since there is no mechanical pumping motion, thus nullifying flow pulses. The ability to manipulate media input waveforms by regulating the pressures placed on each reservoir via simple software manipulation of the vacuum pumps is unachievable by gravity feeding. Hydrostatically driven fluid flow may be the simplest method of flow control; however, it offers unparalleled flexibility and is incredibly useful for microfluidic device testing.

1.5: Goals

A great deal of effort in systems biology is focused on the development and refinement of numerical models for biological systems [15] in order to predict a system’s response in the presence of signal triggers. The ability to characterize these biological systems has only recently become a viable possibility due to an exploding wealth of genetic and proteomic data. However, the advent of new technologies is necessary to acquire sufficient dynamic information to specify the sheer number of parameters that accompany these mathematical models. In this work I set forth to provide a platform that will provide a wealth of experimental data to complement mathematical computation, model generation, and refinement.

This work began by expanding on previous work done on microfluidic platforms that monitored gene expression over long periods of time. By parallelizing that platform, my goal was to produce a high-throughput microfluidic system for large data acquisition. The integration
of a gradient, leading to homogenous concentration levels within each of the eight chambers, allowed up to eight independent experiments to be carried out simultaneously.

This device was then applied to the characterization of an induction curve and the variability of a regulatable gene network in *S. Cerevisiae* to test the feasibility of such a device, as described in Chapter 3. This paper highlights the benefits of a parallelized device and the application of a gradient generating chip by scanning across a wide spectrum of concentrations of inducer for the model gene network. Conclusions from the platform development and induction curve study are presented in Chapter 4. Finally, a look into the future direction and possibilities for application of my platform on other studies are presented.
2

Platform Development and Methodology

2.1: Cells, Constructs, and Cell Conditions

*S. cerevisiae* was chosen to test the microfluidic device because of the extensive information known about the galactose utilization pathway in yeast. More specifically, a *S. cerevisiae* K699 yeast variant was used because it contained deletions of several metabolic pathways providing auxotrophic selection markers to facilitate genetic manipulations.

To observe the galactose utilization processes, a variant of the K699 strain was developed that expressed combinations of yEVenus (YFP) and yCerulean (CFP) (Table 2.1). To create an indicator of the cellular response to an inducer, a yeast-optimized cyan fluorescent reporter protein (yCerulean) [12] was fused to the endogenous GAL1 protein of the K699 strain [27] and yEVenus was fused to the endogenous GAL2 protein. GAL2 is a transporter protein that localizes to the cellular membrane, therefore, the GAL2 fluorescence reporter proteins are localized to the outer edge of the cell (Fig 2.1). Cells were first grown in raffinose, which is
called a neutral sugar because it neither activates nor represses the galactose network [28]. The galactose utilization pathway is induced in the presence of galactose and repressed by glucose carbon sources. Glucose regulation is dominant to galactose regulation in this pathway, and K699 yeast cells only consume galactose in the absence of glucose in the medium [29].

GAL1 proteins were marked with yCerulean fusion proteins to allow for spectral separation from sulforhodamine 101 (S101, Sigma), a red fluorescent dye used to mark inducer reagent concentrations. S101 is hydrophilic and the similarity in molecular weights of the dye (~600 Da) and galactose (~200 Da) make it a suitable tracker to observe channel concentrations and flow procedures.

2.2: Microfluidic Device Fabrication

This work utilized PDMS rapid prototyping via replica molding for the fabrication of microdevices. The process outlined here utilizes a well established three part procedure involving photolithography, soft lithography/ PDMS replica molding, and post processing.

For photolithography, photomasks were designed in vector-based drawing program such as AutoCAD 2005 (Autodesk Inc.), and printed at 20,000 DPI on phototransparency film as clear and opaque regions for the photolithographic process. A comfortable margin of error was incorporated in the designs of features with close proximity to avoid unwanted overlapping of key features. In the event where channels did overlap in the design, a margin of error of roughly

![Fig 2.1: GAL1 and GAL2 expression in *S. Cerevisiae* K699. GAL1 is tagged with a yCerulean fusion protein (CFP) and GAL 2 is tagged with a yEVenus fusion protein (YFP).]
15-30 μm was included to improve the ease of manufacturing. This also allowed for minor cases of photolithographic misalignment, which was a common problem when features were difficult to see due to the ratio of photoresist layer heights exceeding 3:1 between consecutive layers. Patterned features printed as clear regions on black opaque background were used with negative-tone photoresist, a curable epoxy that crosslinks to a solid state when exposed to UV light. The devices described in this section only used SU-8 patterned masters.

Printed phototransparencies were trimmed into individual masks and pasted onto borosilicate glass plates using adhesive Loctite formula 495 superbonder. The glass plates served as a support for the mask so that it could be mounted onto a photomask aligner. The printed (emulsion) side of the photomask was oriented such that it came in direct contact with the photoresist to ensure appropriate pattern masking by reducing the possibility of UV light diffraction between the masks and the photopatternable film.

Although it is common to use glass as a substrate for photolithography, silicon wafers were used in this work. Silicon wafers were prepared by using a wafer spinner at 2000 rpm to solvent clean with acetone, isopropanol, methanol, followed by deionized water, and heated at 100°C using a contact hot-plate. This process removed all debris and moisture from the substrate surface to prevent detrimental SU-8 adhesion in future steps. Due to the microscale nature of the devices, even fine debris located on an important feature could render the whole chip useless.

Photoresist was deposited on the silicon wafers by spin coating at various speeds to achieve a wide range of thicknesses (2 – 40 μm). This process allowed for multiple featured lay-

<table>
<thead>
<tr>
<th>Protein</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>yEGFP</td>
<td>488</td>
<td>507</td>
</tr>
<tr>
<td>yEVenus (YFP)</td>
<td>515</td>
<td>530</td>
</tr>
<tr>
<td>yCerulean</td>
<td>434</td>
<td>477</td>
</tr>
<tr>
<td>S101*</td>
<td>586</td>
<td>605</td>
</tr>
</tbody>
</table>

Table 2.1: Excitation and emission spectral maxima of fluorescent proteins. *S101 is a sulforhodamine 101.
ers to be leveled on top of one another through repeated spin-coating, exposure to UV light, and removal of unexposed photoresist using a photoresist developer. Negative tone photoresist that had undergone UV crosslinking and thermal curing was resistant to overdevelopment. This allowed for a stepwise layer production since developed topology of previous layers were unaffected by subsequent development steps. Once a multilayered master mold was complete, it was treated with vapor deposition layer of organo-silane for 2-5 min under a vacuum hood. This vapor layer on the mold surface aided in the monolith release of PDMS replicas by reducing the surface friction.

