Single Cell Culture Wells (SiCCWells)

Dissertation

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Abstract

Single cell analysis is an increasingly important part of biomedical research and diagnostic medicine. Cells are known to be dynamic organisms and when they are influenced by the cellular micro-environment produce a spectrum of responses. Bulk cellular techniques can lead to the loss of information by averaging signals together from populations that are inhomogeneous. Bulk cellular techniques also typically require the disruption or destruction of cellular process. Because of this there are few, if any, options to monitor a cell in real time \textit{in vitro}. The dichotomy of single cells analysis is that the bulk signal cannot be neglected for the individual cellular response. Rather the bulk signal must be built out of the individual responses. Due to this, single cell resolution must be coupled with high through put automated system that can discretely isolate cells, deliver chemistry, sample the microenvironment, and maintain cells in long term cell culture.

We present an integrated platform for high volume single cell analysis built on the femtopump technology. The femtopumps use a combination of micropatterned materials to restrict electro-osmotic flow to a small cross section. The result is a robust platform technology on which a variety of microchemical total analysis systems can be built. Because of the patterned electro-osmotic technology these devices offer a high degree of precision and repeatability for \textit{in vitro} experimentation.

We present a femtopump-based micro total analysis system, the Single Cell Culture Well or SiCCWell. The SiCCWell design provides for vacuum assisted cell seeding, long term culture, manipulation of the microenvironment through fluid delivery, and continuous monitoring of cellular processes through fluid sampling. The SiCCWells have demonstrated the ability to capture and deliver fluid to cells \textit{in vitro} at a rate of approximately 10
The SiCCWells have also demonstrated the capacity for electro-osmotic generation of microdroplets. The SiCCWell design provides for high throughput single cell monitoring. Current designs are thought capable of generating data at single cell resolution from up to 12500 \( \text{cells/cm}^2 \).

In addition, the theory, design, and practical implementation of an electro-osmotic gradient generator is presented. Chemotaxic gradients are a valuable way to model the "in vivo" cellular microenvironment to understand how chemical and environment cues affect the behavior of cells. The gradient generator uses a highly modified SiCCWell fabrication to give discrete activation sites to which differing volumes of fluid can be delivered. The gradient generator is capable of varying the flow rate per channel to create geometric and arbitrary gradient patterns with a high level of precision and repeatability.

The femtopump-based technologies are an exciting new platform technology that is highly customizable for wide variety of applications. The SiCCWell microchemical total analysis platform is an example of the opportunities in single cell analysis presented by this technology.
Dedicated to my Nephews and Nieces:

Michael, William, Matthew, Kaitlyn, and Abigail.

May you always have the courage to follow your dreams.

–Uncle Jeremiah
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Chapter 1

Background and Significance

1.1 Introduction

Research at the single cell level has gained significant momentum in the last 25 years. Initial experiments with intracellular sampling were conducted in the early 90s and now the methodologies for single cell analysis have become common place in advanced research. More recently there has been an additional push for further development and commercialization of high throughput single cell analysis systems [1, 2, 3, 4, 5, 6, 7, 8].

Biomedical microdevices have risen to the challenge to provide robust platforms that allow implementation of single cell analyses. Microdevice designers attempt to stay one step ahead of researchers by providing tools with capabilities that proceed in a naturally reductivist manner from current techniques [9, 10, 11]. The architecture of these devices is based on the needs of the single cell researcher both today and tomorrow. This, in turn, lends itself to commercialization. Interogation, sampling, ability to be multiplexed, and high throughput are all considered in the design of an optimal microfluidic single cell system [4].

Our lab presents the design and implementation of the Single Cell Culture Well (SiCCWell). This device is based on a patented technology, the femtopump, which is used to control femto-scale ($10^{-15} L$) volumes of fluid. The SiCCWell is a specific morphism of the femtopumps that provides for fluid handling to captured cells in culture. This device allows for delivery to and sampling of the cellular microenvironment for meaningful single cell experimentation.
This text is outlines the process by which a novel biomedical nanofluidic system is fabricated and tested. In Chapter 1, the literature is reviewed to show the need for single cell analyses, microfabrication techniques, and the basis of the core technology (electro-osmotic flow). In Chapter 2, the fabrication of the femtopump-based devices will be explored. In Chapter 3, the SiCCWell (Single Cell Culture Well) cellular staining trials are reviewed. Chapters 4 focuses on SiCCWell based microdroplet generation and Chapter 5 explores the technology’s utility for generating chemotaxic gradients. Chapter 6 concludes with future designs for new femto-pump based devices.

1.2 History of Single Cell Experimentation

Scientific advancement has always required the innovation of technology. From the beginning of cellular experimentation, biotechnology progresses have shared a symbiotic drive/driven relationship with technological progresses. Cole, Hodgkin, Huxley, Southern, and Eberwine are examples of researchers that have provided advancements in technology for both their own research and for the development of technology used by cellular scale molecular biology [12, 13, 14, 15]. The desire to obtain information from a single cell has been consistent since subcellular structures were first discovered in biology [16]. Historically, there have been many significant developments in single cell research, however, there is still a substantial amount of room for improvement of bulk and single cell techniques.

The desire to measure the responses of a single cell begins in the early 1900s. Arguably, the first time single cell experimentation was performed was when the first cell was reported by Robert Hooke in the 1600s. However, it is more likely to fit the nature of this discussion to look at experimentation done after cell theory was established in the mid-1800s. It was not long after establishing cell theory that the discovery of individual intracellular organelles was referenced by Mobius in 1884 [17]. By the turn of the 20th century, scientists described mitochondria, centrosomes, and the golgi apparatus [18, 19, 20]. Specialized organelle references began making appearances by the 1920s and it is at that time that the first true single cell experiments were being performed [21, 16].
In the earliest cited works, researchers made efforts to circumvent the absence of precise single cell tools by focusing on larger cells such as *Nitella flexilis* and *loligo* [16], [12]. Far from simple, cells for investigation had to be surgically removed by hand without destroying the samples [12]. While relatively large, these cells were still on the order of 500 µm. Lawrence Blinks focused on the isolation and investigation of the cellular properties of *Nitella flexilis* using a system by which a cell from the algae was contained within a conductive chamber shown in Figure 1.1 [16].

Kenneth Cole, a doctorate in physics responsible for bringing many of the physical sciences to the application of biology, began working on the giant squid axon after having such specimens recommended to him by John Young [12]. Possibly the most prominent of these experiments is the 1952 work of Alan Hodgkin and Andrew Huxley in which they probed and modeled the reactions of the *loligo* giant axon [13, 22, 23, 24, 25]. The *loligo* experiment was performed using an early single cell analysis tool built for the Hodgkin Huxley experimentation shown in Figure 1.2.

In a few years, the voltage clamp used by Hodgkin and Huxley for their landmark experiments had evolved into a more site-specific patch clamp, though it would not be
Figure 1.2: Original diagram of the single cell apparatus used by Hodgkin and Huxley [13]
referred to as such for several years. The patch clamp used in Strickholm’s first experiment was a small pipette drawn to a 20 micrometer opening. This heat treated glass pipette could be brought into contact with a membrane and held there via suction. In 1961, Strickholm reported the use of these patch clamps in the measurement of the membrane resistance of an isolated area of the sartorius muscle from a frog. Strickholm used this to develop a better model of the electrophysiology of a muscle [26]. The technique was refined by Neher and Sakamann [27] to provide a giga-ohm seal that improved upon the previous models. Further improvements have been suggested and researched since, in the form of microfabricated high throughput patch clamp systems using microfluidic technology [3].

1.3 Bulk Cellular Analysis Methods

Analysis techniques have been developed to monitor the changes of DNA, RNA, surface proteins, and cell morphology. While gross cellular morphology was relegated to histology, techniques were widely developed to understand the mechanisms that occurred within cells by reviewing the analyte responses to various challenges. These techniques often had significant draw backs such as lack of temporal resolution but they were, nonetheless, powerful techniques that allowed for the progress of molecular biology. The microscale adaptation of these techniques is the basis of much of micro total analysis systems (microTAS, µTAS) development.

Bulk cellular analysis typically uses either a chemical or physical system to identify target analytes. These systems can be used to locate and characterize a host of intracellular structures.

1.3.1 Polymerase Chain Reaction

The identification of mutant and expected DNA and RNA sequences is an essential task for molecular biology. Polymerase chain reaction, or PCR, is a mechanism by which DNA and RNA sequences can be identified and quantified. There are many variants of polymerase chain reaction which represent a versatile library of methodologies. While the number
Basics of Polymerase Chain Reaction

Polymerase chain reaction is a method by which small amounts of DNA, cDNA, or RNA can be amplified to detectable levels. Polymerase chain reaction works on the principle of modulating the temperature of the baths in the presence of oligonucleotide probes, in which the target DNA is suspended. Precursors to the DNA are added to the solution in which the DNA can unzip and rezip forming additional copies of the DNA.

Polymerase chain reaction proceeds in several iterations of steps. First the DNA polymer is denatured into single strands of nucleotides, primers are then annealed to the original strands to provide for new double stranded sequences, and the final step is extension of the new sequences. By repeating these steps the number of copies of a target sequence of DNA can be multiplied exponentially, doubling with each temperature cycling.

Alterations to the PCR protocol have led to increased functionality. Each of the refined techniques build on a previous development in sequence detection. A diagrammatic representation of this relationship can be seen in Figure 1.3.
Reverse Transcriptase Polymerase Chain Reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) is used to probe cellular expression of RNA sequences, particularly messenger RNA (mRNA). This is done by adding precursors to the sample analyte that will prime the sequence with a complementary DNA sequence (cDNA). This cDNA sequence can then be expanded upon via typical polymerase chain reaction. Reverse transcriptase polymerase chain reaction is a method of qualitative analysis but the sample can be expanded upon by using quantitative polymerase chain reaction methods.

Quantitative Realtime Polymerase Chain Reaction

Both PCR and RT-PCR are semi-quantitative technologies, which provide an increase in the copy number of sequences of DNA. This allows the detection of the presence of a sequence or the purification of a sample for characterization in another process like blotting or ELISA. However, neither of these techniques, independent of another process, can provide a quantification of the original copy number of a sequence. Quantitative real time polymerase chain reaction (qPCR) is a type of polymerase chain reaction that allows a researcher to appropriately determine the relative amount of a sequence present in a sample.

In qPCR the amount of precursor is limited and is consumed as the reaction proceeds. There are several noted sections of the qPCR reaction curve. Initially, the qPCR reaction shows the typical exponential growth associated with PCR. As the number of reactions that can occur becomes more limited, the curve enters a linear region. In the final phase the reaction enters the plateau phase. These phases make-up an amplification curve that can be compared to additional control DNA or house-keeping genes an example of which can be seen in Figure 1.4. This comparison provides the relative value of the amount of gene expression prior to amplification [28].

The quantitative PCR precursors utilize a stem-and-loop probe sequence containing a bound fluorescent molecule on one end and a quenching molecule on the other as shown in Figure 1.5. When in initial solution the fluorescent molecules are in close proximity
to the quenching molecules and are incapable of producing detectable fluorescence. As the PCR reaction proceeds the fluorescent and quenching molecules are hybridized to the target sequence. Once the fluorescent and quenching molecules are separated the fluorescence can be detected and assigned a numerical value based on intensity. The intensity values are measured in each step of the PCR reaction. By plotting the fluorescent intensity per step for both the unknown and housekeeping genes, a relative value for the unknown quantity can be provided.
Digital PCR

Digital PCR further enhances the capabilities of real time polymerase chain reaction by more directly measuring the amounts of amplified nucleic acids. Digital PCR is substantially more precise than PCR and RT-PCR. Digital PCR is, essentially, a qPCR assay broken down into many individual samples. These individual samples are then probed in much the same way a typical RT-PCR process would be. The result is that samples are reported as target positive or target negative. Based on assumptions about the distributions of proteins within a sample the researcher can very tightly calculate the initial quantity of a sequence [30].

Applications in Microfluidics

Because digital PCR uses subdivisions of samples microfluidic handling is a considerable advantage for high throughput polymerase chain reaction analysis of samples. Polymerase chain reaction has been implemented in a number of ways in microfabricated systems.

The most widely adapted dPCR mechanism has been the attachment of probe sequences to a surface, creating a digital PCR chip. This was one of the earliest manifestations of digital PCR in microfabricated systems. More recently, fluidic systems have gained more traction with commercial products that can automatically subdivide and run digital PCR processes (e.g. Fluidigm’s qdPCR 37K IFC). Additionally, droplet microfluidics have been adapted to run emulsion based digital PCR with the digital PCR subdivisions being accomplished with droplet encapsulation. This technique is known as emulsion PCR [31].

1.3.2 Electrokinetics

Electrokinetic systems are commonly used to characterize bulk cellular properties. There are several types of electrokinetic systems including electrophoresis, dielectrophoresis, and electroblotting. These common protocols form a large number of systems that can separate and characterize cells and subcellular particles [32, 33, 34].

Electrokinetics for cellular characterization offer some specific advantages over other systems. Principally, electric fields can be maintained and modulated with relative ease
Many of the applications of microfabricated electronics are easily implemented within molecular biology analysis. However, these systems encounter specific complexities unique to electrode-fluid and cellular interfaces. To this end electrically based solutions to particle, or more specifically, cellular characterization, have an inherent level of specificity and accuracy not afforded to other systems.

Basics of Electrokinetics

Many of the electrokinetic systems by which cells and subcellular molecules are analyzed work on several basic principles of electrostatics and electrodynamics. Electrokinetics are complex systems, governed by equations for electrostatics, electro-osmosis, electrophoresis, dielectricphoresis, and, typically, fluid dynamics.

Electro-osmotic flow

Electrokinetic systems use an electrical potential to cause the movement of analytes and fluid in a system. Capillary electrophoresis, dielectrophoresis, and gel electrophoresis are the most prominent systems which use electrokinesis to deliver or separate analyte samples.

When a capillary of diameter $d$ and length $L$ is introduced to an electrolyte solution, a boundary of ions is created due to the surface properties of the capillary and the free ions in the electrolyte itself. This boundary is referred to as the electric double layer. Within the electric double layer there is a slip plane at which the counter ions generated by the charge of the bulk material can move past one another. This effect can be seen in Figure 1.6.

When a voltage, $V$, is applied across the capillary, ions move within the electric double layer according to the zeta potential $\zeta$ in the electric double layer as seen in Figure 1.7. The magnitude of the flow velocity can be calculated through the use of the Helmholtz-Smoluchowski equation (eq. 1.1) for systems in which the length of the capillary is suffi-
Figure 1.6: Unit capillary for electro-osmotic flow

\[ \dot{j}_{\text{Coulomb}} = \rho_e \vec{E} \]

Figure 1.7: A diagram of the electric double layer for a surface-electrolyte solution interface. Blue line represents the potential as a function of distance from the material surface. Stern layer is the slip plane of the EDL where the Zeta potential occurs

Blue line represents the potential as a function of distance from the material surface. Stern layer is the slip plane of the EDL where the Zeta potential occurs.
Electro-osmotic flow has considerable advantages over mechanically driven flow. It is easily controlled and readily adapted to lab-on-chip systems. However, electro-osmotic flow has some significant obstacles to implementation. Most importantly is the potential for the evolution of gas bubbles from the surface of the electrodes. Gas evolution will either add experimental noise or act as a source of cytotoxicity by either raising the pH of the solution, or directly acting upon cells. Additionally, there is the possibility of degradation of the activation electrodes.

Because of the nature of electro-osmotic flow, the distance between the electrodes is important to determine the voltage needed to drive the electro-osmotic process. The distance between electrodes relates to the creation of uniform electric field. In the case of an electro-osmotic pump of electrode separation \( y \), the voltage at a point \( \Delta y \) is calculated by the equation

\[
 V_{\Delta y} = (V_2 - V_1) \frac{\Delta y}{y} \quad \text{for the condition where } V_1 = 0 \text{ and } V_2 = V_{app} \text{ the equation reduces to }
\]

\[
 V_{\Delta y} = V_{app} \frac{\Delta y}{y} \quad \text{(1.3)}
\]

in which \( V_{app} \) is the applied voltage. Electro-osmotic delivery systems follow two general patterns: membrane and planar electro-osmotic pumps.

The planar based electrode uses a shallow arrangement of electrodes to drive fluid through a channel. This style of electro-osmotic flow produces movement by providing an electromotive force to fluid passing in contact with the electrodes. This can be done in a microchannel to act as an electro-osmotic pump.

Membrane based electro-osmotic flow has been characterized for a variety of materials, principally, silica (SiO, SiO\(_2\)) and aluminum oxide (Al\(_2\)O\(_3\)). Several factors go into the
efficacy of the electro-osmotic flow. Arguably of highest importance is the zeta potential \( \zeta \) of the capillaries. In any electro-osmotic system the zeta potential is defined as the potential of the electric double layer at the slip plane in the electrolyte with respect to the bulk fluid surrounding the electric double layer.

There have been many attempts to decrease the effects of gas evolution in electro-osmotic systems. To decrease pH disturbances, the amount of fluid present can be increased to dilute the change in pH. As noted by Piwowar, this would not be practical in a microscale lab-on-chip system [41].

Several groups have explored the use of pulsating or alternating voltages in order to decrease the negative side effects of electro-osmotic flow reactions [46, 41]. One group looked at the modulation of signal to give a net zero current which should result in a negligible amount of gas evolution and electrode degradation. This was expanded upon to show a substantial increase in electro-osmotic flow by using an asymmetric wave form given by:

\[
V = \begin{cases} 
V_+ : & \frac{1}{t_+ + t_-} t < \frac{t_+}{t_+ + t_-} \\
V_- : & \frac{1}{t_+ + t_-} t > \frac{t_+}{t_+ + t_-} 
\end{cases} 
\]  

(1.4)

where \( V \) is the applied voltage across the electro-osmotic pump. Piwowar saw significant increases in fluid flow from anodized alumina electro-osmotic using this pulsed field function. There was a substantial decrease in gas evolution when comparing the average voltage applied (given by 1.5) with a constant positively biased field applied at the same voltage [41].

Further discussion of electro-osmotic flow can be found in Section 3.1.2

**Blotting and Gel Electrophoresis**

Gel electrophoresis is a technique used for separating proteins based on molecular weight, size, and charge. Samples are pulled through a gel by electrophoretic forces on particles.
Electrophoretic Gel Pore Size Range (wt %)
---
Agarose 200 - 1000 nm 0.2 - 3 %
Cellulose 10 - 100+ nm 3 - 9 %
Polyacrylamide 0 – 20 nm 0 – 20 %

Table 1.1: Properties of common electrophoretic gels [48, 49, 50, 51, 33, 52]

The gel electrophoresis provides both a particle velocity as well as a drag force by which each particle’s molecular weight can be determined. Blotting is then used to transfer molecules to another media which can be stained via antibodies or imaged through x-ray transmission. Blotting is arguably one of the most powerful techniques for probing the presence of biomolecules.

In gel electrophoresis, the gel is approximated as a porous media. The porosity is dictated by the polymer comprising the gel solute and the concentration of this polymer. Cellulose, agarose, and polyacrylamide are used as porous media for gel electrophoresis. Each polymer has a typical pore diameter associated with the gel structure. Table 1 shows a list of the three most common electrophoretic gels and their associated porosities.

Electrophoretic gels can be approximated as a porous media and the flow through them approximated, again, via the electrophoretic migration. In many circumstances, the process of electro-osmotic flow is unwanted and is purposefully minimized so that separation based solely on the electrophoretic migration is possible.

Electrophoretic gels are typically time consuming processes. The unavoidable step in all the detection of nucleic acid sequences is the collection of the nucleic acid sequence. In the case of both DNA and RNA analysis, samples must undergo some amount of purification. In order to prevent contamination of one sequence from the other sequence type, material must be sacrificed and information lost (i.e. if a researcher wants DNA they must sacrifice RNA and vice versa). Many methods of separation of DNA from RNA exist but are beyond the scope of this discussion [52].

An electrical field is held across the gel typically around 100 volts which causes repositioning of the DNA to an “end-on” orientation in line with the electric field. Single stranded
sequences trend towards moving more slowly through the electrophoretic gels. After gel electrophoresis, blotting is performed either by an electrically driven flow or a capillary force which draws the samples up to a sheet of nitrocellulose. Each of the major alterations of the original system of electrophoresis and capillary transfer are named according to a direction based convention in recognition of the original creator of the technique Sir Edwin Southern. As such, in the case of pressure driven capillary action for DNA, the technique is known as a Southern Blot assay. If the system is applied, instead, to RNA, the technique is known as a Northern Blot. When an electrophoretic flow in a second axis is used to “electroblot” the nitrocellulose film by applying a second voltage it becomes a Western Blot. Western blots are also typically used to probe proteins and other biomolecules. Westerns also commonly use immunohistochemistry for identification. There are many variations to these three principle techniques that probe an even wider variety of analytes.

Electroblotting has not been widely adopted within microfluidic systems. The primary purpose of electroblotting is to narrow the signal provided from individual proteins by isolating a smaller number of the proteins separated in the electrophoretic gel. Microfluidic systems have an inherent accuracy not afforded to the mechanisms which are used in electroblotting. As such, the necessity for transferring samples to a secondary sheet to be labeled via antibodies is unnecessary.

Even though electroblotting is not necessarily for microfluidics, the principle of protein and particle separation by electromotive force in a restrictive media is used in the principle of capillary electrophoresis. As the length of the separation chamber becomes very large with respect to its diameter, electrophoresis is governed by the behavior of capillary electrophoresis.

**Capillary electrophoresis**

Capillary electrophoresis aims to distinguish cells from one another by simple separation of molecules within, as the name implies, a capillary. Samples can be isolated from one another based on their electrical motility and size. There are a number of variations on the mechanism of capillary electrophoresis which serve to better isolate and characterize
samples in an automated method with a single device [55].

Capillary electrophoresis moves particles according to both the bulk flow due to electroosmotic flow within the capillary as well as the electromotility of the particles within the media. As a potential, $\vec{E}$, is held across the capillary, the sample is imparted with a velocity according to equation 1.1 due to the electroosmotic flow in the capillary. The analytes will also be separated by the potential field according to the difference in charge on the analyte which is represented as the electrophoretic migration velocity, $v$, in equation 1.6

$$v_{ep} = \mu_{ep}E$$  

where

$$\mu_{ep} = \left( \frac{L}{t \cdot v_{ep}} \right)$$  

where the $\mu_{ep}$ is the experimentally determined electrophoretic mobility, $V$ is the experimental voltage, and $L$ is the migration distance over a time $t$. The value $\mu_{ep}$ has units of $m/V \cdot s$. Electro-osmotic flow is typically of much greater magnitude than the electrophoretic migration velocity and so the sample will travel towards the cathode, a property known as electroendosmosis. The time in which it takes them to reach a detection point, $t$, is then used to characterize and compare the sample [48].

Capillary electrophoresis is unable to reliably distinguish between uncharged particles, as these particles will only be moving via electro-osmotic flow and will not have significant distance between sample readings [55].

Capillary gel electrophoresis is an alteration to the process of electrophoresis in which the free medium is replaced with a gel, typically polyacrylamide, to enhance analyte characterization by restricting movement due to electro-osmosis. This restriction to analyte movement can be used to further characterize samples according to structure and immunohistochemistry. An excellent example of both is the work by Apori and Herr utilizing discontinuous concentrations of polyacrylamide gels to provide a gradient of porosities through which analytes would move. This system was enhanced by preparing samples with an antibody which caused aggregation to specific antigens and further restricted motility, a process known as
immunosubtraction [56].

