Micro-Patterning Cells on Polyacrylamide Hydrogels

A Major Qualifying Project Report
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Abstract

Stiffness of the extracellular matrix (ECM) influences cancer progression and metastasis. Understanding cell-cell and cell-ECM signaling and behavior can significantly contribute to finding alternative cancer treatments. The purpose of this project was to develop a device and method for micro-patterning cells on an ECM-mimicking substrate that would allow the study of cell-cell and cell-ECM interaction. Polydimethylsiloxane (PDMS) stamps were designed and created using photolithography to act as the micro-patterning device. Polyacrylamide (PAA) hydrogel was identified as an appropriate substrate for stiffness manipulation as well as for cell seeding. Using the PDMS device, collagen patterns were patterned on PAA hydrogels of different stiffnesses onto which NIH/3T3 cells were seeded. Cell growth and behavior was observed and analyzed in response to the different PAA stiffnesses. We demonstrate that our device is a useful tool in patterning cell populations of varying sizes, ranging from as few as 5 cells to 200 cells on single PAA hydrogels of specific stiffness to study the effect of ECM stiffness on behavior of different sized cell populations.

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Authorship

All four members of the team wrote and edited different sections of this report. Below is an outline of the author(s) of them, followed by the editor(s). The initials are as follows:

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Abbreviations:

ACL: Acrylamide
AFM: Atomic Force Microscopy
ECM: Extracellular Matrix
PAA: Polyacrylamide
PDMS: Polydimethylsiloxane
WPI: Worcester Polytechnic Institute

Chapter 1—Introduction

The extracellular matrix (ECM) is the non-cellular component present within all tissues and organs, and provides not only essential physical scaffolding for the cellular constituents but, also initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis [1]. The ECM is an important field of study because it houses the building blocks of life: cells. In the human body there are trillions of cells, all of which have different functions. Human cells are heavily influenced by their microenvironments including: chemical signals, ECM architecture, and mechanical properties [2]. In this report, we describe the design and development of a device that would allow researchers to test the influence of discreet cell populations in response to the changes in the mechanical properties of an ECM, its stiffness, and how it influences cell-cell interactions, cell signaling and changes in contractile properties of discrete groups of cells.

Cellular responses to mechanical cues from their ECM are known to be factors in several human diseases including tumor progression and formation [3]. Additionally, the stiffness of the ECM affects the proliferation, adhesion, and structure of a cell [4]. Previous cancer research shows that the stiffness of cancer cells and their ECM play an essential role in affecting the rate at which cancer cells will proliferate and spread, with stiffer cancer cells spreading at a slower rate than softer cancer cells [5]. New research suggests that there is crosstalk between cells and their ECM, indicating that neighboring cells affect the sensitivity of cells to the ECM stiffness [6]. The full extent of the molecular mechanisms that cause this effect are not known [7].

Cancer is the second leading cause of death in the United States [8]. Current research

on cancer cells and their ECM stiffness is limited, however new research exploring and understanding cell-cell and cell-ECM interactions and how they influence cancer progression can lead to the development of more effective cancer treatments. This research first begins with understanding the effects of cells in cell aggregates, or groups, in response to changing ECM stiffness.

The goal of this project was to design and fabricate biocompatible substrates that enable the studies of how a group of cells respond to substrate stiffness and how does the cell population influence the sensitivity of cells to substrate stiffness. In order to accomplish this goal, the team designed and fabricated a micro-patterning device in the form of a PDMS stamp using photolithography. The stamp had a unique design that allowed for a large range of areas suitable for cell adhesion to be printed on a substrate. Using a collagen solution and the PDMS stamp, the team was able to microprint areas of collagen on Polyacrylamide (PAA) hydrogel substrates that varied in stiffness. The micro-patterning design, the stamping techniques, and the PAA hydrogel fabrication, formed a unique system for observing cell behavior in aggregates in response to changing ECM stiffness. In the following chapters, the design process as well as the verification and validation of the designs and methods used to create this system will be discussed in further detail.

Chapter 2—Literature Review

2.1 The Extracellular Matrix

The extracellular matrix (ECM) is a complex and dynamic network that surrounds cells in all tissues of the human body. In addition to providing structural and mechanical support, it also mediates diverse biological processes that are crucial for supporting tissue formation and function [9]. In order to support the formation and function of tissues, the ECM first works to provide an environment of strength and resilience to cushion cells [10].

The ECM is composed of viscous proteoglycans, collagen fibers and multiadhesive proteins. The multiadhesive proteins are responsible for binding the proteoglycans and collagen fibers to receptors on the surfaces of cells. Because these receptors link the interior of cells to its surrounding ECM, the properties of cells are dependent on the composition and make up of their surrounding matrix [11]. In fact, the elastic and collagen fibers that make up the ECM dominate the physical responses that cells have to different mechanical forces [9]. The ECM can also transmit signals that affect cell proliferation, differentiation, and even death. Ultimately, the ECM can control the fate of all cells [10].

2.2 ECM Stiffness

Cells can sense a multitude of signals from their surrounding ECM. Among other matrix characteristics, the stiffness of an ECM has proven to be an important factor in cellular functions. A stiff matrix, for example, provides more resistance than a softer matrix. Accordingly, research has shown that cells on stiffer substrates have more organized cytoskeletons and more stable focal adhesions [4].

Stiffness within the human body can vary over many orders of magnitude. Whereas the stiffness of the brain typically ranges in a scale of several hundred Pascals, the stiffness of muscle can exceed 12 KPa and the stiffness of tendon or cartilage ranges in the scale of several mega Pascals [12]. When mimicking a physiological stiffness, it is important to keep these ranges in mind. For example, the stiffness of a plastic or glass cell culture dish can range in the scale of GPas—much stiffer than any surrounding ECM found in the human body [4].

One technique that is used to measure stiffness on the microscopic level is atomic force microscopy (AFM). The stiffness of cells and matrix fibers is oftentimes measured this way [4]. By using AFM to measure the stiffness of a substrate, one can observe cellular behavior in response to the stiffness of that substrate.

2.3 Collagen & the ECM

Collagen is the most abundant protein found in the human body. It is present within major tendons and ligaments, the organic matrix in bones, in skin and arteries as well as in the ECM [13]. In fact, collagen is the largest component that makes up the ECM. It provides tensile strength, regulates cell adhesion and migration, and directs tissue development [14].

Other than being found in the human body, collagen can also be used in a variety of applications ranging from food to medical uses. Because it can be so easily processed into various physical forms, such as sponges, powders and solutions, it is especially popular among various biomedical applications. These applications include drug delivery, tissue engineering, wound treatment and even tumor treatment [15]. Applying such research is important because understanding the interplay of a cellular microenvironment can lead to the development of better treatments and therapies for different diseases.

2.4 Hydrogels & Polyacrylamide

Hydrogels are polymer networks produced by the reaction of one or more monomers. These water-swollen polymeric materials are able to maintain a distinct 3D structure, making them a great substrate for cell culture [16]. As compared to 2D cell cultures, the cells on 3D hydrogel cultures have demonstrated more natural behaviors similar to behaviors of cells *in vivo*.

ECM-based hydrogels in particular have been developed as one method of studying cells *in vitro*. These ECM-based gels use collagen to provide a similar culture condition to mimic physiological stiffnesses found in the body [17]. Because collagen has very low antigenicity and immunogenicity, its biocompatibility makes it an ideal material for cell culturing and observations [15].

Polyacrylamide is one example of a polymer that has been popular for hydrogel formation for these purposes. The stiffness of PAA can be varied by changing the concentrations of the acrylamide monomer and the bis-acrylamide cross-linker that are used to make its solution [17]. By varying the stiffness, the mechanical properties of PAA hydrogels also change. Its easy manipulation of stiffness as well as the gel's transparency, make it a great ECM-mimicking substrate and an ideal substrate for observing cells.

2.5 NIH/3T3 Cells

NIH/3T3 cells are a type of mouse fibroblast immortalized cell line [18]. The murine cell line has been used as a model system in a multitude of different studies since its first description in 1963 and was established from *Mus musculus f. domenstica* ("Swiss mouse") embryo fibroblasts [19]. The cells, which immortalized spontaneously, were designated "3T3" according to "3-day transfer, inoculum 3×10^5 cells," "NIH" for "National Institutes of Health" was added later [19].

NIH/3T3 cells are used in a variety of biological and clinical studies, ranging from molecular cytogenics genetics to DNA testing [18]. Even more significant, NIH/3T3 cells are widely used in cancer research for studies examining the oncogene- an abnormal gene that predisposes cells to develop into cancers and if uninterrupted, help drive the uncontrolled growth that underlies tumors [20]. Examining oncogenes and their behavior can be demonstrated *in vitro* using cultured cells in which various oncogenes are introduced, and also *in vivo* using genetically altered mice [21]. Figure 1 below illustrates the collaboration of oncogenes *in vitro*:

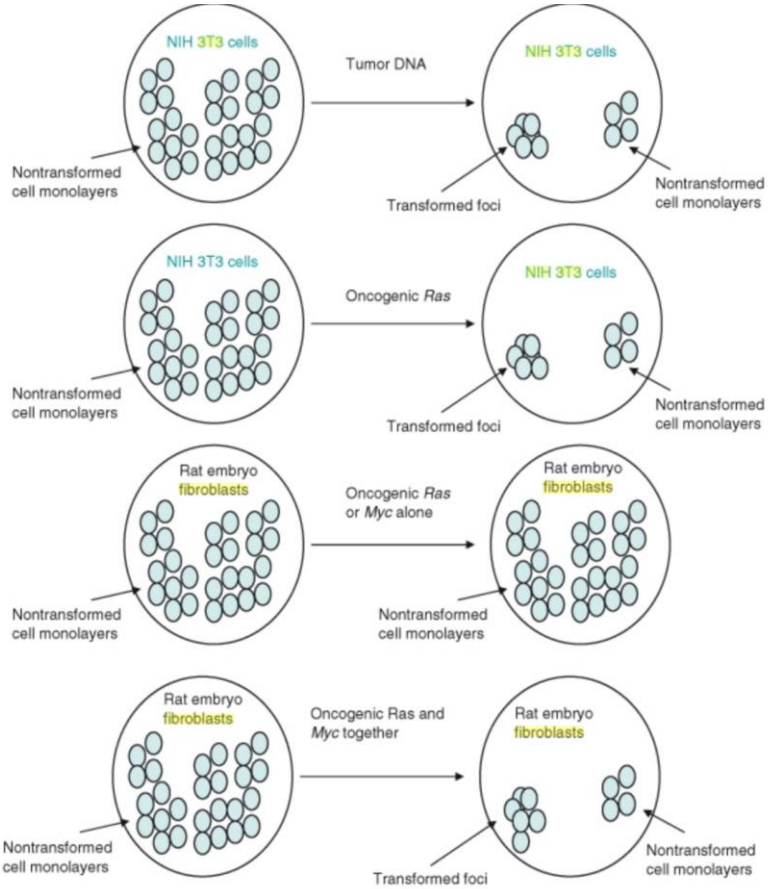


Figure 1: Oncogene collaboration *in vitro* transfection of NIH 3T3 cells with DNA [21]

Figure 1 above demonstrates the behavior of oncogenes after being transfected, causing the cells to be transformed, showing behaviors and features of cancerous cells.

NIH/3T3 cells are further used in cancer research and their applications are explored extensively in the literature review titled, “Physical cues that guide cell adhesion and migration” [22]. In the literature review there is a detailed summary of how tumor cells translate and respond to physical cues through mechanotrasnduction and how NIH/3T3 cells exhibit similar behaviors in similar tests. Results from dimensional control of cell traction forces indicated that, for some classes of tumor cells, traction force generation plays a reduced role during migration in confined spaces in tissues. Consistent with these observations, NIH/3T3 fibroblasts also exert significantly reduced traction forces along 1D lines compared with 2D substrates [22]. Understanding the wide applications and uses for NIH/3T3 cells was important for the team in choosing a cell line suitable for the research presented in this report.

2.6 Cell Signaling

Cells communicate with each other by sending and receiving signals [23]. There are numerous pathways that receive and process these signals, which can originate from the external environment, other cells, and from different regions within a cell [24]. There are four different types of signaling and are pictured below in Figure 2:

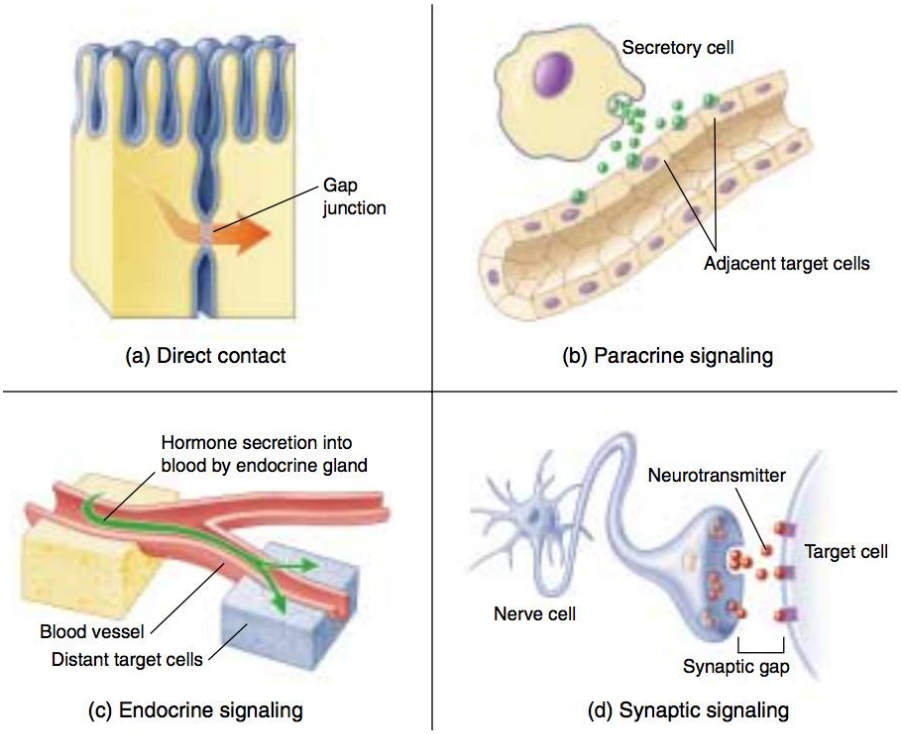


Figure 2: Four types of cell signaling [25]

For the purpose of this research, direct contact signaling (a) was the most important type of signaling to understand. This is because the overarching goal of this project was to observe groups of cells in controlled areas; as a result direct contact signaling became a major point of concern. In order to observe cell groups in their controlled areas, the team needed to control direct contact signaling between the groups of cells to avoid cell intrusions in other surrounding cell areas and groups.

Intracellular signaling describes the behavior of cells communicating with each other by direct contact [26]. This signaling occurs through the release of cytokines and chemokines and once a cell releases these signaling molecules, they are secreted and diffused through a surrounding medium which bind to a neighboring cell's receptors, at this moment the signal is

received by the neighboring cell [26]. The following image in Figure 3 illustrates a single cell secreting cytokines and chemokines and the corresponding concentration gradient:

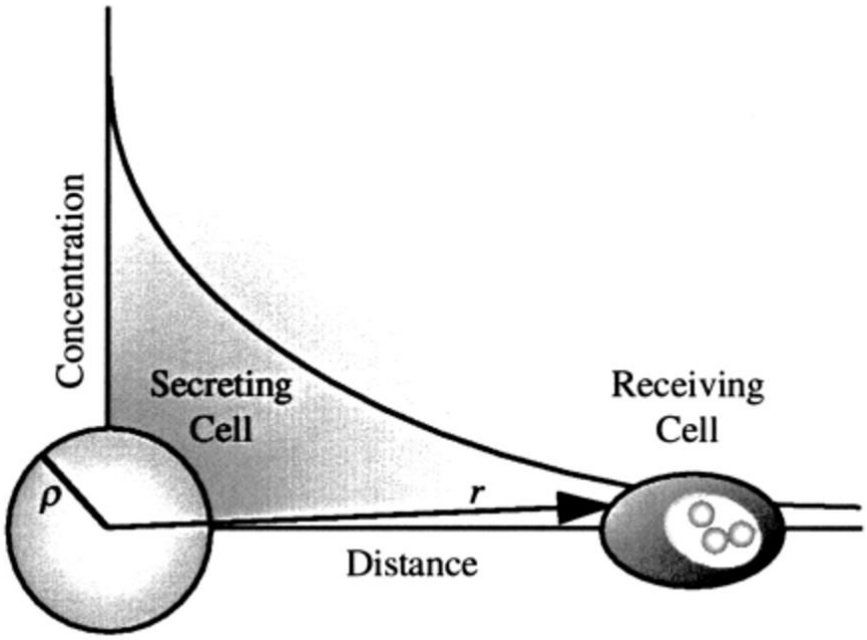


Figure 3: Schematic of secretion of cytokines & chemokines and the concentration gradient [26]

It is important to note the distance (r) pictured in Figure 3. This distance was a major driving characteristic of the teams approach to observing individual cell groups, as this distance determined how far away the controlled areas needed to be in order to avoid direct contact cell signaling. A major finding in a study on intracellular communication, by Fancis and Palsson, was that effective intracellular distances at which cell signaling occurs are ~50 cell radii, or 25 cell diameters [26]. A similar study, focusing on cell intrusions on micro-patterns, also found that cell signaling and intrusions on neighboring cell patterns occurred at approximately 20-25 μm [27]. Figure 4 below demonstrates the intrusion distance plotted against the time of post plating the cells:

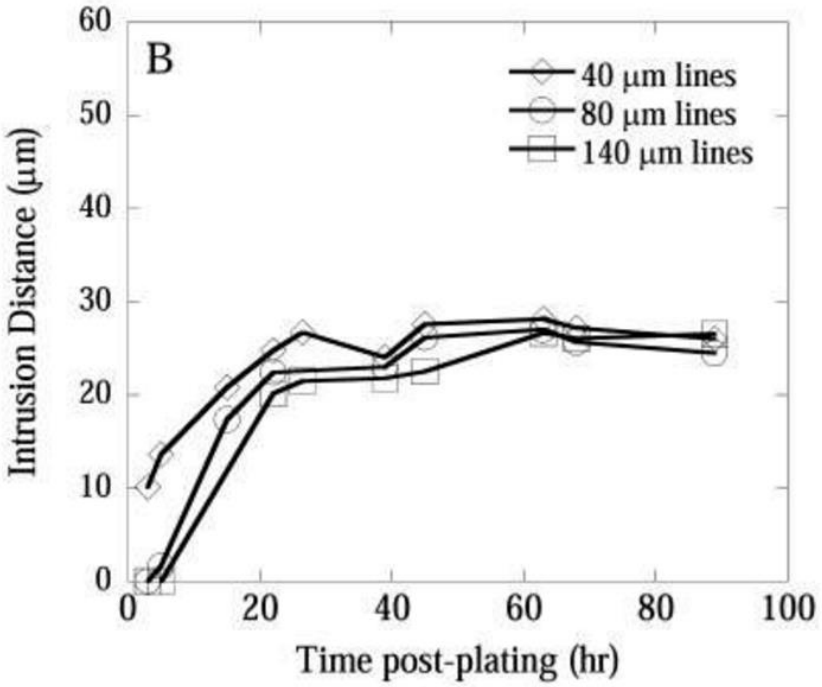


Figure 4: Intrusion distance vs. Time (hr)-post cell plating [27]

With the major findings from these studies, the team had a greater understanding of cell signaling and cell intrusions, which were important factors in the design of the device that would allow for the observation of cell groups.