Replica molding was done using degassed liquid PDMS, which were produced by mixing 10 parts base to 1 part curing agent, equaling a total mass of 33 grams and cured at 80°C for 1.5 hours. Fluid access ports were then bored through the replica, and cleaned of PDMS debris using Scotch tape and isopropanol. PDMS devices which now contained a negative topology of the mold’s image were then sectioned off into six individual devices and prepared for post processing.

Post processing involved adhering sectioned PDMS to glass coverslips using irreversible oxygen plasma bonding. This produced a seal between the PDMS and the coverslip that could withstand pressures as high as 25 psi. Pressures of this magnitude were unattainable when using hydrostatically driven flow for the media loading operation stages, but the ability to withstand such high pressures allowed for increased flexibility in cell loading procedures and prevented fluid leakage.

The microscopic nature of the essential features of a microfluidic platform required strict procedures to minimize manufacturing error. Misalignment (Fig 2.2a) of layers was a common occurrence but was minimized by increasing the number of alignment patterns within the photomask design. Master molds were fabricated in a class 100 clean room to ensure minimal debris contamination. The slightest dust particle or piece of hair (~50 μm) trapped within a
PDMS chip may ruin the functionality of the chip (Fig 2.2b). These were a few of the considerations taken into account during the fabrication process.

2.3: Microfluidic Design

2.3.1 Cell Chamber and Gradient Generating Network

Several microdevices were created for this work, beginning with the design of simple flow channels and ending with more complex parallel gradient platforms. As the work progressed, the devices became capable of probing several concentrations for long periods of time while maintaining single-cell resolution in imaging cellular populations. The two key elements in the design of an integrated microfluidic chip involved the cellular growth chamber and the delivery mechanism for medium and inducer reagents.

Confinement of cells was crucial to cell-tracking, without which it would be nearly impossible to extract single-cell trajectories. The direct applications of many microfluidic designs for microchambers failed when microbial cells such as yeast and bacteria were employed to the device. Trapping nonadherent cells in a microchamber was a critical design challenge because cells that were not localized were flushed away in the presence of fluid flow. Flow chambers
used to investigate biofilm formation in adherent cells were not appropriate for nonadherent cells without surface adhesion.

*S. cerevisiae* presented obstacles to studying time evolved single-cell temporal data due to its affinity towards yeast flocculation, or the clumping together of cells. As cells began to grow in multiple layers, aggregation of cells rendered quantitative single-cell data extraction very difficult when using wide-field microscopy (Fig 2.3). While this effect was not an obstacle in early stages of cell growth, it became increasingly difficult to distinguish individual cells and quantify their responses as these cells began growing in log phase. Thus, the design criteria for cellular chambers had to address two primary concerns; the ability to trap microbial cells, and the ability to restrict cellular growth in the vertical dimension.

The second key component of a microfluidic device was the design of a channel network for transporting medium to the cell chambers. The objective of this device was to probe different colonies of cells with various concentrations of chemical inducer. This necessitated a means to produce and transport distinct concentrations of medium to individual chamber that were different from the others. The solution to producing intervals of varied medium concentration was the development of a concentration gradient generating network.

**Fig 2.3:** Cells growing within the gradient chambers. The chamber height is 4μm while the input media channels are 12μm. Cells that grow outside of the chamber are more difficult to resolve while cells within the chamber are clearly distinguishable.
Gradient generation has strong potential for applications in chemotactic testing and there are many forms of gradient generating networks. The key criteria for gradient generation include small sample displacement volumes, high temporal and spatial stability of gradients, and small total device size. The gradient-forming network is typically placed between two or more inputs and the cell growth chambers; using various mixing levels or controlled mixing ratios to generate different shaped gradients. Early microfluidic work involving gradient generators included arrays of cross intersections and sample shunting for serial dilutions and mixing tees with varying channel resistances to create different combinatorial mixtures for parallel dilutions [30]. The primary concerns regarding gradient generating networks included scalability towards a greater number of chambers and the ability of the network to create precise and stable gradients.

The first integrated devices produced for this work contained microchambers based on the fully enclosed bacterial Tesla microchemostat (TμC) developed by Cookson et al [6]. Fully enclosed chambers trapped cells while media and nutrients were transported to microcolonies within the chamber via diffusion. A low ceiling trapping region [6, 31] constricted the cells from growing in the vertical direction and forced the colony to grow into a monolayer. For yeast devices, microchambers were scaled to heights of 4 μm which is approximately the diameter of a S. cerevisiae cell.

A serial gradient generator was designed by placing 5 TμCs in series. A gradient network diluted a high concentration stream with a lower concentration buffer multiple times after each chamber, producing a non-linear gradient profile (Fig 2.4a). The gradient generator exploited laminar flow and diffusive mixing to generate ratios of two inputs (cell culture medium and inducer solution). Matched flow rates at T junctions ensured a 1:1 mixing ratio between adjacent streams. Therefore, a 10% inducer reagent mixed four times with H2O would form a series of dilutions of 10%, 5%, 2.5%, 1.25% and 0.625%, induced in each chamber respectively (Fig 2.5a).
Fig 2.4: Schematic of the Serial Array: based on the Tesla microchemostat (TμC) developed by Cookson et al. Cells were introduced into the device via port 4 and media through port 1. Port 2 acted as a common waste line during cell loading. During media loading, buffer is loaded via port 3 and acted to dilute the media concentration from port 1 after each chamber, thus creating a serial dilution.

Fig 2.5: Serial Array Concentration profile. (a) This gradient profile was produced by the serial array with media inducer of 100% and a serial dilution of H2O. A linear profile was unachievable with this serial dilution array. (b) A serial dilution array scaled up to 10 chambers shows the limited utility of the last 5 chambers which display roughly the same concentrations.
While the development of the serial TμCs provided a means to localize cells and constrain cell growth, it was hampered by long cell loading times. The Tesla diode loop in the TμC required a passive loading process which demanded cells to be situated at the lip of the chamber before it could be “pushed” in by added external pressure. This procedure of passive cell seeding was a relatively long process and it was difficult to determine how many cells would pass by the lip of the chamber before a few cells could be trapped. This was inefficient and could not be adapted to be used in a serialized or parallelized device design. Coupling two or more of these microchemostats in serial or parallel resulted in long cell seeding times and obtaining cells in all five chambers was extremely difficult.

Moreover, as the serial dilution profile was an inverse function, it became clear that the serial dilution had limited effectiveness beyond a low number of chambers. Significant reorganization of the dilution network channel lengths and resistances was necessary to create a more functional profile. The limited effectiveness in the cell loading process and the marginal utility of adding more chambers reduced the scalability of serial dilutions towards higher throughput design (Fig 2.5b).

New cell chambers were developed to facilitate improved cell loading. The new designs maintained the low ceiling trapping characteristic of the TμC but elongated the trapping region. Adopting a simplified rectangular chamber geometry resulted in single-entry and single-exit chambers that limited cell passage (Fig 2.6) such that the cellular suspension had no alternate path except to the edge of the chambers. Unlike the TμC, cell loading with this new chamber was not a passive process but could be controlled. This resulted in increased efficiency and significantly reduced cell seeding times.