Dielectrophoresis

Dielectrophoresis refers to the phenomena by which a particle is subjected to a non-uniform electric charge. When subjected to an electric field a particle establishes a dipole. Based on the particle one pole or another will be favored in terms of attraction or repulsion from the electric field source.

Dielectrophoresis has been applied to cellular sorting both microscopically and within biomedical systems. Because of the small forces on cells, dielectrophoresis is of particular use in microfluidic separations of cells. Particles in dielectrophoresis are subjected to force related to a non-uniform electric field.

In dielectrophoresis, particles can be sorted on the basis of the relationship of dielectric constant of the media and the particles. These can be well characterized for cells and other biological analytes [57].

Single Cell Impedance Spectroscopy

Microfluidics have allowed particle impedance spectroscopy to become more focused on the single cell or small volume cell changes. Impedance spectroscopy measurements have been classically applied to particles to determine the composition in a non-invasive and label free system.

Impedance measurements are based on the complex permittivity of a particle in solution. A comprehensive review of the principles of the mechanisms is provided by Sun et al. [58]. Impedance spectroscopy can be used to determine the variation of intracellular contents through models based on the Maxwell Garnet mixing theories for dielectric materials shown in the equations:

\[
\epsilon_{eff} = \epsilon_{m}\frac{2(1 - \delta_i)\epsilon_{m} + (1 + 2\delta_i)\epsilon_i}{(2 + \delta i)\epsilon_{m} + (1 - \delta_i)\epsilon_i} 
\]  

where \( \epsilon_{eff} \) is the effective permittivity of a mixture of dielectric media in which \( \epsilon_m \) is the
permittivity of suspending media, $\epsilon_i$ is the permittivity of the particle, and $\delta_i$ is the volume fraction of the particle.

From this the constituents of a cell can be determined by using the simplified model presented in Figure 1.8 which follows the impedance equations of:

$$R_m = \frac{1}{\sigma \left( \frac{1-3\Phi}{2} \right)} G_f$$  \hspace{1cm} (1.9)

$$C_m = \epsilon_\infty G_f$$  \hspace{1cm} (1.10)

$$C_{mem} = \frac{9\Phi R C_{mem,0} G_f}{4}$$  \hspace{1cm} (1.11)

$$R_i = \frac{4 \left( \frac{1}{2\sigma_m} + \frac{1}{\sigma_i} \right)}{9\Phi G_f}$$  \hspace{1cm} (1.12)

Where the permittivity $\epsilon_\infty$ is given by:

$$\epsilon_\infty \simeq \left[ 1 - 3\Phi \frac{\epsilon_m - \epsilon_i}{2\epsilon_m + \epsilon_i} \right]$$  \hspace{1cm} (1.13)

and $C_{mem,0}$ is the specific membrane capacitance per unit area:

$$C_{mem,0} = \frac{\epsilon_{mem}}{D}$$  \hspace{1cm} (1.14)

where $R_m$, $C_m$, $R_{mem}$, $C_{mem}$ are the media resistance, media capacitance, membrane
resistance, and membrane capacitance respectively; $G_f$ is a geometric factor; and $\Phi$ is the volume fraction given by:

$$\Phi = \frac{V_i}{\sum_j V_j} \quad (1.15)$$

where $V_i$ is the volume of species $i$ summed over the total volume $V_j$.

Particle impedance measurements have been used to analyze single particles since around 1910, the most prevalent method being the Coulter Counter in the 1950s [58]. In an impedance spectroscopy system, a particle is approximated by a current pulse which correlates to a particle passing through an aperture in the device. The magnitude of this pulse is directly related to the volume of the particle and the permittivity relative to the suspending media. These systems are widely available commercially and can be implemented for cellular analysis [58].

1.3.3 Immunohistochemical Analysis

An immunoassay, as the name implies, uses antibody binding to capture or mark a specific antigen. Antigen and antibody sites can be highly specific within a given system but non-specific binding often complicates antibody use by requiring additional treatment steps. There are tens of thousands of antibodies listed in catalogs that allow for the specific detection of analytes.

**ELISA**

Immunobinding can be used in many ways to detect the presence of molecules. The most common of these techniques is ELISA or Enzyme Linked ImmunoSorbent Assay. ELISA is another commonplace tool for the detection and examination of analytes from cells. One of the drawbacks to the ELISA is that a researcher must have a priori knowledge of the target analyte which they are targeting.

ELISA is used in molecular biology to detect subcellular analytes, however, antibody binding can also be used to isolate individual cells of known morphology and chemistry.
through a similar mechanism. This can be combined with other such systems as flow cytometry or gel electrophoresis.

### 1.3.4 Cell Sorting

Antibody binding has also been used to separate cells through chemophysical means. By “tagging” a surface or a second particle of known properties, immunobinding can be used to accomplish sorting of cells.

This principle is highlighted by the work of Toner in which antibodies were bound to an array of pillars that could be used to capture samples of circulating tumor cells within samples of blood that were passed through the device. This technique allowed for circulating tumor cell populations to be enriched for quantification and study. This method of turbulent flow facilitating the chemical adhesion of analytes to a treated surface has been used in other methods [59].

**Magnetic Traps**

The use of magnetic traps is a relatively new method of immunobinding separation in which cells are antibody bound to a magnetic bead that can then be manipulated through the use of controlled magnetic fields. A diagram of a magnetic tweezer setup can be seen in Figure 1.9.

The magnetic tweezers are able to be inherently massively multiplexed, however, the magnetic tweezers require an upstream labeling step which bears consequences for an automated platform [60].

### 1.3.5 Micromixing

Due to the nature of microfluidic fluid flow it is regularly necessary to include a micromixing platform to allow for efficient combinations of reagents. This is particularly true in any cell labeling systems. While micromixing is a significant issue regarding microfluidics, mixing, as presented, is outside of the scope of this project. It is anticipated that it will be revisited in future implementations of the femtopump technology.
1.4 Single Cell Analysis Techniques

As previously stated, single cell analysis techniques have been pursued since the discovery of the cell. While bulk cellular techniques have advanced considerably significant information can be lost when multiple response signals are treated as representative of single cells.

1.4.1 The Burden of Bulk Signal Averaging

The use of bulk analytics to probe cellular responses tends to obfuscate information when individual responses are averaged. “Signal averaged masking” is the phenomena by which independent or unique signals are treated as a single response due to the average of their responses. This is a detriment to the understanding of cellular processes. When viewed at the single cell level, groups of cells previously believed to be homogeneous show distinct variations. Under the scrutiny of single cell analysis the concept of cellular homogeneity
breaks down.

In a discussion on single cell experimentation, it is necessary to define the response, or the actual cellular reaction, and the signal, the recordable response shown by the probe of the sample population. In all of these systems, information loss is seen when multiple responses correspond to a single signal. This is the underlying principle of signal average masking [61].

Signal averaged masking is little different from a binning error using too few bins. Signal strength can easily be misconstrued. As Altschuler argued, a classic example would be the response of 10 cells in a close proximity in which two show strong fluorescent signal [61]. The signal averaged strength is represented as $1/5$th the total intensity, however, this is not indicative of any one signal.

Other examples of signal averaged masking are shown in a series of intensity curves in Figures 1.10 - 1.13. In Figure 1.10 a smaller, but discrete signal shifts the mean to the right on the intensity curve. This is the effect of a heavy-tail distribution in the signal. The error propagates on the assumption that there is only a single averaged signal that is relevant to the population of cells. This signal could be from a system that operates on signal intensity, not unlike qPCR automated systems.

In Figure 1.11 the presence of two discrete signals causes the shift of the mean signal
without detecting the second signal. This results in the signal intensity being viewed as reduced for the system while not properly identifying the position of the mean of either signal.

Another example, Figure 1.12, shows the presence of two signals that show the mean in between the actual peaks of the signals. In this case a significantly smaller signal would be represented when, in actuality, two signals are present. This is represented in a three dimensional case in Figure 1.13 in which two very different signal intensities are represented as the same, but incorrect, signal.

Cellular, or any biochemical, processes typically occur stochastically, at least in the terms of the view of bulk cellular processes. Bulk cellular responses represent the number of cells out of a population that are providing a response. However, because of the stochastic nature of these signals they do not necessarily represent the state of a cell in terms of the individual behavior.

1.4.2 Dynamic Cells

The cellular microenvironment has a large number of factors that contribute to the individual responses of cells. The cellular responses are not static, but rather occur dynamically (as a function of time) 62. Most, if not all, of the previously mentioned bulk platforms
Figure 1.12: Two discrete responses resulting in a mean signal corresponding to neither response mean. The mean represented has a substantial reduction in magnitude. Adapted from [61]

Figure 1.13: Mapping error showing distinguishable responses represented by the same indistinguishable signal. Adapted from [61]
do not easily allow continuous recording at the single cell level. The closest approximation is, instead, sampling at time points which usually result in the disruption of the cellular microenvironment which would result in contamination of data.

An example of the a time dependent process is show in Figure 1.14 in which dynamic redistribution is tracked via TIRF (total internal reflected fluorescence) in single cells. Individual cells show substantial differences in the time based response to GPCR agonists [63]. This highlights the need for single cell monitoring. Without continuous monitoring the time based response would be lost in both data averaging and, more simply, a lack of data [62].

1.4.3 The Impact of Single Cell Analysis

Single cell analyses are being pursued for a number of applications in a broad range of industries. The largest, and most captivating of these industries, is in pharmaceutical development. As a snapshot of the utility of single cell analyses, this will review aspects of
Pharmaceutical Development

The pharmaceutical industry is arguably the most heavily influenced industry by single cell analysis. High throughput screening has become the accepted norm for hit-to-lead generation [64]. Hit-to-lead generation refers to the high throughput evaluation of chemical compounds as medicinal candidates [65]. Newer approaches use fragment based drug discovery which is more combinatoric in nature [66]. Fragment based drug discovery is a more targeted form of previously used high throughput drug screening [67]. This method breaks down molecules into fragments that are known to have affinities for a binding site and then building upon those fragments to form a suitable therapeutic compound [66].

Cost Mitigation

The number of candidate therapeutic targets is increasing due to greater understanding of the molecular basis of diseases. However, the number of pharmaceutical compounds being brought to market is declining [69]. The amount of money spent to bring a single drug to market approaches a staggering $1.1 billion dollars. This includes the cost of testing and
eliminating thousands of drug candidates [70]. A break down of these expenses is shown in Figure 1.15.

The slowing of approved drugs brought to market is a multi-faceted problem loosely reviewed by Pammolli et al. [69]. The author reports that there is a growing trend of attrition rates since the mid-to-late 90s. The highest attrition rates were seen in Phase III trials which is potentially the most expensive of the regulatory phases. This decline in productivity is, as reported by Pammolli, not simply an issue of supply and demand but rather a movement of the industry into higher risk areas with a lower probability of success while at the same time the number of potential therapeutic targets has dramatically increased [69].

Despite the level of cost associated with clinical trials (Phase I - Phase III), it is suspected that the number of drugs that are identified to enter the new compound discovery pipeline is a critical factor which can not be easily monetized [68].

As referenced in Paul et al. a significant amount of cost savings can be established by approaching drug discovery through a “Quick Win, Fast Fail” paradigm in which drugs are eliminated as early as possible to reduce expenditures later in the compound discovery process. However, this bears the risk of greater false negative which could potentially eliminate any early cost savings [71].

**Orphan Drug Evaluation**

The Orphan Drug Act of 1983 provided for the economic incentivization of developments of “orphan drugs” including market exclusivity, waivers of application fees, and various tax credits for development. [72]

Orphan drugs are drugs that are not expected to reach market due to inadequately sized markets. The drugs and diseases they treat must be identified as orphan status, typically under 200,000 cases identified, in order to pursue economic benefits. However, the application of orphan drug status does not limit the further development of a drug beyond the orphan status. As such, orphan drugs are looked at as an attractive possibility for pharmaceutical development. To highlight this point several companies, starting in 2010, have developed business divisions specifically for the research and development of orphan
drugs [73].

**Microfluidic Promises**

Microfluidic platforms for single cell analysis offer a level of discretion that is not currently provided in compound evaluation systems. A device capable of delivering precise chemistry and monitoring cellular or target behavior in real time would provide for a decrease in false negatives by providing a complete picture of cellular interaction. The ability to implement a cost effective microfluidic platform for drug discovery is not a trivial matter [1].

Because of the allure of the big pharma concerns regarding research and development cost, pharmaceutical development is an exceptionally attractive target for microfluidic cellular interrogation systems. However, the demands placed on such a microfluidic system are very high. A device or system must, at a minimum, be capable of maintaining discretized cellular (or target) population, tightly controlled chemical delivery, and ability to read information from the target in real-time.

**Personalized medicine**

Personalized medicine refers to the customization of medical treatment and diagnosis to the individual. Personalized medicine can readily benefit from the development of a single-cell or cell-cluster interrogation system. Diagnostics, dosages, and prognostics can be linked directly to rapid cellular results from patients.

Particularly in oncology, resistances to therapies are overcome with the use of sequentially administered therapies. An example of this is the delivery of sequential chemotherapeutics to treat highly aggressive tumors as demonstrated by Sahin *et al.* [74]

### 1.5 Microfabricated systems for Microfluidics and MicroTAS

As shown, there is a need for higher precision than that provided by bulk cellular molecular biology techniques. Microfabrication methods have improved upon the precision of these highly developed bulk cellular techniques. Many, if not all, of the bulk cellular techniques
have a microscale counterpart which seeks to automate and refine the results of these robust tests.

Devices and fabricated systems are categorized, somewhat clumsily, into three major categories: chemical, physical, and hybrid systems. In some cases, like hydrodynamic trapping, the dependency is clearly set to the physical nature of the system. Other systems, such as chemisorbed cell patterning, are obviously a chemical system. Hybrid devices benefit from both chemical and physical characteristics.

1.5.1 Chemical Systems

Chemical systems in this review are defined as those systems which interact with cell principally through a chemical reaction. Selected technologies include chemical adsorption of proteins for patterned cellular adhesion, microfluidic concentration gradients, immunohistochemical capture, and magnetic traps.

Chemical adsorption for patterned cellular adhesion

Promotion or inhibition of cellular adherence is thought to provide better insight to the factors that determine how cells respond to their environment. Altering cellular density, adhesion strength, or other chemical interactions with the environment develops a better model of how a cell behaves \textit{in vivo}. To address this researchers have turned their attention to patterned chemical surfaces. \cite{75, 76}.

The surface of cellular substrate can be modified to increase the selective adhesion of cells. This selective adhesion can be patterned to isolate cells from one another. This can be implemented in several ways. In general, the formation of cellular micropatterns is accomplished by physically masking the cellular substrate in a desired pattern, treating with a surface modification, and removing the mask. \cite{75}.

In the absence of a mask chemicals for surface modification can be deposited via microcontact printing. In microcontact printing, or stamping, a chemical is brought into contact with a substrate through the use of an elastomeric mold. A diagram of this process is shown in Figure \ref{fig:16}. This can be done in either a roll-to-roll fashion or using elastomeric stamps.
Figure 1.16: Three examples of microcontact printing.  

a. Direct stamping with an elastomeric mold produced with soft lithography.  
b. Roll microcontact printing with the deposition material on the roll.  
c. Combination of elastomeric stamp and roll microcontact printing with deposition material on mold.

formed from soft lithography [76].

A unique example of surface modification delivers the surface treatment through a microfluidic sealed to the substrate. The chemical is given time to react with the surface and then the bulk fluid and the mask are then removed, leaving a surface modification that replicates the geometry of the fluidic channels. This process a variant of micromolding in capillaries or MiMIC [76]. An example of this implementation is shown by the work of Tourovskaia et al. in a complex microfluidic pattern was used to deliver chemistry to deter cellular attachment [77].

1.5.2 Physical Systems

Physical systems are those systems that use a physical principle (fluid dynamics, phase separation, etc) to manipulate or interrogate cells. This can include systems like optical tweezers, hydrodynamic traps, and microdroplet encapsulation.

Optical Tweezers

Optical tweezers for microscale manipulation of cells work by exerting a force on small objects at the interface between two transparent media.

Difficulties exist with the implementation of optical tweezers for several reasons. First and foremost is that over powered or under attenuated signals can result in localized heating which is significantly injurious to cells. Because optical tweezers exploit a force based on the
difference in the index of refraction of materials, a considerable amount of power is required for manipulating materials that have similar indices of refraction. In terms of cells, these indices of refraction can be quite similar to culture medium or saline. In the event that the media surrounding a cell is changed or the system is used for cell separation, small changes could result in cellular damage or inadequate force generation.

Second, high throughput systems (over 10,000 cells/min) have yet to be established in optical tweezers though commercial systems exist. In effect, optical tweezers can, laboriously, focus on a single cell and isolate it but can not do so in a timely fashion for large populations of cells. High throughput is inherently hindered because of the position of cells or particles in the optical tweezers. For a high throughput system to be effective the optical tweezer operator or program must be privileged to a priori knowledge of cell position. The problem of cell position is two fold with the second part of the difficulty being in aberrant focal effects due to unknown cell location. In the event that the cell is not located where expected, the cell can be pushed out of the focal area or damaged.

Finally, multiplexing optical tweezers can be a challenge since many fluidic systems are not exclusively comprised of media and cells. Each addition of a microfluidic channel or sensor is an aberration in the continuity of the indices of refraction. As such, simple impurities in a PDMS microchannel could result in a non-functional cellular interface. Further, in encapsulation systems, such as microdroplets (see below), the optical tweezers lose sensitivity because of the change in the index of refraction of the external phase. These difficulties result in large hurdles for optical tweezers to overcome before they can be widely adopted.

Hydrodynamic Traps

A very common theme in cellular manipulation is hydrodynamic trapping. Hydrodynamic trapping refers to the ability to trap a cell, after which the fluid flow dynamically changes to ignore or move around the trapped cell as shown in Figure 1.17a&b. In effect, hydrodynamic trapping can be viewed almost as a type of size exclusion system. A review of several dynamic trapping techniques is discussed by Di Carlo in the preceding reference.

Figure 1.17 shows an example of hydrodynamic trapping. In this device a grid of hy-
hydrodynamic traps are established in a microfluidic channel. A fluid flow a constant rate is introduced to the microchannel. Cells are trapped within the structure and the fluid flow dynamically changes. With this dynamic change in the flow profile additional cells now pass around the trapped cell and into downstream traps. These systems can be used to address or isolate cells, either by changing the chemistry or reversing flow to remove the cells [62].

It stands to reason that hydrodynamic trapping can be used beyond cells as well. It has been demonstrated that hydrodynamic trapping can be used for capture microdroplets [78]. This shows that the utility of hydrodynamic traps goes beyond simply a cellular isolation platform.

**Microdroplet Encapsulation**

Droplet microfluidics are an extension of emulsion based techniques where the “droplet” is a single, stable unit in the emulsion system. Droplet microfluidics are particularly well suited for the analysis of single cells due to their small droplet volumes and capacity for high throughput microfluidics [79]. The basic principle of droplet microfluidics is manipulating the interface of two immiscible liquids. In cellular applications this is typically a flourinated oil, a stabilizing surfactant, and aqueous solution.
In two-phase microdroplet systems there are two different types of systems, the water in oil (W/O) system or the oil in water system (O/W). In both emulsion systems the contained media is referred to as the internal phase and the media in which it is contained is the external phase \[80\]. In microemulsions for cell culture, the system is typically water in oil with a surfactant added to the oil. The surfactant in the oil contains a polar head and a non-polar tail that, in the proper concentration, are able to interact with both phases, thereby stabilizing the microdroplet \[8\].

**Manufacture of droplets in microfluidics**

Emulsions can be made by a variety of methods. These emulsions typically contain a narrow, but polydisperse droplet distribution \[80\]. However, many of the large scale techniques used to create emulsions in bulk fail to allow for tight control over the makeup of the droplets contained with the emulsion. This can lead to unstable and unpredictable droplet behavior. Microfluidic systems to produce droplets are exceptionally consistent and are capable of producing well defined monodispersions.

Flow focusing is the formation of microdroplets through multiple microchannels which result in combining flows of oil and aqueous solutions. These are of particular interest when encapsulating reagents and biological materials because the oil simply envelops the aqueous media passed through the center channel. Flow focusing uses a three channel design with a single outlet.

Microfluidics can also use T-junctions to accomplish droplet formation \[81\]. Within a T-junction, the shear force of outer phase acting perpendicular to the flow of the inner phase “shears off” the fluid to be encapsulated \[82\].

Both of these systems generate microdroplets that can be altered in size by adjusting flow rates. This principle allows microdroplets to be tightly controlled by regulating flow velocities \[82, 8, 81\].

Microdroplets are referred to as metastable since, given enough time, the droplet will coalesce with neighboring droplets. Droplet stability is of high importance when working with emulsions at the microscale. There are several considerations that must be taken into account for the formation of stable microdroplets.
Criteria for Microdroplet stability

Long term metastability in droplet microfluidics is accomplished by the use of a surfactant, oil, and aqueous media. Coalescence in a surfactant, oil, and aqueous system is affected by the probability of collisions and the probability of the rupture of the surfactant “envelope” around the microdroplet [83].

Cell Culture in Microdroplets

The applications for droplet microfluidics in small molecule reactions and capture is of particular value. By manipulating cells into a microdroplet, relatively low-number analytes are, by virtue of the small volume, maintained at a relatively high concentration.

A typical cell diameters range from 5 - 15 $\mu$m and microdroplets can be produced that can contain both the cell and enough media to keep the cell alive [84]. Through the use of emulsion mixing systems, it is possible to combine microdroplets to refresh the amount of nutrients in the microdroplet [8]. The prospect of single cell capture within a droplet is a promising application.

Analyte detection in microdroplets

Reactions within the microdroplet can further enhance small molecules through reactions such as emulsion PCR [31]. The limitations on the polymerase chain reaction occurring within the microdroplet is hindered only by the practical considerations of interacting with the microdroplet.

1.5.3 Hybrid Physical and Chemical Systems

Many microscale systems developed for the manipulation and observation of cells can be easily divided into physical or chemical systems. But there are many technologies for which the line between physical and chemical systems is not obvious. These systems typically have a multistage interaction with cell in which they are able to perform multiple functions. An example would be magnetic tweezers which use immunohistochemistry to identify cells and a magnetic field to trap and manipulate the cells [60].
Magnetic Traps/Tweezers

Magnetics have been used to manipulate cells as early as the turn of the 20th century [83]. Of particular note is a discussion on the manipulation of protoplasm in the 1950s when the technique was discussed by Dr. Francis Crick of double-helix reknown [86]. Early studies were able to identify properties such as the protoplasm properties/behavior [86] and elasticity of DNA [87]. Magnetic tweezers have been used for the direct manipulation of cellular and subcellular molecules [60]. As microfabrication techniques continue to advance, devices that directly manipulate cells via magnetic fields have become more common place.

Magnetic traps and tweezers exploit non-uniform fields in patterned materials. The choice of materials is subject to how the electric field is required to behave. Groups have fabricated magnetic traps that have been micropatterned using permalloy [60], cobalt-nickel, and nickel-cobalt-boron [88].

Most magnetic traps/tweezers rely on an anti-body bound superparamagnetic bead attached to the cell. A superparamagnetic bead is non-magnetic bead (in the absence of an externally applied magnetic field) made from a magnetic material. When this bead interacts with applied magnetic field it can be drawn to local field maximums and thus be used to move the cell through an array of microfeatures.