2.7 Micro-Patterning

Micro-contact printing, otherwise called micro-patterning, is a method for producing patterns on biocompatible substrates for the purpose of restricting cells to specific areas of various shapes. From this, observations of cellular behaviors can be studied.

In order to create a desired micro-pattern on a topological master mold, a process known as photolithography is used. After the micro-pattern is created onto a master mold, it is

transferred on a silicon wafer using either positive or negative photoresist [28]. Below, Figure 5 displays both these ways.

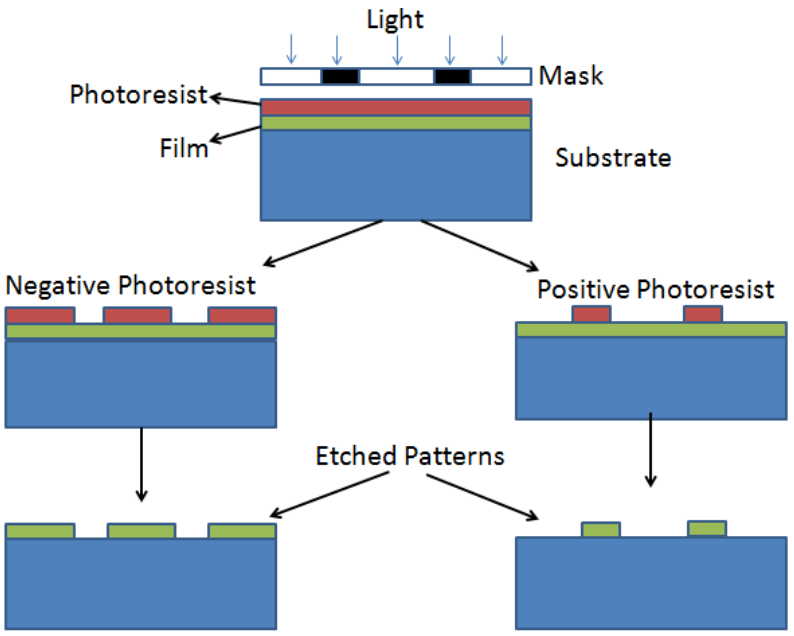


Figure 5: Photolithography Photoresists: Negative (left) & Positive (right) [29]

From the silicon wafer, it is then possible to create polydimethylsiloxane (PDMS) stamps that can be used as a micro-pattern transferring device. One such purpose could be to transfer monolayer coatings of a protein that would enable for cell adherence and growth, as explored by the team for this project.

Currently, most micro-patterning techniques used for cell research use some form of micro-contact printing. Other methods, however, have also utilized stereolithography, which uses direct printing with the silicon wafer into hydrogels for the purposes of creating 3D hydrogels that enable multi-layered wells or specifically designed scaffolds [30]. Most recreated 3D scaffolds have in fact used hydrogels as the choice material. As explained in Section 2.4, this is

because of their ability to encapsulate cells within defined compartments as well as their similarity to native tissues and ECMs.

2.8 Tumors

As previously described, micro-patterns are being used more and more to actively study the growth and development of cell aggregates. Micro-patterns are also being used to not only study individual cells, but also tumorigenic cells.

One study in particular looks at the optimum 3D matrix stiffness for cancer stem cells on polyethylene glycol diacrylate (PEGDA) hydrogels [31]. The results of this study showed that cancer cell types grew faster in the PEGDA hydrogels that possessed stiffness most similar to those of their respective tissues of origin. As the stiffness increased or decreased farther away from this value, the speed of the tumor growth also progressively decreased [31].

There is much more research that can be conducted to further understanding the behavior of normal as well as tumorigenic cells., The more research that is conducted about how different cell responses to different controllable variables, the more accurately the cells can be modeled and their behavior predicted. This kind of information would not only improve diagnostic capabilities, but would also identify physiological conditions and parameters that could control the growth and metastatic properties of cancer cells. With this information, new drug treatments could emulate these environments slow or halt cancer progression.

3— Project Strategy

This chapter provides information on the strategy the team developed after extensive research and collaboration in order to meet the needs of the project. After receiving the initial client statement, the team listed the most important background topics that needed to be researched in order to develop objectives. Identifying the objectives of the project led to determining their associated constraints. These steps were necessary for the team to implement in order to gain a better understanding of the project, which, lead to the revision of the original client statement. Developing a strategic project approach allowed the team to delegate and prioritize the different completion of tasks in order to complete the project. These major steps in framing and structuring the project will be will be discussed in further detail in the following sections:

By the end of Chapter 3 the following should be understood: the needs of the project, how the team planned to address these needs and their strategy for completing the project.

3.1 Initial Client Statement

"Cells sense mechanical cues from their ECM. It has been demonstrated that the ECM stiffness can affect the adhesion, migration, and differentiation on individual cells. In real tissues, cells also send signals to their neighbors. It is unclear whether the signaling from neighboring cells would affect the ability of cells to sense the cell-ECM signaling. Therefore, we hypothesize that the neighboring cells affect the sensitivity of cells to the ECM stiffness. To test this hypothesis, we study the mechanosensitivity of a group of cells.

The needs of this project are:

1. *Develop a method to fabricate hydrogels with controlled regions for cell adhesion. And the size of the region should be varied to allow the formation of cell aggregates containing different cell numbers (ranging from single to hundreds of cells).*
2. *The gel should be transparent so that the cells can be imaged using a regular microscope.*
3. *The gel should be mounted on a transparent microscope coverglass and uniform in thickness of about 100-500 microns, preferably in the 100-200 micron range.*
4. *Vary the gel stiffness and characterize the sensitivity of cell aggregates to gel stiffness."*

The main topics the team research after receiving this client statement were:

A. Current research on mechanosensing ability of cells
B. Interactions between cells and their ECMs
C. Approaches to imitating an ECM with the ability of altering the stiffness
D. The relationship between tumor progression and ECM stiffness

The literature review gave the team a better understanding of current practices on observing groups of cells, the influences of ECM stiffness on cells, and how this project could contribute to cancer research in tumor progression. This led to the development of the following objectives.

3.2 Design Requirements

In this chapter, the objectives, constraints, specifications, and functions of the project and its two designs elements are identified in greater detail. This chapter provides guidance to a better understanding of what the team decided was important, and how they decided to complete the project accordingly.

3.2.1 Objectives

The objectives for this project are listed in Figure 6 in the form of an objectives tree.

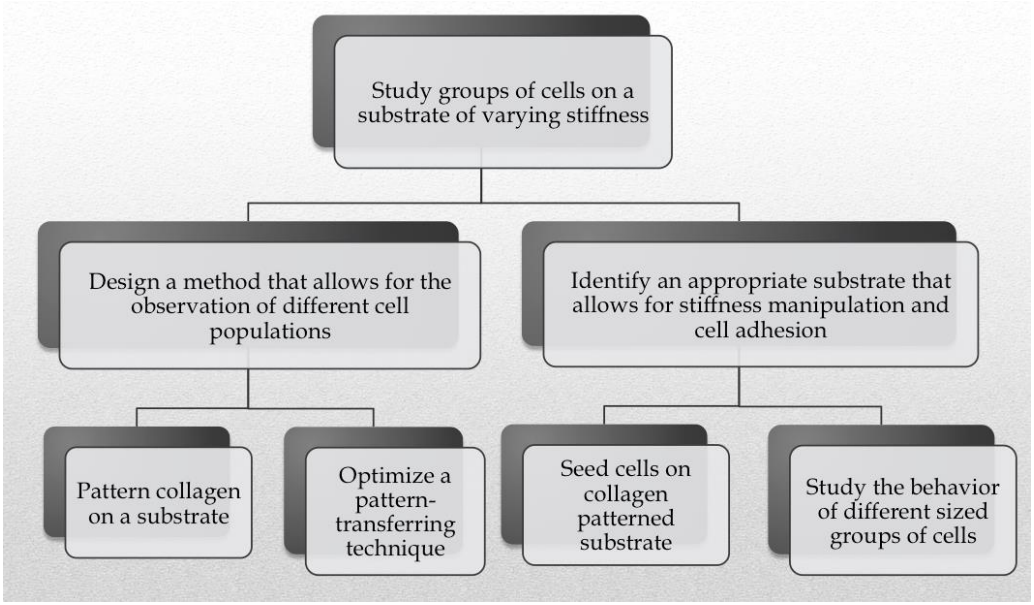


Figure 6: Project Objectives Tree

The objectives are listed in order of importance and chronological occurrence. It is important to understand that the major steps in beginning the project were:

1. Exploring different methods to observe groups of cells
2. Identifying a substrate that allowed for stiffness manipulation and cell adhesion

Because there were several objectives dependent on these two main objectives, they needed to be completed first so that the team could move forward. Below in Figure 7 is the objectives tree for the Micro-Patterning Device and for the Substrate.

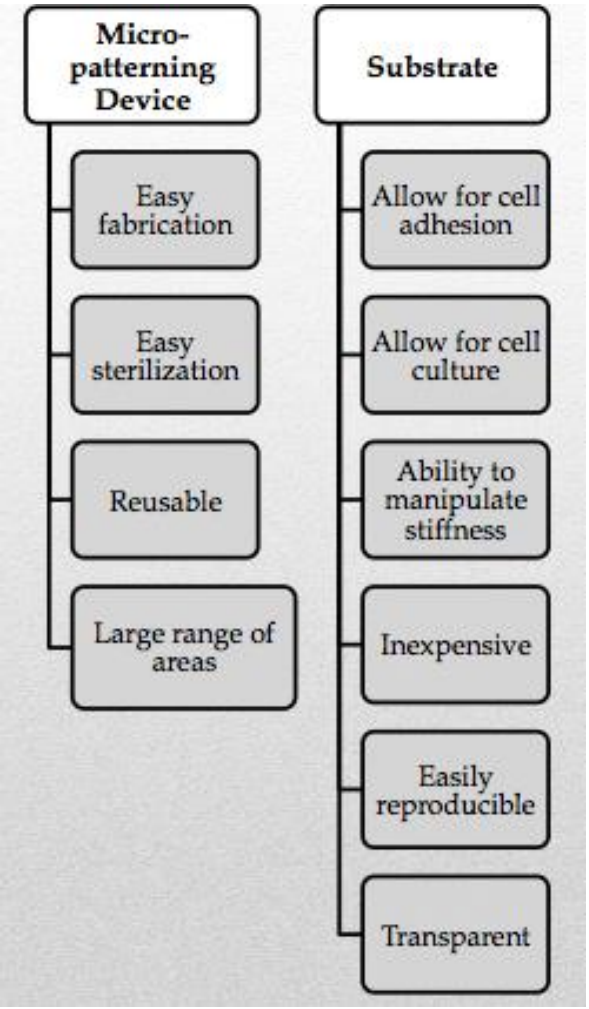


Figure 7: Objectives Tree for the Micro-patterning Device (left) & Substrate (right)

3.2.2 Constraints

This section discusses all of the constraints associated with the overall project, the micro-patterning device, and the substrate. Understanding the constraints of the project enabled the team to analyze and determine the feasibility of the project, which was instrumental for framing a management strategy. In this report, constraints are described as any economic, environmental, social, political, ethical, health/safety, manufacturing and sustainable considerations that would limit the team's strategy and approach in any way [32].

The team identified the following project constraints:

A. Project Budget. The team had a total budget of ~$500 (~$125/person).

B. Time. This project was introduced to the entire team in September 2015 had until May 2016 to complete it. Total time: 8 months

C. Equipment Use. The observations that needed to be gathered in order to test the hypothesis required the use and handling of specialized equipment and chemicals. Additional training was provided for use of:

    i. Atomic Force Microscope
    ii. Fluorescent Microscope
    iii. Oxygen Plasma Cleaner
    iv. Ultrasonic Cleaner
    v. Glutaraldehyde

Time, budgeting, and the different equipment needed to obtain results and observations limited the team. The budget limited the team because the cost of fabricating the micro-patterning device and the substrate, as well as the cost of other lab equipment and materials were all shared. With the budget covering all of these costs, the team was

limited to inexpensive methods of fabrication for the micro-patterning device and the substrate that were within the budget.

In addition, the need for using the lab equipment and chemicals that required additional training mentioned above, limited the flexibility of experiments. Most of the experiments would have to be designed around the availability of this equipment and inventory of materials in both Salisbury and Gateway labs at WPI.

The following constraints were identified as having the greatest impact on the development of the micro-patterning device:

A. The device had to be small enough to pattern on a glass coverslip. The original client statement requested that the substrate should be mountable on a glass coverslip. Therefore, the stamp should also be mountable on a glass coverslip in order to get an optimal pattern transfer.

B. The cost of fabricating the device should be low. Since the budget was limited, the fabrication method for the device had to be low in order to refrain from exceeding the budget.

The following constraints were identified as having the greatest impact on the development of the substrate:

A. The substrate has to be suitable for cells to live on. This limited the amount of materials and chemicals the team could use based on their toxicity and potential harm to cells

B. The substrate had to mimic the behavior of an ECM. Mimicking an ECM limited the type of hydrogel the team would be allowed to use, as the hydrogel had to be biocompatible but also vary in stiffness like an ECM

3.2.3 Functions

After having a clear understanding of what needed to be done, and identifying all of the potential limiting factors, the team was then ready to identify the functions of the micro-patterning device and the substrate. Identifying the functions of each design enabled the team to move forward and create design specification, which was the last step before creating designs. Below are the functions for each design:

Micro-Patterning Device Functions

A. Contain a pattern that has specific areas for cells to grow in
B. Compatible with a chosen substrate

Substrate Functions

A. Promote cell adhesion, cell survival and cell proliferation
B. Mimic the mechanical behavior of an ECM
C. Have the ability to absorb the solution used from the micro-patterning device to transfer the pattern on its surface

3.2.4 Specifications

This section lists the specifications of each design: the micro-patterning device and the substrate. These specifications were made after understanding all of the objectives, constraints, and functions of each design.

Micro-Patterning Device Specifications

A. Pattern should have circular areas ranging from a minimum of 100 μm in diameter to a maximum of 500 μm in diameter
B. The device should be biocompatible

C. The device should be reusable for a minimum of 50 uses
D. The cost of fabricating the device should be a maximum $150.00 USD
E. The dimensions of the device should be a maximum of 25 x 25 mm

Substrate Specifications

A. The substrate should be transparent
B. The cost of fabrication should be a maximum $150.00 USD
C. The minimum stiffness the substrate should be is 5 kPa
D. The maximum stiffness the substrate should be is 40 kPa

Once the specifications of the design elements were identified, the team revised the initial client statement, which best reflected the goals and needs of the project accordingly.

3.3 Revised Client Statement

"Design and develop a device and appropriate technique for micro-patterning cells on a hydrogel that allows for the study of cell-cell signaling in response to changing ECM stiffness."

Hypothesis

Based on the revised client statement, the anticipated results of this project are that with increasing cell populations, the effect of stiffness on cell behavior would be reduced. In order to test this hypothesis, the team developed a list of the necessary steps for meeting the needs of this project:

1. Design and fabricate a micro-patterning device.

2. Use micro-patterning device to develop a stamping technique that would successfully transfer a pattern that would allow for cell adhesion.
3. Fabricate a hydrogel that would allow for cell culture and uniform pattern transfers through use of the micro-patterning device and stamping technique.
4. Seed cells on hydrogels and observe cell attachment by measuring cell populations in the designated areas created by the pattern.

3.4 Project Approach

The duration of this project was from August 2015-April 2016. Figure 4 below is an image of a Gantt chart representing the schedule the team followed in completion of this project.