Turning to parallel networks, designs were developed involving multilevel ladder structures which separated and joined mixtures through a series of inter-connecting channels [32, 33]. Alternative designs optimized parallel networks using a combination of bifurcated and trifur-
Fig 2.6: Schematic representation of device operation (a) Schematic illustration of the microchambers. The filter barrier located at the junctions between the gradient generating network and the cell culture modules were 2 μm in depth to avoid entry of cells to upstream channels. (b) Representation of the main stages of device operation. The three main stages include cell loading, cell culture, and cell-based assay. Fluid flow is represented by blue arrows and the green boxed region shows a typical field of view. (c) Representative images of cells within the growth chamber. From left to right, brightfield, red fluorescence, cyan fluorescence. Large circular region in the top right of the image is a support pillar in the chamber.
cated channels to reduce the number of discrete steps necessary to generate the same gradient [34]. Parallel networks have demonstrated a capability for generating linear gradients with immense accuracy and stability. More complex functions such as saw tooth and parabolic gradients are achievable through combinations of multiple pyramidal networks [32], while parallel flow dividers of different lengths and patterns within a flow channel can produce gradients of any functional profile [35].

Pyramidal ladder networks provided a fixed number of output channels at different concentrations and the use of more microchannels produced smoother and more stable gradients to their macroscopic counterparts [34]. As the fluid streams flowed down the network, they were repeatedly split at the nodes and recombined. Neighboring streams carried different concentrations of diffusible particles mix diffusively in proportion to the flow rates at each node.

There were two basic design criteria regarding the gradient generation network: 1) the fluid flow rate must be equal when two streams joined at a T junction 2) the pressure drop across any branched system level must be constant across each channel. To calculate the concentration level in each of the microchambers, it was necessary to know the relative flow rates at which the streams are mixed together in the serpentine channels. The flow rates governed the splitting ratios of the streams at the branching points in the network. A network with $X$ inlet ports and $Y$ chambers formed a $X$-input $Y$-output network. Each level of the pyramid contained $N$ serpentine channels and each channel was labeled from left to right starting at $i=1$ and ending at $i=N$. Nodes after the serpentine channels were considered part of the previous level, such that order of each network branch in a network was equal to its level $N$. The nomenclature for this paper is summarized in Fig 2.7.

All serpentine channels within a branched system had equivalent dimensions and the number of serpentine channels within a branched system increases by a single unit between subsequent levels. The fluidic resistances of the horizontal channels connecting the serpentine chan-
nels were negligible in comparison to the longer serpentine channels. In an equivalent electrical circuit analogy, \( R_H \gg R_v \), where \( R_H \) is the resistance of the horizontal channels and \( R_v \) is the resistance of vertical serpentine channels. Since all the serpentine channels were equivalent in dimension, the entire fluidic mass was forced to exit one stream and distributed evenly among the channels in the following branched level. As a result, mass was conserved and the bulk flow entering each level was equal to the total mass exiting. The stepwise increase in serpentine channels causes a consequent reduction in flow rate from one level to the next. This relative change in flow rate is given by the ratio of \( N/(N+1) \). However, the change in flow rate did not affect the concentration profiles as long as complete diffusion occurred in all serpentine channels.

**Fig 2.7:** Schematic explaining the nomenclature used for the mathematical characterization of the network. (a) Channels are numbered starting from \( i=1 \) on the left to \( i=N \), where \( N \) is the number of serpentine channels in that level. (c) Application of equations 3.1 and 3.2 governing the mixing ratios at the branching points. The dotted line indicates the boundary of the two combining fluid streams.
The following governing equations describe the splitting ratios at the branching nodes. The portion of the stream that came from the left channel (eq 2.1) and the portion that came from the right channel (eq 2.2) is given by the following ratios:

$$\frac{N - i}{N - 1} \quad (2.1)$$
$$\frac{i - 1}{N - 1} \quad (2.2)$$

Chambers $i=1$ and $i=N$ only have a single contributing stream, while the rest of the chambers are a mixture of a stream that turned left and a stream that turned right. The concentration in any given serpentine channel where $1 < i < N$, can be determined by the sum of eq. 2.1 and 2.2 multiplied by the corresponding concentration entering into each channel such that

$$\frac{(N - i_L) \cdot C_1 + (i_R - 1) \cdot C_2}{N - 1} \quad (2.3)$$

At the end of the network, channels carried intervals of different concentrations of chemical reagents to the modified cell chambers.

The design of the cell chamber and the gradient generating network were crucial to the success of the new microfluidic device. The designs explained in this section aims to address some of the key obstacles in expanding microfluidics towards higher throughput. Coupling these two components would set the stage for a new microfluidic device.

2.3.2: A quantitative, long-duration imaging platform

A microfluidic device featuring a parallel gradient generating system was developed specific to the analysis of budding yeast *S. cerevisiae*. This microfluidic chip integrated the generation of inducer concentration gradients and a series of cell operations such as cell seeding and induction. Expansion of microfluidic gradient generating networks from single to multiple chambers offers great utility in high-content cell systems biology research. *S. cerevisiae* serves
an excellent prototypical test subject for the device as it is a model eukaryotic organism for cell biology research. Yeast offered several benefits over *Escherichia coli*, most notable was the relative size of each species and its corresponding geometric restraints. *S. cerevisiae* is significantly larger than *E. coli*, thus placing fewer restrictions and complications in the design and fabrication of microchambers.

This design borrowed the key features of the pyramidal-ladder geometry of the concentration gradient generator developed by Dertinger *et al*. For the cellular chamber, a reduced ceiling height localized yeast cells that was mentioned previously in chapter 2.3.1. The flexibility of PDMS allowed the ceilings of PDMS microchannels to bulge upwards and allowed cells to flow into the chambers during cell loading procedures. Once the gauge pressure was decreased, the chambers were returned to its fabricated height of 4 μm, effectively localizing the trapped cells.
and restricting movement of the cells in the presence of fluid flow. Chambers were designed following general design guidelines that maintained cell chamber height to width ratio of 30:1 to prevent chamber collapse. Vertical columns were placed in the chamber to provide mechanical support whenever the design ratio was exceeded. These support columns in the chamber also provided a simple but effective pattern for image autofocusing.

The constriction of 2-D growth allowed for wide-field epifluorescent imaging for single cell fluorescence quantification. While specialized techniques such as confocal microscopy could resolve three dimensional imaging, these microscopy techniques were limited due to the rapid timescales of cellular processes. Fig 2.3 shows how extraction of single-cell data was more difficult on colonies with proliferation in all three dimensions while those confined in the 4μm chamber grew in a lateral monolayer with distinguishable cellular borders. The open end of the trapping region allowed for peripheral cells at the end to escape when the microcolony filled up the chamber. The large size of the cell chamber (450μm x 120μm) coupled with the free escape of cells allowed the cell chamber to maintain a relatively constant population of cells.