Magnetic tweezers have exceptional advantages in terms of the overall accessibility of technology. The manipulation of the magnetic gradients can be tuned to give operators intuitive control over the movement of cells without damaging the cell. In one case the magnetic tweezer gradients were tailored so that a cellular manipulation system could be controlled through the operation of a video game controller [60].

One of the inherent concerns with any system that uses immunohistochemistry for the identification of cells is that the antibodies will sterically block other probes and potential reactions. Immunohistochemistry at the microscale is an excellent identification system but it bears consequences for the further interrogation of an unadulterated cell as well as requires a priori knowledge.

The ability to capture non-tagged cells with magnetic traps also exists. Winkleman
showed that the local minimum of an electric field could be used to trap diamagnetic particles, including cells, through the use of a paramagnetic buffer. While the study showed that the paramagnetic solution did not display negative effects to cell growth at the concentrations used for the study, it did have negative affects at higher concentrations on yeast cells and 3T3 fibroblast cells [89]. Low molecular-weight chelates have shown no gadolinium negative impacts and are widely considered to be safe. However, this is significantly affected by the clearance efficiency of these low molecular weight chelates [90]. Studies have shown that the presence of macromolecule albumin-Gd(DTPA) have resulted in negative side effects in vivo and in vitro [91]. This calls into question the capacity of this unlabeled magnetic trap to culture uninfluenced cells.

**Cellular Plinko - Hydrodynamic Immunohistochemical Traps**

Hydrodynamic systems can be enhanced by using antibodies bound to a surface. One particularly good example is in Toner’s work with circulating tumor cells. In this system, antibodies to circulating tumor cells were attached to a grid of pillars. Whole blood cycles through this grid of pillars and the circulating tumor cells bind to the immobilized antibodies [92].

1.6 Conclusions

The need for increased precision for development of pharmaceuticals, personalized medicine, and other biotechnology fields requires precise single cell measurements. Researchers working with micro- and nanofabricated total analysis systems have made significant strides in the development of a wide variety of devices and mechanisms to interface with cells and the cellular microenvironment. However, the need still exists for an integrated, inexpensive, and reliable platform for the delivery of chemistry, culture of cells, and analysis of responses in a single device. The femtopump series of microTAS systems provides a new and unique system which can vacuum seed cells, address individual cells with chemical delivery, provide for long term cellular culture, and export the contents of the cellular micro-environment for
further analysis.
Chapter 2

Design and Fabrication

2.1 Introduction

Fabrication for each of the devices presented in this dissertation follows a similar process as described below. The distinguishing steps that make up each individual device are described in their respective chapters. The design of each device was built to mimic previous macroscopic analogous methodologies presented in the literature (see Chapter 1).

Each device outlined in this work is built off of the same femtopump technology. The SiCCWells and SiCCWell based microdroplet generator share the same morphology but use differing fluidic surface chemistries to affect delivery (SiCCWells) or sampling (Droplet Generator). The gradient generator has a distinct morphology and is, if nothing else, a representation of the robust nature of the femtopump fabrication process. A relationship of the devices to the femtopump technology is seen in Figure 2.1.

2.2 Background - How to make a SiCCWell

The SiCCWells are built from several well known fabrication techniques supplemented with adaptations to allow their success on a nanoporous substrate. The major processes are photolithography, liftoff, and soft lithography.
Figure 2.1: Femtopump technology as the foundation for all three devices presented. The SiCCWells were designed for delivery of fluids to the cellular micro-environment. The Gradient Generator provides insight into migratory cellular behavior. The microdroplet generator provides sampling from the cellular microenvironment.

2.2.1 Photolithography and Liftoff

Photolithography is at the heart of the development of the SiCCWells and femtopump-based technologies. It is used as the first step for patterning the membranes, the microfluidics, and the insulating layers for each device. A diagram of the exposure is shown in Figure 2.2.

Photolithography uses a light sensitive polymer, known as a photoresist, to transfer a pattern on a high resolution mask to the polymer. The photoresist is either primed for removal or strengthened against removal by exposure to light. In the case of a polymer that is primed for removal it is referred to as a positive tone photoresist. For a polymer strengthened against removal is referred to as a negative tone photoresist. The application and the tone of the mask determines which resist will be chosen for use.

Photoresist is, typically, spun on to a substrate from a premixed solution. Spin coating photoresists provides a uniform layer of resist to expose the resist consistently across the substrate. The resist is then soft baked to remove solvents from the photoresist. After the soft bake, the resist is exposed to UV light through the mask. Typical exposure values are usually provided by the manufacturer of the photoresist.
Figure 2.2: Representation of photolithography for positive and negative tone resists. From [93]
After exposure, the resist is commonly baked again, referred to as the post-exposure bake or PEB. The PEB typically increases the resolution of the features and anneals the resist, which decreases cracking. After the PEB, the resist is cooled and developed. In development, the photoresist is exposed to a chemical which selectively removes the regions exposed (positive tone) or the regions unexposed (negative tone). The type of developer is largely dependent on the type of resist being used [93].

**Liftoff**

After development is patterned regions of the substrate are selectively exposed for processing based on the pattern of the photomasks. At this stage many processes are available to further treat the substrate. However, these grossly fall into either removing or adding material according to the patterned photoresist. For removal, a variety of etches can be used and for addition there are several mechanisms of material deposition [95].
In the case of the SiCCWells, lift off processing is used in which a metal layer is deposited through a physical deposition system. The layer is, ideally, discontinuous at the patterned features of the photoresist due to either a high aspect ratio or an undercut of the resist. The metal and resist are introduced to a solvent which dissolves the polymer resist and the metal on top of the resist is removed (for a positive resist) [95]. A schematic diagram of a liftoff is seen in Figure 2.3.

2.2.2 Soft Lithography

Soft lithography was developed in the late 90s and revolutionized the development of microfluidic systems by allowing more rapid development of silicone based fluidics. Whitesides’ seminal [76] paper has been referenced more than 7800 times at the time of writing which shows the level of impact this technique has had on microfluidic and polymer fabrication (Google Scholar as of July 22nd, 2014).

Soft lithography uses a photolithography, typically with SU-8 as the resist which is patterned using a chrome or transparency mask. The photoresist is developed and treated with a release layer which prevents irreversible bonding to the silicon wafer or photoresist. In Whitesides’ discussion this is a silanization, although other materials can be used, such as hexamethylene disilazane. Poly(dimethyl siloxane), PDMS, is cast on to this surface, cured, and released. In the case of microcontact printing, the PDMS layer will be used as a stencil or stamp for further processing. In the case of microfluidics, the PDMS will be used directly with another substrate to form a microfluidic structure. A brief diagram of the process of soft lithography is seen in Figure 2.4 [76].

2.3 Materials

Several materials were used in the fabrication of the femtopump based devices. These materials are commonly used in either microfabrication or biocompatible systems.
Figure 2.4: Diagrammatic representation of Soft Lithography. Taken from [96]
2.3.1 Polydimethyl Siloxane

Poly(dimethyl siloxane), PDMS, is a room temperature vulcanizing silicone rubber. Two types of PDMS were used in this study. The first, used for mold making and microfluidics, is T-2 Silastic (Dow Chemical Xiameter RTV-4232-T2). The second is Sylgard 184 (Dow Chemical Sylgard 184). Sylgard is more optically transparent than T-2 and, more importantly for this study, has a significantly lower viscosity. For thin films of PDMS, Sylgard is preferred as it can be spin coated to a lesser thickness due to the lower viscosity.

2.3.2 Nanoporous Material

The nanoporous alumina membranes used in this dissertation were Whatman Anodisc 47 mm membranes with a nominal pore diameter of 100 nanometers. These pores are stated by the manufacturer to have no more than 50% more than the nominal pore size, and no less than 25% less than the nominal pore size. The membranes were previously reported to have a zeta potential, $\zeta$, of $+19\text{mV}$ [43]. The membranes are nominally reported to be 50 $\mu\text{m}$ in thickness.

Zeta potential of Nanoporous Anodized Alumina

The Whatman Anodisc membranes were previously reported by Ai et al. to have a zeta potential, $\zeta$, of $+19\text{mV}$ [43]. However, reports have shown that, at cellular pH levels, alumina shows a significantly higher $\zeta$ potential. Sources have reported the $\zeta$ potential as having an isoelectric, $\zeta = 0$, point at a pH of 8. Original data from Sprycha is shown in Figure 2.5.

This relationship dictates the voltage bias to be held across the alumina membrane during device activation to provide a given flow rate. In this case the voltage bias should be positive to produce forward flow.
2.3.3 Polystyrene

The insulation layer for the SiCCWells was polystyrene (\([\text{CH}_2\text{CH}(\text{C}_6\text{H}_5)]_n\)). Polystyrene was procured from Sigma-Aldrich (Cat # 441147) with a molecular weight of 350,000. Polystyrene was chosen as it had been previously used in work on vacuum assisted cell seeding and is commonly used for cell culture. Polystyrene is able to be surface modified and easily adhered to the surface of the alumina membranes regardless of the patterned metallization layer. The polystyrene was diluted with anisole (Sigma ReagentPlus Cat #123226) at a concentration of 15% by weight. The polystyrene was then filtered through a 0.45 µm syringe filter overnight to remove any large aggregates.

2.3.4 Photolithography - Liftoff Resists

Two resists were chosen for photolithography. Both are based on the same general chemistry and are positive tone lift offs. Both are soluble in N-methyl pyrrolidinone (NMP) (Fisher
Bioreagents Cat# BP117 2 4). The crucial difference in the results is the thickness of the photoresist which contributes to resolution. Both resists used for the development of the SiCCWells are Novolac based resins. This raised some issues during deposition (see Fabrication Results).

One resist used is Microposit Shipley S-1813 photoresist. At a spin speed of 3000 RPM it provides a thickness of 1.3 micrometers. This resulted in a higher resolution for the microfeatures when used with a poly(vinyl alcohol), PVA, base layer as described below. S-1813 also benefits from a substantially shorter processing time which will be explained in the description of the second photoresist. For S-1813 there is no post exposure bake and a very short soft bake.

The second resist used is SPR-220-7.0 which is of similar chemical make up. This resist is significantly more viscous and was originally chosen prior to the use of the PVA for pore blocking as the lift off results were more stable. SPR-220 has a refractory period after exposure of a minimum of 2 hours for best resolution. Because of this step, the throughput for photolithography is lower. SPR-220 has a nominal thickness of 10 microns at 3000 RPM. This makes it more attractive for thicker lift off depositions (such as the silica lift off for the gradient generator).

The developer used was Microchem MF-CD-26 (Microchem) which is a high speed low resolution developer. For the purposes of eliminating membrane contamination the high speed developer is preferable as it removes the resist more quickly than the PVA, in the case of a lower speed higher resolution developer (MicroChem MF-319 was tested) the developer removes the PVA faster than it dissolves and removes the resist resulting in significant membrane staining.

**PVA for reversible pore blocking**

Poly(vinyl alcohol) ([-CH₂CHOH]-ₙ) was used to reversibly block the nanoporous membrane. The method of pore blocking is further discussed in Section 2.4. The PVA was procured from Sigma Aldrich (Cat #P8136) and had an average molecular weight of 30 - 70,000. PVA was mixed to 10% by weight with de-ionized water and mixed at 37°C for
2 hours. The PVA solution was then centrifuged at 2000 RPM for 5 minutes. The supernatant was removed and the remaining PVA was discarded. This final solution was a PVA solution of less than 10% by weight but free of large aggregates.

2.3.5 Platinum for Electrodes

Platinum was chosen as our electrode material because it is inert in saline. The electrode contacts are expected to come in direct contact with cells and will have to withstand a variety of chemicals during processing. The platinum was shown to adhere to the alumina without the need for a secondary adhesion layer, as is necessary with silica or silicon. Adhesion layers are typically less inert materials (chromium, titanium) and are thought to be a potential avenue of electrode damage. If hydrogen evolution were to occur at the electrode surface, the adhesion layer could be removed even if the platinum electrodes were not affected. This would result in electrode failure if the pH were lowered or raised locally. Because the platinum adheres directly to the anodized aluminum oxide, it creates a more robust electrode for repeat experiments.

2.3.6 Photolithography - Soft Lithography Resists

Two variants of the Microposit SU8-2000 series resists were used. The first used was SU8-2025 which was chosen for a nominal resist thickness of 25 microns at 3500 RPM. This resist was used for the production of microwells in the SU-8 which would later result in PDMS micropillars to spin coat with polystyrene. The second resist was SU8-2100 which was chosen for the microfluidic channels. This resist is nominally 100 microns thick when spun at 3000 RPM. This resulted in large channels which were well formed and discrete from one another.

SU8 must be developed using SU-8 developer (MicroChem) which is a solvent based developer. To test for completion of the development step, isopropyl alcohol can be sprayed across the surface. If the alcohol runs clear, the development process is finished; if it is a murky white then the development must be continued. This makes SU-8 development considerably more robust than that of other photoresists used in this study as complete
Figure 2.6: Isolation of bond pads from negative electrode to prevent a short circuit from forming when silver paste is applied.  

- **a.** Original activation and counter electrode relationship.  
- **b.** Modified relationship to prevent short circuit across membrane. Alumina membrane is omitted from this image but would normally be between the two electrodes, under the activation electrodes and obscuring the counter electrode.

development can be accurately gauged.

### 2.3.7 Specific Materials for Packaging

A variety of materials were used for packaging that do not fit categorically into another subsection. The most important of these is the silver-bearing two stage conductive epoxy. The epoxy chosen was 124-08 A&B (Creative Materials 124-08 Part A & Part B). This epoxy has considerable mechanical strength and excellent conductivity. This material was used to attach the negative (or bottom) electrode to the conductive contacts of the packaging. The same epoxy is used to make individual electrical connections. The epoxy shows a high degree of thixotropy and as such is capable of entering the nanopores despite the viscous nature of the two constituent parts. This increased the area of attachment for electrical connection but required the isolation of the bond pads from the negative electrode to prevent shorting. The alterations to the positive and negative electrode geometry to prevent a short circuit at the bond pads can be seen in Figure 2.6.

Silver solder was also used (Digikey #KE1128-ND) to further decrease impedances between materials. Because the silver bearing epoxy was a crucial component in the development of the devices it was determined that silver based materials would be used anywhere
that resistances could increase due to the size scale of features. This particular silver bearing solder is 3% silver with a water soluble rosin. As acetone can damage or alter the structure of the polystyrene microwells it was decided that having a water soluble rosin was the most appropriate choice for the integrity of the microfeatures.

All interconnects, wires, bread board, and circuitry were purchased through Digikey.

2.4 Fabrication

Fabrication is broken down into several major steps which are then combined into the final device through several joining steps. These steps are patterning electro-osmotic pumps, microfluidic fabrication, microwell fabrication, microwell lamination, microfluidic lamination, and packaging.

For each of these major steps the differences for the SiCCWells, Gradient Generator ($\mu\nabla$Gen), and the SiCCWell-based microdroplet generator will be discussed.

2.4.1 Patterned Electro-osmotic Pumps

Alumina membranes of 47 mm diameter with a pore diameter of 100 nm (Whatman Anodisc) were mounted to a carrier wafer using Kapton tape. The alumina membrane was spin coated with PVA at 6000 RPM (3000 RPM/s), for 60s and then hot plate dehydrated at 90°C for a minimum of 300 s. The membranes were then spin coated with a Shipley S-1813 using a 300 RPM (100 RPM/s) spin spread for 8s followed immediately by a 3000 RPM (5000 RPM/s) spin coat. The carrier wafer and membrane were then baked in contact with a hotplate at 115°C for 60s. Samples were removed to a wafer cartridge to air cool.

Samples were removed from carrier wafers by removing the Kapton tape. Samples were exposed using an EVG 620 contact aligner. Nominal exposure energy was 31.2 J/cm².

Samples were developed using a combination of spray and bath development. Both used CD-26 metal ion free developer to remove exposed photoresist. Spray development was done at a rate of 50 mL/min for 45 seconds. Samples were immediately removed to a bath for 90s. Samples were flipped in the bath to allow full development of the photoresist as air
has a tendency to be trapped inside the nanoporous structures and prevents full infiltration of developer. The membranes were then carefully rinsed and the patterned surface was nitrogen-dried to remove residues from patterned features.

Samples were dried on an aluminum foil sheet at a steep angle (minimum 45° to horizontal) to prevent residual fluid from drying on the patterned features and to prevent sticking to petri dishes or other polymer surfaces. The polypropylene ring of the alumina membrane was pH sensitive and could, effectively, adhere to other polymers strongly enough to cause membrane damage. Samples were then dried at a similar angle in a nitrogen-purge oven for a minimum of 2 hours.

Samples were coated with a nominal 80 nanometers of platinum using a Denton e-beam evaporator. Samples were initially coated at low evaporation rates (0.1 - 0.2 Å/s) for the first 10 nanometers and then increased to 1 Å/s. Samples were cooled in a nitrogen purged low pressure environment (evaporator chamber at standby) for 20 minutes.

Samples were removed and the annular propylene ring removed from the membrane. The samples were masked using glass slides over all features except the contact pads and a 300 nanometer layer of silver was evaporated onto the contact pads at 5.0 Å/s.

Lift-off was performed via a vacuum liftoff system using N-methyl-2-pyrrolidone (NMP) heated to 120°C on hotplate. The patterned surface was placed down in a glass frit supported vacuum filtration assembly (Sigma-Aldrich #Z290432). Isopropanol was used to decrease sticking between the membrane and support frit as the funnel top was set in place which, in turn, decreases the potential for cracks. Vacuum was applied at -15 in.Hg for 300s. Care was taken to maintain membrane hydration throughout liftoff process to prevent redeposition. Samples were rinsed with isopropanol to dislodge platinum. The membranes were then sonicated as needed for brief periods (5 - 15s), repeated as necessary, to remove remaining metal from the membrane surface. Membranes were rinsed with isopropanol to remove remaining NMP. Samples were returned to an aluminum foil "tent" at a minimum angle of 45° to horizontal. The samples were placed, still on the aluminum foil in an oven at 60°C for 4 hours to dry the membrane.

The membrane backside was masked using glass slides to prevent deposition directly
under the contact electrodes. A second platinum layer was deposited on the backside of the membrane (opposite the patterned layer) to 80 nm using the Denton E-beam evaporator. The deposition rate was set to 1.0 Å/s. The samples were cooled in a nitrogen environment for 20 minutes.

2.4.2 Microfluidic Fabrication

Microfluidics were fabricated using soft lithography as previously described in Whitesides’ seminal paper [76]. SU-8 2100 negative tone photoresist was spin coated onto a p-type ⟨100⟩ test grade silicon wafer at 3000 RPM (500 RPM/s) for 60s. During the application step of 500 RPM, edge bead remover was applied to the outermost centimeter of the silicon wafer.

2.4.3 Microwells

The microwells’ fabrication also uses soft lithography. A photomask with 15 micron circular holes with a pitch of 30 microns in a square arrangement was used. The porosity, $\Phi_{ps}$, of this pattern is given by the equation:

$$\Phi_{ps} = \frac{\pi}{P_x P_y} \left( \frac{d_{mw}^2}{4} \right)$$

where $P_x, y$ is the pitch between microwells in orthogonal axes on the mask, and $d_{mw}$ is the diameter of the circular holes. In a square, $P_x = P_y$ geometry, the equation simplifies to

$$\Phi_{ps} = \frac{\pi}{P^2} \left( \frac{d_{mw}^2}{4} \right)$$

where $P$ is the pitch of the circular holes.

SU-8 2005 is spin coated at 6000 RPM and soft baked at 95°C for 120s. The SU-8 2005 was flood exposed at 130 J/cm² and post exposure baked at 95°C for 120s. SU-8 2025 was spin coated onto a p-type ⟨100⟩ test grade silicon wafer with a 500 RPM (500 RPM/s) application spin followed immediately by a 2000 RPM (500 RPM/s) spin coat. Edge Bead Remover (Microchem EBR) was applied to the outermost centimeter during the application step.
The SU-8 layer was soft baked at 65°C for 180s immediately followed by an increase to 95°C for 6 minutes. Wafer was cooled in contact with a metal surface maintained at 18.5°C. Resist was exposed using an EVG contact aligner with 168 J/cm². Resist was then post exposure baked for 60s at 65°C followed by an increase to 95°C for 6 minutes. Wafer was then cooled in contact with a metal surface maintained at 18.5°C. Development was performed using SU-8 developer bath (Microchem) for 6 minutes or until an isopropanol rinse drains clear from the patterned features. Wafers are then placed in an HMDS deposition oven at 150° for 37 minutes to deposit HMDS on the surface to act as a release layer.

T-2 Silastic (Dow-Corning, Xiameter RTV-4332-T-2) was combined with curing agent at a ratio of 10:1. The mixture was then mixed in an orbital vacuum mixer at 1250 RPM with a vacuum of 2.0 torr for 60s, atmospheric pressure for 60s, and 2.0 torr for 30s. The mixed T-2 was cast onto the SU-8 features and degassed in a dessicator for 10 minutes. The PDMS and mold was then placed in an oven at 60°C for 2 hours.

The PDMS was removed from the mold and diced into 1 x 2 cm stamps. A filtered polystyrene/anisole solution (10% by weight) was pipetted on to the stamp covering the surface. The stamp and solution were vacuumed for 60s. The stamp was then spin coated at 3500 RPM (500 RPM/s) for 60s. Anisole was baked out of the polystyrene by placing the samples in an oven for 5 minutes at 60°C.

2.4.4 Microwell Lamination

The polystyrene sheet was removed from the stamp using a pair of fine tipped tweezers and positioned on the surface of a patterned membrane. The membrane was brought into contact with a hot plate at 150° for 30s. Lamination could be determined by a shade change on the surface of the membrane.

2.4.5 Microfluidic Attachment

Microfluidics were bonded to the backside of the membrane by using a PDMS adhesion layer. A standard glass slide was scribed and broken in half. The slide was then spun coated with Sylgard 184 (Dow-Corning) at 4000 RPM (1000 RPM/s). The microfluidics were stamped
into the Sylgard liquid thin film and weighted to 200 grams for 300s. The microfluidic block was then removed from the Sylgard and left to settle for 20 minutes. The microfluidics were then brought in to contact with the base layer of the membrane, perpendicular to the electrode channels. A pressure of 50 kPA was applied to the microfluidic at 60°C for 15 minutes after which the temperature was raised to 90°C for 2 hours until fully cured.

2.4.6 Packaging

Several methods of packaging were used. Silver epoxy (Creative Materials 124-08 A/B) was applied to the counter electrodes adjacent to two opposing sides of the microfluidic and circumscribed by a small amount of Sylgard. The adhesive laden membrane was brought into contact with a frame constructed of patterned silver on glass slides. Contact electrodes were connected using a silver epoxy to draw a short line of conductive material from the bond pad to the silver pattern. The epoxy was baked at 60°C for 4 hours. The macro well was cut from some 5 mm thick cured PDMS and bonded using the same method as the microfluidic attachment.

The entire device was potted level with T-2 PDMS to secure the microfluidics directly to the glass frame. The PDMS was cut to the edges of the glass frame. Silver solder was used to connect wires (26 AWG, E-Z-Hook) to the positive and negative electrodes.

2.4.7 Gradient Generator

The gradient generator was fabricated according to a process similar to the SiCCWells. The base layer was a nanoporous electro-osmotic membrane. However, the fabrication of the gradient generator required an additional lift off step.