Tasks	A-Term 8-27-15 - 10-15-15	B-Term 10-27-15 - 12-17-15	C-Term 1-14-16 - 2-4-16	D-Term 3-14-16 - 5-3-16
Revise Client Statement				
Revise Objectives & Constraints				
Preliminary Conceptual Designs				
Budget Analysis				
Revise Conceptual Designs				
Explore Fabrication Methods				
Begin Preliminary Experiments				
Finalize Designs				
Fabricate Final Designs				
Finalize Experimental Methods				
Conduct Experiments				
Collect Data & Observations				
Conduct Final Experiments				
Data Analysis				
Data Interpretation				
Finalize Report				

Figure 8: Project Schedule Gantt Chart

The team allocated approximately three months to designing and fabricating the substrate and micro-patterning device as they each had several associated design specifications and limitations. After identifying a suitable hydrogel, the team then wanted to focus on creating a simple technique for transferring a pattern onto those hydrogels. After finalizing a technique that would produce consistent patterns, the team then wanted to implement the use of the micro-patterning device, stamping technique, and hydrogel, in order to seed cells and verify that the patterns transferred onto the hydrogel resulting in cell adhesion and cell growth.

Chapter 4— Design Process

This chapter will provide insight on the decision process that the team followed in order to finalize the design of the micro-patterning device and the substrate. Before selecting any final designs the team first had to prioritize the objectives of each design. Ranking these objectives provided a better understanding of which characteristics were most important for the final designs of both the micro-patterning device and the substrate. Afterwards, the team was ready to explore alternative designs, which finally led to choosing a final design. The reasoning behind the different design options will be discussed in this chapter in the following order:

4.1— Needs Analysis
4.2— Alternative Designs
4.3— Selection of Final Designs

By the end of this chapter, the design process as well as the major decisions made for the final designs should be understood. This process led the team to prepare for the next step of the design process, which will be discussed in Chapter 5.

4.1 Needs Analysis

In this report, the properties that the designs must incorporate, to ensure the intended results, will be referred to as "needs." Similarly, the properties that the team would like the designs to have, but may not be possible to achieve considering the associated constraints, will be referred to as "wants" [32]. Both the needs and wants of each design will be discussed in further detail below.

Micro-Patterning Device

Recall from Figure 9 below, that the objectives of this device were:

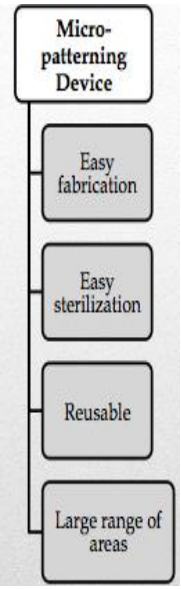


Figure 9: Micro-Patterning Device Objectives

These objectives were further analyzed in a decision matrix in order to distinguish the wants from the needs. Table 1 below demonstrates the results:

Table 1: Decision Matrix Ranking the Objectives of the Micro-Patterning Device.

Micro-Patterning Device Decision Matrix					Total
	Easy fabrication	Easy sterilization	Reusable	Large range of areas	
Easy fabrication	**X**	0	0	0.5	**0.5**
Easy sterilization	1	**X**	0.5	1	**2.5**
Reusable	1	0.5	**X**	1	**2.5**
Large range of areas	0.5	0	0	**X**	**0.5**

Needs:

According to the results from Table 1, the following objectives were prioritized as the needs of the device:

A. Easy sterilization
B. Reusable

These objectives were ranked the highest and prioritized as needs based on the nature of the functionality of the device. Recall that the device was identified as needing to be used for a minimum of 50 uses, highlighting the need for easy sterilization and reusability.

Wants:

From the same results demonstrated in Table 1, the following objectives were ranked the lowest and therefore categorized as wants:

A. Easy fabrication
B. Large range of areas

These objectives were ranked the lowest because they were not as pertinent to yielding the final intended results as the higher ranked objectives were. Even so, the team still tried to incorporate the wants into the final designs to meet all of the client's needs.

Substrate

Recall from Figure 10 below, that the objectives of the substrate were:

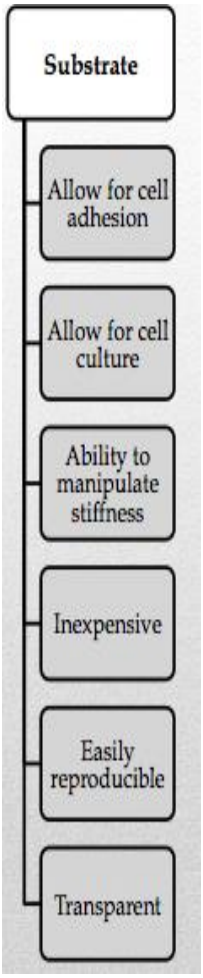


Figure 10: Substrate Objectives

The same process used for distinguishing between the needs and wants from the micro-patterning device objectives was applied to the substrate objectives. Table 2 represents the results of the decision matrix for the substrate:

Table 2: Decision Matrix Ranking the Objectives of the Substrate.

Substrate Decision Matrix							Total
	Allow for cell Adhesion	Allow for cell culture	Ability to manipulate the stiffness	Inexpensive	Easily reproducible	Transparency	
Allow for cell Adhesion	**X**	0.5	0	1	1	0.5	**3**
Allow for cell culture	0.5	**X**	0.5	1	1	0	**3**
Ability to manipulate the stiffness	1	0.5	**X**	1	1	0.5	**4**
Inexpensive	0	0	0	**X**	0	0.5	**0.5**
Easily reproducible	0	0	0	1	**X**	0	**1**
Transparency	0.5	1	0.5	0.5	1	**X**	**3.5**

Needs:
According to the results from Table 2, the following objectives were prioritized as the needs of the substrate:

A. Ability to manipulate the stiffness
B. Transparency
C. Allow for cell adhesion and Allow for cell culture (tie)

These objectives were ranked the highest and prioritized as needs based on the nature of the functionality of the substrate. Recall that the substrate needed to mimic an ECM, and the

needs ranked above reflect the most important characteristics needed for the substrate to have in order to carry out that function.

Wants:

According to Table 2 above, the following objectives were ranked the lowest and therefore categorized as wants:

A. Easily reproducible
B. Inexpensive

These objectives were ranked the lowest because they were not as pertinent to yielding the final intended results as the higher ranked objectives were. Even so, the team still tried to incorporate the wants into the final designs to meet all of the client's needs.

4.2 Alternative Designs

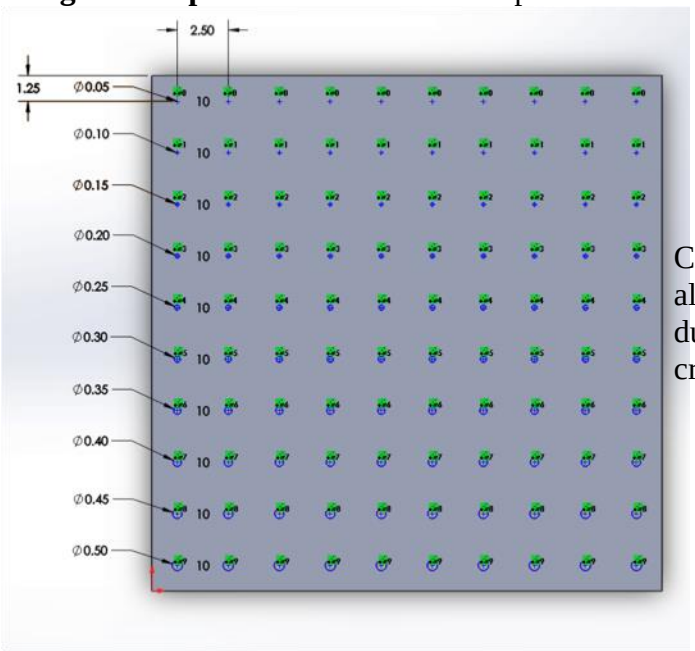
In this section, all of the alternative designs for the micro-patterning device and the substrate will be presented. First, different alternative designs for each will be explored. The advantages and disadvantages of each will be explained in table as well as the feasibility of the concepts.

Micro-Patterning Device

Tables 3, 4, and 5 present alternative designs for the micro-patterning device. The advantages, disadvantages, and feasibility are also discussed— based on the "needs" and "wants" of the device.

Table 3: Modified Coverslip Alternative Design

Design Description: Modified Coverslip



Creating a coverslip that would alter the shape of the hydrogel during cross binding for the creation of pillars for cell growth.

Figure 11: Modified Coverslip Conceptual Design

Advantages	Disadvantages
• Would allow for the specific placement of collagen suitable for cell adhesion	• Size of pillars would be too small for precise placement • Limited contact area for cell adhesion • Materials needed would have to be suitable to withstand cross bonding conditions

Feasibility:
This conceptual design was the least feasible mainly because of the time constraint the team had (~8 months). Modifying a coverslip would involve continuous iterations of experimental procedures and protocols that would demand time and attention that the team did not have to spend. Recall that designing a micro-patterning device was only one of the several objectives the team identified in order to obtain the intended results.

Table 4: 3D Printed Stamp Alternative Design

Design Description: 3D Printed Stamp

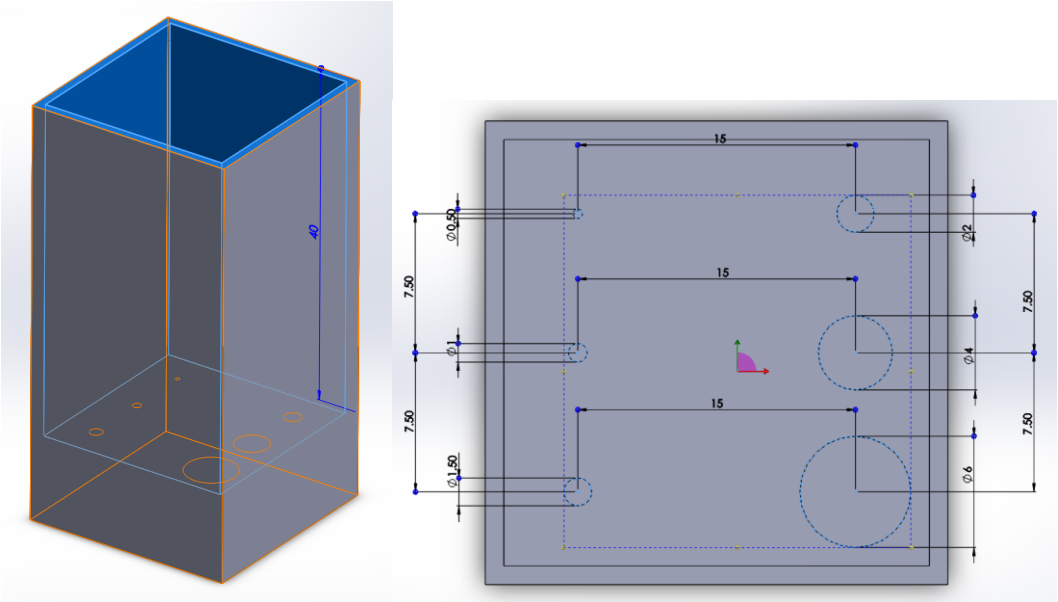


Figure 12: 3D Printed Stamp- Solid Works Alternative Design Model

A 3D printed stamp that would allow for the placement of specific cell grouping sizes on a hydrogel with the use of a collagen coating solution.

Advantages	**Disadvantages**
• Would allow for the placement of specific cell grouping sizes on a hydrogel	• Finding a fabrication process that would create the range of areas needed observation (very small and difficult to accomplish)

Feasibility: From the literature review, photolithography was identified as a method to produce microfluidic devices used in various applications. Since the team was under a constrained budget, alternative methods for fabricating a stamp similar to a microfluidic device were explored. The optimal alternative method was 3D printing the stamp, as 3D printer were readily available at the school and only required a pattern designed in SolidWorks. Below is an image of the design of the stamp pattern the team 3D printed:

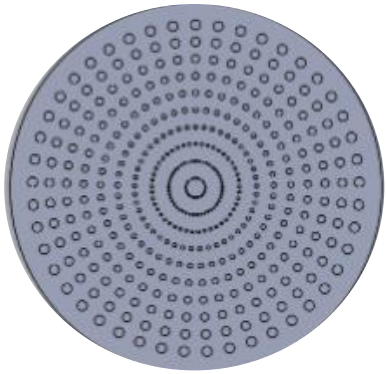


Figure 13: Stamp pattern designed in SolidWorks

The pattern in Figure 13 was 3D printed using a MakerBot MP04948, with a standard 0.4mm nozzle and Polylactic acid (PLA) filament. This alternative fabrication method was deemed unfeasible because the 3D printer was not able to extrude the filament uniformly and therefore not able to produce the small area ranges needed. The team had the option of designing a smaller nozzle that would be compatible with the 3D printer, in order to print smaller areas. This was an option but it was also infeasible because it added additional design parameters and associated costs with no guarantee that it would work.

Table 5: Cone Shaped Mold Alternative Design

Design Description: Cone Shaped Mold

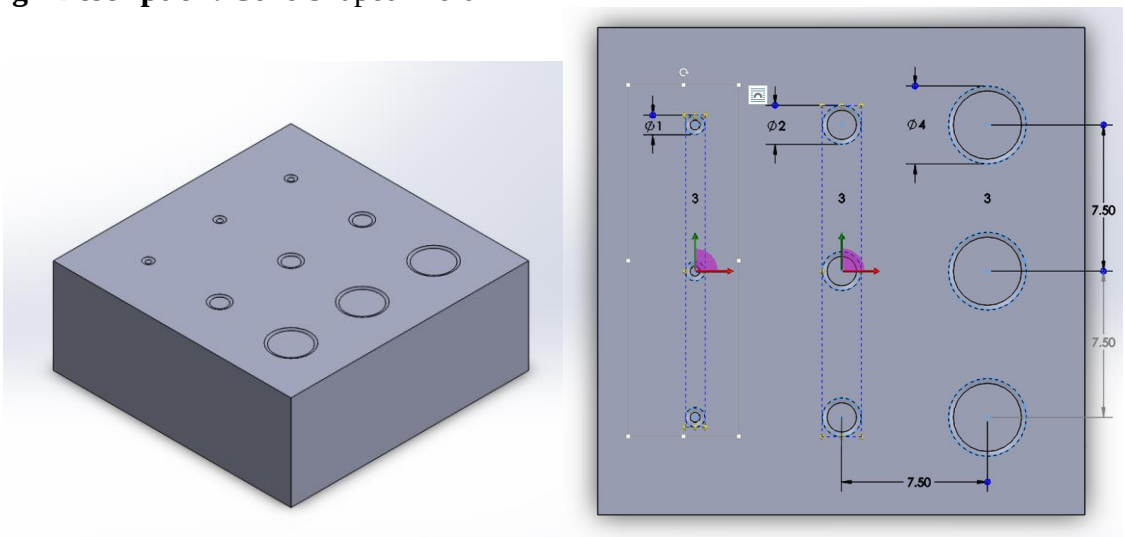


Figure 14: Cone Shaped Mold- SolidWorks Conceptual Design Model

A mold that has cone shapes to modify the hydrogel and can be adjusted for different area sizes for the placement of certain cell groups with the use of a collagen coating solution.

Advantages	**Disadvantages**

• Cone shapes would allow for specific cell areas • Adjustable sizes when using the device while creating hydrogels	• Creating cone shapes, the geometry is complex to fabricate on such a small scale • Creating a flat surface on the cone would also be a challenge
Feasibility: This mold design was deemed infeasible because it required an additional dynamic system in order to function. The idea behind this mold was that a hydrogel solution would be flushed into the mold, and the height of the pillars would be determined by the amount of hydrogel solution being pumped in. This approach was out of the design specification range as the client statement did not require a system, just a device that would transfer patterns. Including the pump in order for this mold to function properly would only add additional costs that the team could not afford.	

Substrate

Table 6 presents the conceptual designs for the substrate. The advantages, disadvantages, and feasibility are also discussed— based on the "needs" and "wants" of the substrate.

Table 6: Polyacrylamide as an Alternative Design for the Substrate

Design	Advantages	Disadvantages
Gelatin	• Transparent • Inexpensive • Readily available and easy access • Biocompatible • Easy fabrication	• Too stiff (insert average stiffness here) • Forms at colder temperatures, not suitable for cell culture • Entire surface would promote cell adhesion instead of certain areas
Polyacrylamide	• Transparent • Biocompatible • Easy fabrication • tunable stiffness (5-40kPa)	• Cost of fabrication • Varying reproducible stiffness
PDMS	• Transparent • Biocompatible • Inexpensive	• High average stiffness greater than desired

Feasibility: All of these substrate options were feasible in terms of the constraints listed in the previous chapter but in terms of the "needs" and "wants" of the substrate, some were not. Specifically, the gelatin and PDMS because they were too stiff and modifying their stiffness would be difficult. Modifying the stiffness of the substrate was the most important "need" and therefore the other alternatives were not considered in the final design because the goal of the team was to satisfy the "needs" of the project.

Moving forward the team evaluated all of the conceptual designs and began finalizing the details and methods for producing the final designs. These steps were crucial as the micro-patterning device and the substrate needed to be fabricated in order for the team to move forward and begin experimenting.