Each microfluidic chip consisted of an up-stream gradient generating module and a down-stream cell culture module. Chemical reagents of two different concentrations are loaded into the top inlets of the gradient-generating module and driven through the microchannel network by hydrostatic pressure. Figure 2.9 shows a graphical representation of the microfluidic network used to develop gradients. The two input channels at the top of the graphic contain blue (left) and red dye (right). The branched serpentine channels were designed to split, join, and mix fluid streams, generating eight distinct concentrations.

In order for eq. 2.3 from the previous section to hold true it was necessary to design the serpentine channels with sufficient length for adjacent laminar streams to diffuse and equilibrate over a wide range of input pressures. As established in chapter 1.4.1, fluid flow at the microfluidic scale was strictly laminar and this aided in calculating the serpentine channel lengths. In
fluid flow where $Re$ is high, turbulence aids in chaotic mixing of particles and chemical mixing occurs relatively quickly. However, a direct result of laminar flow is that mixing of two conjoining fluids is entirely dependent on diffusion alone.

Consider the T junction in which two incoming fluids flowed adjacent to one another, as in Fig 2.9. An estimate of the time can be approximated using $\tau_D \sim w^2/D$, where $w$ is the width of the channel. The distance $Z$ necessary is given by $Z \sim U_0 w^2/D$ and the relationship between the distance required for complete mixing and channel width is given by the dimensionless number known as the Péclet number,

$$Pe \equiv \frac{Z}{w} \sim \frac{U_0 w}{D} .$$  \hfill (2.4)
The Péclet number expresses the importance of convection to the diffusion process [22] and gives the number of channel widths necessary for complete diffusive mixing. Initial models used 10kDa dextran conjugated Rhodamine B dye as a model solute particle to determine serpentine channel length. Since the largest particle in medium was on the order of ~300 Da, modeling with a larger particle dye ensured complete diffusive mixing. The diffusion constant of the dye was estimated to be $79 \, \mu m^2/s$ using the Stokes-Einstein equation for large particles [22],

$$D_{AB} = \frac{k_B T}{6\pi \mu_B r_A},$$  

(2.5)

where $D_{AB}$ is the diffusion coefficient of solute A in solvent B, $k_B$ is Boltzman’s constant, $T$ is the ambient temperature, $\mu_B$ is the dynamic viscosity of solvent B, and $r_A$ is the spherical radius of solute A. For dextrans, the value of $r_A$ was determined based on a previously reported molecular weight relationship [36]

$$r_A = 0.488(MW)^{0.437}.$$  

(2.6)

With the estimated diffusion coefficient of $79 \, \mu m^2/s$, typical flow rates ranging from 200 to 500$\mu$m/s, and a channel width of 20$\mu$m, $Pe$ was determined to range from 50.63-126.58. Channel lengths were required to be 2.6mm to ensure the resultant stream to be a homogenous mixture. Further elongation of serpentine channels within the network provided increased network resistance and shielded the cellular cultures in the microchambers from dramatic changes in gradient profile.

At the end of the gradient module, streams carrying different concentrations of media reagent perfused the cell culture chambers (120 $\mu$m x 450 $\mu$m). The chambers were separated from the reagent delivery channels by a reduced height capillary. At a height of 2$\mu$m, the filter barrier was too small for yeast cells to pass into the delivery channels and prevented cell blockage of media channels. The width of the filter barrier was kept similar to the cell chamber width
to avoid large increases in flow rate in the media from the channels into the chamber. Once the cells were loaded, nutrients and inducers were delivered through a combination of diffusion and advection. Advective transfer of media to the cells in the chamber occurred in the initial stages of media loading. However, as the colony grew, the fluidic resistance in each chamber increased throughout the trapping region and diffusion dominated the transport process. Channels reconvened after exiting the cell chambers and medium left the device through a common waste port connected to a vial line.

2.4: Microscopy

2.4.1: System Description

All image acquisition was performed using a Nikon Eclipse TE2000-U advanced research grade inverted epifluorescent microscope equipped with automated fluorescence excitation and emission filter wheels, and Uniblitz VS35 high-speed shutters (Vincent Associates, Rochester, NY) mounted onto the fluorescence and transmitted light paths. A motorized XY stage and fine focus motor were directed by a hardware-based autofocus Proscan-II controller (Prior Scientific, Rockland, MA). Images were acquired with a Hamamatsu ORCA-ER cooled CCD firewire camera. An EXFO X-Cite 120 halide arc lamp was use as the fluorescent light source and an Ushio Eva halogen lamp was used as a transmission light source. The fluorescent lamp illumination power was reduced to 12.5% of the maximum lamp power using the adjustable iris during image acquisition to avoid photobleaching effect of the cells. Image acquisition was controlled using NIS-Elements Advanced Research (Nikon) and fluorescence visualization was performed with narrow band-pass excitation and emission filters for CFP, GFP, and mCherry for cells, beads, and dyes.
2.4.2: Thermal Maintenance

Image acquisition was done using a Nikon CFI Plan Apochromat 60x magnification, 1.40 N.A. oil immersion objective. Since the stage and objective were thermally coupled to the microfluidic chip, ambient air was controlled to reduce thermally induced drift in the focal plane. Chip temperatures were maintained using incubators (In Vivo Scientific) at 30.0 ± 0.1°C.

2.4.3 Image processing and analysis

Fluorescent images were analyzed using WCIF ImageJ 1.37c software. Fluorescence correction was done for medium and device fluorescence, as well as illumination nonuniformities, by subtracting the average local background fluorescence.
Experimentation

3.1: Introduction

Synthetic and naturally occurring gene networks are mediated by internal and external chemical stimulation [37]. Understanding the dynamics between chemical stimulations and their consequential cellular responses may give insight into uncovering basic mechanisms that are essential to regulating certain cellular activities. Studies of dose-dependant cellular responses to chemical stimulation would certainly benefit from platforms that could generate stable concentration gradients similar to chemical concentrations in vivo [38].

This work aimed to develop a microfluidic cell culture array for long-term high-throughput monitoring of single cells in culture. A microfluidic device was constructed to incorporate the advantages of a concentration gradient into a series of processes typically used in cell culture experiments on a single microfluidic platform. The functionality of the platform includes the ability to perform a series of cell operations such as cell seeding, culture, stimulations, and imaging within a precisely controlled environment. A 2-input 4 chamber microfluidic array, capable of carrying out four independent experiments simultaneously (Fig 2.5), was employed for this study. Here, this device was applied to monitor the response of the S. cerevisiae galac-
tose gene network to various concentrations of galactose.