Fabrication

The alumina membrane was attached to the carrier substrate (typically 100mm wafer). The attached membrane was spin coated with a prepared 10% polyvinyl alcohol (PVA) solution at 6000 RPM for 60 seconds. The carrier substrate and membrane were baked at 90°C for 5 minutes.
The PVA solution was mixed to 10% by weight with de-ionized water. The solution was then centrifuged at 2000 RPM for 5 minutes. Once centrifuged the super-natant solution was removed and the remaining solid discarded.

The PVA coated alumina membrane was then spin coated with Shipley Microposit S-1813 (Dow Chemical) at 300 RPM, 100 RPM·s$^{-1}$ for 5 seconds followed by a 3000 RPM, 5000 RPM·s$^{-1}$ spin coat for 60s. The S-1813 layer was then soft baked at 115$^\circ$C. S-1813 spin and softbake were completed on a Cost Effective Equipment 100CB Spin Coater/Hotplate. The photoresist was exposed using an EVG 620 Contact Aligner. Hard contact was used with an exposure energy of 31.2 J/cm$^2$.

The exposed resist was developed via continuous spray development using CD-26 (Microchem) for 45s after which it was immediately removed to a bath of clean CD-26. The membrane was submerged with the patterned side up for 30s, then flipped to patterned side down for 30s, then returned to patterned side up for another 30s. The membrane was immediately removed to a water bath, rinsed with DI water via spray, and then briefly dried with a nitrogen gun. The membrane was then returned to the developer bath and the rotation of the membrane repeated for a total of 90s. The membranes were again rinsed and the patterned surface nitrogen gun dried.

The membranes were then dried at a 60$^\circ$ angle to horizontal, on a hotplate set to 90$^\circ$C until any remaining water droplets have been removed. Membranes were then removed to a nitrogen purge oven and baked at 95$^\circ$C for a minimum of one hour.

Membranes were placed in a Denton E-beam evaporator. The evaporator chamber was evacuated to a pressure of 10$^{-6}$ torr and platinum was deposited to the membrane surface. Platinum deposition was held to 0.2±0.1 Å·s$^{-1}$, as previously discussed, to prevent thermal damage to the photoresist layer. After the deposition of 10 nanometers of platinum the deposition rate could be increased to 1.0±0.1Å·s$^{-1}$.

Lift off proceeds as previously referenced in Section 2.4.1. Membranes were placed on a vacuum liftoff chamber with the patterned surface closest to the vacuum source, so as to pull the fluid across the membrane and the 80$^\circ$C n-methyl-2-pyrrilidone (NMP) was introduced to the upper vacuum chamber. A vacuum was applied across the membrane at -15 in.Hg.
for 10 minutes. The membrane was then sonicated in a bath of 80°C as needed to remove any additional photoresist and platinum. The membrane was washed with an isopropanol spray bottle to remove any platinum particles, to prevent redeposition. The membranes were dried at an angle (minimum of 30° to horizontal) on a hotplate set to 90°C.

Membranes were re-attached to a new carrier substrate and re-spin coated with PVA and S1813 as in the previous steps. The photoresist was exposed again, this time masking the active gradient generation region and the electrical contacts. The resist was developed for 20 seconds using spray development and an additional 10 seconds in a bath of CD-26 developer. The membrane was immediately spray rinsed and then nitrogen-gun dried. The abbreviated development was to reduce the effects of under- and back-cutting of the PVA layer (see Fabrication Results).

The second pattern was dried according to the same steps as previously described. The membranes were returned to the e-beam evaporator and silica was evaporated onto the surface to a thickness of 300 nm at a rate of 5Å·s⁻¹.

Lift-off is performed in a large NMP bath (200 mL) to dissolve the masking photoresist and to clean the membrane. Membranes were introduced to the NMP for a minimum of 10 minutes to release the silica. Sonication is used only in the event of incomplete lift-off and is avoided as it has been shown to damage the micropatterned metalization.

Membranes were then coated on the back side with an unpatterned layer of 80 nm of platinum and 20 nm of silica at 1.0Å·s⁻¹ and 5Å·s⁻¹ respectively. Selectively patterned regions for electrical contact can be made by covering some regions with glass slides. The membranes were then bonded to a polydimethyl siloxane microfluidic, fabricated using soft lithography [76], via plasma bonding of 30 SCCM oxygen and 40 Watts for 60s. The adjoining surfaces were brought together and weighted to 1 kPa for a minimum of 12 hours.

The membrane was coated with a small amount of silver bearing epoxy, then brought into contact with a glass frame with patterned silver on the surface. The epoxy was cured in an oven at 60°C for 6 hours after which additional PDMS was added to mechanically support the membrane by attaching it to the glass frame.

The upper channel was bonded in a similar manner orthogonally to the lower microflu-
Figure 2.7: Representation of the orthogonal relationship between the feeder channel (blue) under the membrane and the gradient carrier channel (red) on the opposing side of the membrane.

Wires were attached using silver bearing epoxy and further mechanically stabilized with additional PDMS.

2.5 Fabrication Results

Fabrication results are presented in the same order as the fabrication steps with a discussion of the results in Section 2.6.

2.5.1 PVA effects on resolution

The PVA masking of the pores in the nanoporous membrane was expected to minimally affect the resolution of the photolithography. Photolithography results are compared to the photolithography pattern and methods on silicon wafers. The silicon wafers were treated with HMDS to improve adhesion in samples with only S-1813 positive tone photo resist. Silicon samples with a PVA coat were not treated with HMDS with no decrease in adhesion between the PVA and S-1813.

Samples were treated in the same manner of steps as the alumina membrane development described previously in Section 2.4.1. Treatment of alumina and silicon samples is listed in table 2.1. Metalized layers were compared at the center millimeter of the delivery channels.
Table 2.1: Silicon and Alumina resolution comparison

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>Substrate</th>
<th>HMDS</th>
<th>PVA</th>
<th>S-1813</th>
<th>Spray or Bath</th>
<th>NMP Bath</th>
<th>Relative Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Silicon</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Both</td>
<td>NMP Bath</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Silicon</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>Both</td>
<td>NMP Bath</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Silicon</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>Spray</td>
<td>NMP Bath</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Alumina</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>Both</td>
<td>Vacuum</td>
<td>2</td>
</tr>
</tbody>
</table>

for the SiCCWell pattern and the activation electrodes of the gradient generator. The HMDS treated silicon showed the highest resolution (Fig. 2.8.A), as expected, followed by membranes using PVA and S-1813 spray developed and bathed in CD-26 (Fig. 2.8.B). The silicon samples with PVA and S-1813 which were spray developed had the next highest resolution (Fig. 2.8.C). Silicon samples that were spray developed and bath developed showed the lowest resolution with a complete loss of discreet activation electrodes (Fig. 2.8.D).
Figure 2.8: A. Lift-off performed with S-1813 on HMDS treated silicon as a resolution control. B. Lift off performed with S-1813 on PVA coated silicon wafer. C. Lift-off performed with S-1813 on PVA coated anodized alumina (Al₂O₃) membrane. Red arrow shows redeposition of platinum during lift-off processing. Developer bath and spray used. D. Lift off performed with S-1813 on PVA treated silicon. Developer bath and spray used resulting in a loss of features. Yellow arrows indicate similar regions between techniques.
Electrode silvering was performed at a high rate of deposition which decreased the average pore diameter, reducing the overall porosity, $\phi$, by up to 50% or more. Figure 2.9 shows the changes in porosity of a bond pad before and after thickening via silver deposition. Changes in porosity were compared by thresholding images of the same region (within several microns) and calculating the percent change in the density of black pixels in the image. This increases the exposed surface area as well as increasing the bond pad depth which relates to the contact resistance of the electrode channels (see Discussion).

Soldering was performed after potting in PDMS as the heat from the soldering iron may result in thermal cracking of the glass slides which could propagate through the alumina membrane. Membrane cracking results in device failure.

### 2.5.2 Gradient Generator Specific Results

Aside from the obvious changes to the fabrication protocol, such as the inclusion of an additional liftoff step, changes were necessary to several key steps. In particular, the polyvinyl alcohol layer was expected to be removed due to an isotropic etch from the aqueous CD-26.
However, the removal rates of the PVA were considerably faster than expected. This is thought to be due to a “back etch” in which the aqueous material is wetting the hydrophilic membrane either through the nanopores or forcing itself under the PVA. This would result in the thin layer of PVA being released unexpectedly early as schematically represented in Figure 2.10. This was compensated for by reducing the development time. However, reduction of development time resulted in membrane contamination by the dissolved photoresist. However, this could be largely removed by extending the time spent in the NMP bath and resulted in only a minor discoloration of the membrane with no effects on porosity.

**Silica Liftoff**

Overall, silica liftoff proceeded favorably with a strong interaction between the silica and alumina layers. An image of all three regions of patterning can be seen in Figure 2.11. There were no indications of weak bonding or aberrant geometry on any of the features.

The silica also appropriately sealed the nanopores and maintained a close bond with the alumina substrate. An image of a micrograph of the SiO$_2$, Al$_2$O$_3$ interface can be seen in Figure 2.12.

### 2.5.3 Geometric Resistance

The generator’s geometric gradient of resistances was established following the pattern of

$$ R_N = R_1 + (N_C - 1) \cdot R_\Delta $$

(2.3)
Figure 2.11: Three regions of the gradient generator patterned depositions: a. SiO$_2$ deposited to Pt deposition, b. SiO$_2$ deposited to Al$_2$O$_3$, and c. Pt deposited on Al$_2$O$_3$. Arrow shows location of curled silica.

Figure 2.12: Micrograph of the Al$_2$O$_3$, SiO$_2$ interface. Close connection is seen throughout the interface and a complete loss of porosity is seen at the silica surface.
where $R_N$ is the resistance of the path length to the activation electrode $N_C(n = 1, 2, ..., 10)$, $R_1$ is the resistance of the first electrode, and $R_\Delta$ is the resistance difference between each channel. The resistance of this circuit increases linearly. The inverse relationship of the voltage to the resistance in parallel was neglected during the design. As such, the voltage at each channel behaves more as $\frac{V_{\text{applied}}}{N}$ which results in the greatest difference between channel one and two. The difference between channels shows a steep drop off as $N_C$ goes to 10.

2.6 Discussion

Several techniques were developed to specifically overcome the challenges associated with micropatterning a nanoporous membrane. Additionally, some factors are believed to be directly responsible regarding the success or failure of particular steps.

2.6.1 Spray Development & PVA pore masking

A large issue with the membrane development was the problem of pore contamination. Pore contamination resulted in patterned membranes that were either discolored, clogged with residues, or a combination of the two. Due to the very small features in our initial 20 nm samples the pores could become clogged with only ambient dust or scum. Since the purpose of the device is to maintain porosity this was a significant problem. The first solution was to turn to a spray based development.

In theory the spray development would wet, develop, and be removed quickly which would reduce the potential for the photoresist to move into the pores. This was partially successful but was substantially aided by the addition of the PVA pore masking layer. As previously mentioned, because the PVA masking layer is water soluble, it does not change the development process appreciably. Since the PVA is the last layer of material to be removed by the developer all large particles in the photoresist have already been removed and the pores are opened after the PVA dissolves. After dehydrating the process can continue as if the PVA were not used.
2.6.2 Liftoff failures

Two types of liftoff failure were experienced during the liftoff stages. The first of the failures was attributed to the platinum deposition. The second was seen as the redeposition of material onto the surface of the alumina substrate.

**Thermal Liftoff Failure - Platinum and Novolac Photoresists**

The femtopump-based devices use two Novolac resin based photoresists, S-1813 and SPR-220, as referenced in Section 2.3. An unexpected difficulty that was encountered was that the deposition temperature of platinum, even at low pressures, is very close to the melting temperature of the Novolac based resin. When the platinum impinges upon the surface of the device it is possible for it to melt through the photoresist. Poly(vinyl alcohol) has a similar melting temperature at $200^\circ\text{C}$ [98]. The thin layer of PVA and photoresist is believed to be compromised by the high rate of platinum deposition. The photoresist reflows and the metal layer is able to come into contact with the alumina or otherwise fully encapsulate the photoresist.

The solution to this problem was to lay down the deposition of platinum at a very slow deposition rate for the first 10 - 20 nanometers. A typical deposition rate was less than $0.1 \, \text{Å/s}$. This creates a layer of platinum that is capable of thermally shielding the critical features. This solution resulted in the elimination of thermally based liftoff failures.

**Redeposition Failure - Wet Gilding**

Redeposition failures were rarely as dramatic as with the thermal effects. However, the redeposition failures were the source of a large number of shorts that affected the individual activation of electrodes. This, unfortunately, was responsible for a number of complications in device testing, including short circuits, the resultant electrolysis, and aberrant impedance measurements.

The short circuits were caused when electrodes were connected to the negative terminal in order to prevent flow recruitment from inactivated channels. When this occurred the
electrical impedance would typically drop by an order of magnitude and the current flow was unregulated across the phosphate buffered saline resulting in gas evolution. This is not typical as more recent experiments were run with a floating negative terminal and ground, so the potential between electrodes were typically smaller than the voltage required to cause electrolysis.

Similarly, when multiple electrodes were activated due to shorts, the impedance across the membrane would be reduced to

\[ R_s = \frac{R_{memb}}{N_s} \]  

where \( R_s \) is the resistance across the membrane accommodating \( N_s \) shorts between channels that would initially have a resistance of \( R_{memb} \). Under constant voltage this again could result in a resistance drop of a large enough magnitude to result in gas evolution.

To clarify, it is not the current or the voltage alone that causes gas evolution but rather the movement of electrons at a potential above the electrolytic voltage of water 1.29V.[99][100]. In an electrolyte solution like phosphate buffered saline the presence of voltage does result in a measurable current unless significant measures are taken to prevent such current. In Figure 2.13 gas evolution can be seen due to several channels shorting (5) and causing a very large and dramatic evolution of gas on the surface of the device.

The problem of redeposition was largely alleviated by using the vacuum filtration assembly to draw the NMP bath across the membrane. This pulled the metal layers and the photoresist away from the surface of the device. Additionally, the first devices were developed by using acetone as our liftoff agent. This is a relatively common process and acetone is more widely available. However, the use of acetone is discouraged as it can result in an increase of redeposition. When the solvent fully evaporates it leaves a clean and unimpeded binding site on the alumina to which the metal can adhere. This is not unlike the process of wet gilding. The solution was to switch to a less volatile solvent and only allow the substrate to dry after lift off was satisfactorily complete. As redeposition was actively avoided, less tests exhibited signs of electrolysis.
Figure 2.13: Gas evolution caused by short circuit between channels. Channels still showed the ability to deliver fluid during gas evolution but the delivery is not useful as the gas has affected the entire device.
2.6.3 PDMS Stamping

The PDMS stamping used in this process is continually undergoing revision and development. It is a particularly important technique when the materials chosen for a device or system can not be tailored to be compatible with more traditional plasma bonding techniques. The most recent development is that of vacuum sealing the PDMS microfluidic to the surface of the membrane. The Sylgard 184 (Dow-Corning) used is of a low enough viscosity that it can readily permeate the nanoporous membrane. This has been a trait that we have exploited in other areas of our fabrication (particularly in packaging). We have attempted to vacuum seal the PDMS wetted microchannels onto the backside of the membrane rather than applying a pressure. This was left in place for up to 1.5 hours and was then removed to the hot press where it was heated to 90°C under a pressure of 50 kPa.

A picture of the initial results can be seen in Figure 2.14. In this image the negative impression of the nanoporous structure can be seen in PDMS which has been acid etched to remove the nanoporous alumina membrane. This is an encouraging result as the sample in the figure was only vacuum sealed for 15 minutes prior to pressing at a lower pressure than 50 kPa.

The vacuum assisted sealing is expected to further strengthen the bond between the
microfluidic channels and the alumina. This will further improve the delivery of chemicals as well as future efforts in vacuum seeding cells into the device.

2.6.4 Silica onication

It is believed that the process could be enhanced through the use of a thicker positive photoresist. At liftoff boundaries a curling of the silica layer was seen which is thought to be an artifact of a sloped geometry with respect to the photoresist. The photo resist is only an 1.4 optimal µm and the silica layer is deposited at 300 nanometers. It is possible due to the brevity of the development steps associated with the silica layer that incomplete removal of the PVA layer may also contribute to a curved geometry at the liftoff boundary. This can be seen in Figure 2.11 (arrow).

In the current process this is relatively negligible as since it affects each activation site equally. However, less development or less removal of the PVA layer has resulted in significant difficulties during the liftoff process. To encourage liftoff, which was unlikely to proceed further, devices were sonicated in NMP for 15 minutes at 80°C. This resulted in substantial damage to the micropatterned silica. A comparison between an briefly sonicated (≈15s) sample and a heavily sonicated sample (≈15 min) can be seen in Figure 2.15.

2.7 Conclusions

The fabrication techniques outlined in this chapter provide the basis for technologies encompassed by the title “femtopumps” for their ability to control small and discrete volumes of fluid. It is anticipated that further manipulations of the geometry of the electrodes, insulating layers, and microfluidics will provide for even more solutions for cellular interaction.

The femtopump systems (SiCCWell, Gradient Generator, and Droplet Generator) are complex systems built on techniques that represent a merger of the microfabrication, electrochemistry, and biomedical engineering. The fabrication processes are significantly laborious when performed by hand but operational scale up of the devices is expected to drive down fabrication time per device substantially. The femtopump platform is a significant step
Figure 2.15: Comparison between (a) heavily sonicated samples and (b) samples which were briefly sonicated forward in mass manufacturing of high precision femtofluidic total analysis systems.
3.1 Background

As implied by the name, the manipulation of fluids is crucial to microfluidic total analysis systems. Small, precise and system integrated pumps are designed for use with microscale analytical devices. There is a wide variety of microscale fluid handling which are largely classified into mechanical and electro-osmotic pumps.

3.1.1 MEMS Pumps

There are many types of electromechanical pumps but the basic principle behind each of them is typically similar. An oscillation of a mechanical feature creates a pressure head that is pulsatile in nature that drives flow forward \[101\]. Mechanical pumps offer several drawbacks over other methods of pumping. Peristaltic and rotary pumps can cause a pulsatile flow rather than a continuous flow. In contrast, electro-osmotic pumps are capable of a sustained flow resultant from a constant electro-motive force. Mechanical pumps are also inefficient and require higher power to operate at length. Because of this mechanical pump implementation in lab-on-a-chip applications are more difficult than lower powered systems.

3.1.2 Electro-osmotic Pumps

Electro-osmotic pumps are a class of pumps that make use of an electric field to produce a bulk fluid flow through a system. As described in Section \[1.3.2\] a layer of charge exists at the interface of an electrolyte liquid and most materials known as the electric double layer.
Figure 3.1: Several approaches to fabrication of the patterned alumina for the SiCCWells

EDL. When a potential is held across a capillary in which the EDL can be maintained, due to pH or ionic movement, excess ions in the electric double layer are subjected to an electrophoretic force. Ions at the slip plane of the electric double layer, known as the Stern layer, are imparted with a velocity due to the electrophoretic force. This motion within the electric double layer subsequently results in a bulk fluid velocity.

Electro-osmotic pumps are well suited to biomedical applications because of the ability to be easily adapted to low voltage lab-on-a-chip applications.

**Single Cell Culture Wells (SiCCWells)**

The SiCCWells are an extension of electro-osmotic flow created by using microfabrication to restrict the movement of liquid to specific regions. These activation sites are limited, in principle, to micro and nanofabrication methods. Because the SiCCWell is a membrane based electro-osmotic pump it provides considerable advantages in terms of “gating” between two fluidic chambers.

As discussed in the previous chapter, several alterations to the SiCCWell fabrication have occurred as processes were developed. The specificity of activation sites and adhesion of microfluidics is directly affected by these changes. Several of the major morphological changes to the SiCCWells are shown in Figure 3.1.2.
3.2 Theory

Electro-osmotic flow is a complicated subject without the use of patterned nanoporous membranes. The introduction of the patterned electrode, microfluidic, and insulating polystyrene significantly alter how the theoretical model of fluid delivery is represented.

3.2.1 Activation Sites and Cell Density

Regions where the microfluidics cross with the activation electrodes are referred to as a single activation site as shown in Figure 3.2. Each activation electrode forms 10 activation sites and each microfluidic forms 19 activation sites for a total of 190 activation sites.

The number of microwells in an activation site is not restricted to a specific number due to inconsistencies in alignment. The number of microwells in an activation site, $N_{\text{cas}}$, is reported as a range. This is because the number of microwells is related to the alignment of the polystyrene sheet to the activation site. Figure 3.2a & b show different values for $N_{\text{max}}$. In both cases, the porosity of the polystyrene insulation, $\Phi_{\text{ps}}$, is the same found by using equation (2.1) in Section 2.4.3. For the SiCCWells presented, the number of microwells

![Figure 3.2: Two diagrams of the activation site showing the minimum (a.) and maximum (b.) number of microwells contained within the boundaries of an active site for a given porosity.](image-url)
per activation site is \( \{4 \geq N_{\text{mas}} \geq 9\} \).

The maximum cell density for a given device can then be calculated as the number of activation sites per unit area multiplied by the number of microwells. This can be found for the general case of rectilinear spaced wells using

\[
\left\{ \frac{N_{\text{as,low}}}{P_{\text{as},x}P_{\text{as},y}} \geq D_{\text{cell}} \geq \frac{N_{\text{as,upp}}}{P_{\text{as},x}P_{\text{as},y}} \right\}
\]

(3.1)

where \( D_{\text{cell}} \) is the cell density in \( \text{cells/cm}^2 \), \( N_{\text{upp}} \) and \( N_{\text{low}} \) are the upper and lower number of cells, and \( P_{\text{as},(x,y)} \) are the orthogonal terms in the rectilinear pitch of the activation sites.

For the SiCCWells the cellular density is \( \{5000 \cdot \text{cells/cm}^2 \geq D \geq 11250 \cdot \text{cells/cm}^2\} \).

### 3.2.2 Resistance Model of the Nanoporous Membrane

In the model of the SiCCWells, the membrane and the ionic fluid within the nanopores can be approximated via a parallel resistance network. A circuit diagram can be generated which represents the voltage applied across each pumping region. The description of the behavior can be evaluated through the voltage and resistance equations for the parallel resistor circuit for each position where the electrodes cross with the microchannels on the far side of the membrane as shown in Figure 3.3.