After exploring different alternative designs for a micro-patterning device and a substrate, the team decided to focus on the most feasible design for each: a PDMS stamp and Polyacrylamide hydrogel. By focusing on a PDMS stamp as the micro-patterning device, the team now had to create different designs of patterns for the stamp. These different stamp patterns will be presented as alternative designs in Tables 7, 8 and 9. By focusing on Polyacrylamide as the substrate, the team now had to determine which stiffnesses of Polyacrylamide would work best. These alternative designs for the substrate are presented in Table 10. For each alternative design there will be a design description as well as a list of advantages and disadvantages associated with it.

Micro-Patterning Device: PDMS Stamps

Table 7: Set of Square PDMS Stamp Patterns Alternative Design

Design Description: Set of Square PDMS Stamp Patterns

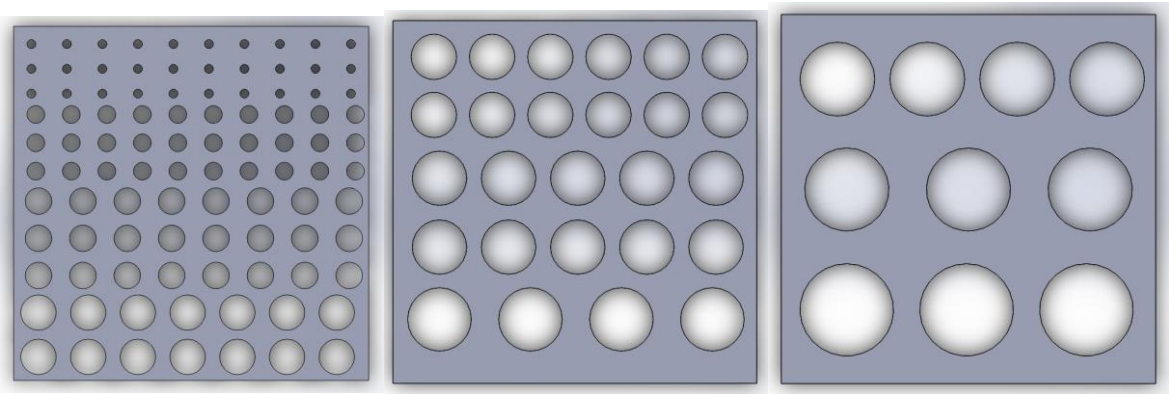


Figure 15: Set of Square PDMS Stamp Patterns

A set of three stamp patterns was designed in SolidWorks. The circle diameters on the set ranged on the patterns from 500-950 microns. The first stamp design contained circle diameters of 500 microns (30 circles), 550 microns (30 circles), 600 microns (24 circles), and 650 microns (14 circles). The second stamp design contained circle diameters of 700 microns (12 circles), 750 microns (10 circles), and 800 microns (4 circles). The third stamp design contained circle diameters of 850 microns (4 circles), 900 microns (3 circles), and 950 microns (3 circles).

Advantages	**Disadvantages**
• Distinguishing between the circle diameters after micro-patterning would be easier because not all sizes are one stamp and therefore can be easily determined based off of the specific stamp that was used	• The amount of circles for each diameter significantly varied from stamp to stamp • Micro-patterning would take longer as all three stamps would need to be used to stamp all the desired circle diameters • Cost: three separate stamps would have to be fabricated

After designing the set of the three PDMS stamp patterns, the team realized that the range of circle diameters used was incorrect. Rather than having the smallest circle area

start at a diameter of 500 microns and increasing the diameter, the team needed to start at a diameter of at least 100 microns, as specified in the micro-patterning device specifications. The team also determined that in order to later be able to perform AFM on a micro-patterned substrate, the pattern had to be in the shape of a circle rather than in a square in order to fit under the AFM microscope.

Table 8: Circular + Inner Square PDMS Stamp Pattern Alternative Design

Design Description: Circular + Inner Square PDMS Stamp Pattern

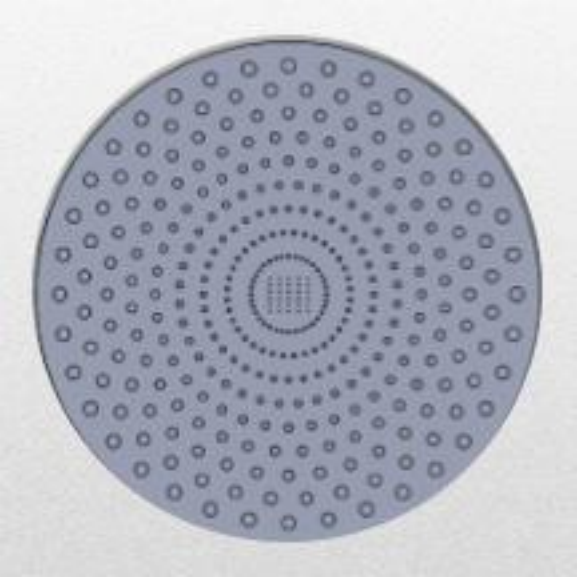


Figure 16: Cicrcular + Inner Square PDMS Stamp Pattern

This Circular + Inner Square pattern was designed in SolidWorks. The circle diameters ranged from 50-500 microns. This stamp pattern design was one of the first initial designs created for a circular shaped PDMS stamp. When designing this stamp, the goal was to utilize all the space by fitting as many circle areas as possible. In trying to create similar amounts of circle areas for each diameter size, the team determined that it would be best to have the smallest circle diameter (50 microns) in a square shape in the middle of the stamp to be consistent and fit as many circle areas as possible rather than just a ring of 50 micron diameter circles of a smaller count.

Advantages	**Disadvantages**
• The design utilizes the maximum amount of the space	• The pattern of the smallest circle diameter (50 microns) completely differed from the rest of the circle diameters

Table 9: Compacted Circular PDMS Stamp Pattern: Advantages & Disadvantages

Design Description: Compacted Circular PDMS Stamp Pattern

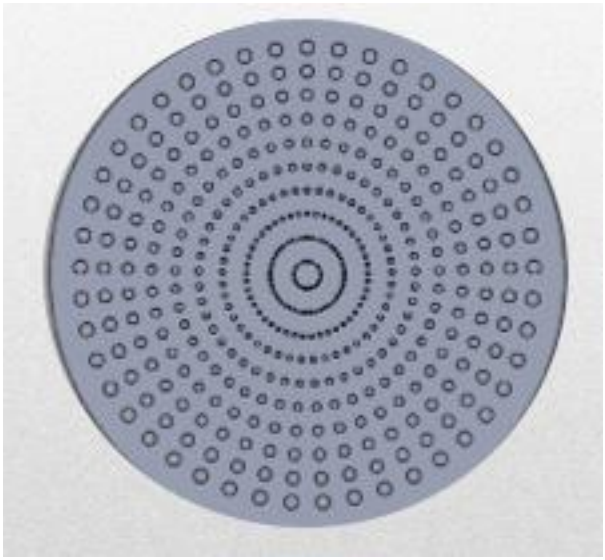


Figure 17: Compacted Circular PDMS Stamp Pattern

This Compacted Circular pattern was designed in SolidWorks. The circle diameters on this design also ranged from 50-500 microns, as on the "Circular + Inner Square PDMS Stamp Pattern." Trying to keep the pattern of the different circle diameters consistent for this pattern, the amount of circle areas decreases as the circle diameter decreases.

Advantages	**Disadvantages**
• Consistent pattern shape for each circle diameter size	• Varying amounts of circle areas for each circle diameter • Very little space between circles

Substrate: Polyacrylamide

Table 10: 10% PAA & 12% PAA: Advantages & Disadvantages

Design	Advantages	Disadvantages	Predicted Stiffness [4]
10% PAA For 1 mL hydrogel: 250 uL ACL 60 uL bis 690 uL HEPES	• Hydrogel solution components are readily available • Provides another stiffness value	• Because it is a stiffer percentage of PAA, it gels rather quickly • Quick gel time will limit allowed experiment time and affect experiment	12 kPa
12% PAA For 1 mL of hydrogel: 300 uL ACL 70 uL bis 630 uL HEPES	• Hydrogel solution components are readily available • Provides another stiffness value	• Gels very quickly, even faster than 10 % PAA • Quick gelling prevents spreading of the gel solution for uniform distribution • Quick gel time will limit allowed experiment time and affect experiment	20 kPa

The team determined that 10% and 12% PAA would be considered as alternative designs because since all the hydrogel solution components were readily available, it would be possible to experiment and make PAA hydrogels at these stiffnesses. However, due to the

quick gelling, the PAA hydrogels at these stiffnesses would affect the experiment by limiting the allowed time to perform it. For example, if gel formation occurs too quickly, the micro-pattern would not be transferred onto the substrate.

4.3 Selection of Final Designs

In this section, the final designs for both the micro-patterning device and substrate are discussed. With each final design, the reader will understand why the design was chosen and how it proved to be superior to the previously discussed conceptual and alternative designs.

Out of all of the possible technologies and methods appropriate for creating a final design, a combination of photolithography and soft lithography was the most realistic option for satisfying all of our device objectives. In photolithography, the silicon topological master can only be fabricated with the correct equipment. From this master, several iterations of the design can be produced in PDMS molds using soft lithography methods. These devices partially satisfy the need for easy fabrication and possessing a large range of areas, but they are extremely reusable and easily sterilized. Other techniques for micro-patterning, such as stereo-lithography, micro-serigraphy, or even direct photolithography, were not easily accessible options. The technology and equipment required to perform these methods were not within the budget, nor were they available for continued use throughout the duration of the project.

Before the creation of the devices, the determination of the final micro-pattern designs was necessary. Doing this involved extensive literature research into the behavior of cellular micro-patterns, as well as the range of sizes that collagen can be consistently

reproduced on hydrogels. The team decided upon the "Concentric Circles" and "Alternating Circles" patterns as the final designs for the PDMS stamps to use as the micro-patterning device for the project. Both these pattern designs sufficed the micro-patterning device specifications as well as the functions that the device needed to have.

Micro-Patterning Device Specifications

A. Pattern should have circular areas ranging from a minimum of 50 μm in diameter to a maximum of 500 μm in diameter
B. The device should be biocompatible
C. The device should be reusable for a minimum of 50 uses
D. The cost of fabricating the device should be a maximum $150.00 USD
E. The dimensions of the device should be a maximum of 25 x 25 mm

Micro-Patterning Device Functions

A. Have a pattern that has specific areas for cells to grow in.
B. Use a solution that is biocompatible and promotes cell adhesion when transferring the pattern
C. Have several areas in one single pattern

Two final designs were chosen to satisfy the device specifications and functions because it was known that it would be possible to integrate more than one micro pattern into the topological silicon wafer prior to fabrication of the photoresist. Spacing between pattern elements, as well as the number of elements, were important details that were taken into consideration before developing the following final designs.

Table 11: Final Design: Concentric Circles PDMS Stamp: Advantages & Disadvantages

Design Description: Concentric Circles PDMS Stamp Pattern

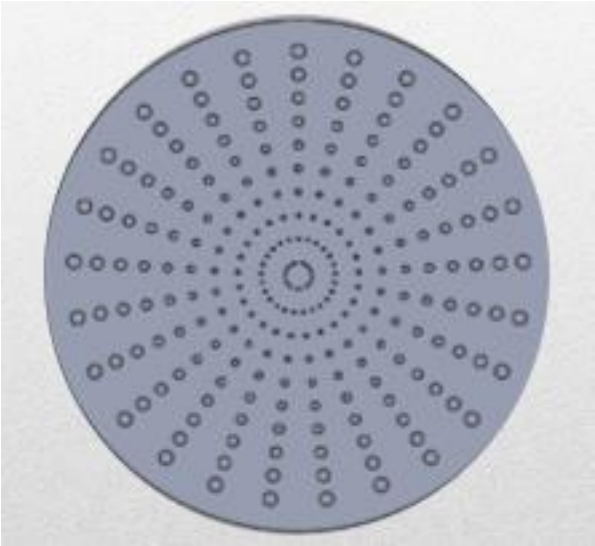


Figure 18: Concentric Circles PDMS Stamp Pattern

Advantages	**Disadvantages**
• Wide range of circle diameters • All circle diameters present on one design • Pattern shape is the same for each circle diameter	• May be difficult to distinguish circle diameters apart from one another after micro-patterning

Table 12: Final Design: Alternating Circles PDMS Stamp: Advantages & Disadvantages

Design Description: Alternating Circles PDMS Stamp Pattern

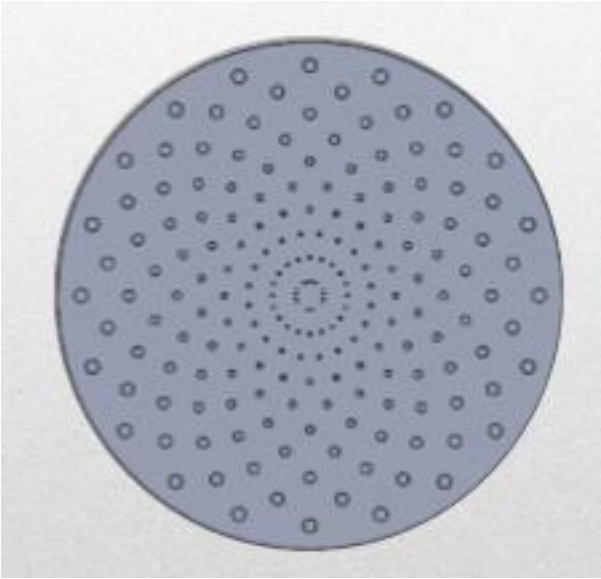


Figure 19: Alternating Circles PDMS Stamp Pattern

Advantages	**Disadvantages**
• Wide range of circle diameters • All circle diameters are present on one design	• May be difficult to distinguish circle diameters apart from one another after micro-patterning • The alternating pattern starts to disappear as the circle diameters decrease

Substrate Specifications

A. The substrate should be transparent
B. The cost of fabrication should be a maximum $150.00 USD
C. The minimum stiffness the substrate should be is 5 kPa
D. The maximum stiffness the substrate should be is 40 kPa

Substrate Functions

A. Promote cell adhesion and cell development

B. Mimic the mechanical behavior of an ECM in vivo

C. Have the ability to absorb the solution used from the micro-patterning device to transfer the pattern on its surface

Table 13: Final Design: 5% PAA & 8% PAA: Advantages & Disadvantages

Design	Advantages	Disadvantages	Predicted Stiffness [4]
5 % PAA **For 1 mL of hydrogel:** **125 uL ACL** **40 uL bis** **835 uL HEPES**	• Gel solution is spreadable without quick formation, allowing for uniform distribution	• Limits the range of experimental stiffness	3 KPa
8 % PAA **For 1 mL of hydrogel:** **200 uL ACL** **50 uL bis** **750 uL HEPES**	• Gel solution is spreadable without quick formation, allowing for uniform distribution	• Limits the range of experimental stiffness	8 KPa

4.4 Final Design Considerations

As discussed in section 2.6 of the literature review (Cell Signaling), cells are able to communicate to each other by sending and receiving signals. It was important to take this into consideration when the team designed stamp patterns as to avoid any possible cell signaling between the discreet groups of cells. It was also important to keep the groups of cells far apart to avoid the groups from coalescing. Through research it was found that cell signaling and intrusions on neighboring cell patterns occur at approximately 20-25 μm. [27] Therefore, when designing the stamp patterns, the team ensured that any distances between individual features were greater than 20 μm.

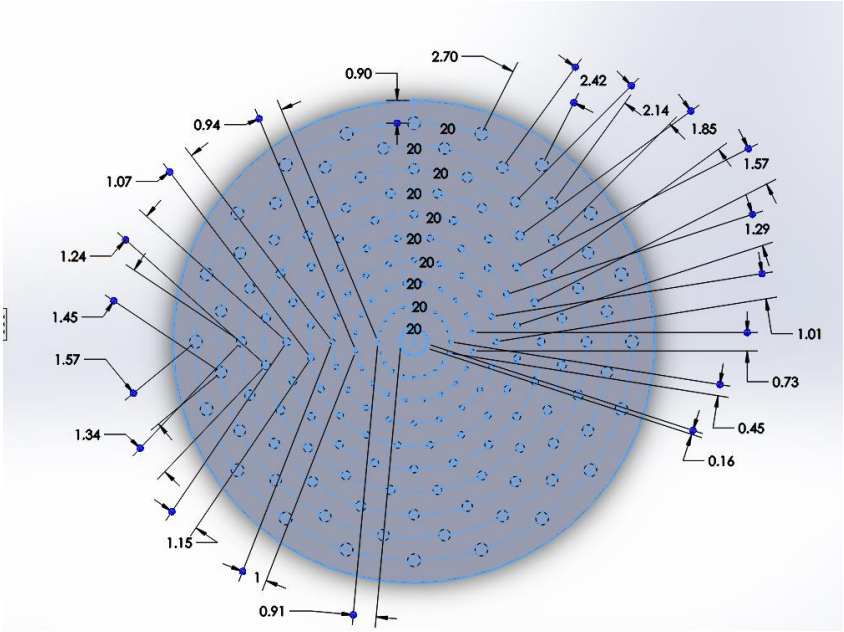


Figure 20: Distances between Circle Diameters on Alternating Circles Stamp Pattern

Figure 20 displays the stamp design and the distances between features on the stamp Table 14 and 15 shows the feature sizes and the distances between adjacent features for the Alternating circle stamp patterns and the Concentric circle patterns respectively.

Table 14: Distances between Circle Diameters on Alternating Circles Stamp Pattern

Circle Diameter Size (μm)	Number of Circles in Ring of that Circle Diameter	Distance between Circle Diameters in Ring of that Circle Diameter (μm)	Distance between Circle Diameter and Circle Diameter of Next Ring (μm)
50	20	160	910
100	20	450	940
150	20	730	1000
200	20	1010	1070
250	20	1290	1150
300	20	1570	1240
350	20	1850	1340
400	20	2140	1450
450	20	2420	1570
500	20	270	900

Below, Figure 21 displays the distances between the circle diameters of the circles on the Concentric Circles stamp pattern.