Due to the logarithmic scale of media concentration necessary to capture the full induction response, three four chamber arrays were used instead of eight chamber arrays to better capture the minute changes in response. Each microchip contains an upstream gradient generating network, a downstream cell culture array of four or eight chambers, and three ports for fluidic access. The device was designed to generate distinct concentrations within a linear range through a network of channels that promoted complete diffusive mixing. The four resultant media streams were then transported to a monolayer growth chamber (Fig 2.2), which was designed for long-term single-cell data imaging of yeast cells. Filter barriers were also incorporated between the gradient generating network and the microchamber to prevent cell blockage of the upstream network. The microchambers were continuously perfused with a fresh supply of media from the gradient generating network.

To create a fluorescent indicator of the cellular response to an inducer, a yeast-optimized cyan fluorescent reporter protein (yECFP) was fused to the endogenous GAL1 protein in *S. cerevisiae* [27]. Yeast cells will only consume galactose in the absence of glucose in the medium [29]. The galactose utilization network is a well researched network that contains many regulatory mechanisms that are common to higher organisms and has become a model system for eukaryotic gene regulation studies [27]. To showcase the capabilities of the microfluidic platform, we use *S. cerevisiae* as a test organism to quantify the dynamics of network activation in response to galactose carbon sources.

### 3.2: Materials and Methods

#### 3.2.1: Microfluidic Device Fabrication

The microfluidic cell arrays were fabricated using well established soft-lithography techniques and the University of California San Diego Nano3 facility as described in Chapter 2.
Photolithographic photomasks were drawn using Illustrator (Adobe) and AutoCAD (Autodesk Inc.), printed on transparent film (Output City, Poway, CA), and mounted onto borosilicate glass plates (McMaster-Carr, Los Angeles, CA). To produce master molds, SU-8 negative photoresist (Microchem, Newton, MA) was spin coated using a single wafer spin processor (Laurell Technologies Corp, North Wales, PA) and patterned via UV exposure. Photomasks were oriented using a contact mask aligner (HTG, San Jose, CA). The first layer consisted of a filter barrier at a height of 2μm and the second layer was spun to 4μm to create the low ceiling trapping chamber for confining monolayer growth in S. Cerevisiae cells. Media channels were fabricated to a height of roughly 12μm. After all photolithographic steps were finished, feature heights of the mastermold were verified using a DEKTAK 2020ST profilometer (Sloan Technology Corp., Santa Barbara, CA). The mastermold surface was coated with a vaporous organo-silane ([Tridecafluoro-1,1,2,2-tetrahydrooctyl]-1-trichlorosilane, C₈H₄Cl₃F₁₃Si, United Chemical Technologies) for 5 minutes. PDMS (Sylgard 184, Dow Corning) was mixed in a 10:1 ratio between the polymer and cross-linking agents and degassed in a vacuum cleaner for 1 hr before curing in an 80°C oven for 1.5 hrs.

The hardened PDMS monolith was then carefully released from the mastermold and fluidic ports for media and cell loading were punched using 20-gauge Luer stub adapters (McMaster-Carr). The monolith was then sectioned into individual chips using a razor blade and cleaned with isopropanol and 0.2-μm-filtered deionized water. Scotch 810 (3M) office tape was used to remove any remaining particles from the chip surface. 24×40 mm, #1-1/2 coverslips (Corning Inc., Corning, NY) were cleaned using HPLC grade methanol and deionized water. The PDMS chips were irreversibly bonded to the glass coverslips by exposing oxygen plasma treatment from a UVO Cleaner (Jelight Company Inc, Irvine, CA) on the bottom of the device and the coverslip for 3 minutes. Sealed chips were left at room temperature overnight to ensure maximum bonding.
Two different arrays were fabricated: a 4 chamber array and an 8 chamber array. In this study, we will be using the 4 chamber array to demonstrate the capabilities of a parallel chip. Prior to experimentation, chips were wetted using sterile water injected into the cell loading reservoir before attaching media and cell culture lines.

### 3.2.2: Cell Preparation and Culture

Successfully transformed K699 yeast cells were grown on synthetic dropout plates at 30°C for 2 days and stored at 4°C for no more than 2 weeks. 24 hours prior to the experiment, an isolated colony was inoculated in liquid minimal medium containing 2% raffinose at 30°C at 300 rpm overnight. Approximately 4 hours prior to the experiment, the overnight culture was diluted to an OD₆₀₀ of 0.1 (if necessary) and grown to OD₆₀₀ 0.2-0.3 to ensure that the cellular batch was in log phase growth.

### 3.2.3: Experimental Conditions

Each experiment tested three growth conditions, with the concentration of inducing reagent tailored to the range desired in each individual experiment. All selective medium contained 2% (w/v) raffinose as the carbon source. For example, an experiment probing the galactose utilization response to a array of induction levels ranging from 0.01% to 0.1% would have the following media concentrations: loading media, 2% raffinose; high inducer media, 2% raffinose + 0.1% galactose; low inducer media, 2% raffinose + 0.01% galactose. In order to characterize an entire induction curve, the medium used for the reagent loading stage of the chip contained raffinose and small amounts of galactose, ranging from 0.001% to 0.5%. Negative and positive control experiments with 0% galactose and 2% galactose, respectively were also carried out to measure both ends of the spectrum. All media used synthetic drop out based minimal media with appropriate supplements for auxotrophic selection. The higher inducer media contained
0.01 mg/mL of sulforhodamine 101 to track on-chip galactose concentration and ensure proper gradient distribution.

Microfluidic array devices were mounted on a motorized stage and the enclosed incubator was maintained at an optimal growth temperature of 30°C. The device was initially prepared by wetting the chip with a 0.2-μm-filtered dH2O via a 10 ml syringe, to remove dead volume and air bubbles inside the device. Following the filtered water priming, an open 10 ml syringe containing the cell suspension was placed in the cell loading port and suspended 10 in above the chip. When it was confirmed that 5-10 cells were trapped in each chamber, the cell suspension was swapped out with a 10ml syringe and 3 ml of dH2O. After cell loading, 2 separate media syringes containing 3ml of media and inducer of interest were brought to their run-time heights. Media reservoirs were fixed at a height 20 in above the media port and waste reservoir was fixed at a height 10 in above the chip. This differential in heights provided the hydrostatic pressure to create directional flow from the media loading ports to the waste port. The chip was then imaged under these conditions to ensure a linear gradient profile across the chambers as well as establish a non-induced baseline of fluorescence. Finally, the microfluidic array was run for 48 hrs and each chamber was imaged every 15 minutes in cyan, red fluorescence, and transmitted light.