The nanoporous membrane can be approximated by the porosity and the linear resistance of the filling fluid, in this case the phosphate buffered saline, both of which can be determined experimentally or from literature references. For the resistance of the membrane it will follow

\[
R_{\text{mem}} = \frac{R_{\text{PBS}}}{N_{\text{pores}}}
\]

(3.2)

where \( R_{\text{mem}} \) is the membrane resistance as a function of the number of pores in a microwell, \( N_{\text{pores}} \). \( R_{\text{PBS}} \) is given by

\[
R_{\text{PBS}} = \frac{4}{\pi} \frac{t_{\text{mem}}}{d_{\text{pore}}^2} \tilde{\rho}_{\text{pore}}
\]

(3.3)

where \( \tilde{\rho}_{\text{pore}} \) is the average pore diameter, \( t_{\text{mem}} \) is the thickness of the nanoporous membrane,
and $\rho_{\text{pore}}$ is the unit resistance of phosphate buffered saline. The term $N_{\text{pores}}$ is given by

$$N_{\text{pores}} = \frac{4}{\pi} \frac{A_{\mu\text{well}} \phi_{\text{Al}_2\text{O}_3}}{d_{\mu\text{well}}^2}$$

(3.4)

where $A_{\mu\text{well}}$ is the area of an individual microwell and $\phi_{\text{Al}_2\text{O}_3}$ is the porosity of the alumina membrane. $A_{\mu\text{well}}$ is given by

$$A_{\mu\text{well}} = \frac{\pi}{4} N_{\mu\text{wells}} d_{\mu\text{wells}}^2$$

(3.5)

where $d_{\mu\text{wells}}$ is the average diameter of the polystyrene microwells and the number of microwells per activation site is given as a range of values. Combining equations 3.4 and 3.5 resulting in $N_{\text{pores}}$ being related to the square of the ratio of the mean pore diameter and microwell diameter. The number of pores is now stated as

$$N_{\text{pores}} = N_{\mu\text{wells}} \left( \frac{d_{\mu\text{wells}}}{d_{\text{pore}}} \right)^2 \phi_{\text{Al}_2\text{O}_3}$$

(3.6)

where $d_{\text{pore}}$ is the mean pore diameter, $\rho_{\text{PBS}}$ is the unit resistance of PBS, $t_{\text{pore}}$ is the
Figure 3.4: Resistances overlayed on a figure of the SiCCWell in cross section. Yellow regions represent the conductive path between the positive and negative electrodes for a system without individualized counter electrodes.

The effective resistance of a single channel $N_{\mu\text{wells}}$ is the number of microwells in an activation region, $d_{\mu\text{wells}}$ is the diameter of the microwells, and $\phi_{\text{Al}_2\text{O}_3}$ is porosity of the nanoporous membrane.

For a device that does not have individual cathodes to create a fluid delivery “pixel” a continuous cathode is used. Due to the effects of diffusion, continuous cathode can be problematic as all pores across all channels can be expected to fill with conductive electrolyte as seen in Figure 3.3. In the case of this system, the circuit diagram for the system is modeled as resistors in parallel. For this circuit, the voltage at each activation region can be calculated by the equation

$$V_N = \frac{V_{\text{App}}}{R_c} \left( \frac{R_{\text{node}}R_{\text{mem}}}{R_{\text{mem}} + R_{\text{node}}} \right) \left( \frac{R_{\text{mem}} + R_{\text{node}}}{R_{\text{mem}}} \right)^N$$

in which $V_{\text{App}}$ is the applied voltage, $R_c$ is the electrode resistance from the contact to the first node, $R_{\text{node}}$ is the resistance between nodes. Both $R_c$ and $R_{\text{node}}$ are given from the
equation

\[ R_e = \rho_{pt} \frac{L_e}{w_e} \]  

(3.8)

where \( L_{\text{electrode}} \) and \( w_{\text{electrode}} \) are the dimensions of the thin film electrode lead and \( \rho \) is the sheet resistance with units of \( \Omega/\square \), or “ohms per square”. The minimum dimension, in this case the electrode width, is used to define the “square” of the electrode which is used for calculations regarding sheet resistance.

The theoretical resistance is given by the solution to a resistance ladder for \( N \) segments. The solution can be determined by using a recursive function for the parallel and serial resistances. The first three terms being displayed in equation 3.9

\[
R_{\text{eff}}(N) = \begin{cases} 
N = 1 : R_{\text{eff}} = R_c + R_{\text{mem}} + R_- \\
N = 2 : R_{\text{eff}} = R_c + \left( \frac{1}{R_{\text{mem}} + \frac{1}{R_{\text{node}}} + \frac{1}{R_{\text{node}}}} \right) + R_- \\
N = 3 : R_{\text{eff}} = R_c + \left( \frac{1}{R_{\text{mem}} + \frac{1}{R_{\text{mem}} + \frac{1}{R_{\text{node}}}} + \frac{1}{R_{\text{node}}}} \right) + R_- 
\end{cases}
\]

(3.9)

where the term \( R_- \) is the resistance of the negative electrode, which is a non-negligible but small contribution to the total resistance. The recursive Matlab code for determining the resistance for \( N \) ladder rungs is given in Appendix A. In devices that are patterned on both sides of the device, the effective resistance and voltage drop for all nodes goes to \( N=1 \), and the only variable becomes the sheet resistance to the activation site.

### 3.3 Materials and Methods

The fabrication of the SiCCWells forms the basis of the fabrication for devices outlined in this work. Patents have been filed for the fabrication of a masked, micropatterned electrode and fluidic feeding chamber designs. The fabrication and materials for the SiCCWell were fully described in the previous chapter, Section 2.4.
3.3.1 Cellular targets

Several cell lines were used for vacuum seeding. The purpose of the earliest cellular studies was to provide for a two-stage dye, as a cellular dye would not fluoresce until after binding with DNA of a cell. For use with live/dead stains this could be any mammalian cells. Specifically, cells that would be maximally adherent were used to test device function. 293-T human embryonic kidney cells were cultured in 5% CO$_2$ at 37°C in Dulbecco’s modified eagle medium with 1% penicillin and strepavidin. Cells were released from culture using 1% trypsin in media for 5 minutes and immediately vacuumed seeded.

3.3.2 Live-Dead Assay for Mammalian Cells

Life Technologies Live-Dead Assay for mammalian cells (Life Technologies Catalog #L-3224) was prepared in phosphate buffered saline according to protocols from Life Technologies. The calcein was combined to a molarity of 2 mM and the ethidium homodimer-1 was mixed to a molarity of 4 mM [102].

Calcein is transported into live cells and the fluorescent site exposed by intracellular esterases which results in a strong green fluorescence which is contained within the cells. As dead cells can not produce esterases which explode the fluorescent site, this stain will only stain regions with live, or at one point live, cells [102].

Ethidium homodimer-1 is a membrane impermeable dye which binds strongly to DNA with a maximum fluorescence of 617 nanometers when excited with a maximum of 528 nanometers. The ethidium homodimer fluoresces weakly until it is bound to DNA after which there is a 40-fold increase in the fluorescence of the molecule [102].

To track cell location after seeding with a third dye, the samples could be stained with a 4’,6-diamidino-2-phenylidole (DAPI) stain by suspending the cells in the stain for 20 minutes prior to cellular seeding. The cells were trypsinized, removed from the culture plate and resuspended in PBS with DAPI at a concentration of 300 nM which provides a fluorescent wavelength of 500 nanometers (maximum). DAPI binds to the AT minor groove through the displacement of water in both molecules [103].
3.3.3 Vacuum Priming

Devices were vacuum primed using a decreasing concentration of ethyl alcohol in a solution of ethyl alcohol and phosphate buffered saline. 200 $\mu$L of solution was placed in the macrowell and a vacuum held across the microfluidics at -15 in.Hg for 5 minutes. Vacuum pressure was removed from the microfluidics and the solution was aspirated. The next solution was pipetted into the macrowell and the vacuum reapplied. After the PBS was pipetted and the vacuum removed, the solution was left in the macrowell to maintain the PBS hydration.

3.3.4 Vacuum Cell Seeding

24 hours prior to cell seeding, PBS was removed and a 0.1% solution of gelatin was applied. 293A cells were seeded into the device at a cellular density of $10^6$ cells/mL. Vacuum was applied at -15 in.Hg for 5 minutes. With the vacuum still applied, the cellular solution was aspirated from the chamber. 200 $\mu$L of culture media was added to the macrowell and the device was incubated at 37$^\circ$ with 5% CO$_2$ for 8 hours. Media was replaced every 8 hours after initial cell seeding until fluid delivery.

3.3.5 Fluid delivery

Fluid delivery was calculated by reducing the fluid passage through the microfluidics to a function of time. The fluid was controlled via a laboratory syringe pump. The syringe pump controlled the volume flow rate (i.e. $\mu$L per minute). By running the fluidic pump in reverse for a controlled amount of time to “load” the Tygon tubing with live/dead assay, the amount of fluid passed through the microchannels, in contact with the negative electrode, could be accurately gauged. The total plug volume was given by:

$$V_{contact} = r_V \cdot t_c$$

(3.10)

where $r_V$ was the volume flow rate in $\mu$L/min and $t_c$ was the time of contact required. For the SiCCWell experiments, $t_c$ was chosen to be 15 s, to deliver a contact volume of 2.5 $\mu$L.
In order to completely ignore the effects of diffusion through the membrane, the fluid volume of PBS must displace the live/dead assay sufficiently after electro-osmotic delivery. This was accomplished by calculating the amount of time required for the live/dead plug to be cleared from the microfluidics. The volume of the microfluidic channels ($V_{\mu F}$), connection joint $V_{\text{joint}}$, and inlets ($V_{\text{inlet}}$) can be found according to the original designs and the thickness of the SU-8 used for fabrication.

The total fluid volume, $V_{\text{tot}}$, was given by:

$$V_{\mu F} + V_{\text{joint}} + V_{\text{inlet}} = V_{\text{tot}} \quad (3.11)$$

Combined with equation 3.10 this allows the determination of the amount of time for the passage of the fluid beyond the membrane as the clearance time, $t_{cl}$

$$t_{cl} = \frac{V_{\text{tot}} + V_{\text{contact}}}{r_V} \quad (3.12)$$

The fluidic connections result in a substantial dead volume due to the significantly larger cross sectional area. Reducing the length of the injection joints can reduce the amount of dead volume but increases the difficulty of manipulating the devices.

Initially, the clearance time was estimated to be 300s seconds for the first experiments. However, this length of time subjects the cells to too much uncontrolled exposure as well as provides too much opportunity for gas evolution when using a DC signal. The calculated clearance time was reduced to 60s.
Because of this, the system was primed using bursts of fluid of load time, $t_A$, spaced by a second load time of PBS, $t_B$. This method is shown in a diagram in Figure 3.5. Using a continuous flow skews the boundaries of the individual plugs due to the potential for diffusion at the interface of the two liquids. The diffusion can be lessened by decreasing the cross sectional area of the connection tubing. This increases the linear distance between the interfaces, $d_p$, given by:

$$d_p = \frac{t_B \cdot r_L}{A_{fl}}$$  \hspace{1cm} (3.13)

where $A_{fl}$ is the cross sectional area of either the microchannels or the tygon tubing, $r_L$ is the linear flow velocity, which can be found by converting the volume metric flow rate.

In this system, the fluid contact will be maintained while limiting diffusion across the membrane. The diffusion both between plugs in low Reynold’s number flow and through the nanochannels can be estimated using Fick’s law of diffusion in one dimension [104].

During the clearance time, the voltage was maintained across the membrane at 1 volt which corresponds to a voltage applied of 6 volts at the source. This provides maximal flow across the membrane for the duration of the clearance time.

After fluid delivery and electrode activation, medium (DMEM) was added to the macrowell and the devices were returned to incubate at $37^\circ C$ with 5.0% CO$_2$.

### 3.4 Results

The results of the SiCCWells fluid delivery were largely favorable. However, certain functions, specifically patterned vacuum cell seeding, will require more data to gauge their efficacy and use.

#### 3.4.1 Vacuum Priming & Seeding Results

Vacuum cell seeding was seen in several devices, however, the overall cellular seeding efficiency was not considered to be statistically significant.
Figure 3.6: A. DAPI Staining of cells vacuum seeded to SiCCWell device B. Green fluorescent stain (Calcein AM) shown being regionally delivered C. Red fluorescent stain (ethidium homodimer-1) regionally delivered to cells vacuum seeded to SiCCWell device

3.4.2 Cellular Results

293A human embryonic kidney cells were stained using a live/dead stain at 2mM for calcein AM and 4mM for ethidium homodimer-1 (Life Technologies L-3224). Controls showed no staining for live/dead assay while activated electrodes showed significant cellular staining.

Fluid delivery was recorded in all devices electrically activated for femtopump delivery. Samples were predominantly stained for dead cells along activation channels. Channel leakage was responsible for a considerable amount of staining outside of the specified pixel region. In one circumstance it was shown that the cells were stained only on the bottom of a large cell mass.

Cellular Staining

Channel leakage was seen as the activation of lines of electrodes rather than a discrete “pixel” of cellular staining. In Figure 3.6 the regional staining of cells with live-dead assay can be seen. The cells were previously stained with 4',6-diamidino-2-phenylindone (DAPI) stain for imaging after cell seeding. In the figure, a defect in the polystyrene layer can be seen which provided a reference position for earlier cellular experimentation.

Cells were seeded at a density of $10^6$ cells/mL which resulted in large cell masses in early trials. These cellular masses did not prevent the flow of live-assay but rather exemplified
the “bottom-up” nature of the pumping within the microfluidic system. Figure 3.7 shows an image of a region stained with live/dead assay prior to the introduction of propidium iodide (PI) along side an image of the cell mass after being stained with PI.

This shows that the live/dead stain is originating from below the cellular mass as opposed to being delivered through another mechanism.

Due to low fluid delivery, the voltage and delivered volume was increased to 6 volts and 150 µL. This resulted in an excess amount of live/dead stain being delivered. Combined with channel-to-channel leakage entire electrodes were activated showing a decreasing fluorescent intensity as distance from the delivery fluidic channel increased. This effect can be seen in Figure 3.8.

3.4.3 Signal Attenuation

A regular effect during cellular staining is that the fluorescence directly above the delivery channel is absent from images. This effect is demonstrated in Figure 3.10. This is thought to be an effect of the microscope setup that was used to image the cells. An inverted
Figure 3.8: A predelivery image with red fluorescence filter B. Live dead assay delivered to entire electrode due to channel leakage.

Figure 3.9: Regional fluid delivery of ethidium homodimer-1 directly adjacent to fluid delivery microchannel. Green box is the region examined in Figure 3.10.
fluorescent transmission microscope was used to image the cells. In the current design of the SiCCWells, if the channel is full of phosphate buffered saline the light will pass from layer to layer relatively unencumbered. However, it is known that when the microfluidics are disconnected that the resulting vacuum removes a large portion of the fluid. During imaging, further evaporation of fluid has been imaged actively occurring and is shown in Figure 3.11.

Color enhancement has shown that the Fluorescence does, in fact, exist but has been significantly reduced in relative intensity due to much stronger fluorescence in regions directly over the PDMS microfluidic. A color enhanced image is shown in Figure 3.12.

3.5 Discussion

3.5.1 Contact resistance and non-uniform sheet resistance

To decrease contact resistance in future implementations of the SiCCWells, the conduction lines between activation sites can be selectively thickened with platinum. Alternatively, a thick film of silver could be patterned to form an encapsulated low resistance conduction line adjacent to the activation electrodes. The benefit of this is that the electrodes across
Figure 3.11: Bubble formation in microfluidic channel. Yellow arrow indicates the interface of the bubble (dark region) and remaining PBS (light region)

Figure 3.12: Color enhancement of cells showing fluorescence obscured by relative intensity of surrounding signals.
the membrane will, potentially, have a negligible difference in the voltage drops across each activation region, which will result in an increase in repeatability at differing sites.

Contact resistance between the wire and the bond pad is a function of the thickness and area of the bond pad. The wire, significantly larger than the bond pad, can be described as a square contact of finite radius and infinite length. It is assumed that the silver epoxy covers the entire surface area of the contact pad as the epoxy has a low enough viscosity to enter the nanopores. The contact resistance in a thin film contact of dissimilar materials is given by

\[
R = \frac{\rho_2 L_2}{2W h} + \frac{\rho_2}{4 \pi W} \bar{R}_c \left( \frac{a}{b}, \frac{a}{h}, \frac{\rho_1}{\rho_2} \right) + \frac{\rho_1 L_1}{2 W a}
\]

(3.14)

where:

\[
\bar{R}_c \left( \frac{a}{b}, \frac{a}{h}, \frac{\rho_1}{\rho_2} \right) =
\]

\[
2 \pi \frac{\rho_1}{\rho_2} \sum_{n=1}^{\infty} B_n \coth(F_c(h)) \frac{\sin(F_c(a))}{F_c(a)} - \frac{2 \pi (b - a)}{h}
\]

(3.16)

where:

\[
F_c(x) = \left( n - \frac{1}{2} \right) \frac{\pi x}{b} \bigg|_{x=a,h}
\]

(3.17)

where the variables are define according to the block diagram of dissimilar thin film contacts in Figure 3.13. \( L_1 \) is the thickness of the connecting terminal of material I with resistivity \( \rho_1 \), \( L_2 \) is the width of the thin film contact pad \( b \) minus the width of the connecting terminal \( a \), \( h \) is the thickness of the bond pad of material II with resistivity \( \rho_2 \).

In a contact resistance with the 80 nanometer platinum contacts the contact resistance term of \( a/h \) is significant, whereas the term \( a/b \) is assumed to be unity. This reduces equations 3.16 and 3.17 to:

\[
\bar{R}_c \left( \frac{a}{b}, \frac{a}{h}, \frac{\rho_1}{\rho_2} \right) = 2 \pi \frac{\rho_1}{\rho_2} \sum_{n=1}^{\infty} B_n \coth(F_c(h)) \frac{\sin(F_c(a))}{F_c(a)}
\]

(3.18)
Figure 3.13: Block diagram of the general case of contact resistance between two dissimilar materials I & II \[105\]

\[ F_c(a) = \pi \left( n - \frac{1}{2} \right) \]  

(3.19)

Our model for the contact pad also ignores the constriction resistance that would be included due to the porosity of the nanoporous membrane which is believed to be a significant factor. For simplicity, this has also been omitted in this discussion.

The silvered layer decreases the \( a/h \) factor to a fifth of the previous value. The term \( a/b \) remains the same. Porosity decreases 50% or more with an additional 300 nm layer of silver as seen in Figure 2.9. In addition to lowering the contact resistance directly through the thickness of the bond pad the resistance decrease due to the reduction of a constriction resistance, or the resistance caused by a relatively large current moving through a, cross-sectionally, small interface.

**Effect of the Electric Double Layer on Charge Transfer**

In addition to the contact resistance of the electrical bond pads. There exists a resistance between the electrodes and the electrolyte solution. In theory, for a DC voltage there is no
conductance at this junction as the electric double layer is modeled as a capacitor with a voltage-dependent capacitance.

However, this model assumes that the charged surface does not pass any current. Since current can pass through the platinum electrodes the system is instead modeled as a “leaky” capacitor, or a capacitor and resistor in parallel. This modifies the previous model for our resistor network by replacing the resistors in parallel with parallel RC circuits in parallel. This model is shown in Appendix C The electric double layer is a dominant resistance in the model. This correction can be inserted into the voltage equations and recursive resistance equation to give the total system resistance. In a direct current system there is little change except when the model capacitors are charging. However, for alternating current the system shows a significant difference in voltage drops and resistances.

**Alternating Current Revisited**

An idealized system would tune the frequency of the applied voltage to allow the capacitor to be, effectively, neglected in the equation. The frequency dependent impedance of the electric double layer, \( Z(\omega)_{edl} \), becomes:

\[
Z(\omega)_{edl} = \frac{1}{i\omega C_{edl} + \frac{1}{R_{edl}}}
\]

where \( \omega \) is the frequency, \( C_{edl} \) is the capacitance associated with the electric double layer, and \( R_{edl} \) is the resistance associated with the electric double layer. The charge transfer reaction is modeled by the Frumkin correction:

\[
j_c = -nFk_s c_0^* \exp \left[ -\frac{z_i F(\Phi_2 - \Phi^S)}{RT} \right] \exp \left[ -\frac{\alpha_c n F(\Phi^M - \Phi^S)}{RT} \right]
\]

where \( \Phi^S \) is the inner potential of the bulk phase, \( \Phi_2 \) is the potential at the outer Helmholtz plane, \( \Phi^M \) the potential at the electrode surface, \( R \) is the gas constant, \( T \) is the absolute temperature in Kelvin, \( F \) is Faraday’s constant, \( k_s \) is the standard heterogeneous rate constant, \( c_0^* \) is the bulk concentration of component electrolyte in solution, \( z_i \) is charge number of the ion of species \( i \), \( \alpha_c \) is the transfer coefficient, and \( n \) is the charge number.
of the electrochemical reaction \cite{106}. Based simply on Ohm’s law the impedance is simply $V = IR$ where the current is the area multiplied with the current density, $j_c$, from the electrolyte which gives the

$$
\frac{j_c A_{pt}}{V_{edl}} = i\omega C_{edl} + \frac{1}{R_{edl}} \tag{3.22}
$$

where $V$ is the voltage applied at the electrode when the EDL. This can be calculated based on the previous voltage drop equations modified with the currently discussed impedances.

The area of the platinum electrodes can be estimated as the non-porous region of the patterned membrane by

$$
A_{total} = A_{elec}(1 - \phi_{Al_2O_3})(\Phi_p) + \bar{d}_{pore} t_{pt} \{N\} \Phi_{ps} \pi \tag{3.23}
$$

where

$$
\{N\} = \frac{4 A_{elec} \phi_{Al_2O_3}}{\pi d_{pore}^2} \tag{3.24}
$$

where $A_{elec}$ is the area of the electrode/microfluidic cross section (100x100 µm), $\phi_{Al_2O_3}$ is the porosity of the alumina membrane, $\Phi_p$ is the porosity of the polystyrene insulation, $d_{pore}$ is the average pore diameter on the alumina membrane, and $t_{pt}$ is the thickness of platinum deposited.

The alternating current discussed by Piwowar can be tuned to maintain an “in phase” relationship with the electrical double layer resistance. This allows more efficient charge transfer to the electrolyte. This results in a more tightly controlled EDL resistance. However, with electro-osmotic flow equations subjected to a symmetric wave will simply result in no net flow \cite{41}.

Piwowar demonstrated the ability to overcome this by using an asymmetric wave to, effectively, discharge the electrodes while minimizing back flow. This method allowed for the disruption of continuous hydrogen gas by discharging the electrodes while driving the current in the reverse direction in such a way as to reverse any hydrogen evolution that had occurred \cite{41}.
This can be further enhanced by developing a system that uses the alternating current in such a fashion as to allow the voltage drop across the solution in the pores to contain the largest voltage drop in the system which requires tuning the system to a frequency at which the electric double layer offers a resistance of $R_{edl}$.

$$\lim_{\omega \to \infty} Z_{edl} = 0 \quad (3.25)$$

This being said, infinite frequency is not realistic. In addition, increases in frequency will, at certain values, increase impedance in other structures. To this effect the frequency should be tuned to minimize the overall resistance except across the nanoporous membrane.

### 3.6 Conclusions

The SiCCWells represent a powerful combinatoric tool for the generation of large data sets for cell analyses. The ability to interface with a cell in real time is of great importance to many fields of research and medical diagnostics. The SiCCWells will continue development to improve upon the resolution of the devices down to the single cell.

In addition, designs can be altered, using common electrical engineering techniques, to implement systems such as a parallel bus for distribution of voltages to the activation without a drop in voltage between each element.
Chapter 4
SiCCWell Microdroplet Production for Analyte Detection

4.1 Introduction

Microdroplets refer to a phase based compartmentalization of materials. The most recognizable of these compartmentalizations is a water in oil emulsion. An emulsion refers to the break up of one liquid phase inside another [80]. Through the use of surfactants these emulsions can be stabilized for long term compartmentalization [8].

Microdroplets have seen increasing interest from the microfluidic community. Microdroplets allow for small volumes of liquid or even entire cells to be encapsulated in a traceable form that can be easily manipulated using a variety of technologies [82].

Microdroplets are able to maintain higher relative concentration of analytes. This allows for more precise detection of indicators from biological processes. Several bulk cellular techniques have already been investigated for use in microdroplet technology, the most prominent of these being emulsion polymerase chain reaction (PCR), in which PCR can be performed in controlled microdroplets [31].