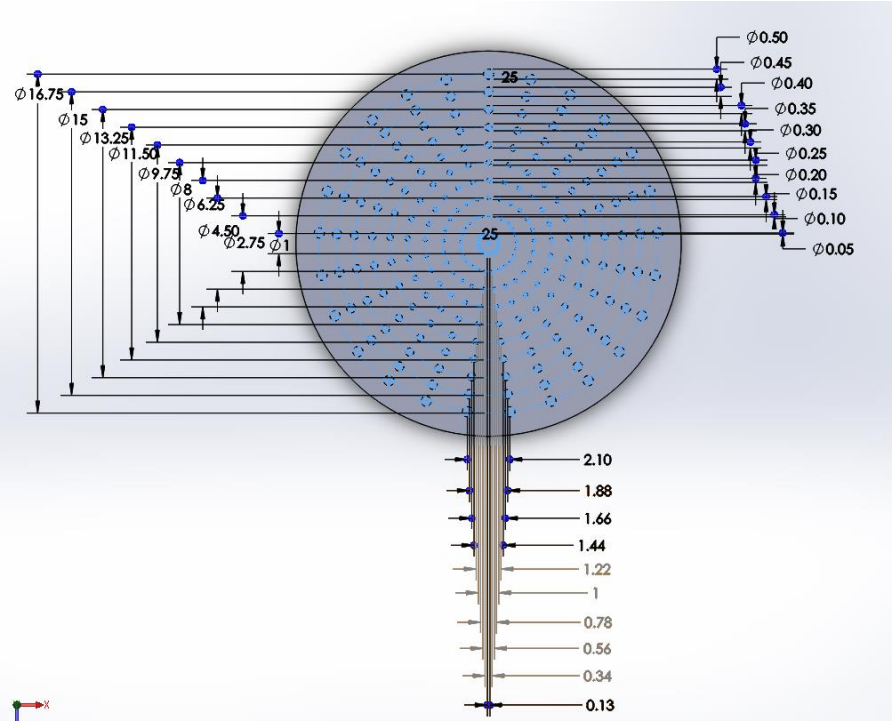


Figure 21: Distances between Circle Diameters on Concentric Circles Stamp Pattern

Table 15: Distances between Circle Diameters on Concentric Circles Stamp Pattern

Circle Diameter Size (μm)	Number of Circles in Ring of that Circle Diameter	Distance between Circle Diameters in Ring of that Circle Diameter (μm)	Distance between Circle Diameter and Circle Diameter of Next Ring (μm)
50	25	130	900
100	25	340	880
150	25	560	880
200	25	780	880
250	25	1000	880
300	25	1220	880
350	25	1440	880
400	25	1660	870
450	25	1880	880
500	25	2100	900

The distances in Table 14 and Table 15 show that the distances used were much greater than the researched distances of 20-25 μm. This overcompensation for space between circles on the stamp patterns therefore would prevent any possible cell-to-cell signaling or cell intrusions over to the next pattern.

Through the use of digital software (ImageJ, ref), the nuclei area and the cytoplasm area were measured. It was determined that the nuclei to cytoplasm ratio (N:C) was 0.19. With this, the average nuclei area was determined to be 255 μm^2 and the average cell area was 1287 μm^2 (data not presented). Using these numbers, the team was able to determine the theoretical number of 3T3 cells with cytoplasm that would fit on each of the different diameter patterns. The theoretical cell numbers are shown in Table 16 on the next page.

Table 16. Theoretical number of cells that will fit in each pattern based on the size of NIH/3T3 cells grown on polystyrene cell culture dishes

Diameter size (μm)	Theoretical Number of NIH/3T3 cells per pattern
50	2
100	6
150	14
200	25
250	38
300	54
350	75
400	98
450	124
500	152

In regards to the team's final selection of PAA as the substrate for this project, the team was able to satisfy the functions of substrate based off of the research presented in section 2.4 of the literature review (Hydrogels & Polyacrylamide).

Chapter 5— Final Design

The methodology of the fabrication of the micro-patterning device and substrate will be explained in this chapter. In addition, the experimental methods for utilizing these designs to verify their functions will also be explained in further detail. After creating the micro-patterning device, the team developed an optimal stamping technique for transferring the pattern on the substrate. The team then developed a method for seeding cells onto the substrate for examining the stamp transfer and for further observation of cell attachment measured by cell populations. The combination of these designs and methods created a system for observing cells in aggregates in response to chaining ECM stiffness. The verification of this system will be evaluated in a later section of this chapter.

5.1 Methodology

This chapter will explain the methods used to create the micro-patterning device in the form of PDMS stamps and the formation of the substrate—the PAA hydrogel. The methods for utilizing these designs as systems to observe cells will also be explained.

5.1.1 Fabrication of PDMS Stamps

The final stamp patterns for the micro-patterning device were chosen and the fabricated stamps can be seen in Figure 22 below:

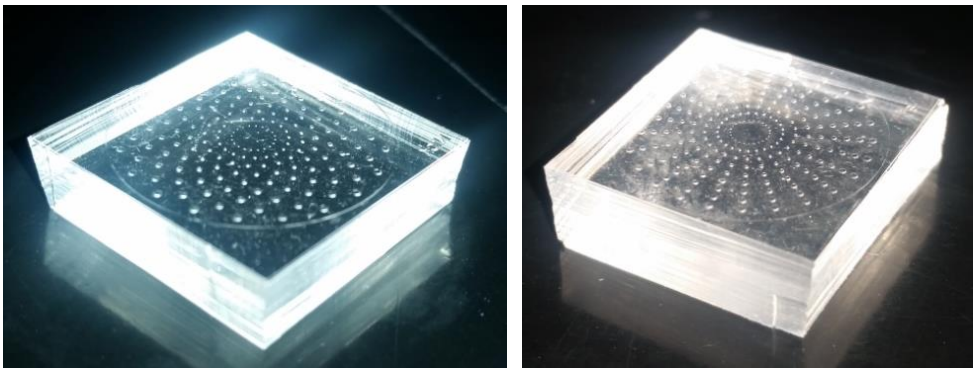


Figure 22: Final PDMS Stamps: Alternating Circles (left) & Concentric Circles (right)

The final design that was chosen to perform micro-patterning involves two processes to create the final devices; photolithography and soft lithography. As mentioned previously, photolithography is a process that involves transferring geometric shapes on a mask to the surface of a topological master wafer. The procedure used for creating the topological master is in accordance with the Standard Operating Procedure (SOP) used by the Micro-fabrication Lab at WPI, headed by Dirk Albrecht PhD (Appendix VII). In general, the main steps used for creating the final silicon wafer are as follows:

1. Dehydration Bake
2. Spin-Coating
3. Prebake
4. UV Exposure
5. Post-Exposure Bake
6. Development
7. Inspection
8. Post-Bake

The team outsourced to a Cad/art service company to create the photomask from the SolidWorks models previously shown in Figures 14 and 15. After receiving thephotomask, the silicon wafer and PDMS stamps were then created in Professor Albrecht's lab at WPI.

After creating this silicon master mold, PDMS is used to mold into it in accordance to the SOP for soft lithography that is also used by the Micro-fabrication Lab (Appendix VIII). This PDMS acts as a stamp for transferring the micro-pattern onto a desired substrate. In general, the steps required for generating iterations of the design as PDMS stamps are as follows:

1. Fluorination of Micro-patterned Substrate
2. Preparing the PDMS Mixture
3. Casting and Curing PDMS
4. Preparing a PDMS Device
5. Plasma Bonding

5.1.2 Micro-Pattern Transfer

Using the PDMS stamps, the team tried a combination of various techniques for efficiently and effectively transferring a pattern on to a substrate. The team used a technique adopted from the published paper: "Technique for Micro-Patterning Proteins and cells on Polyacrylamide Gels" [34] as a baseline for finding an optimal method for transferring the stamp patterns. A brief summary of this technique can be found in Appendix IV. The major steps from this technique involved:

1. Using a protein mixture as a stamping solution
2. Immersing a stamp in the stamping solution
3. After soaking the stamp, it would be placed on a 25 X 25 mm glass coverslip

The team modified this technique by first trying to modify the glass coverslip in order to promote cell adhesion. The most optimal method was to make the glass coverslip hydrophilic by using oxygen plasma cleaner, with a complete method of doing this detailed in Appendix II. The PDMS stamp was dipped into the petri dish that contained the solution mix of 3.1 mg/ml pureCol, 1M Acetic Acid, MilliQ water and 1 mg/mL fluorescein isothiocyanate (FITC). The stamp was then hand air pumped to remove the excess liquid. The stamp was then placed on the top slide, which was resting in a petri dish. A ~50g circular weight was placed on top of the stamp to allow for an equal pressure distribution on the stamp. The petri dish containing the top slide, stamp and weight was then placed on top of a slide warmer with an approximate temperature of 37°C for 10 minutes. The weight was then removed and the stamp peeled off from the top slide. The process of stamp transfer on to the surface of a glass coverslip is demonstrated in Figure 23 below:

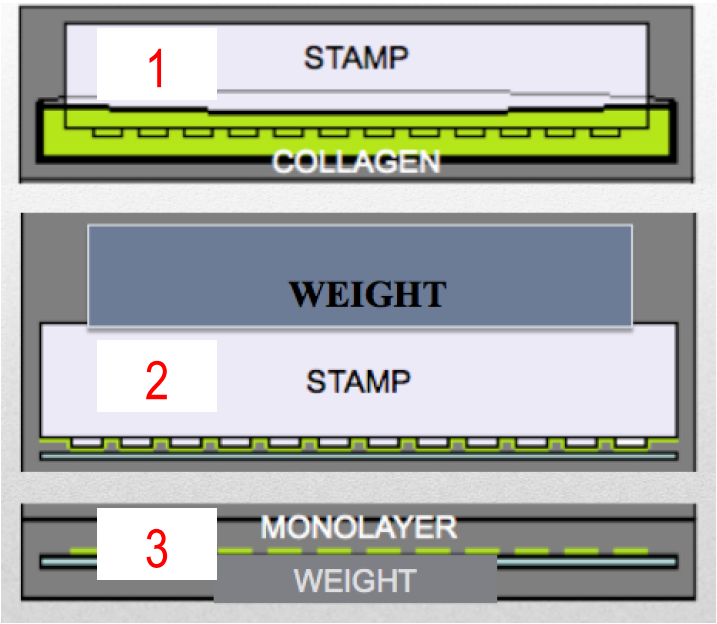


Figure 23: Micro-pattern Transfer Schematic

After this process was complete, the glass coverslip were imaged using a fluorescent microscope. The patterns created from the stamping technique above are presented in Figure 24 below:

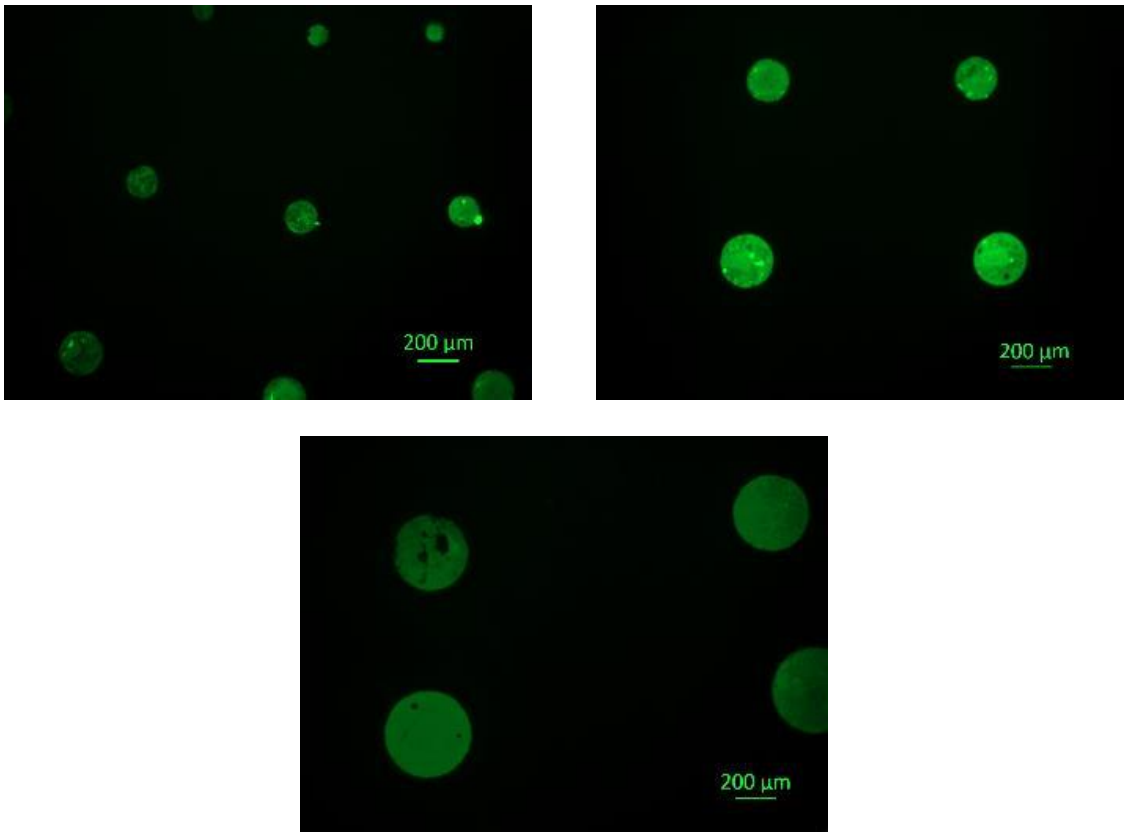


Figure 24: Images of collagen patterns on top slides

After it was confirmed that the pattern has transferred successfully to the top coverslip, the glass coverslips with the micro-patterns were then stored in a refrigerator at 4°C for storage until further use. For a detailed protocol on this micro-patterning technique, please refer to Appendix IV. The team then focused on fabricating a substrate to see if the pattern would allow for cell adhesion and the creation of cell aggregates on its surface.

5.1.3 Polyacrylamide Hydrogel Formation

The team chose to fabricate PAA hydrogels that would serve as an ECM substrate. The team used a protocol from Professor Wen's lab as a baseline for fabricating gels. This detailed protocol can be found in Appendix V. After weeks of experimentation, the team modified Professor Wen's protocol that yielded the most uniform hydrogels. The main steps of this protocol were:

1. Preparing a glutaraldehyde bath for 25 x 25 mm glass coverslips
2. Immersing the glass coverslips in the bath for treatment, approximately 24 hours.
3. Preparing a PAA hydrogel solution based on the desired stiffness.
    a. Pipetting the PAA solution onto the surface of a glutaraldehyde treated glass coverslip, which was glued to the bottom of a petri dish.
4. Placing the glass coverslip with the micro-pattern from the previous protocol onto the surface directly on the surface of the hydrogel.
    a. Placing the petri dish on a slide warmer at 37°C for 30 minutes.

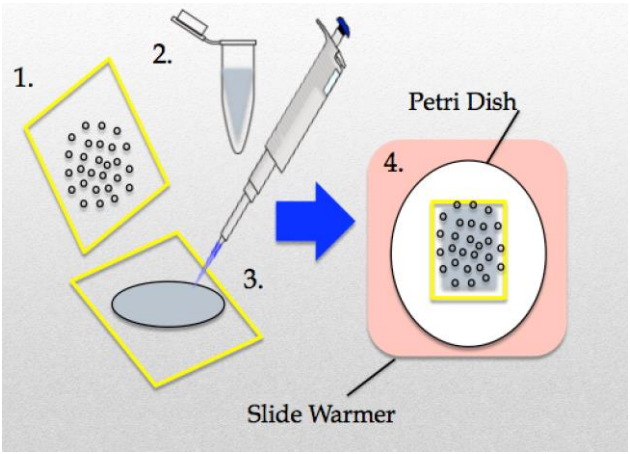


Figure 25: Schematic of the PAA Hydrogel Formation Process

After the PAA hydrogel was formed, the top glass coverslip would be removed and the hydrogel would be submerged in HEPES and were stored in a refrigerator at 4°C until further use. At this time the hydrogels would be prepared for cell seeding.

5.1.4 Cell Seeding & Incubation

Standard cell passaging procedures were used for isolating the desired amount of cells to be seeded. The cell passaging protocol used in this project can be found in Appendix I. Approximately 250K to 500K NIH/3T3 cells were seeded on each hydrogel. In order to ensure that every hydrogel being seeded contained the same amount of cells, they were placed inside the incubators so that cultures dishes did not have to be moved into incubators. Moving the dishes before allowing the cells to seed onto the collagen would sometimes spill a significant amount of cells over the edge of the glass coverslip and allow the cells to grow on the culture dish. This would sometimes cause cells to grow underneath the coverslip or on its bottom side so that being able to observe the individual cell aggregates became difficult due to the blurring interference of nearby cells on a different plane of visualization.

After seeding, the cells were allowed to adhere on the collagen patterns for 2-3 hours. After cell adherence was confirmed via microscopy, additional media was pipetted into the petri dish and used to rinse the hydrogel of dead cells and cells that were unable to properly adhere. This media was then aspirated and additional media is added to fully submerge the coverslips and hydrogel. At this point, the cell aggregates are ready for observation. A detailed protocol of this process can be found in Appendix VI.