3.2.4: Data Analysis

After cells were loaded onto microchambers, they were allowed to grow five to six generations before they were analyzed for fluorescence induction. Images were analyzed in WCIF ImageJ 1.37C by measuring the mean intensity within each cell. Bright field images were overlaid with cyan fluorescent images to further enhance cell boundaries. Since only a single snapshot of fluorescence was needed to quantify the induction response, all the cells in each picture were manually measured.
3.3 Results and Discussion

3.3.1: Gradient Generation

Sulforhodamine 101 was used to test the gradient generator network as a fluorescence probe for estimating the gradient profile generated by each gradient generator. The fluorescence intensity was proportional to the concentration of inducer in each medium input, thus the degree of diffusive mixing was able to be monitored throughout the gradient network. Due to the reduced height of the micro-scale chambers (4 μm) and the high demand for fabrication precision, slight variations in channel height resulted in wider variations in imaging. Therefore, the fluorescent profile was imaged and analyzed by fluorescence microscopy at the exit channels of the gradient generator right before it entered the microchamber. The concentration profile of three independent experiments obtained from the two-inlet 4 chamber generators were compared to the profiles derived from theoretical calculations (Fig 3.2). The correlation coefficient between the theoretical estimates and the experimental data ranged from 0.9993-0.9998, validating the ability of this microchip to generate high-precision gradient profiles.

The duration in stability of the concentration profile generated by this device was also verified. Initial growth of the newly seeded cells exhibited a longer division time (~4.5 hrs) but
quickly settled into a steady doubling rate between 3 and 3.5 hours within two generations. Preliminary testing of this chip resulted in a relatively short duration due to chambers quickly filling up in a 24 hour time-span. As cells in each chamber grew, the fluidic resistance of each channel changed and thus had a significant impact on the consistency of the gradient profile. A non-uniform distribution of cells during the cell loading procedure lead to varying rates of increased resistances and changes to gradient profiles (Fig 3.3).

The 2μm filter barrier was useful in preventing cells from migrating into the gradient generating network during cell loading. However, it did not prevent cells from blocking the entrance to the cell chamber as the colony filled the chamber. The flexibility of PDMS allowed new cells which were smaller than 2μm to grow and force up the ceiling to the filter barrier. Thus, inevitably the entrance to the gradient generator was blocked and ultimately stopped flow as cells filled up the media transport channels. Without fresh media, unhealthy cells were observed to have quick degradation of fluorescence and abnormalities in cell shape.

A malfunction in one channel thus had a significant effect on the operation of the other channels. However, it was determined that a change in procedural cell loading could increase the duration of experimental runs. Reducing the density of cells loaded into the device and reducing the number of cells loaded into each chamber ~3-4 led to significantly longer experimen-
tal runs with healthy yeast cells fluorescing for more than 48 hours (roughly 15-16 generations).

### 3.3.2: Characterization of fluorescence induction response

The effect of chemical stimuli can be determined through dose-dependant cellular responses [38]. In the presence of galactose, K699 cells exhibited a sigmoid increase in GAL1 gene expression (Fig 3.4). Cells stimulated with low (less than 0.001%) and high (more than 0.008%) galactose concentrations responded quickly to inducer stimulation and reached a steady state after 9 h. However, cells were much slower to respond to the chemical stimulation at intermediate levels of galactose.

At low concentrations, stochastic dynamics were observed between two stable expression states, a phenomenon also observed in batch cell culture measurements [28]. The vast majority of cells at low (less than 0.005%) galactose levels exhibited low GAL1 activity (OFF) but a low number of cells exhibited high GAL1 activity (ON) as seen in Fig 3.5. Bimodal distribution of fluorescence was seen in intermediate levels of galactose and led to greater variability in cellular response. Previous flow cytometry data was acquired with sample sizes of ~10^5 cells [27], while microscopy offered a significantly reduced data set, thus leading to higher statistical
Fig 3.4: Galactose induction for *S. Cerevisiae* K699. Shown are the experimentally obtained data from optical microscopy using the gradient array (red squares) and flow cytometry (blue triangles) and the numerical fit from the induction curve model (dashed blue line) for K699 developed by Bennett et al.

Microscopy imaging was able uncovered data previously masked by flow cytometry data. Cellular memory of expression states were passed down each generation as a few ON cells at low galactose concentration gave rise to daughter cells that were ON as well. The reverse was true as well, in some cases the progeny of an OFF cell remained OFF for a few generations until gradually induced. Optical microscopy allowed cell tracking of these events and the analysis of phenotype heritability.

3.4: Conclusions

In this work, the utility of a microfluidic platform featuring an integrated gradient generating network with parallel micro-scale arrays was demonstrated. The parallel microchip had the advantage that each set of channels was independent of the others, thus allowing the genera-
Fig 3.5: Fluorescence induction in the gradient array. (a) Representative images of microcolonies in corresponding dilutions (0.001, 0.0033, 0.0057, 0.008, 0.01, 0.023, 0.0367, and 0.05%). Brightfield images were overlaid with fluorescence images in ImageJ and quantified for mean fluorescence per cell. (b) Histograms depicting percent population of the colony with normalized fluorescence.
tion of a gradient with well-defined resolution in multiple chambers for a single operation. The results show that the process of parallelizing microchambers has great potential for applications in the analysis of synthetic gene networks and offers an unprecedented increase in research efficiency. This assay exploits the fundamental advantages of microfluidic technology by requiring minimal sample consumption and no additional instrumentation for liquid dilutions. Thus using this platform, it is now possible to probe networks of model organism in multiple independent experiments while maintaining a sterile and highly controllable environment to reduce variability between experiments. This direction towards high-throughput microscopy experimentation coupled with computational analysis tools will lead to significant advances in the quantification of dynamic gene expression and cellular regulatory mechanisms.

3.5: Acknowledgements

Summary and Future Directions

4.1: Review

The development of a microfluidic cell culture array offers a large potential for better applications in biotechnology research such as drug screening, quantitative biology, and biosensor development [39, 40]. A central focus of this study was to develop a platform that would be adaptable for a large number of research investigations. Microfabrication techniques and microfluidic technology is ideal for high-throughput experimentation due to its rapid prototyping and inexpensive production costs. This work highlighted the development of a gradient generator device capable of carrying multiple independent experiments on the same microfluidic chip. The gradient generator network created distinct concentrations of uniform medium within each microchamber using only two fluidic inlets and is compatible with most types of optical microscopy.

This microfluidic platform is capable of integrating cell seeding, culturing, drug or reagent stimulation and cellular response monitoring. In this study, the microfluidic device was
applied to the characterization of the induction response of a K699 \textit{S. cerevisiae} yeast variant in the presence of different concentrations of galactose. This microchip assay served as an example of high-throughput analysis that was previously considered too inefficient for microfluidic technology.