In addition to analyte detection, whole cells have been encapsulated in microdroplets [8] [82] [84]. While this is outside the capacities of the device described in this chapter it is important to note that this is a possibility for future interactions. The ability to encapsulate and maintain cells is also possible as shown by Koster in their paper using 2C6 hybridoma
cells. These cells showed viability of up to six hours on chip [84].

Again, the microdroplets can be easily manipulated using a series of technologies. These include a variety of particle separation techniques. This was touched on briefly in a discussion regarding hydrodynamic trapping [78]. It is also possible to adapt flow cytometry systems to perform high speed sorting of microdroplets as one would sort individual cells or particles in an analogous system [107]. In effect, the microdroplets represent a new “cell” that is fabricated and maintained artificially.

There are two classifications of single emulsion microdroplet generation which are, oil in water or water in oil. In both of these systems the external phased does not need to be specifically a pure “oil” or the internal a pure “water” but rather these relate to the distinction of the phases as aqueous and non-aqueous. In cellular systems, they are largely dominated by water in oil systems as the cellular environment is, in fact, aqueous. The access to fluorinated oils has substantially altered how these systems are produced as such oils allow for the transfer of nutrients and gases across the interfaces [8].

The perceivable weaknesses in microdroplet technology come from microdroplets that are not monodisperse, or rather they have a significant distribution to their size and composition [8] [82]. This is problematic when microdroplets are combined or otherwise treated as a consistent size. Many techniques have been shown to allow for the production of monodisperse microdroplet populations and are discussed in Section 1.5.2.

4.2 Background

4.2.1 Design Criteria for microdroplet production

Layers of silica have been deposited over the electrodes on both sides in an effort to achieve two goals: increased electrical isolation and the ability to make surfaces hydrophobic through self assembling monolayers.

The first goal is to increase the electrical impedance for currents inside the bulk phosphate buffered saline (PBS) held on the surface. This conductance is changed according to the open surface area exposed to the PBS in situ. If one takes the resistance of the platinum
to be directly attributed to the open surface area of the platinum electrodes then the initial area “seen” by the PBS is

\[ A_{total} = A_{elec}(1 - \phi_{Al_2O_3})(\phi_{ps}) + \bar{d}_{pore}t_{Pt} \{N\} \phi_{ps}\pi \] (4.1)

where

\[
\{N\} = \frac{4A_{elec}\phi_{Al_2O_3}}{\pi d_{pore}^2} \] (4.2)

where \( A_{elec} \) is the area of the electrode/microfluidic cross section (100x100 \( \mu \)m), \( \phi_{Al_2O_3} \) is the porosity of the alumina membrane, \( \phi_{PS} \) is the porosity of the polystyrene insulation, \( d_{pore} \) is the average pore diameter on the alumina membrane, and \( t_{Pt} \) is the thickness of platinum deposited. Typically for a porosity of 50% in the alumina and 20% in the polystyrene insulating layer (based on a square grid of 15 \( \mu \)m wells with 30\( \mu \)m pitch) the resistance is dominated by the first term which is the open surface of the electrode exposed to the media. If an insulated layer of silica is deposited over the electrode, the first term is reduced to zero and the second term becomes the dominant term. Thus the resistance is governed only by the thickness of the platinum while maintaining the same relationship to the electro-osmotic pump. A diagrammatic representation of the pore can be seen in Figure 4.1.

Since the second term, resulting from the area of a ring of deposited platinum is now the dominant term, the equation to calculate the effective area is now:

\[ A_{total} = t_{Pt} \left\{ \frac{4A_{elec}\phi_{Al_2O_3}}{\pi d_{pore}^2} \right\} \phi_{ps}\pi \] (4.3)

The second goal is to provide for a neutral or hydrophobic surface in the microchannel into which the microdroplets will be generated. Droplet coalescence or, conversely, metastability is determined by the interstitial forces of the emulsion as well as the surface energy of the surfaces in which the emulsion is carried [8].

The properties of a microdroplet are governed, principally, by the Young-Laplace equation:
Figure 4.1: Block Diagram of nanoporous membrane with metalization layer and electric double layer. The arrows, label First and Second Term, reference the regions of the platinum layer that are associated with the respective terms in equation 4.1

\[ \Delta p = -\gamma \nabla \cdot \hat{n} \]  
\[ = \gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right) \]  
\[ = \frac{2\gamma}{H} \]  

where, \( \Delta p \) is the pressure difference between phases, \( \gamma \) is the surface tension, \( \hat{n} \) is unit normal, \( H \) is the mean curvature, and \( R_1 \) and \( R_2 \) are the principal radii of curvature. The model for the surface tension in this case is shown in Figure 4.2. The equations for the interfacial surface tensions are given by

\[ \gamma_{1,2} = \gamma_1 + \gamma_2 - 2\Phi \sqrt{\gamma_1 \gamma_2} \]  
\[ \gamma_{L_1,S} - \gamma_{L_2,S} = \gamma_{L_1,L_2} \cos \theta_1 \]  

for liquid interfaces and

for liquid-liquid-solid interfaces. \( \gamma_{L_1,S}, \gamma_{L_2,S}, \gamma_{L_1,L_2}, \theta_1 \) are defined as shown in Figure 4.2.
Figure 4.2: The interface of a solid and two liquids at a contact angle of $\theta_1$ (adapted from [80]).

Figure 4.3: Progression of microdroplet formation in a t-junction channel. 
1a - 1b. Forward flow in the internal phase pushes fluid into the external phase. 
2. External phase flow velocity begins to pinch off the internal phase from the bulk. 
3. Cohesive forces of internal phase complete pinching off. 
4. Internal phase and external phase in equilibrium.
The electro-osmotic flow velocity is given by the Helmholtz-Smoluchowski equation:

\[ \vec{v}_{EOF} = -\frac{\varepsilon_w \varepsilon_0 \zeta}{\eta} E_{Applied} \] (4.9)

As such the formation of microdroplets is governed by the difference between the surface tension of the microchannel, external phase, and that of the internal phase as well as the interfacial pressures imparted by the flow velocities. The flow velocities for an incompressible fluid can be calculated by the simplified Bernoulli’s equation:

\[ \frac{v^2}{2} + \Psi + \frac{p}{\rho} = C \] (4.10)

where \( v \) is the flow velocity, \( \Psi \) is the force potential, \( p \) is the pressure, \( \rho \) is the fluid density, and \( C \) is a constant. This allows us to equate the surface tensions at the interface \( \gamma_{Al_2O_3}, \gamma_{ext}, \gamma_{int} \) and the flow velocity of internal phase (given by Equation 4.9):

\[ p_{int} = -\frac{v_{int}^2}{2} \cdot \rho_{int} \] (4.11)

\[ p_{EOF} = \frac{\rho_{int} E_{app}^2}{2} \left( \frac{\varepsilon_w \varepsilon_0 \zeta}{\eta} \right)^2 \] (4.12)

Dividing by the area gives the force exerted by the electro-osmotic driven plug gives:

\[ F_{EOF} = \frac{2\rho_{int} E_{app}^2}{d^2 \pi} \left( \frac{\varepsilon_w \varepsilon_0 \zeta}{\eta} \right)^2 \] (4.13)

where the term \( \bar{d} \) is the mean diameter of the pores in the nanoporous membrane.

In Figure 4.3.1a, the electro-osmotic pressure does not exist, so a balance of pressures appears as the contact angle at equilibrium. Once the electro-osmotic flow has been activated (4.3.1b) the radius of the plug is given by the Young-Laplace equations combined with the electro-osmotic pressure:

\[ R_{drop} = \frac{2\gamma L_1 L_2}{\rho E_{app}^2} \left( \frac{\eta}{\varepsilon_w \varepsilon_0 \zeta} \right)^2 \] (4.14)

The radius becomes of relevance when the droplet begins to exit the nanopore as shown in Figure 4.3.2. As the droplet leaves, the droplet will grow to a radius \( R_{drop} \) balanced
by the pressure from the forward flow velocity in the channel below. This radius can be
attained quickly in the case of closely spaced nanochannels as interdroplet coalescence is
expected to be near instantaneous. Due to this it is assumed that small “droplets” exiting
the nanopores will coalesce to attempt to reach a lower internal pressure. The effects of
the nanochannel surface presented to the external phase are considered negligible and are
ignored.

The pressure from the forward flow is, again, given by the Bernoulli equation. The
droplet is “pinched” off (Fig. 4.3.3) when the pressure from the flow velocity of the external
phase is able to equal or exceed the pressure of cohesion at the membrane surface.

After the droplet has completely “pinched off” the system will return to equilibrium as
shown in Figure 4.3.4.

4.2.2 Voltage effects on microdroplet stability

Microdroplet stability has been shown to be dynamically disrupted in the presence of small
voltages (1 – 3V) [108]. This is of particular concern when contemplating the generation of
droplets using the femtopumps, as the voltages applied in the femtopumps are on the same
order of magnitude as the voltages used by Priest et al. [108]. An analysis of the electric
fields are not suspected to leave the system open to voltage based coalescence. The use
of electro-osmotic flow for the generation of microdroplets has been previous demonstrated
though not through the use of a nanoporous membrane based electro-osmotic pump [109].

4.3 Materials and Methods

In this section the materials and methods chosen for microdroplet generation are explored.
The first section discusses the choice of self assembling monolayers, the second discusses the
alterations to the fabrication process for the devices, and lastly, the methods of microdroplet
production are described.
4.3.1 Self-assembling monolayers for hydrophobic substrates

Natively, the alumina membrane is largely hydrophilic which results in a significant problem regarding droplet coalescence inside the microchannel. Because of this, the platinum layer of the device must be treated in order to make the surface hydrophobic, see Section 4.2.

Two materials were chosen for this, OTS and ODT.

The first material chosen is octadecyltrichlorosilane, OTS, was chosen for its use in several other publications regarding microdroplet experimentation. When OTS comes in contact with an metal oxide surface it forms a bond with the silane group. The principal concern with the use of a silane is that it would require the use of an anhydrous carrier fluid, such as toluene, which would result in the swelling of the PDMS. In addition, the silane group would require the deposition of a silica layer on to the surface of the platinum ground electrode, a process that did not prove successful (see Results, Section 4.4)

1-Octadecanethiol, ODT, was also selected as it is possible to bind ODT directly to semi-noble and noble metals. Using ODT, the self assembling monolayer loses a hydrogen-sulfide bond or a sulfur-sulfur bond with the sulfur binding instead to the metal layer. The functional group then behaves as a hydrophobic substrate.

This second method was preferred as the carrier fluid for the ODT was ethanol, which did not cause swelling of the PDMS. It also allowed the SiCCWell fabrication described previously to remain largely unchanged. The silane group was ultimately abandoned as the silica layer did not behave as anticipated and the process was deemed too complicated to be chosen over the thiol functionalized surface.

4.3.2 Fabrication

SiCCWell based microdroplet generator fabrication used modified fabrication of the SiCCWell cell culture platform. Two different models of the SiCCWell were prepared to test two separate methods of self assembling monolayers (SAMs), a thiol and silane.

Samples that were to be treated with a thiol functional group were unchanged from the previously described fabrication process. Samples that were to be treated with a silane
functional group underwent a 100 nanometer deposition of silica over the surface of the negative electrode. The negative electrodes had regions blocked, using glass slides, to allow electrical connections during packaging. The microfluidics were then bonded to the back side of the membrane using plasma bonding as it was expected that this would facilitate a stronger bond between the PDMS and membrane.

Prior to delivery of self assembling monolayer solution the macro well was filled with phosphate buffered saline (PBS). The macrowell was then capped with a block of poly(dimethyl siloxane) (PDMS) and taped down tightly to form an airtight seal. This provided a pressure bias on the nanochannels to deter flow into the nanochannels by the ethanol/SAM solution.

Twelve hours prior to microdroplet production 1 mM 1-octadecanethiol (ODT) (Sigma Aldrich 08158) in ethanol was introduced to the microchannels via syringe pump. The fluid was delivered at 1 $\mu$L/min for 15 minutes. The channels are then closed off using Kapton tape over the inlets.

Vacuum priming was conducted similarly to the SiCCWells. Remaining PBS from the ODT treatment stage was removed via aspirating pipette. A 75% solution (by volume)of PBS and ethanol was introduced to the top well until full, in this case 200 $\mu$L. Vacuum was applied at -15 in.Hg through the microchannels for 5 minutes and then released. The solution was removed via aspirating pipette and the next solution was added. This was repeated for dilutions of 75%, 50%, 25% and pure PBS. The device was then recapped and the inlets sealed with Kapton tape until the experiment was ready. An upper limit to this pre-test phase has not been investigated.

4.3.3 Microdroplet production

After 12 hours of treatment with self assembling monolayers in ethanol solution the devices were flushed with Flourinert FC-40 in solution with 1% PTFE oil as a stabilizing agent. A generic, but chemically identical, form of Krytox was used for the PTFE oil. This solution was maintained at a continuous flow of 10 $\mu$L/min throughout experimentation. Waste was collected in a 12 well plate and labeled according to time. 2.5 mM Flourescein in phosphate buffered saline was used in the top macrowell. 6 volts was applied to the terminals of
Figure 4.4: Microdroplets in an Al$_2$O$_3$ PDMS microchannel imaged via inverted microscope. Droplets marked by arrows are out of the focal plane adhering to the under side of the device.

the device. This was expected to result in a 1 volt potential across the membrane. The negative and positive terminals were reversed to provide electro-osmotic flow away from the microwells. The voltage was held constant throughout the experiment. Unlike the electro-osmotic flow in the SiCCWells, it was not necessary to modulate the voltage as the microdroplets are formed discretely.

4.4 Results

The SiCCWell based microdroplet generator successfully produced monodisperse microdroplets in a 100 $\mu$m x 100 $\mu$m fluidic channel. Droplets were approximately 25 - 30 microns in diameter which corresponds to an internal droplet pressure of 50 dynes $\cdot$ cm. This verifies the balance of equations presented in Section 4.2. The results of the droplet production can be seen in Figure 4.4.
4.5 Discussion

While the formation of microdroplets was observed, two major changes to the experimental setup are expected to improve on the imaging of the microdroplet formation. The resolution of the system was hindered by the fluorescence of two adjacent fluidic channels for unknown reasons. Additionally, the ability to resolve microdroplets during formation was affected by the significant height of the channel. Lastly, it is believed that the minimum flow rate provided by the syringe pump resulted in a pulsatile flow rather than a continuous flow.

4.5.1 Competitive Fluorescence

In the initial trial of the microdroplet generator the two adjacent microfluidic channels in the device emitted a strong fluorescence at the same emission wave length of the fluoroscein isothiocyanate (FITC). The reason for this fluorescence was not immediately apparent. One suggestion was that during the thiol treatment of the microfluidic channel, ethanol was able to contaminate the adjacent channels. When the FITC was introduced, it is possible that the ethanol was able to interact with the flourescein and increase the diffusion of FITC across the membrane. FITC is substantially more soluble in ethanol (20 mg/mL) than in water (0.1 mg/mL) or, consequently, PBS [110]. This may explain why the effect was seen in only the channels adjacent to the thiol treated channel. To reduce this effect in future trials, prior to the introduction of FITC, all channels will be filled with flourinated oil and resealed to prevent aberrant signals.

4.5.2 Channel Height

Results of the microdroplet generator were generally favorable with microdroplets of approximately 15 microns formed in the microchannels. The microdroplets were only able to be resolved after they had been fully developed and descended (towards the camera in Fig. 4.4). This is likely due to the depth of the fluidic channel which is roughly 100 µm in height. This height is unnecessary and was developed largely to assist in fluidic bonding and fluid delivery. It is possible to reduce the channel height to allow imaging of microdroplets during
formation. However, this would also increase the linear velocity within the microfluidics for a defined volume flow rate.

### 4.5.3 Flow Rate

The minimum flow rate provided by the syringe pump used (Harvard Apparatus Pump 33) was 0.1 mL/min. However, due to equipment limitations this flow was pulsatile rather than continuous. With the channel geometry presented and the number of steps per second (roughly 10) a fluid plug in the channel would be expected to travel between 10 and 20 µm per second. It is expected that this resulted in significant difficulty when visualizing microdroplets while the external fluid is in motion.

### 4.5.4 Bubbles during trial

During the trials, there was a significant difficulty with images because of the presence of air bubbles entering the microfluidics frequently but irregularly. Steps have been taken to further the efforts to remove air bubbles from any systems as well as reduce dead volumes that may have contributed to air in the fluidics.

### 4.6 Conclusions

The SiCCWells ability to reverse a voltage bias to pump into the microchannel provides a valuable mechanism for sampling from the cellular microenvironment during experimentation. By utilizing well documented microdroplet production techniques we have been able to produce microdroplets using an applied electric field. Coupled with the inherent ability to chemically address individual cells or groups of cells, the SiCCWell platform becomes a very attractive solution for microTAS applications.
Chapter 5

Gradient Generators

5.1 Background

Microfluidic gradient generators have been produced to mimic cellular signaling and environmental cues which cells encounter in vivo. Many methods for creating gradients have been produced. Methods used include, micromixers with fluidic flows [111, 112], assymetric electric fields [113], microvalving [114], and diffusion [115]. At its core a gradient generator is a device that can produce a step between concentrations of fluid according to a predeter-mined pattern. These gradients can be linear, exponential, or arbitrary based on the needs of the researcher [111]. Many of these gradient generators are designed to understand the effects of chemotaxis [116].

Chemotaxis, a response to chemical stimuli, are responsible for a large number of biologi-cal functions. The growth of cells is largely governed by both chemical and physical migration cues. The ability to understand these cues is a significant issue within regenerative medicine, drug development, and oncology [111, 112, 113, 115]

5.1.1 Chemotaxis for tumor migration inhibition

The balance between chemotaxis (movement in response to chemical cues) and mechanotaxis (movement in response to physical cues) is believed to be at the heart of biological processes such as the migration of highly diffuse brain tumors, in this case glioblastoma multiforme. Typically, one “taxic” effect dominates the other, for instance mechanotaxis is typically the dominant effect for GBM [117]. By developing in vitro chemotactic gradients it is possible
to pursue the development of mechanotaxic inhibiting pharmaceuticals to effectively halt mechanotaxic migration.

Many steps have been taken to develop chemical gradients to probe the migration of cells. The most common is exploiting the laminar flow properties of microfluidics, coupled to a step-wise dilution mixing pattern, to create discrete concentration flows within the same channel. Some methods are content to use diffusion between discrete flows to determine chemotaxic effects. Other methods have involved non-linear electric fields.

5.1.2 Diffusion

An inherent problem with the use of diffusion based chemotaxic gradients is that the effects of diffusion as function of time are considerable over short distances but drastically lower at larger distances [104]. Because of this, generation of chemical gradients can be somewhat slow. Diffusion behaves as described by Fick’s first and second laws

\[ J_i = -D \nabla \phi \]  
(5.1)

and

\[ \frac{\partial \phi}{\partial t} = D \frac{\partial^2 \phi}{\partial x^2} \]  
(5.2)

where \( J_i \) is the diffusion flux of the \( i \)th species, \( D \) is the diffusion coefficient, and \( \phi \) is the concentration in amount of substance per unit volume. Fick’s second law describes the time based concentration at a point a distance \( x \) away from the initial concentration of fluid. At short, sub-micron scales, this gradient is appropriate for chemotaxic investigations. However, at substantially large scales, hundreds or thousands of microns, the amount of time dedicated to establishing a chemical gradient will become a significant factor in the study of cellular interactions with said chemical gradient. [104].

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5.2 Theory

The gradient generators of this dissertation were based on the concept of being able to exploit the inherent material properties of the metal coated nanoporous membrane. Specifically, the device makes use of the enhanced sheet resistance of the nanoporous platinum. By patterning regularly spaced electrode leads the voltage drop to the electro-osmosis activation region varies as a function of resistance. Nanoporous metal has a significantly higher sheet resistance than that of a non-porous metal thin film. Because of this higher sheet resistance it is easier to tune the voltage drops based on the thickness of the platinum on the membrane surface.

5.2.1 Sheet Resistance

The resistance of a conductive thin film is typically referred to as a sheet resistance in units of “Ohms per Square” $\Omega/\square$. Sheet resistance is established by the equation:

$$R = \rho \frac{L_{tf}}{A_{cs}}$$

(5.3)

where $R_{tf}$ is the thin film resistance, $\rho_{tf}$ is the thin film resistivity, $L_{tf}$ is the length of the thin film, and the cross sectional area, $A_{cs}$, can be represented by the thin film thickness, $t_{tf}$, and the electrode width, $W_{tf}$.

$$R_{tf} = \rho_{tf} \frac{L_{tf}}{t_{tf}W_{tf}}$$

(5.4)

From this equation the width and length can be expressed as a ratio, $L_{tf}/W_{tf}$, which represents the number of squares, $\square$. The ratio $\rho_{tf}/t_{tf}$ then becomes the sheet resistance in units of $\Omega/\square$. The bulk resistivity can, if necessary, be recovered from the sheet resistance if the thickness of the material is known.

For the femtopump devices there are three dependent parameters that can be varied: thickness, conductivity (or $1/\rho$) and porosity. The SiCCWells were designed to push the boundary between porosity and conductance to prevent unnecessary voltage drops. In
contrast, the gradient generator fabrication reduces the conductivity to control the voltage drops based exclusively on the geometry of the conduction lines.

### 5.2.2 $V_{\text{app}}$ determination

The applied voltage is determined by using National Instruments MultiSIM to model the circuit diagram with assumed values for areas that could not be directly experimentally determined (i.e. the resistance and capacitance of the electric double layer).

An annotated representation of the circuit diagram of the gradient generator can be seen in Figure 5.1. The connections with the phosphate buffered saline for the reference and generation electrodes are modeled as shown in Figure 5.2.

A transient analysis was run in the simulation to view the current and voltages across the active channels during the electro-osmotic pumping.
5.3 Methods

Testing of the electro-osmotic gradient generator was initiated by vacuum priming the nanoporous membrane at the pumping sites. After this was done, samples were examined for their ability to generate a series of flow models in addition to simply providing a linear gradient from the geometry of the generator.

5.3.1 Vacuum Priming

The gradient generators, as with many electro-osmotic devices require a vacuum priming step. While electrowetting was possible, the rapid change in resistance as the membrane is wetted can cause uncontrolled current exchange resulting in electrolysis. Vacuum priming was conducted by preparing dilutions of ethanol in phosphate buffered saline (PBS). The dilutions ranged from 75% to 0% ethanol in steps of 25%. The ethanol dilutions were introduced to the upper (gradient) channel via syringe pump at 50 \( \mu \text{L.s}^{-1} \) while a vacuum of 15 in.Hg. was held in the lower (feeder) channel. Each dilution was vacuum primed for 300 seconds.
Figure 5.3: Polarization of bulk PBS causes “recruitment” of adjacent electrodes which resulted in an associated flow across inactive channels.

Figure 5.4: Grounded reference electrode resulted in a circuit which can discharge the PBS polarization without inducing appreciable flow.

5.3.2 Geometric Gradient Generation

Samples for a simple geometric gradient generator were wired to a prototyping board. The positive leads of the generator were connected to a 6 volt supply of a modular power supply (Agilent E3681A). The negative electrode was connected directly to the negative terminal of the power supply. The reference electrode was connected to the negative terminal of a 6V potentiostat with the positive terminal being attached to the positive leads of the gradient generator. Another configuration was connecting the reference electrode to ground.