5.2 Verification

The following images in Figure 26 were taken 12 hours after NIH/3T3 cells were seeded onto 8% PAA hydrogels.

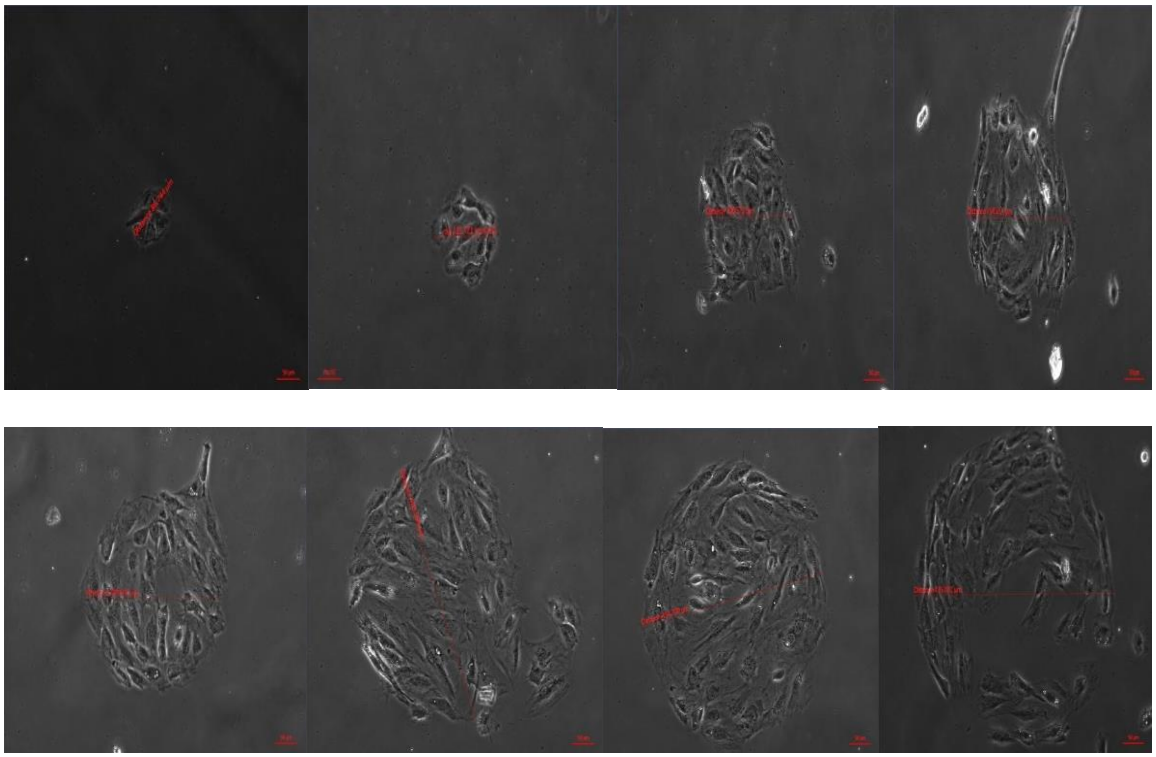


Figure 26: Patterns of collagen on 5% PAA after 12 hours, Observed at 20x magnification, Diameters (Top, left to right) 150um, 200um, 250um, 300um, (Bottom left to right) 350um, 400um, 450um, 500um at 20x magnification.

The images in Figure 26 verify that the different sized patterns allowed for different sized cell populations to adhere to the stamped patterns on PAA substrate. Figure 27 below, shows different sized cells adhered on 5% PAA 24 hours post seeding—also verifying that cells adhered in the stamp pattern.

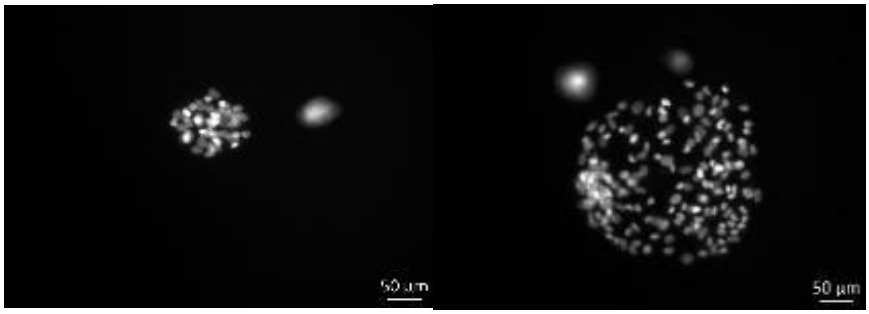


Figure 27: Different Sized Cell Patterns on 5% PAA after 24 hours. The image shows Hoechst stained nuclei on two patterns of (100 and 300 micron in diameter) 20x Magnification

The images in Figure 25 above verify the system created by the team, which allowed for the observation of cell aggregated in response to varying substrate stiffness. After this system was verified through several iterations demonstrating its effectiveness, the team was then ready to observe the cellular behaviors in response to the mechanical cues of its stiffness.

The engineering standards used in the experimental phase of this project were in accordance with the standards set forth by the International Organization for Standardization (ISO) [35]. The following ISO standards used by the team are reflected in Table 17 below:

Table 17: ISO standards and their intended purpose that were used by the team

Standard	Purpose
ISO 8655:2002	Measuring Equipment
ISO 8037-1:1986	Microscope Slide Characteristics
ISO 8578:2012	Microscope Characteristics
ISO/NP 20391-1	Cell Counting Procedure
ISO 8255-1:2011	Glass Coverslip Characteristics

This specific project does not have any significant economic, environmental, social, political, health, or sustainability, influences, impacts, or concerns.

Chapter 6— Final Design Validation

6.1 Results

This section describes the results from this project and validation after quantifying several measurements taken across experiments. The validation results presented in this section are:

6.1.1 Validation of the device

6.1.2 Collagen Patterning

6.1.2 Cellular parameter on hydrogels of varying stiffness

6.1.1 Validation of the device

The dimensions of the PDMS stamps are determined by the final dimensions of the silicon wafer that is fabricated through photolithography. The dimensions of this silicon wafer are also determined by the final dimensions of the photoresist that is manufactured by _outsourced company_. The height of the wafer is in accordance with the original thickness of the SU8 2035 silicon wafer used during photolithography; 80µm in thickness. The resulting radial dimensions of the PDMS stamps were obtained using a Zeiss microscope to record phase contrast images, accompanied by the Zen microscope imaging software as shown in Figure 28 below.

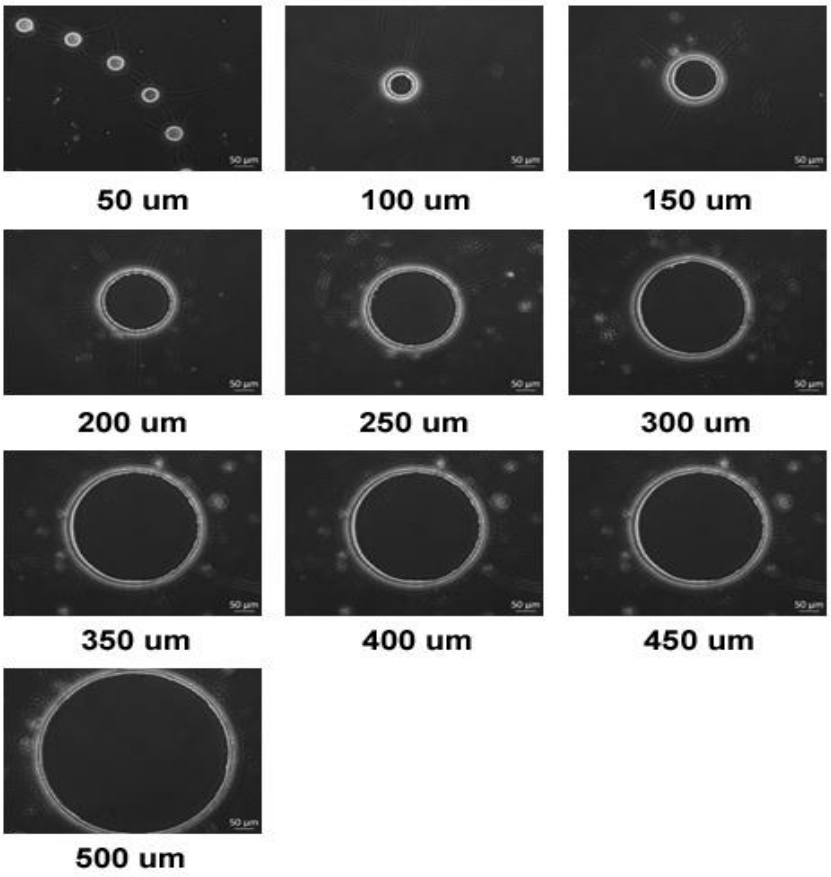


Figure 28. PDMS Pillars at 20X Magnification

The dimensions for each respective pattern size in both designs used to create the PDMS stamps were averaged together, having yielded the same values post-fabrication. Their averages and the standard deviations are shown in the Figure 29 below.

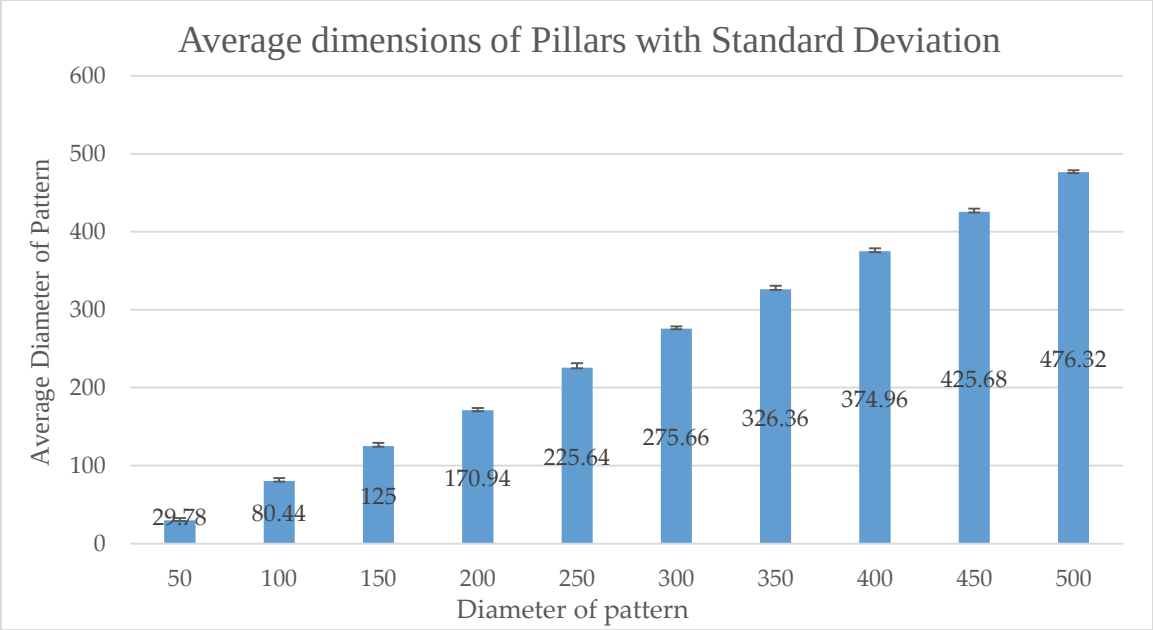


Figure 29. Average dimensions of pillars on PDMS stamp with Standard Deviation (SDV)

The theoretical calculations of the PDMS area diameters should be as noted above, so the standard deviations that accompany those values represent possible differences in the actual measurements as suggested by imaging and the calculate averages of the measurements.

6.1.2 Collagen Patterns

The first of these products was the fluorescent collagen stamped areas on the top slides. The application of the collagen to the slides was done in a variety of methods until the most consistently reliable method was produced.

The resulting pattern from the optimal stamping method, detailed in Appendix IV, is shown in Figure 30 below.

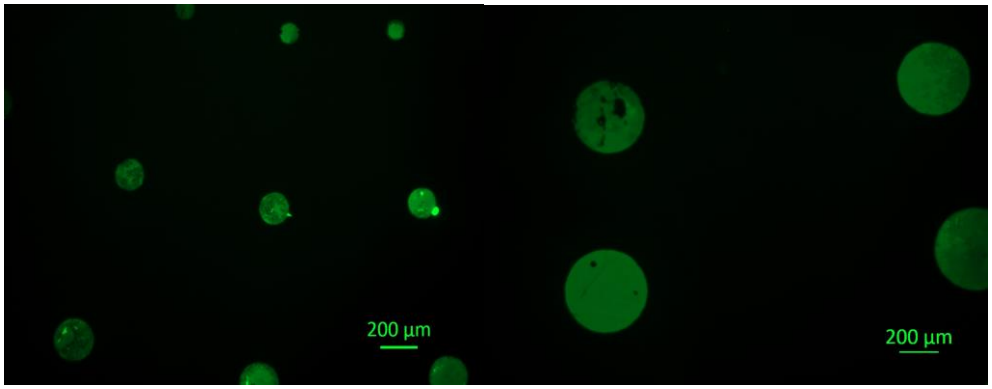


Figure 30: Monolayer Stamped Collagen with diameters of (left) 100um, 150um, 200um (right) 400um, 450um (5X Objective)

In Table 18 below presents how many islands of each diameter there are per stamp and also the average number of these islands that transferred with each stamp.

Table 18. Average Number of islands that transfer per stamp

Diameter (μm)	Number of Islands on PDMS stamp	Average Number of Islands that successfully transferred to coverslip	N = Number of Stamps Recorded
50	25	12.5	N=2
100	25	24	N=2
150	25	21	N=2
200	25	17	N=2
250	25	16	N=2
300	25	16	N=2
350	25	16	N=2
400	25	16	N=2
450	25	17	N=2
500	25	19	N=2

We observed that the transfer of the 50 μm diameter patterns from the PDMS stamp to the top coverslip was inconsistent. As this process is not an automated process, there can be some very large variations between both how many islands are printed, as shown in the table above, and what the actual diameter of these islands are, which is represented in figure 31 below.

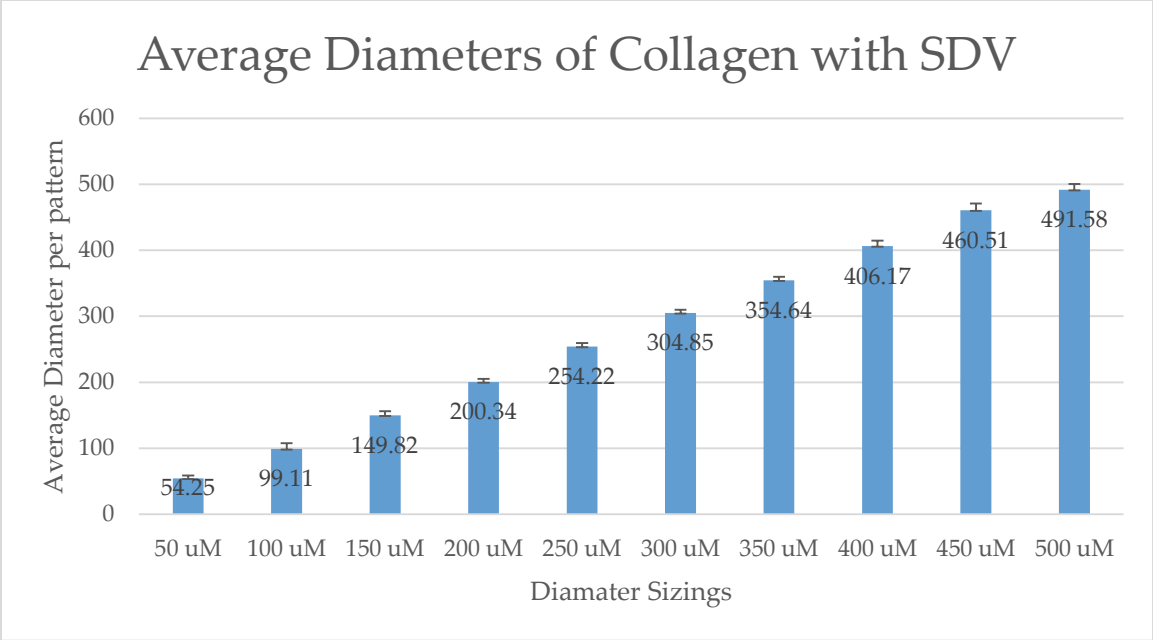


Figure 31. Average Diameters of Collagen Islands Transferred for Cell Attachment

The average diameters for most of the island sizes is well within 5um of the desired diameter. This difference would only result in the attachment of only a few more cells at the smaller sizes while only having a larger impact on the attachment size at the progressively larger sizes though not anything significant enough to grossly affect data.