4.2: Significance

There are many obstacles in the adaptation of microfluidic technologies to systems biology application. Chief amongst them is the experimental compatibility of large-scale applications with established methodologies [19]. While many studies currently focus on validating the advantages of microfabricated systems, there is a need for implementation of engineering principles in order to increase efficiency and exploit the true advantages of microfluidic technology. For instance, many microfluidics boast the ability to save time and resources, but most microfluidic devices lack the methodology for true low volume delivery of cells. Because of this, microfluidics makes high-throughput systems a theoretical novelty but not a practical reality. Some microfluidic devices require immense amounts of set up time, with dozens of ports and reagent loading vials. Such devices offer novel approaches towards biological quantification but lack the adaptability to broad experimental techniques. The continued advancement of microfluidic technology is crucial to their application in systems biology for high-throughput studies will quickly gain widespread acceptance in biological experimentation in the near future.

4.3: Future Directions

The 2-input 4 chamber array signifies a major step towards the development of high-throughput microfluidic platforms and this work is currently used within my research group for probing other synthetic networks in \textit{S. cerevisiae}. However, several areas still have yet to be
addressed.

One of the fundamental obstacles facing adaptations of novel microfluidic techniques is the integration of living organisms while maintaining the stability of the primary chip function. The dynamic increase in resistance of the chambers due to the growing cell culture may cause the gradient profile to change over time, thus changing the environment of the microchamber. Future work on this chip requires long-term imaging without changing the dynamic profile of the gradient. This could be solved by redesigning cell chambers that would maintain more stable populations of cells. Multi-level PDMS structures have a great potential in providing improved trapping mechanisms.

The 2μm filter barrier should also be redesigned such that cells are physically unable to block the concentration gradient network. Instead of one wide channel for the filter barrier, a series of microchannels about 0.5μm in width would allow for rapid transport of media throughout the growth chamber and provide better protection against cell overgrowth.

This work showed the development of a parallel array of up to 8 parallel chambers and the number of chambers could be increased without great difficulty. If this chip were to be expanded to a greater number of chambers, a new method of cell loading may be required to evenly distribute cells to every chamber. As mentioned in chapter 3.3.1, uneven cell loading may cause varied resistance changes and changes to the gradient profile.

4.4: Closing

This paper serves as an example of optimizing designed microfluidic devices to increase the efficiency of these platforms and applying fabrication engineering principles to research. In many cases biological lab research is burdened by inefficiency and redundant processes. The tools developed in this study will serve to provide a foundation for a new type of experimenta-
tion in which high-throughput analysis in microscopy is now achievable. This integration of mi-
crofluidic devices and systems biology will lead towards a future of unimaginable advances in
predictive and preventative therapies [41].
Appendix

Microfluidic Devices

A.1: Parallel Neurospora Crassa Microdevice

A.1.1: Fabrication

Table A.1: Master mold feature height specifications. †Photoresists are SU-8 unless otherwise specified.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Thickness (µm)</th>
<th>Photoresist †</th>
<th>Spin Speed (rpm)</th>
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</thead>
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<tr>
<td>Spore Boundary</td>
<td>1.9</td>
<td>2001</td>
<td>1000</td>
</tr>
<tr>
<td>chambers</td>
<td>4</td>
<td>2003</td>
<td>2000</td>
</tr>
<tr>
<td>flow channels</td>
<td>12</td>
<td>2007</td>
<td>1650</td>
</tr>
<tr>
<td>access port</td>
<td>38</td>
<td>2015</td>
<td>1000</td>
</tr>
</tbody>
</table>
A.1.2: Device Schematic and Port Assignments

**Figure A.1:** Device schematic for serial T\(\mu\)C. Inset displays a magnified view of the growth chamber and autofocusing pattern.

<table>
<thead>
<tr>
<th>Port</th>
<th>Abbr</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>input, cell suspension</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>input, media</td>
</tr>
</tbody>
</table>
A.2: Serial TµC: Tesla micro-Chemostat and integration of a concentration gradient

A.2.1: Fabrication

Table A.3: Master mold feature height specifications. †Photoresists are SU-8 unless otherwise specified.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Thickness (µm)</th>
<th>Photoresist†</th>
<th>Spin Speed (rpm)</th>
</tr>
</thead>
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<tr>
<td>chambers</td>
<td>4</td>
<td>2003</td>
<td>2000</td>
</tr>
<tr>
<td>flow channels</td>
<td>12</td>
<td>2007</td>
<td>1550</td>
</tr>
<tr>
<td>access port</td>
<td>38</td>
<td>2015</td>
<td>1000</td>
</tr>
</tbody>
</table>
A.2.2: Device Schematic and Port Assignments

Figure A.2: Device schematic for serial TμC. Inset displays a magnified view of the growth chamber and autofocusing pattern.

Table A.4: Port assignments for serial TμC

<table>
<thead>
<tr>
<th>Port</th>
<th>Abbr</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>input, media 1 (inducer)</td>
</tr>
<tr>
<td>2</td>
<td>W</td>
<td>output, common waste</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>input, media 2 (buffer)</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>input, cell suspension</td>
</tr>
</tbody>
</table>

49
A.3: 2-Input 4-Chamber Array

A.3.1: Fabrication

Table A.5: Master mold feature height specifications. †Photoresists are SU-8 unless otherwise specified.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Thickness (µm)</th>
<th>Photoresist †</th>
<th>Spin Speed (rpm)</th>
</tr>
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<td>Filter barrier</td>
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<tr>
<td>chambers</td>
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<td>2003</td>
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</tr>
<tr>
<td>flow channels</td>
<td>12</td>
<td>2007</td>
<td>1550</td>
</tr>
<tr>
<td>access port</td>
<td>38</td>
<td>2015</td>
<td>1000</td>
</tr>
</tbody>
</table>
A.3.2: Device Schematic and Port Assignments

Figure A.3: Device schematic for 2-input 4-chamber array. Inset displays a magnified view of the growth chamber and filter barrier.

Table A.6: Port assignments for 2-input 4-chamber array

<table>
<thead>
<tr>
<th>Port</th>
<th>Abbr</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>input, media 1 (inducer)</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>input, media 2 (buffer)</td>
</tr>
<tr>
<td>3</td>
<td>C/W</td>
<td>cell loading: input, cell suspension</td>
</tr>
<tr>
<td></td>
<td></td>
<td>media loading: output, common waste</td>
</tr>
</tbody>
</table>
A.3.3: Operational Protocol

Required Materials

bonded microfluidic device
microfluidic lines (XX cm), 3 total
microfluidics lines (XX cm), 1 total
fluid reservoirs, 3 total
10 mL syringe, 1 total
inverted microscope with imaging camera, shuttered light sources (transmitted and fluorescence), and appropriate acquisition software.
Heat maintainer

Naming and Media Conventions

There are three fluidic ports on the device and three corresponding fluid reservoirs. There is 1 reservoir that serves both as a “cell suspension” input as well as a “waste” output, e.g. where fluid flow exits the device, and 2 “media/input” reservoirs where fluid enters the device. Waste reservoirs are denoted by a “W” label. The input reservoirs are labeled: I and B, for loading “induction” media and buffer media.