In Figure 5.3 the diagram shows the charging of the phosphate buffered saline that was in contact with the activation site. This results in a potential across adjacent activation sites (as well as the reference) through the conductive fluid. This potential, though lower than the activation site, was expected to produce a flow across the membrane. The previ-
ously discussed grounded reference electrode prevents flow between activation electrodes of differing voltage potentials. This concept is shown in Figure 5.4. Once the potentiostat or ground is attached to the reference electrode it adjusts the voltage to provide a small but oppositely charge field, which, geometrically prevents the flow through adjacent activation sites.

For the purpose of the current experimental setup, it was reasoned that a grounded connection was capable of serving the same purpose as a potentiostat connection. In the event the potentiostat was used it was set to maintain a voltage-driven current towards the reference electrode (1 nanoamp).

The gradient solution was introduced to the feeder channel at a rate of 10 µl·s⁻¹ via syringe pump. Fluid was drive by a negative pressure bias on the draw syringe in order to limit pressure based flow. The upper channel was filled with phosphate buffered saline at a rate of 1 µl·s⁻¹.

5.3.3 Fluid Delivery

A variable power source was wired in series to an ammeter and a voltage controlled output was slowly increased (≈ 3V/min) and the channels imaged for gradient generation using a Leica microscope at a 10x magnification. The voltage output was increased to drive a 100 µA current across the membrane. Video was taken using a green filter to image the dilutions of fluoresein as they enter the primary channel of the gradient generator.

5.4 Results

The gradient generators are an ongoing project which has not resolved a distinct and discrete gradient. There are several factors that are believed to be at play, the most likely was the inability to reliably image the surface of the device and detect flowing concentrations of fluids.

Several experimental runs have resulted in ambiguous results in which the presence or absence of a gradient was not obvious. Such an example is shown in Figure 5.5. As seen in
Figure 5.5: Gradient Generator *in situ*. Blue and Red lines correspond to spectra values in previous image.

in this image the presence of a gradient-like intensity is only able to be detected during post processing. A mild $\frac{1}{x}$ relationship can be extrapolated from the data however, it is still not conclusive as all activation channels were not functional (6, 7 were not activated and 8, 9, 10 did not show resistance) and background fluorescence was a significant problem.

Data analysis using imageJ (FIJI) was performed. The gray scale intensity of fluorescence across the electrodes and the conduction lines directly adjacent were analyzed. The results of this analysis can be seen in Figure 5.6.

It is expected that these results will have to be verified on a pristine device with post-processing planned for *a priori* as data has been lost due to the method of collection.

### 5.5 Discussion

The gradient generator was tested using orthogonally flowing fluids coupled with an electro-osmotic pump fabricated from a nanoporous aluminum oxide membrane. The pumps were fabricated in such a way that the sheet resistance of the conduction lines would provide a native geometric ($\frac{1}{x}$) gradient.

The flow can also be varied in a point-to-point style by varying the input impedance. The system is limited in the “shape” of the gradient created as the current model has 10 electrode positions. In the event that a complex shape is desired it would require more
Figure 5.6: Intensity Spectrum from FITC gradient. Red line is the spectrum across the activated electrodes. Blue line is spectrum across the conductive lines directly above. Spectra were taken with respect to the lines shown in Figure 5.5.
electrodes. The effects of diffusion and convection in the short distances between electrode contacts are believed to “smooth over” the discontinuities in the fluid flows.

5.5.1 Geometric Gradient

The geometric gradient generation was tested experimentally and in simulation. In simulation there were significantly more assumptions that needed to be made in terms of the properties of the device. The effects of the electric double layer were, again, considered a hindrance to the expected behavior of the gradient generator but no more so that in any of the other devices previously presented.

Initial efforts to generate a geometric gradient were unsuccessful because of a short across the electrodes. This led to the path of lowest resistance being through the first channels. Increasing the voltage resulted in electrolysis. The electrolysis was seen to increase sequentially through channels 1 - 3 as the voltage was increased.

It became necessary to supplement the geometric gradient resistances as the contact resistance between the electrodes and the electrolyte became the dominant resistance in the equation. Voltage drops are typically calculated via the ratio of the resistors which can be expressed as

\[ V_{drop} = \Phi_R \cdot V_{App} \]  

(5.5)

where

\[ \Phi_R = \frac{R_n}{R_{tot}} \]

and

\[ V_n = V_{App} - V_{drop} \]

where, \( V_{drop} \) is the voltage drop, \( \Phi_R \) is the ratio of channel resistance to total resistance, \( R_n \) is the resistance of channel \( n \), \( V_n \) is the voltage immediately after the resistance of channel \( n \) with respect to 0, and \( V_{App} \) is the voltage applied. \( R_{tot} \) is the total resistance given by Ohm’s law.

Figure 5.7 show the relationship of the ratio \( \Phi_R \) with respect to the resistance across

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Figure 5.7: The effects of membrane resistance on the ratio $\Phi_R$ from equation set 5.5. Dashed lines represent the values of the resistance of the channels $R_1$ and $R_{10}$.

The viability of a single, on-membrane, resistance network to provide for gradient generation is likely impractical due to variations in the membrane resistances. These resistances can be caused by variations in electrolyte dilution and alumina surface charge. These, however, can be accommodated through integrated circuitry on the chip directly adjacent to the membrane.

### 5.5.2 Arbitrary Gradients

Arbitrary gradients maybe generated by using a series of potentiometers. Because the devices were designed to exploit the sheet resistance of nanoporous platinum conduction lines to vary the fluid flow between electrodes it was necessary to customize additional resistances in such a way that the effects of the geometric generator do not dominate the
Figure 5.8: Values for potentiometers for linear gradient generation. Inset shows the regime where the membrane resistance is equal to that of one of the native resistances of the electrodes.

trials. To do this, potentiometers can be used to “dial-in” calculated resistances. Using the equations from above and experimental impedance values of the membrane resistance the value of the resistance to be added in series can be identified. Some sample values are presented in Figure 5.8.

Some variability in the gradients is still expected due to the dependence of the incremental calculation on the resistance of the first electrode resistance. As shown in Figure 5.9 the maximum value of $\Delta \Phi_{R_{j,k}} \ (j, k = N; j \neq k)$ does not occur at the same membrane resistance for each channel. For simplicity, the incremental adjustment is based off of $R_1$.

5.5.3 Membrane color variance, contrast

Flow was not seen in the geometric generator when the gradient fluid was dye laden phosphate buffered saline. As shown with previous femtopump experiments, the limitations of the available imaging systems do not allow for high enough contrast to discern dye laden fluid on top of the alumina membranes. This was the original motivation for reducing the contamination of the membranes by dyes in the photoresist. S-1813 and SPR-220 are both
Figure 5.9: a. The difference between the ratio (with respect to total membrane resistance) of $R_j$ and $R_k$ as a function of $R_{memb}$. b. The magnitude of the difference between the ratio (with respect to total resistance) of $R_1$ and $R_N$ ($N \neq 1$).

distinctly red in color. It has been noted that, in the gradient generation region, it is possible to expose the membrane to very weak dilutions of commercially available bleach. This does, however, pose a potential danger to the platinum, silver, and alumina and as such has not been fully explored at the time of this writing.

5.5.4 The Voltage-Dependent Electric Double Layer

The simulated models can be found in Appendix C with the annotated model in Section 5.2.2. The model breaks down where the model is needed most, at the voltage dependent electric double layer. As previously mentioned the Frumkin equation is used to show the current due to the charge transfer reaction. In the case of a perfect conductor, the model of the electric double layer would have a resistor and capacitor in parallel but with a resistance approaching 0. In this case there would be no voltage drops across the electric double layer and $V_{app}$ would be the voltage held at each activation electrode across the nanopores. In the opposite case, as the resistance goes to infinity, no charge transfer would occur and the
voltage across the nanopores would be zero.

The resistivity of the nanoporous platinum is exploited to provide a geometric gradient explicity from the geometry of the electrodes. This may increase the effects of the electric double layer resistance by decreasing the effective current flow through the platinum. In this manner, as the thickness of the platinum decreases, the resistance of the platinum may have a significant effect on the resistance of the electric double layer. This phenomena will require considerable work on a total model of electric fields on nanoporous membranes at electrolyte interfaces in order to be fully defined.

The immobility of charge causes the electric double layer to form on dielectric materials. In the case of conductive materials, it is still possible to develop an electric double layer. However, the electric double layer in this case is governed by the voltage, the resistivities of the electrolyte and electrodes, and finally mass transport of the ion species across the electric double layer \cite{106}. It is expected that this can be modeled and a total simulation of thin film metalized ammodized alumina membranes for electro-osmotic flow provided. However, this requires a significantly more complex model of the electric double layer which is expected to be revisited in future work.

5.6 Conclusions

The electro-osmotic gradient generator is a novel solution for delivering a chemical gradient to cells \textit{in vitro}. The start-stop ability of the gradient generator gives it an inherent advantage over devices that require gradients be continually delivered while establishing fully developed flow. The gradient generator will proceed to cellular experimentation to test the effects of anti-migratory candidates against aggressive types of invasive cancers. The expectation is that the capacity to generate a wide variety of flows will result in crucial information regarding anti-metastasis drugs.
Chapter 6
CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

The SiCCWell platform is a powerful new MicroTAS platform for cell capture, cell culture, and analysis of cellular processes. The group of devices presented can be further expanded upon by refining the techniques of micro- and nanofabrication by exploiting processes already in place for the semiconductor and electronics industries.

As presented, the core criteria for an integrated stand-alone microTAS system are:

- Single cell chemical delivery
- Continuous (or near continuous) single cell monitoring
- Long term cell or cell culture viability
- Discretization of cells or cell groups

Many back end or down stream technologies exist for the amplification (PCR), detection, and manipulation (microfluidics, surface modification, etcetera) of biomolecules. However, to the best of this author’s knowledge, no single technology exists that can properly carry out these tasks for the individual cell. The SiCCWell and Gradient Generator are tools that address this problem directly. The SiCCWell has demonstrated the potential for Cell isolation through vacuum cell seeding, manipulating the microenvironment through electro-osmotic bottom-up pumping, sampling the microenvironment through electro-osmotic top-
to-bottom pumping, and the design and materials support long term cell culture experimentation.

This chapter will explore some of the proposed morphisms and optimizations of the femtopump technology. Many of the applications are limited by fabrication techniques. The largest issue restricting the ability to reduce feature sizes is the ability to then interface with those features. For example, the silver epoxy method would need to be either altered or replaced with a more precise method of bonding electronics.

6.2 SiCCWell One - Single Cell Delivery

Theoretically, the ability to scale the SiCCWell down to the individual cell is strictly an issue of fabrication. The method of lamination of the original SiCCWell does not lend itself to the ability to align the microwell with the electrode surface. The reduction of the electrode area to accommodate fluid delivery to only a single cell also results in technical hurdles regarding the sheet resistance of the electrode channels. Designs for the SiCCWell One adopt the serial bus arrangement mentioned in Section 3.6 to reduce current resistance across the conduction lines.

A mockup of what this might look like is shown in Figure 6.1. The bus is represented as the blue lines bordering the activation regions. These would be molded in to the insulation layer shown in the insert.

The SiCCWell One presented in the image has a pitch of 62.5 microns which is a reduction in cellular density from the current device. This still allows for a density of 256 delivery regions per square millimeter. These sites are, however, independently addressable for both delivery and sampling. For a 1 cm$^2$ region, 25600 active sites are available for continuous monitoring. At this point, the limitations would be largely dependent on packaging as 80 lead wires would be required from each side of the device. Our current CAD systems will not allow us to render this level of complexity.
Figure 6.1: Possible candidate for the SiCCWell One. Blue lines represent highly conductive silvered bus.
6.3 SiNCNet - Single Neuron Activation

The Single Neural Cell Network, or SiNCNet is a near future design for the SiCCWell technology. The fabrication parameters for the SiNCNet vary only slightly but may require a move to a different mechanism of insulation attachment. Dr. Malkoc of our group has previous presented a micropatterned neural network device. The results of this work can, briefly, be seen in Figure 6.2. This device utilizes vacuum seeded neural cells and dendritic guides to form active neural connections. It is the intent of our group to exploit this work by applying the neuronal network guides directly over a patterned electro-osmotic membrane.

This device is replicated in the design shown in Figure 6.3. In this design, an electrode is placed beneath each cell in the network. This would allow for the delivery and sampling from neural cells after a neural network has formed or to encourage the network to form in a particular manner.

In this device the cellular density is further reduced from that of the SiCCWell One. However, in the case of neural networks it is a question of quality over quantity. The cellular density in the current design is 100 cells per mm² with a dendritic guide of 175 µm.

This design is expected to significantly benefit from improvements in packaging and automated fabrication. The electrode density is currently designed to be wired by hand which makes it significantly inefficient. A standard cell culture plate (6, 12, 24, etc. well)
is roughly $109 \text{ cm}^2$. It is anticipated that with a 12.5% packaging density the SiNCNet would be able to maintain over 300 neural networks with 64 individually addressed cells per network.

The SiNCNet is expected to be a particularly difficult device to implement due to the tempermental nature of synapse forming neural cells. However, it is also expected that the SiNCNet can be tested and multiplexed in such a way to provide novel and valuable information on the behavior of neural networks.

### 6.4 Non-biological Applications

The use of nanoporous alumina within electro-osmotic pumps for biological applications represents a small part of the utility of anodized alumina. In the past 20 years the material has seen significant interest in the development of gas sensors. The high surface area of the nanoporous structure provides for a large area with which gases can react. Additionally, the alumina is relatively stable in the presence of gases [118].

Within the scope of our technology there is a potential application within gas sensors that are partitioned in to several sensors on the same “chip”. The micropatterning system
outlined would allow for the fabrication of a multi-gas, or high-sensitivity array of sensors fabricated from the same membrane. The microfluidic systems could allow for the control and manipulation of gas flows to sensor surfaces.

The most readily adaptable sensor to the femtopump technology would use patterned membranes for the development of a multistage humidity sensor. Current sensors use a single frequency that is tuned to a difference in humidity. This sensor can be changed dynamically to accommodate different frequency impedance responses [119]. However, with the femtopump technology it would be possible to directly use a wide array of frequencies without the need for frequency modulation which would provide an immediate reading with higher precision.

Both gas flow in microfluidics and nanoporous sensors fabricated from anodized alumina are of significant interest in research presently. As such, it is an optimal opportunity to expand the femtopump technology into additional fields. These studies would likely not be directly engaged by our research group but rather developed by a collaborator with more knowledge of the field.

6.5 Contributions to the Field

Contained within this work are several contributions to fabrication of microfluidic and electro-osmotic fluidic devices. This section will discuss the value of several of those contributions and how they are believed to be further adapted to increase their value.

6.5.1 Fabrication and Methods

The fabrication protocols used in the development of the SiCCWells were driven by the need for precise microfabrication methods on a relatively novel substrate. Both in chemistry and mechanics, nanoporous alumina is significantly different from traditional microfabricated substrates. This is further complicated with the addition of a conductive layer to the alumina membrane. As such, the methods outlined in this paper provide valuable information into the development of other technologies facing similar fabrication hurdles.
Figure 6.4: PVA Spin coating for nanopore blocking  

a. PVA solution is applied to the surface of the nanoporous membrane. A combination of surface tension and viscosity prevents full pore wetting.  
b. PVA is spun at a high speed until only a thin film remains in contact with the surface of the alumina.  
c. Samples are baked to remove water which further reduces PVA layer thickness.  
d. Photoresist is applied normally

### 6.5.2 PVA pore blocking

The influence of the polyvinyl alcohol based reversible pore blocking is of considerable value to small laboratories without access to large scale manufacturing systems. Many commercial systems use laminate photoresist which is capable of “tenting” or “tent-and-etch” which is a procedure for selective closing of an open area of a microelectromechanical system. However, laminate photoresists are not always accessible, require very specific developers, likely result in scumming of the surface during liftoff, and can reduce resolution.

The PVA method has been able to provide for a stable adhesive layer for hydrophobic photo-resists without using a surface modification. The viscosity of the solution is a benefit as it prevents the PVA solution from saturating the nanopores. The high speed spin of the PVA solution removes a majority of the PVA from the surface of the alumina, as shown in Figure 6.4.
The method for the PVA pore blocking is further outlined in Section 2.4.1. The full profile of the PVA blocking system has not been fully developed at the time of this writing but it is expected to be of significant utility to researchers working with nanoporous materials; particularly anodized alumina.

6.5.3 PDMS bonding to porous substrates

PDMS bonding to porous substrates has been a challenge. While adhering PDMS to alumina is not a significant difficulty, doing so while maintaining the integrity of a micropattern is a challenge. To address this we attempted many bonding techniques. Plasma bonding was inappropriate due to the potential damage to the platinum thin films. Pressure and vacuum appeared to work best. In Figure 6.5 a micrograph of the fluidic channels can be seen in which the alumina membrane has been etched away with piranha bath. A magnified view of regions associated with vacuum sealing are shown in the insets of Figure 6.5b. This is thought to be the negative of the features of the membrane which are shown in Figure 6.5b.
6.5.4 Vacuum Liftoff

At the time of writing this, we are unaware of any other references to a vacuum based liftoff. The utility of this step less broad than that of the PVA blocking layer. However, it is thought that this has become a crucial step in both reducing redeposition and membrane contamination. Contamination is a major issue with any porous media but especially for nanoporous alumina. Our vacuum based liftoff is well defined and can be implemented in a series of designs with a variety of porous materials.

6.5.5 Electro-osmotic pumping

Electro-osmotic pumping continues to be looked to as a viable mechanism for the control of small volumes of fluid. Within this work the fabrication of a “femto-pump” has been described which looks at a patterned electro-osmotic pump which allows for the precision of electro-osmosis to be combined with a very small area. The result of this is small fluid volumes that can be regionally delivered to a sample or set of samples. This represents a viable and functional application of electro-osmotic pumps that is directly in contact with the cell.

6.6 Concluding Remarks

The development of new and appreciably novel devices can be a trying path. The ground work to provide accessibility to new regions of research is a major contribution of this document. The femtopump work rarely ever followed the most optimistic path. However, novel solutions have been developed to overcome the hurdles encountered. As such we have been able to outline novel fabrication steps, gather data on exciting new technology, and ultimately pave the way to the next, even more advanced, generation of technological devices. It is our earnest hope that the work that is encompassed provides, not a conclusion, but the start of a road map which will lead us to continue our work. The single cell interface, the chemotaxic gradient, the massive multiplexed humidity sensor, and any other problems we find ourselves solving will become the building blocks for the next generation
of biomedical engineers.


[28] DNA amplification by polymerase chain reaction.


[40] LEMI. Lemi: Laboratory for energy and microsystems innovation at mit — research: Electrokinetics.


[49] Lewis M. Agarose gel electrophoresis (basic method).


[72] Orphan drug act.


[102] Life Technologies. *Live-Dead Viability-Cytotoxicity Kit *for mammalian cell*.*.

[103] Life Technologies. Dapi nucleic acid stain — life technologies.


[118] Ivan K. Schuller. Schuller nanoscience group.


[125] Sivhola A. *Electromagnetic mixing formulas and applications.*


[156] Pei H, Allison S, Xin Y. Electrophoresis of spheres with uniform zeta potential in a gel modeled as an effective medium. 313:328 – 337.


[164] Lee Y Wang W, Chan Y. Biased reptation model with electroosmosis for dna electrophoresis in microchannels with a sub-micron pillar array. 21(085031):1 – 11.

[165] Li D Movahed S. Electrophoretic transport through the nanopores in cell membrane during electroporation. 369:442 – 452.


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[199] Make it from alumina (aluminum oxide).

Appendix A
MATLAB Codes for Resistances and Effective Voltages

A.1 Recursion Equations for Resistances

clear
clc
close all

Rm=2;
Rt=2;
Rbox=2;

n=5;

if n==1
    A=Rt+Rm;
    y=A;
else
    A=Rm+Rbox;
    for i=1:n-1
        Atemp=Rbox+1/((1/Rm)+(1/A));
        A=Atemp;
    end
    y=Rt+A-Rbox
end
end
A.2 Voltage drops for resistance Ladder of N-rungs

clear
clc

% Script to analyze voltage drops across Alumina membrane for SiCCWells

%% define box side
box = 100;

%% define ohms per square
R_box = 100;

%% Define Channel Lengths
% Define Straight section separately
L_channel = 12500-1850;
L_channel_TC = L_channel + 1500;

L_channel_TC1 = L_channel + 1400;
L_channel_TC2 = L_channel + 2300;
L_channel_TC3 = L_channel + 3200;
L_channel_TC4 = L_channel + 4100;
L_channel_TC5 = L_channel + 5000;
L_channel_TC6 = L_channel + 5900;
L_channel_TC7 = L_channel + 6800;
L_channel_TC8 = L_channel + 7700;
L_channel_TC9 = L_channel + 8600;

% Normalize to Box size
Corners = 3;

M = L_channel_TC/box;
M1 = L_channel_TC1/box - Corners;
M2 = L_channel_TC2/box - Corners;
M3 = L_channel_TC3/box - Corners;
M4 = L_channel_TC4/box - Corners;
M5 = L_channel_TC5/box - Corners;
M6 = L_channel_TC6/box - Corners;
M7 = L_channel_TC7/box - Corners;
M8 = L_channel_TC8/box - Corners;
M9 = L_channel_TC9/box - Corners;

R_mem = 100000;

R_CTC = M*R_box;
R_C1 = M1*R_box + Corners*R_box*0.33;
R_C2 = M2*R_box + Corners*R_box*0.33;
R_C3 = M3*R_box + Corners*R_box*0.33;
R_C4 = M4*R_box + Corners*R_box*0.33;
R_C5 = M5*R_box + Corners*R_box*0.33;
R_C6 = M6*R_box + Corners*R_box*0.33;
R_C7 = M7*R_box + Corners*R_box*0.33;
R_C8 = M8*R_box + Corners*R_box*0.33;
R_C9 = M9*R_box + Corners*R_box*0.33;

%experimental
R_C(1) = M*R_box;
R_C(2) = M1*R_box + Corners*R_box*0.33;
R_C(3) = M2*R_box + Corners*R_box*0.33;
R_C(4) = M3*R_box + Corners*R_box*0.33;
R_C(5) = M4*R_box + Corners*R_box*0.33;
R_C(6) = M5*R_box + Corners*R_box*0.33;
R_C(7) = M6*R_box + Corners*R_box*0.33;
R_C(8) = M7*R_box + Corners*R_box*0.33;
R_C(9) = M8*R_box + Corners*R_box*0.33;
R_C(10) = M9*R_box + Corners*R_box*0.33;

% Channel TC at position

%% Set Loop Variables
Inc = 3;

V_app = 6;

% N=0

%% Calculate Voltage Array
for N=0:9
    V_0(N+1) = ((R_mem*R_box*Inc)/(R_mem+R_box*Inc)) \* (V_app/R_CTC)*(((R_mem)/(R_mem + R_box*Inc))^N);
end