6.1.3 Cells on Polyacrylamide Hydrogels

Once the method for creating the top slide for transferring the collagen was perfected, the most efficient method for creating the hydrogel with the pattern on the slide to be determined. s mentioned in the previously in the section, early methods of pattern transferring resulted in much of the collagen either breaking apart or shifting resulting cell attachment that did not fit any pattern or designated cell area. In Figure 32 below, the resulting cell attachment from a fragmented pattern is shown

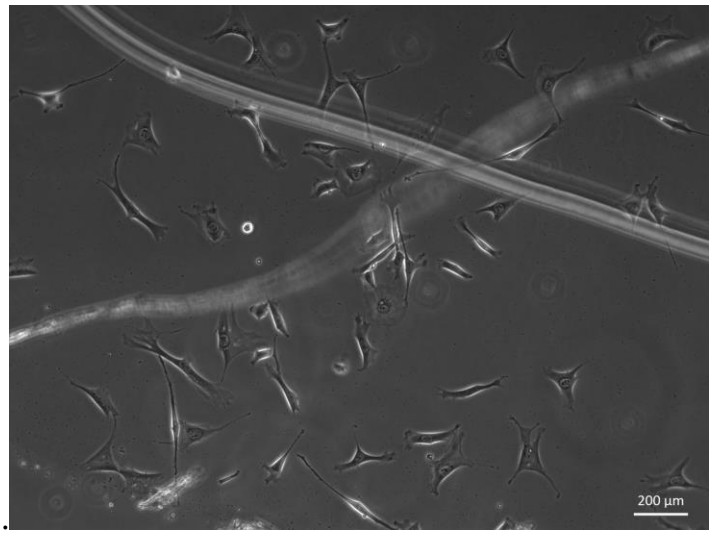


Figure 32: Fragmented collagen seeded on 8% PAA hydrogel

When the method was corrected so that there would be no excess collagen on the stamp forming only a monolayer of collagen, the pattern on the hydrogel started to transfer almost perfectly with some variance in the size of the collagen diameters which have been shown in Table 17, to have a range of variability of sizes. So the sizes that are observed with cell attachment are within the range that is expected to be seen.

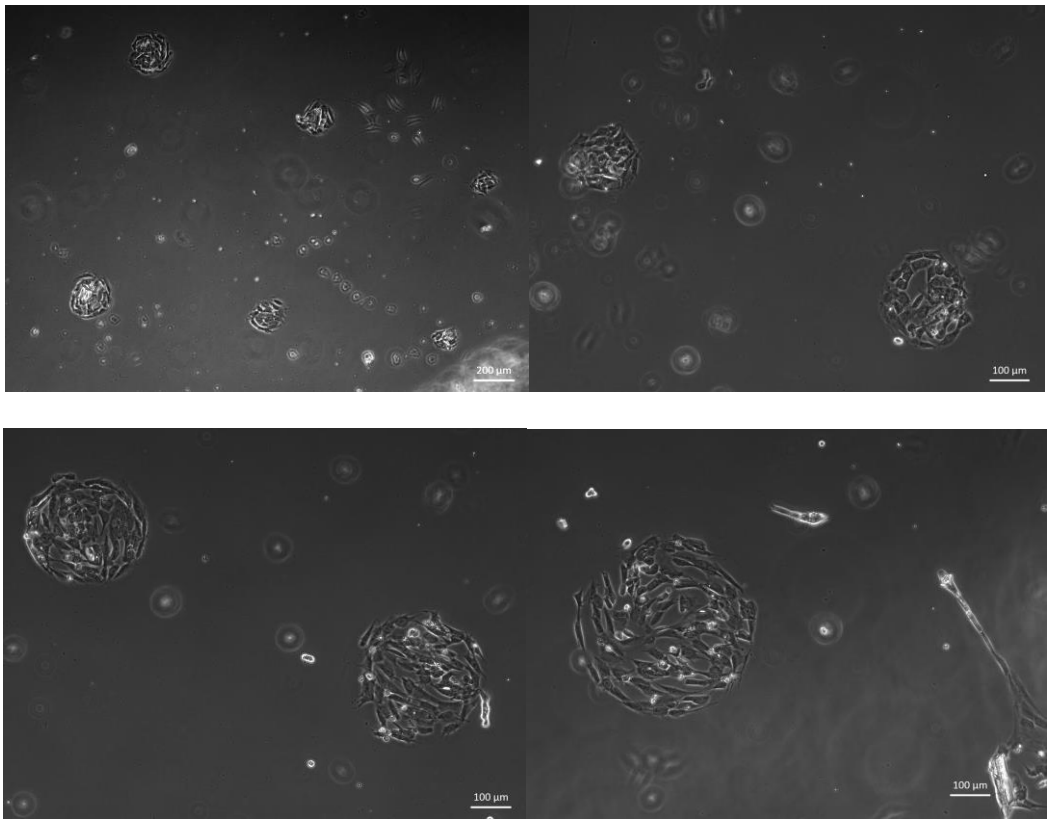


Figure 33: Phase contrast images of NIH/3T3 cells patterned on 8% PAA hydrogels

As the method was optimized and there was a significant pattern transfer with a majority of the hydrogels, we examined the growth and morphology of the NIH/3T3 cells on PAA gels of different stiffness. The cells were stained with a Hoechst dye to observe and count the nuclei on each of the areas for cell attachment. In Figures 31 and 32, the differences between both the nuclei and the cytoplasm is shown at different cell attachment diameters and PAA hydrogel stiffness.

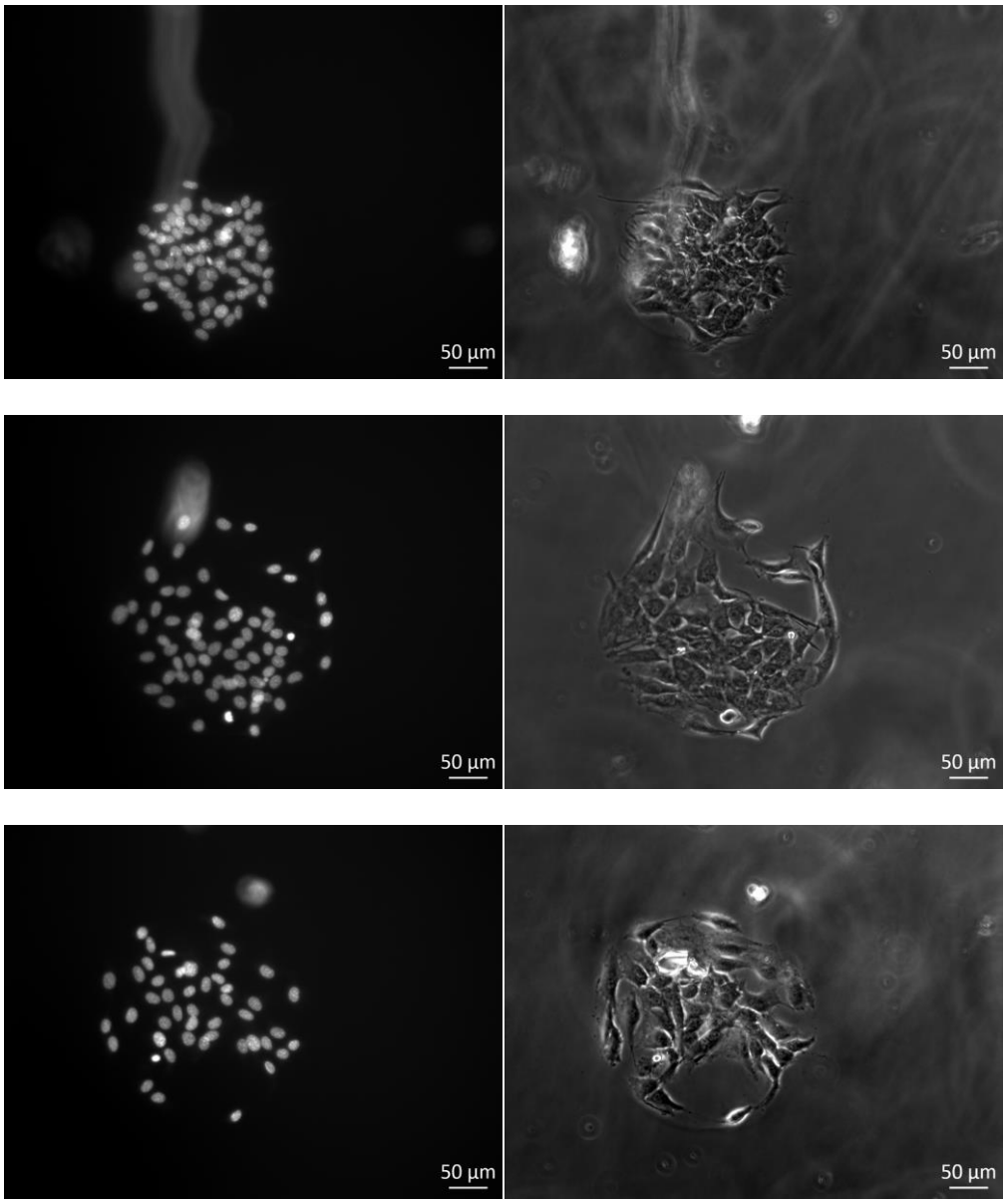


Figure 34: Hoechst stained nuclei (left) & phase contrast image of cells (right) on 8% PAA hydrogel.

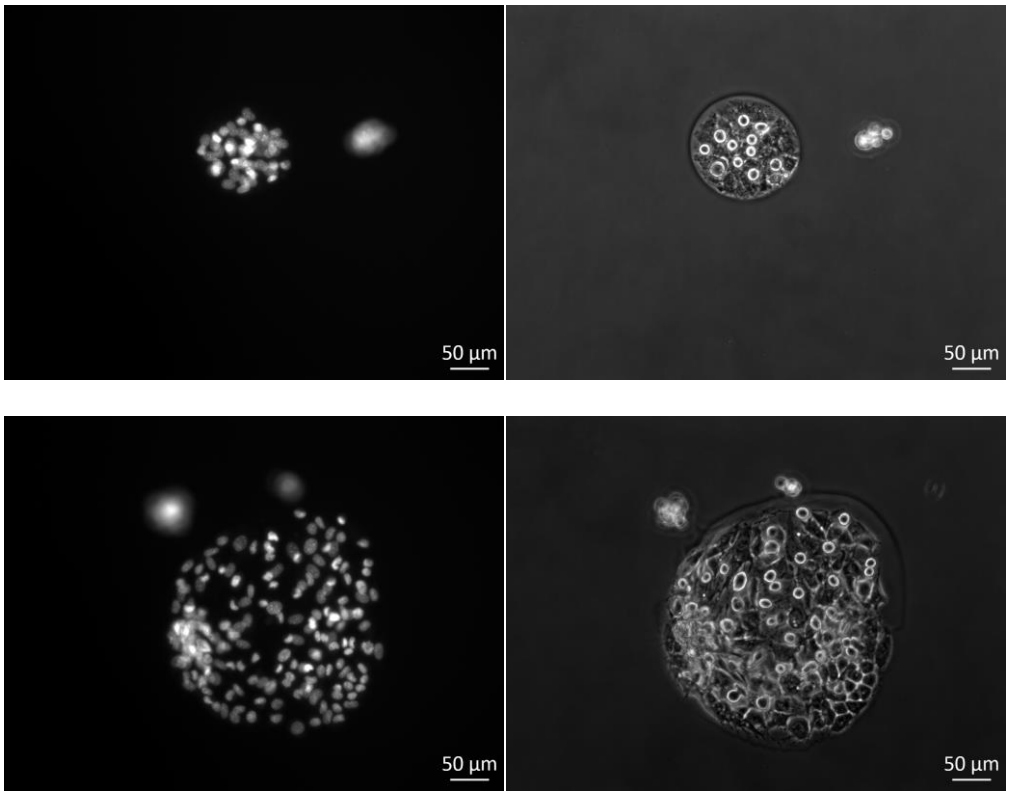


Figure 35: Hoechst stained nuclei (left) & phase contrast image (right) on 5% PAA hydrogel

Comparing how cells developed on the two different stiffness of the hydrogels, the cells that were on the 5% hydrogel have nuclei that are much more rounded, compared to the elongated nuclei that occur upon the 8%. The cells that develop on the 8% also exhibit more cell spreading and cytoplasm development than those that develop on the 5% hydrogels.

6.2 Cell Measurements

Once the method for the formation of hydrogels and transference of the collagen pattern to the hydrogel was optimized, cell counting with Hoechst stained cells was done to determine the number of cells that attached to each cell population diameter for each of the stiffness. In the table below, the results for the cell counting of each diameter taken was averaged out and recorded.

Table 19. Average Number of cells per diameter on 5% PAA & 8% PAA hydrogel

Pattern Diameter (μm)	Average number of Cells on 5% PAA Hydrogels per aggregate	Average number of aggregates observed	Average number of Cells on 8% PAA Hydrogels per aggregate	Average number of aggregates observed
50	11 ± 4	4	10 ± 6	7
100	23 ± 4	6	18 ± 6	10
150	45 ± 15	3	29 ± 8	11
200	50 ± 4	3	33 ± 6	10
250	N/A	N/A	53 ± 7	8
300	107 ± 11	3	71 ± 11	8
350	132 ± 5	4	101 ± 18	9
400	141 ± 9	3	124 ± 17	9
450	185 ± 20	4	145 ± 18	6

We were unable to obtain cell population data points for island diameters of 250 μm size on the 5% hydrogels. Similarly, we were unable to observe cell seeding on the 500 μm diameter features on both 5% and 8% PAA gels.

After observing the number of cells that were on each diameter of the hydrogel, a statistical analysis was done to determine the average nuclei area on each diameter and stiffness. There was insufficient data to determine the average nuclei area on each diameter for the 5% ACL hydrogels, however shown in Table 20 below is the average cell diameter and standard deviation of each diameter size on the 8% ACL hydrogel.

Table 20. Average nuclei area (um^2) for each diameter pattern on 8% PAA

Diameter of Pillar (um)	Average Nuclei Area (um^2)	Standard Deviation +/-	N= # of patterns measured
50	283.7	62.7	N=7
100	340.5	132.3	N=12
150	262	113.1	N=10
200	280	81.6	N=10
250	321	115.1	N-9
300	290	56.9	N=8
350	307	110.5	N=7
400	348	70.1	N=5
450	352	55.3	N=5

Chapter 7— Discussion

The team was able to successfully design, verify, and validate the function and efficacy of a micro-patterning method. The silicon wafer that was fabricated from photolithography allows for several iterations of the pattern to be reproduced as micro-patterning stamps. This is convenient for replacing stamps that break or tear over time and repeated use. The stamps themselves were successfully used as devices for micro-contact printing, which allows for easily reproducible micro-patterns that were consistently printed on glass coverslips. These patterns were also properly transferred onto polyacrylamide hydrogels for the purpose of data analysis. The results obtained from the verification of the design show that our method and device succeeded in creating micro-patterns that control for sizes of cell aggregate areas, the number of aggregates, and substrate properties, which include the stiffness and chemical composition. With the design, the team was able to see clear differences in cell attachment and morphology of different cell populations all on the same substrate. Our project has also been able to delineate the potential for continued use of our project design in conducting experiments on the behavior and development of cellular aggregates.

Validation of the stamp

The team was able to develop PDMS stamps from the silicone wafer in sizes ranging from 50 to 500 µm on a single PDMS stamp. Each PDMS stamp incorporated multiples of pattern sizes for each feature as shown in Figures 18 and 19. Our

verification studies by means of collagen/FITC stamping on coverslips indicate that we were unable to obtain consistent stamp patterns for the 50 μm features. In general, the actual sizes of the features transferred on the coverslips indicated that the features were slightly smaller than the expected sizes as detailed in Figure 29. One of our constraints was the time factor as well as access to manufacturing the silicone wafer on our own. Despite this anomaly we were able to create feature sizes of 100 to 500 μm sizes repeatedly and consistently.

Stamping process

Our stamping process is a unique and simple process wherein we were able to consistently and repeatedly transfer collagen patterns stamped on coverslips to PAA hydrogels as shown in Figure 30.The success of this procedure was demonstrated by the ability of NIH/3T3 cells to adhere and replicate on the patterned areas. Measurements of the patterns created on coverslips indicated that the diameter of the patterns were approximately 5 μm less than the predicted size. As discussed earlier, this may have happened in the process of making silicone wafer. Improvements in quality control can easily fix this issue. This small change in diameter will not significantly affect the number of cells that can adhere to the patterns. The simplicity of our approach lies in the fact that the stamping process does not require special chemicals or expensive set up. Since the patterns are transferred to the hydrogel as the gel is forming the collagen molecules are being trapped and stabilized by the hydrogel. A modified method to quantify the amount of collagen transferred would be desirable.

One of the limitations that we encountered in the research is that we were

unable to discern the differences between smaller patterns on PAA gels because of the lack of a proper marker (florescent or other) to detect the patterns after the transfer of collagen/FITC mixture on to the hydrogel. Since the FITC is not conjugated to the collagen, the FITC gets washed away as soon as the coverslip with the collagen/FITC patterns was placed on the hydrogel. The only means to identifying the pattern transfer and its stability was solely based on cell attachment and growth. This limited our ability to discriminate between 50 and 100 μm, 100 and 150 μm, 150 and 200 μm sizes etc. It is most likely that the 50 μm features did not form or transfer consistently on to the hydrogels. It is therefore important to incorporate a method or reagent to allow the ability to detect the patterns on the hydrogel before seeding the cells. This could help one to validate the success of pattern transfer before cell seeding. We were able to observe cell growth on patterns ranging from 100 to 450 μm. However, after repeated attempts, we were unable to observe cell growth on 500 μm patterns consistently despite the fact that these patterns were observed on the top coverslip. It is possible that the shear force of the hydrogel during the gel formation is fracturing the pattern beyond 450 μm limit. It is important to perform an analysis of this process using more precise method.

The diameters for every element of the pattern were all less than the theoretical dimensions as shown in Figure 34, within their calculated standard deviations respectively. This discrepancy in the pattern dimensions of the PDMS stamps was observed using phase-contract microscopy. The fact that the dimensions of the PDMS stamp elements were consistently lower by the same amount is attributed to either the fabrication of the photoresist used for photolithography or the silicon wafer itself.