The “I” reservoir typically contains unaltered growth media that is the same as the media used to prepare the cell suspension for overnight culture. In addition to this growth medium is any chemical agents that will elicit a cellular response such as inducers, activators, or repressors. The “B” input reservoir is the same as the media in the “I” reservoir without the aforementioned chemical agents. If a gradient of chemical agent X is desired across the four chambers, the only difference in input “I” and “B” should be the difference in concentration of chemical agent X. Reservoirs “I” or “B” should also include a difference in concentration of flow tracing
dye (e.g. 0.01 mg/mL sulforhodamine 101, Sigma S7563) which is used to determine the concentration gradient across each chamber. For most cases, the “I” reservoir has the additional tracer dye.

The remaining input reservoir is used for the cell suspension. This is the only reservoir that is not recycled to prevent cellular contamination in subsequent experiments. During media loading operation, this reservoir is converted to an output (waste) reservoir of the device. This will be explained in more detail below.

Protocol

Line Attachment and Device Wetting

1. Secure the device to the microscope stage by placing the device on the microscope. Using 4x magnification, inspect the device for any defects that could impair its performance — i.e. channel leakage due to poor bonding, or channel blockage due to dust particles. Discard defective devices.

2. Place all reservoirs on the gravity towers and ensure that all the fluid connection lines are sufficiently prepared and void of bubbles.

3. Set the heights of the reservoirs to their operational positions. These positions are specific to each experimental setup, but generally they should be (inH2O): 15, 15, and 28 for I, B, C, which are the heights specified used in MOCA simulations.

4. Fill the syringe with surfactant (Tween +LB) and connected to the XX cm fluid connection line. Connect the surfactant reservoir to the port 3 on the device and gently apply pressure to the rubber piston until fluid is visibly traveling through the channels.

5. After a few minutes, fluid droplets should form at ports 1 and 2 simultaneously. Connect the I and B reservoirs to ports 1 and 2 on the device, respectively. Make sure no air bubbles are present in the fluid lines. Do not attach the cell suspension reservoir at this time.
Secure and lock down the device onto the microscopic stage insert. Inspect all channels for pockets of air or channel leakage. Check to see if the chamber and microchannels that connect it to the integrated media switch are appropriately wetted. If there are air pockets anywhere in the chip, continue to apply pressure to the surfactant syringe. Once all air pockets have been removed from the device, release pressure on the syringe but do not disconnect the fluid lines. Place the syringe on the microscopic stage.

**Predetermining Media Gradient Concentration**

1. Move the field of view (4x or 10x magnification) to the first set of serpentine channels and illuminate using appropriate fluorescence settings for the tracer dye used.

2. At the far left of the middle serpentine channel, you should see an interface form between the dyed and undyed media midway across the T-junction.

3. Monitor the interface and manually adjust the height of the I and B reservoirs if the interface is visibly dislocated from the center of the channel. In most cases this should not be an issue as long as the media reservoirs are placed correctly on the gravity towers.

4. Move the field of view (4x or 10x magnification) to the cellular growth chambers and record the mean fluorescence in each chamber to determine the gradient ratios of each chamber. If a histogram is not available on the DAQ software, capture images and measure mean intensity levels in ImageJ and proceed to cell loading procedures.

**Cell Loading**

1. Detach the syringe containing surfactant and replace with the cell suspension reservoir to port 3 on the device. Make sure that there is a bead of liquid at the port before attaching the cell suspension reservoir. If not, briefly pressurize the syringe until one appears on the port and pro-
ceed. Discard syringe in a waste container.

2. Illuminate the device with transmitted bright light and move to a field of view that displays the fluid port entrance to port 3.

3. Cells should now start to flow into fluid channels leading to the four growth chamber. If cells are retained at the entrance, you may increase the pressure by gently flicking the fluidic lines to help dislodge the cells.

4. The cells will align themselves at the interface between the 12 µm channel and the 4 µm chamber. As soon as there are a few cells at the interface of all four chambers, flick the fluidic lines to provide enough pressure to push the cells into the chamber.

**Media Loading**

1. Once a satisfactory number of cells have entered the chambers, reposition the height of the cell suspension reservoir only. This converts cell suspension reservoir to a waste reservoir because of its base height relative to all the other reservoirs. Loading media will now purge the loading segment to reduce device fouling. The new height of the waste reservoir should be 3 to 4 inches lower than I and B reservoirs.

2. Once cells are loaded and satisfactorily distributed, wait a few minutes to let the media flow into the cell growth chambers. Illuminate the chambers with fluorescence to view the dye concentration to ensure proper operation of the media flow.

3. Maintain incubator cages at a steady 30°C and proceed to data acquisition.

**Data Acquisition**

1. Secure the microfluidic lines and be sure to provide enough slack for any required stage movements.
2. Change to the desired magnification (typically 40x) and move to the desired field of view. If a motorized stage is installed, prepare the operating software appropriately. Typically this requires setting the stage origin and/or defining a scan pattern.

3. Acquire using your desired acquisition settings.

**Cleanup**

1. Remove the device from the stage insert.

2. Remove the fluidic lines from the device by holding the device by the PDMS portion and pulling firmly on the lines. Unlock the PDMS device and discard in a waste container.

3. Collect the lines together with a binder clip and place them so that all flow is collected in a waste collection beaker.

4. Dump the contents of each reservoir into the waste collection beaker and discard fluid lines and reservoirs but making sure to keep the steel tips to be cleaned and reused in subsequent fluid line production.

5. Keep steel tips in a clean Petri dish to be cleaned, primed, and stored in a dry location for future reuse.
A.4: 2-Input 8-Chamber Array

A.4.1: Fabrication

Table A.7: Master mold feature height specifications. †Photoresists are SU-8 unless otherwise specified.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Thickness (µm)</th>
<th>Photoresist†</th>
<th>Spin Speed (rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter barrier</td>
<td>2</td>
<td>2003</td>
<td>2100</td>
</tr>
<tr>
<td>chambers</td>
<td>4</td>
<td>2003</td>
<td>1150</td>
</tr>
<tr>
<td>flow channels</td>
<td>12</td>
<td>2007</td>
<td>1550</td>
</tr>
<tr>
<td>access port</td>
<td>38</td>
<td>2015</td>
<td>1000</td>
</tr>
</tbody>
</table>

A.4.2: Device Schematic and Port Assignments

Figure A.4: Device schematic for 2-input 8-chamber array. Inset displays a magnified view of the growth chamber and filter barrier.

Table A.8: Port assignments for 2-input 8-chamber array

<table>
<thead>
<tr>
<th>Port</th>
<th>Abbr</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>input, media 1 (inducer)</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>input, media 2 (buffer)</td>
</tr>
<tr>
<td>3</td>
<td>C/W</td>
<td>cell loading: input, cell suspension</td>
</tr>
<tr>
<td></td>
<td></td>
<td>media loading: output, common waste</td>
</tr>
</tbody>
</table>
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