V_0

%experimental
for A=1:10
    for N=0:9

\[ V_{1,0}(N+1,A) = \left( \frac{R_{mem}R_{box}\text{Inc}}{R_{mem}+R_{box}\text{Inc}} \right) \times \frac{V_{app}}{R_C(A)} \times \left( \frac{(R_{mem})}{(R_{mem} + R_{box}\text{Inc})^N} \right); \]

\[ \text{end} \]

\[ \text{end} \]

\[ V_{1,0} \]

\[ V_{1,0}.' \] %transpose

%% Plot Voltage vs Distance

\[ \text{plot}(0:400:3600,V_0) \]
\[ \text{xlabel('Distance (Microns)')} \]
\[ \text{ylabel('Voltage (Volts)')} \]
\[ \text{title('linear')} \]

\[ \text{figure} \]
\[ \text{semilogx}(0:400:3600,V_0) \]
\[ \text{xlabel('Distance (Microns)')} \]
\[ \text{ylabel('Voltage (Volts)')} \]
\[ \text{title('logx')} \]

\[ \text{figure} \]
\[ \text{semilogy}(0:400:3600,V_0) \]
\[ \text{xlabel('Distance (Microns)')} \]
\[ \text{ylabel('Voltage (Volts)')} \]
\[ \text{title('logy')} \]

\[ \text{figure} \]
\[ \text{plot}(0:400:3600,V_{1,0}) \]
\[ \text{xlabel('Distance (Microns)')} \]
\[ \text{ylabel('Voltage (Volts)')} \]
\[ \text{title('combined')} \]
Appendix B
MATLAB CODE FOR PORE IDENTIFICATION AND DIAMETER MEASUREMENT

Any ellipses "..." must be removed and code re-joined in editor for code to be functional

clc
clear
close all

% Define symbolic

syms grayThresh TopThresh mudist1 mudist2 mudist3 mudist4 ...
graythresh1 graythresh2
% Code adapted from MatLab Sample code for identification ...
of circular objects and measurement

%%
%Image to compare

Image1 = imread('C:\Users\Jeremiah\Dropbox\Hansford Lab Group Folder ...
\Jeremiah Data\Comparison Images\schley\Pore Comparison...
\Presilvered\Device 2\Si-S-1813 PVA Grad site205.tif');

Image2 = imread('C:\Users\Jeremiah\Dropbox\Hansford Lab Group...
Folder\Jeremiah Data\Comparison Images\schley\Pore Comparison ...
\Post Silvered\Device 2\Device 2 Site 205.tif');
% grayImage2 = imread('C:\Users\Jeremiah\Dropbox\Hansford Lab Group...
Folder\Jeremiah Data\Comparison Images\schley\Pore Comparison...
\Presilvered\Device 2\Si-S-1813 PVA Grad site206.tif');

% Crop out the SEM information
grayImage1 = imcrop(Image1, [0,0,1030,700]);
grayImage2 = imcrop(Image2, [0,0,1030,700]);

% Collect graythresh data for each image

grayThresh1 = graythresh(Image1);
grayThresh2 = graythresh(Image2);

% Set the threshold for everything after section II there...
% are several CASE SENSITIVE choices:
% muNorm1 - muNorm4 is the threshold set to the curve fit...
% of the % differences between both images,
% grayThresh will compute the optimal % threshold for the...
% image (recommended)
% graythresh1 will use Image1’s optimal threshold for all
% graythresh2 will use Image2’s optimal threshold for all
% TopThresh will use the threshold that provides maximum ...
% difference % between the two images (not recommended)

setthresh = grayThresh;

clearvars -except Image1 grayImage1 Image2 grayImage2 ...
grayThresh1 grayThresh2 setthresh grayThresh dotscale ...

mudist1 mudist2 mudist3 mudist4 TopThresh graythresh1 graythresh2

[rows columns numberOfColorBands] = size(grayImage1);

%% Section II: Finding optimum difference threshold between two SEMS
for n = 1:256

threshold = ((1/255))*(n-1);

binaryImage = im2bw(grayImage1, threshold);

numberOfBlackPixels = sum(sum(binaryImage == 0));
numberOfWhitePixels = sum(sum(binaryImage));
totalNumberOfPixels = rows * columns;

[rows columns numberOfColorBands] = size(grayImage2);

% Scale x axis manually

145
binaryImage2 = im2bw(grayImage2, threshold);

numberOfBlackPixels2 = sum(sum(binaryImage2 == 0));
numberOfWhitePixels2 = sum(sum(binaryImage2));
totalNumberOfPixels2 = rows * columns;
percentBlackPixels2 = 100.0 * numberOfBlackPixels2 / totalNumberOfPixels2;
percentWhitePixels2 = 100.0 * numberOfWhitePixels2 / totalNumberOfPixels2;

PercentChange(n) = (numberOfBlackPixels - ... 
   numberOfBlackPixels2)/totalNumberOfPixels;
end

% Properties of Loop for reference later

xlim([0 255]);
[maxdiff, maxi] = max(PercentChange);
Topthresh = (maxi-1)/255;

Disttype = 'gauss1';

% Screwing with Numbers!
Xzero = find(PercentChange>0, 1, 'last');
Atozero = PercentChange(:,1:Xzero);
LAt0zero = length(Atozero);
Xall = [0:length(PercentChange)-1];
Xdiff = length(Xall)-length(Atozero);

% Correction Index to 0 <-- The histogram should run from 0 to whatever but
% the indices run from 1 to whatever + 1
CLAtoYzero = LAt0zero - 1;
Xrange = 1:LAt0zero;
CXrange = 0:CLAtoYzero;

% Curve Fitting and define curve parameters

FitDist = fit(CXrange.', Atozero.', 'gauss1');
mu1 = FitDist.b1;
muDist1 = mu1/255;
FitDist2 = fit(CXrange.', Atozero.', 'gauss2');
mu2 = FitDist2.b1;
muDist2 = mu2/255;
FitDist3 = fit(CXrange.', Atozero.', 'gauss3');
mu3 = FitDist3.b1;
muDist3 = mu1/255;
FitDist4 = fit(CXrange.', Atozero.', 'gauss4');
mu4 = FitDist4.b1;
muDist4 = mu4/255;

% Figures for plotting
close all

% Distribution of differences with respect to threshold
figure;
plot(FitDist, Xall, PercentChange)
line([maxi maxi], [0 maxdiff])
hold on
plot(FitDist2)
plot(FitDist3)
plot(FitDist4)
hold off

% Original Image Cropped
figure;
h1 = subplot(2, 3, 1);
imshow(grayImage1, []);
title('Preprocessed Image', 'FontSize', 12);

% Histogram of Original
[pixelCount grayLevels] = imhist(grayImage1);
h2 = subplot(2, 3, 2);
bar(pixelCount);
title('Histogram of Preprocessed Sample Image', 'FontSize', 12);
xlim([0 grayLevels(end)])
ylim([0 max(pixelCount)])
set(gca, 'Ytick', [])

% Define Threshold
if setthresh == grayThresh;
    GrayThresh1 = grayThresh1;
elseif setthresh == mudist1;
    GrayThresh1 = muDist1;
elseif setthresh == mudist2;
    GrayThresh1 = muDist2;
elseif setthresh == mudist3;
    GrayThresh1 = muDist3;
elseif setthresh == mudist4;
    GrayThresh1 = muDist4;
elseif setthresh == TopThresh;
    GrayThresh1 = Topthresh;
elseif setthresh == graythresh1;
    GrayThresh1 = grayThresh1;
elseif setthresh == graythresh2;
    GrayThresh1 = grayThresh2;
end

GrayThresh1
% Separate image to binary based on optimal threshold
binaryImage = im2bw(grayImage1, GrayThresh1);

% Show threshold image
h3 = subplot(2, 3, 3);
imshow(binaryImage, []);
title('Binary Preprocessed Image', 'FontSize', 12);

numberOfBlackPixels = sum(sum(binaryImage == 0));
numberOfWhitePixels = sum(sum(binaryImage));
totalNumberOfPixels = rows * columns;
percentBlackPixels = 100.0 * numberOfBlackPixels / totalNumberOfPixels;
percentWhitePixels = 100.0 * numberOfWhitePixels / totalNumberOfPixels;
message = sprintf('Total number of pixels = %d
Black pixels ...'
     = 'd = %.1f%%\nWhite pixels = %d = %.1f%%', totalNumberOfPixels,
     numberOfBlackPixels, percentBlackPixels, numberOfWhitePixels, ...
     percentWhitePixels);
% msgbox(message);
%
[rows columns numberOfColorBands] = size(grayImage2);

% Original of second image
h4 = subplot(2, 3, 4);
imshow(grayImage2, []);
title('Postsilvered Grayscale Image', 'FontSize', 12);

% Histogram of second image
[pixelCount2 grayLevels] = imhist(grayImage2);
h5 = subplot(2, 3, 5);
bar(pixelCount2);
title('Histogram of original image', 'FontSize', 12);
xlim([0 grayLevels(end)]);
ylim([0 max(pixelCount)]);
set(gca, 'Ytick',[])

% Define Second image threshold levels
if setthresh == grayThresh;
GrayThresh2 = grayThresh2;
elseif setthresh == mudist1;
    GrayThresh2 = muDist1;
elseif setthresh == mudist2;
    GrayThresh2 = muDist2;
elseif setthresh == mudist3;
    GrayThresh2 = muDist3;
elseif setthresh == mudist4;
    GrayThresh2 = muDist4;
elseif setthresh == TopThresh;
    GrayThresh2 = Topthresh;
elseif setthresh == graythresh1;
    GrayThresh2 = grayThresh1;
elseif setthresh == graythresh2;
    GrayThresh2 = grayThresh2;
end

% Threshold second image
binaryImage2 = im2bw(grayImage2, GrayThresh2);

% Show threshold of second image
h6 = subplot(2, 3, 6);
imshow(binaryImage2, []);
title('Binary Image', 'FontSize', 12);

%% Calculate a whole bunch of values to report
numberOfBlackPixels2 = sum(sum(binaryImage2 == 0));
numberOfWhitePixels2 = sum(sum(binaryImage2));
totalNumberOfPixels2 = rows * columns;
percentBlackPixels2 = 100.0 * numberOfBlackPixels2 / totalNumberOfPixels2;
percentWhitePixels2 = 100.0 * numberOfWhitePixels2 / totalNumberOfPixels2;
message2 = sprintf('Total number of pixels = %d
Black pixels = %.2f%%
White pixels = %.2f%%',
    totalNumberOfPixels2, percentBlackPixels2, percentWhitePixels2);
% msgbox(message);

%% Call the text boxes for data report
xl = xlim(h2);
xPos = xl(1) + diff(xl) / 2;
yl = ylim(h2);
yPos = (yl(1) + diff(yl)) * 8 / 9;
t = text(xPos, yPos, message, 'Parent', h2)
set(t, 'HorizontalAlignment', 'center');

x12 = xlim(h2);
xPos2 = x12(1) + diff(x12) / 2;
yl2 = ylim(h5);
yPos2 = (yl2(1) + diff(yl2)) * 8.95 / 10;
t2 = text(xPos2, yPos2, message2, 'Parent', h5)
set(t2, 'HorizontalAlignment', 'center');

%% Detect pore diameter for optimal

%clear everything we don't want to carry over

clearvars -except Image1 grayImage1 Image2 grayImage2 grayThresh1 ...
grayThresh2 setthresh grayThresh dotscale mudist1 mudist2 mudist3 ...
mudist4 muNorm1 muNorm2 muNorm3 muNorm4 TopThresh Topthresh graythresh1 ...
graythresh2 dotscale

% Input Values for automated pore diameter, effectively: dimension/pixel =
% mlength/npixel. For the standard SEM image mlength can be input as spot
% diameter and the npixels will remain the same.

npixels = 1030;
mlength = 3010;
dotscale = mlength/npixels;

%Define the threshold values again

%Threshold for first image

if setthresh == grayThresh;
    GrayThresh1 = grayThresh1;
elseif setthresh == mudist1;
    GrayThresh1 = muDist1;
elseif setthresh == mudist2;
    GrayThresh1 = muDist2;
elseif setthresh == mudist3;
    GrayThresh1 = muDist3;
elseif setthresh == mudist4;
    GrayThresh1 = muDist4;
elseif setthresh == TopThresh;
    GrayThresh1 = Topthresh;
elseif setthresh == graythresh1;
    GrayThresh1 = grayThresh1;
elseif setthresh == graythresh2;
    GrayThresh1 = grayThresh2;
end
figure;
A = grayImage1;

imshow(A)

h1 = subplot(2, 3, 1);
imshow(A)
title('Original Grayscale Image', 'FontSize', 12);

h2 = subplot(2, 3, 2);
threshold = 0.2
bw = im2bw(A, GrayThresh1);
imshow(bw)
title('Threshold Binary image', 'FontSize', 12);

%subplot(2, 3, 3)
se = strel('disk',5);
bw = imclose(bw, se);
imshow(bw)
title('Curve Close and Invert', 'FontSize', 12);

h3 = subplot(2, 3, 3);
bwinv = imcomplement(bw);
imshow(bwinv)
title('Curve Close and Invert', 'FontSize', 12);

h4 = subplot(2, 3, 4);
[B,L] = bwboundaries(bwinv,'noholes');
imshow(A)
title('Boundaries with Original', 'FontSize', 12);
hold on

for k = 1:length(B)
    boundary = B{k};
    plot(boundary(:,2), boundary(:,1), 'r', 'LineWidth', 1)
end

hold off

stats = regionprops(L,'Area','Centroid','EquivDiameter');
```
circthreshold = 0.94;

h5 = subplot(2, 3, 5);
imshow(A)
hold on
title('Display Pore Centers' , 'FontSize', 12);

% loop over the boundaries
for k = 1:length(B)

    % obtain (X,Y) boundary coordinates corresponding to label 'k'
    boundary = B{k};

    % compute a simple estimate of the object’s perimeter
    delta_sq = diff(boundary).^2;
    perimeter = sum(sqrt(sum(delta_sq,2)));

    % obtain the area calculation corresponding to label 'k'
    area = stats(k).Area;

    % Computer Diameter and export to array
    Diameter(k)= (stats(k).EquivDiameter)*dotscale;
    Centerx(k) = stats(k).Centroid(:,1);
    Centery(k) = stats(k).Centroid(:,2);
    aradii(k) = (stats(k).EquivDiameter)/2;

    % compute the roundness metric
    metric = 4*pi*area/perimeter^2;

    % display the results
    %metric_string = sprintf('%2.2f',metric);

    % mark objects above the threshold with a black circle
    if metric > threshold
        % centroid = stats(k).Centroid;
        % plot(centroid(1),centroid(2),'ko');
    end

    text(boundary(1,2)-35,boundary(1,1)+13,metric_string,'Color','y','FontSize',14,'FontWeight','bold');

end

%Transpose Stuff
```
CenterxT = Centerx';
CenteryT = Centery';

centerradii = ones(1, length(Centery)) * 0;
centerradiiT = centerradii';

% Plot Circles for pore locations
viscircles([[CenterxT, CenteryT], aradii, 'EdgeColor', 'g', ...
  'LineStyle', '-.', 'LineWidth', 0.5, 'DrawBackgroundCircle', 0);
viscircles([[CenterxT, CenteryT], centerradii, 'EdgeColor', 'w', ...
  'LineStyle', '+', 'LineWidth', 0.5);

hold off

Numberpores = length(Diameter)

Dmax = max(Diameter);
Dmin = min(Diameter);
Drange = Dmin:1:Dmax;
DiameterT = Diameter';
Dist = fitdist(DiameterT, 'normal')
sigma = Dist.sigma;
mu1 = Dist.mu;
Normplot = pdf(Dist, Drange);

Axsig1 = (mu1 - sigma):((mu1 + sigma);
Normplotsig = pdf(Dist, Axsig1);
histplop = hist(Diameter, 200);

h6 = subplot(2, 3, 6);
histfit(DiameterT, 100, 'normal');
Titleh6 = sprintf('Pore Distribution at %.3f threshold', GrayThresh1);
title(Titleh6, 'FontSize', 12);
hold on
  % patch(Axsig1, Normplotsig, 'r', 'LineStyle', 'none')
  % Fillinx = [Axsig1(1), Axsig1(length(Axsig1)), ...
  % Axsig1(length(Axsig1), Axsig1(1))]
  % Filliny = [Normplotsig(1 Normplotsig(length(Axsig1)), 0, 0]
  % patch(Fillinx, Filliny, 'r', 'LineStyle', 'none')
Lineymax = max(Normplot);
Lineymin = min(Normplot);
line([mu1 mu1], [Lineymax, 0])
% set(gcf, 'Position', get(0, 'Screensize')); % Maximize figure.
set(h6, 'YTick', []);
AvgD = mean(Diameter);
format short
message = sprintf('Pores = %.f
Avg. Diameter = %.2f
Std. Dev. = %.3f', Numberpores, AvgD, sigma);
x12 = xlim(h6);
xPos2 = (x12(1) + diff(x12))*9.75/10;
y12 = ylim(h6);
yPos2 = (y12(1) + diff(y12)) * 7.75 / 10;
t2 = text(xPos2, yPos2, message, 'Parent', h6, 'FontSize', ...
    8, 'HorizontalAlignment', 'right');
set(t2, 'HorizontalAlignment', 'right');

figure;
imshow(Image1)
viscircles([CenterxT, CenteryT], aradii, 'EdgeColor', 'g', ...
    'LineStyle', '-', 'LineWidth', 0.5, 'DrawBackgroundCircle', 0);
viscircles([CenterxT, CenteryT], centerradiiT, 'EdgeColor', 'w', ...
    'LineStyle', '+', 'LineWidth', 0.5);

%% Pore Diameter for second image
clearvars -except Image1 grayImage1 Image2 grayImage2 grayThresh1 ...
    grayThresh2 setthresh grayThresh dotscale mudist1 mudist2 mudist3 ...
    mudist4 muNorm1 muNorm2 muNorm3 muNorm4 TopThresh Topthresh ...
    graythresh1 graythresh2 dotscale

%Define Threshold valuues
%Threshold for second image

if setthresh == grayThresh;
    GrayThresh2 = grayThresh2;
elseif setthresh == mudist1;
    GrayThresh2 = muDist1;
elseif setthresh == mudist2;
    GrayThresh2 = muDist2;
elseif setthresh == mudist3;
    GrayThresh2 = muDist3;
elseif setthresh == mudist4;
    GrayThresh2 = muDist4;
elseif setthresh == TopThresh;
    GrayThresh2 = Topthresh;
elseif setthresh == graythresh1;
    GrayThresh2 = grayThresh1;
else
elseif setthresh == graythresh2;
    GrayThresh2 = grayThresh2;
end

figure;
A = grayImage2;

imshow(A)

h1 = subplot(2, 3, 1);
imshow(A)
title('Original Grayscale Image', 'FontSize', 12);

h2 = subplot(2, 3, 2);
threshold = 0.4
bw = im2bw(A, GrayThresh2);
imshow(bw)
title('Threshold Binary image', 'FontSize', 12);

h3 = subplot(2, 3, 3);
se = strel('disk', 5);
bw = imclose(bw, se);
imshow(bw)
title('Curve Close and Invert', 'FontSize', 12);

h4 = subplot(2, 3, 4);
[B, L] = bwboundaries(bwinv, 'noholes');
imshow(A)
title('Boundaries with Original', 'FontSize', 12);
hold on
for k = 1:length(B)
    boundary = B{k};
    plot(boundary(:,2), boundary(:,1), 'r', 'LineWidth', 1)
end
hold off

stats = regionprops(L, 'Area', 'Centroid', 'EquivDiameter');
cirthreshold = 0.94;

h5 = subplot(2, 3, 5);
imshow(A)
hold on
title('Display Pore Centers', 'FontSize', 12);

% loop over the boundaries
for k = 1:length(B)
    % obtain (X,Y) boundary coordinates corresponding to label 'k'
    boundary = B{k};
    % compute a simple estimate of the object's perimeter
    delta_sq = diff(boundary).^2;
    perimeter = sum(sqrt(sum(delta_sq, 2)));
    % obtain the area calculation corresponding to label 'k'
    area = stats(k).Area;
    % Computer Diameter and export to array
    Diameter(k) = (stats(k).EquivDiameter)*dotscale;
    Centerx(k) = stats(k).Centroid(:,1);
    Centery(k) = stats(k).Centroid(:,2);
    aradii(k) = (stats(k).EquivDiameter)/2;
end

% Transpose Stuff
CenterxT = Centerx';
CenteryT = Centery';

centerradii = ones(1, length(Centery))*0;
centerradiiT = centerradii';

% Plot Circles for pore locations
viscircles([CenterxT, CenteryT], aradii, 'EdgeColor', 'g', ...
    'LineStyle', '-', 'LineWidth', 0.5, 'DrawBackgroundCircle', 0);
viscircles([CenterxT, CenteryT], centerradiiT, 'EdgeColor', 'w', ...
    'LineStyle', '+', 'LineWidth', 0.5);

hold off

Numberpores = length(Diameter)
Dmax = max(Diameter);
Dmin = min(Diameter);
Drange = Dmin:1:Dmax;
DiameterT = Diameter';
Dist = fitdist(DiameterT, 'normal')
sigma = Dist.sigma;
mu1 = Dist.mu;
Normplot = pdf(Dist, Drange);

Axsig1 = (mu1-sigma):(mu1+sigma);
Normplotsig = pdf(Dist, Axsig1);
histplop = hist(Diameter, 200);

h6 = subplot(2, 3, 6);
histfit(DiameterT, 100, 'normal');

Titleh6 = sprintf('Pore Distribution at %.3f threshold', GrayThresh2);
title(Titleh6, 'FontSize', 12);
hold on
% patch(Axsig1, Normplotsig, 'r', 'LineStyle', 'none')
% Fillinx = [Axsig1(1) , Axsig1(length(Axsig1))], ...
% Axsig1(length(Axsig1)), Axsig1(1)]
% Filliny = [Normplotsig(1) Normplotsig(length(Axsig1)), 0, 0]
% patch(Fillinx, Filliny, 'r', 'LineStyle', 'none')
Lineymax = max(Normplot);
Lineymin = min(Normplot);
line([mu1 mu1],[Lineymax,0])
% set(gcf, 'Position', get(0,'Screensize')) ; % Maximize figure.
set(h6, 'YTick', [])
AvgD = mean(Diameter);

format short
message = sprintf('Pores = %.f
Avg. Diameter = %.2f
Std. Dev. ...
= %.3f', Numberpores, AvgD, sigma);
x12 = xlim(h6);
xPos2 = (x12(1) + diff(x12))*(9.75/10);
y12 = ylim(h6);
yPos2 = (y12(1) + diff(y12)) * 7.75 / 10;
t2 = text(xPos2, yPos2, message, 'Parent', h6, 'FontSize', 8, ...
'HorizontalAlignment', 'right');
set(t2, 'HorizontalAlignment', 'right');

figure;
imshow(Image2)
viscircles([CenterxT,CenteryT], aradii,'EdgeColor', 'g', ...
'LineStyle', '-.', 'LineWidth', 0.5, 'DrawBackgroundCircle',0);
viscircles([CenterxT,CenteryT], centerradiiT,'EdgeColor', ...
'w', 'LineStyle', '+','LineWidth',0.5);
Appendix C
Circuit Diagrams for Devices
C.1 Gradient Generator

C.1.1 Full Circuit Model

A model used for numerical simulation is presented in figures C.1 and C.2. Many of the resistors still have estimated values for the capacitance and resistance of phosphate buffered saline and the electric double layer effects.

C.1.2 Unit Model

A testable unit circuit model is shown in figure C.3. This model can be more rapidly analyzed for changes in the uncontrolled properties and their effect on the voltage and current drops across the electrode regions.
Figure C.1: General Circuit Diagram for Gradient Generator
Figure C.2: General Circuit Diagram for Gradient Generator (cont.)
Figure C.3: Unit Circuit Diagram for Gradient Generator