While observing the cells on the hydrogel, results also showed that all pattern circle sizes could be transferred onto the hydrogel. There was a significant amount of variability throughout the experimental phase in how often different sizes would be transferred and how wholesome they remained in the gel during gelation. These differences could be observed after cell seeding and monitoring over time. These results are specifically shown in Table 18 and Table 19.

The possibility of a decrease in the diameter measurements on each cellular pattern was not considered during the design process of the micro-patterns. In order to understand the causation of the reduction in pattern size, further research was conducted into cellular and collagen behavior during hydrogel formation and cellular adhesion. Studies have shown that decrease in matrix area can be induced by cellular contractions induced after adhesion to the ECM. Mechanical signals from to the ECM, as well as contractile forces to the ECM, are transmitted by focal adhesions. These focal areas are the interface between cells and their surrounding ECM, giving cells an inherent ability to adhere to its ECM and contract [36] [39].

Creation of patterns of discreet cell populations

The idea of creating the patterns of varying sizes on hydrogels was to allow the study of cellular dynamics and behavior of discreet populations of cells. The stamp patterns were able to create islands of collagen stamps on PAA that was able to support cell populations from as low as approximately 10 to about 150 cells based on theoretical calculations based on cell areas calculated from NIH/3T3 cells grown on polystyrene cell culture dishes (Table 16). We observed that the cell numbers on 5%

and 8% PAA hydrogels significantly differs from the predicted numbers (Table 19). This indicates that the cell morphology changed as a result of changes in stiffness. Both 5% and 8% gels carried more than the predicted number of cells. This preliminary data indicates that stiffness plays a major role in cell behaviors. Further studies on the nature of changes in various parameters such as growth rate, cellular viscoelasticity, gene expression patterns, contractile properties and other relevant physiological parameters can advance our knowledge in the area of tumor biology. This can also help advances in the area of studies related to cellular changes for various cell types.

Tracking the cells on individual features

One limitation of the stamp designs became glaringly apparent to the team when studying individual cell aggregates. This limitation was that due to the identical distribution of area units on the pattern, tracking the development of a specific cell aggregate from the pattern over time would be nearly impossible without proper markings of some kind. This makes it very hard to compare the cellular development at certain time points unless a specific location can be recorded and identified on the patterns. To accommodate this need, it would be necessary to incorporate some sort of labeling or micron sized features/etchings on the coverslip that would allow for a researcher to easily identify and track the patterns and the cell aggregates over extended periods of observation and imaging. The design labeled as "Concentric Circles", which has linear columns of circles, has sufficient space between them for organization of the micro-pattern with lines separating the columns and number/letter

labels for each column. By the nature of this pattern, each column has one aggregate of each specified dimension, so identifying the correct group of cells by column label can be accomplished. One suggestion that requires minimal amount of modification is to use thinly-pointed markers to mark the underside of the transparent culture dishes in which the hydrogels are in, although this would require that the coverslips are permanently adhered onto the dishes. This could not work for the design labeled as "Concentric Circles" and label modifications to the actual stamping device would likely be necessary. Completely changing the geometrical designs of the stamps is another potential solution to facility location of specific cell aggregates. Instead of the current circular designs with rings, a square design could be used with the pattern circles organized into rows and columns that would be easily identified and recorded by their position in the pattern matrix. This would make the observations of certain patterns simpler and allow for the user to have an easier time gathering data from individual populations of cells as they develop.

Regarding cellular growth and development, our results show that the cell population on each of the island diameters of the 5% PAA hydrogel had more cells attached to than those of the 8% PAA hydrogel as seen in Table 17. The cells that do attach to the 5% hydrogel however exhibit characteristics that are different from cells grown on tissue culture plastic. The cells that attach to the 8% hydrogels however are similar in both structure and behavior to those on the petri dish. The difference in cell number could be partially attributed to the amount of cell spreading that occurred with cell development on the hydrogels during adhesion. The individual NIH 3T3 cells experienced much more cytoplasmic spreading in the 8% hydrogels in comparison to the

NIH 3T3 cells in 5% hydrogels, which were mostly be clumped and experienced very minimal cytoplasmic spreading. The limitations of this portion of data that were only able to observe the effects of hydrogel on a single cell type at two different stiffness values. Regardless, it was possible to conduct sufficient statistical data analysis on the results yielded from the design, representing a successful proof of concept of our final design.

Being able to study the effects that both cell-cell and cell-ECM interactions have on one another has numerous applications in the development of ways to fight the progression of tumors. We suggest that our method and design can be used improve upon the methodologies of past research such as those that have focused on the role of ECM stiffness in tumor progression. One such study by Levental et al. analyses the role that stiffness of the ECM plays in tumor progression with little to no focus on the effect that cell-cell interactions play. [38] They observed how the crosslinking of collagen which lead to the stiffening of the ECM which promoted tumor progression in the cells observed. This research could be improved by our method by not only enabling the study of how stiffness play a role in the progression of tumors, but also how interactions with between cells affects the progression of tumors. Data analysis on cell conglomerates of increasing sizes would yield models that describe the characteristics of tumors at various stages of growth and populations levels.

Another study that actually used micro-contact printing to define the geometries and perimeters of cancer cell aggregates could also benefit from our design. This study, conducted by Janet M. Tse, et al. studied how mechanical compressions of cancer cells causes them to develop into more invasive phenotypes. Their results of their study could have been more expansive by incorporating a pattern design that was similar to our

approach [39]. All of the sizes of the cancer cell aggregates were uniform, which means they were not able to produce results that could show statistical differences in the cellular responses to the mechanical stimulation as the sizes increased or decreased from their original population size.

Chapter 8— Conclusions & Recommendations

In conclusion, the final methods that the team designed and used for the creation of studying the behavior of discrete cell populations were successful in meeting the goals that the team had designated in the project objectives. The project was successful in partially meeting the goals of the revised client statement, as the team was able to create a method to observe different sized cell populations on a substrate of varying stiffness. This method of using a PDMS stamp for the placement of the collagen patterns on to a top slide allowed for the rapid and efficient production of stamped top coverslips for transfer to PAA hydrogels. The PDMS stamps were also very cost effective with the largest cost being the silicon wafer used for photolithography. However, after the expense of the mold had been paid, it was easy and cheap to rapidly create more stamps. The stamps are also highly efficient, being both able to rapidly produce patterns along with the technique used in the creation of the hydrogel, allowing for the optimal pattern transference and thus a greater rate of successful patterns for data collection.

After creating this method for transferring the collagen pattern onto the hydrogel, the team set out to try and observe the physical properties of the cells that were affected by the change in stiffness of the substrate and the changing cell population sizes. However, due to time constraints and unfortunate mechanical restraints due to the loss of the use of an AFM microscope, the observable cell properties came solely from the team's observations during cell development. The team saw that with increasing gel stiffness, more cells would attach and develop normally compared to those on softer

stiffness. While at larger cell population sizes softer gel stiffness did have more cells seeded on them, these cells did not have the extensive cytoplasm that would be normal for cells that attached and develop as per the norm.

For future projects the team suggests that some additional observations are recorded as to gather more detailed data that could be used in different research. The team suggests that future teams do a time lapse recording of the development of the cells as the project was limited to only observing the cells at intervals, which did not allow for observation of complete development of the cells on the hydrogels. The team also suggests that future groups increase the amount of times that stamps are replaced as the team replaced the stamps approximately every two months. The team suggests that the stamps instead be replaced every two-three weeks, especially if the stamps are consistently being used for stamping. This change would prevent any real chance of pattern degradation due to the damaging of the stamp itself.

For future projects the team also recommends that groups look into seeding more cell types on to the hydrogels, such as various cancerous cell lines, and other cell types such as epithelial, endothelial cells etc. Observing how different cell lines react to the changing stiffness and cell population size is key to understanding the cell-cell and cell-ECM interactions and cell signaling. As it has been observed that cancer cells invade into other cells much more rapidly in which the ECM stiffness is higher. Our approach would allow the study of the behavior of discreet cell populations. This is particularly relevant in the case of cancer cells to measure cellular properties such as contractile forces and cell differentiation. It would allow for the analysis of these properties through the use of AFM to measure changes in the viscoelastic nature of the cells and the concomitant

phenotypic and gene expression changes that cells undergo as the cell acquire the ability to transform from a non-metastatic to a metastatic phenotype. With the team's project, both the stiffness of the substrate along with the population size of the cells that would interact could be varied, allowing for a better understanding of how cell-cell and cell-ECM interactions affect the development and differentiation of cancer cells.

References

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Appendix

I. Protocol for Cell Culture of NIH/3T3 Cells

Materials

- NIH/3T3 Cells
- Complete media
    - 10% FBS
    - DMEM
    - PenStrep
    - GlutaMax
- 15 mL conical tube
- .25% Trypsin
- Hemocytometer
- DPBS

Procedure

- Remove Complete media from Petri Dish
- Rinse with DPBS then aspirate out
- Add .25% trypsin to the petri dish
- Put Petri dish into incubator and check dish under microscope every 2-3 minutes until 90% or more of the cells have detached.
- Add complete media to the dish that is equal to double the amount of trypsin that was added.
- Pipette complete media out of Petri dish and into a conical tube.
- Centrifuge complete media for 5 minutes
- Aspirate complete media out of conical tube (careful to not aspirate the cells out)
- Add complete media to conical tube, repeated pipetting to ensure cells break apart from one another.
- Count cells using a hemocytometer and determine the cell density per mL
- Replate cells at desired cell density

II. Protocol for Treatment of Top Cover Slips

Materials:

- Glass cover slips
- Cover slip holder
- Glass beaker
- Tweezers
- Culture/petri dishes
- Air pump
- 70% ethanol
- Ultrasonic cleaner (exact model needed)
- Plasma cleaner (exact model needed)

Sterilization Procedure:

- Insert the desired number of cover slips into the holder.
- Ensure that all coverslips are not in physical contact and there is sufficient space between them
- Insert cover slip holder into a glass beaker
- Fully submerge cover slips in 70% ethanol
- Activate the ultrasonic machine for 5 minutes
- Remove the holder and cover slips
- Dry the holder and all cover slips with the air pump
- Store each cover slip in a separate sterile container

Plasma Cleaning:

- Make sure each cover slip is isolated in separate containers
    - i.e. culture/petri dish
- Insert the container into the plasma cleaner after removing the cover
- Turn on the machine and pressurize the chamber
- Allow the cover slips to be cleaned for roughly 60 seconds
    - maximum frequency setting
    - ensure purple glow
- Turn off the plasma cleaner and depressurize the plasma chamber
- Extract the dish from inside the machine and cover the dishes
- Repeat procedure for any cover slips to be stamped on

III. Protocol for Treatment of Bottom Cover Slips

Materials:

- Glass cover slips
- Cover slip holder
- 2 glass beakers
- Tweezers
- Culture/petri dishes
- Air pump
- Stir bar
- Stirrer
- 70% Ethanol
- (3-Aminopropyl)trimethoxysilane
- DI (deionized) water
- 0.5% Glutaraldehyde Bath

Sterilization:

- Insert the desired number of cover slips into the holder.
- Ensure that all coverslips are not in physical contact and there is sufficient space between them
- Insert ceramic tray into a glass beaker
- Fully submerge cover slips in ethanol
- Activate the ultrasonic machine for 5 minutes
- Remove the tray with tweezers
- Dry the ceramic tray and all cover slips with the air pump
- Store each cover slip in a separate sterile container

Preparation for Glutaraldehyde Bath:

- Add the cover slip holder with cover slips into the beaker
- Fully submerge the cover slips (~150ml of 70% Ethanol)
- Add 2ml of (3-Aminopropyl)trimethoxysilane to this beaker
- Place the beaker on the stirrer and insert the stir bar
- Turn on stirrer and allow it to spin at the fastest setting for 30 minutes
- Remove the cover slip holder and insert it into a separate container filled with 70% ethanol to rinse for 2-3 minutes
- Remove and insert the holder into a container with DI water for 2-3 minutes
- Use the air pump them to dry each individual cover slip before submerging them in the glutaraldehyde bath
- Ensure that all coverslips are not in physical contact and there is sufficient space between them in the bath.
- After a minimum of 4 hours, the coverslips should be ready to be used for hydrogel formation

- The more time the slides remain in the bath will ensure that they have been fully sterilized and the treatment is preserved, leave slides in the bath overnight.

Extracting and Storing Hydrogel Cover Slips

- Remove the cover slips from the glutaraldehyde bath individually with tweezers
- Use the air pump to completely dry the cover slips
- Store them in a sterile container
    - i.e. culture dish or petri dish
- Keep these cover slips stored in a fridge, at roughly 4°C
- The coverslips should be acceptable for use in hydrogel formation for 2 weeks
- Note that the effects of treatment degrade over time and

IV. Protocol for Stamping of Top Cover Slips

Materials

- Fluorescent dye
- Bovine collagen (3.1 mg/ml) (keep on ice)
- Acetic acid (1mM)
- MilliQ water chilled for ~10 min
- 70% Isopropyl
- Tweezers
- Clean disposable paper towels (i.e. KIMTECH Kimwipes)
- Glass beaker
- Air pump
- Culture/petri dishes
- Heating platform
- 50g-100g weights

Making the Collagen Solution

- Keep container that will have the solution on ice.
- Use a micropipette to combine
    - 250ul fluorescent dye
    - 30ul bovine collagen (3.1 mg/ml) (keep on ice)
    - 100ul acetic acid (1mM)
    - 4.32ml chilled MilliQ water
- Add fluorescent dye as needed to ensure that micro patterns will be visible for imaging and recording the extent of pattern fidelity.

Micro-Contact Printing Procedure

- Place each individual top coverslip in a separate container (culture/petri dish)
- Use sterile tweezer when handling the PDMS stamps
- Soak PDMS stamps being used in 70% Isopropyl for a 5 minutes
- Completely dry the stamp with the air pump
- Soak the pattern side of the stamp in the collagen solution for at least 30 seconds
- Remove the stamp and air pump the excess fluid off of the stamp
- Make sure the entire pattern is in over the cover slip before releasing it.
    - Releasing the stamp instead of placing it prevents double-printing
- Place a small weight (~50g to 100g) on top of the stamp to ensure full conformal contact
- Place the assembly onto a platform heated to 37°C and leave it there for 30-45 minutes
- Remove the weight and peel off the stamp without letting it touch the slip again.
- Replace the coverslip in its container, pattern-side up.
- Keep these cover slips stored in the refrigerator, at roughly 4°C, prior to use.

V. Protocol for Fabricating Polyacrylamide & Manipulating Stiffness

Materials

- 2% Bis Solution
- 40% acrylamide solution
- 10% APS
- TEMED
- Patterned Top slide
- Glutaraldehyde treated bottom slide
- HEPES (8.4 pH)
- Razor blade
- 1 mL conical tube

Procedure

- Mix components of hydrogel into 1 mL tube at desired concentrations for desired stiffness
    - First add 2% Bis solution & 40% acrylamide solution to the 1mL tube
    - Then add 3 ul TEMED per 1mL of solution that is made
    - Finally add 10 ul of 10% APS per 1 mL of solution made
        - This must be done last, vortexing happening immediately after
- After vortexing, immediately place 64 ul of hydrogel solution onto glutaraldehyde bottom slide
- Place patterned top slide onto bottom slide, carefully ensuring the hydrogel spreads over the entire slide
- Place the petri dish with the hydrogel onto a slide warmer set at ~37oC for 30 minutes
- After 30 minutes, remove the petri dish from the heat
- Add HEPES to petri dish, submerging the slide
- Use the razor to remove the Top slide
- Place hydrogel with HEPES into refrigerator

VI. Protocol for Cell Seeding on Polyacrylamide Hydrogels

Materials

- NIH/3T3 Cells
- Complete media
    - 10% FBS
    - DMEM
    - PenStrep
    - GlutaMax
- 15 mL conical tube
- .25% Trypsin
- Hemocytometer
- DPBS
- Hydrogel (Patterned)

Procedure

- Remove Complete media from Petri Dish with Cells
- Rinse with DPBS then aspirate out
- Add .25% trypsin to the petri dish (should coat entire bottom)
- Put Petri dish into incubator and check dish under microscope every 2-3 minutes until 90% or more of the cells have detached.
- Add complete media to the dish that is equal to double the amount of trypsin that was added.
- Pipette complete media out of Petri dish and into a conical tube.
- Centrifuge complete media for 5 minutes
- Aspirate complete media out of conical tube (careful to not aspirate the cells out)
- Add complete media to conical tube, repeated pipetting to ensure cells break apart from one another.
- Count cells using a hemocytometer and determine the cell density per mL
- Determine mL that will allow for ~500k cells to be seeded
- Place the determined volume on to the hydrogel (try not to have the mixture slide off the hydrogel)
- Place the hydrogel with the cells seeded into the incubator for 2 hours
- After 2 hours rinse with complete media then aspirate out the media (don’t touch the hydrogel)
- Add new complete media then image at infrequent time intervals.

VII. Photolithography using SU8 Photoresist: Standard Operating Procedure (Dirk Albrecht, PhD)

1. MICROFABRICATION LAB

Location

Gateway Park 0122, BME MicroFabrication Laboratory (BME-MFL), WPI

Access

Prof. Dirk Albrecht, Dept. of Biomedical Engineering (508-831-4859, dalbrecht@wpi.edu).

Technical Contact

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Document Revision

26-Mar-14 v.0.2 DRA/LA

2. INTRODUCTION

Photolithography is a standard procedure to transfer patterns onto the wafers in the microfabrication process. This Standard Operating Procedure(SOP) provides information on the photolithography process that has been developed at the MicroFabrication Laboratory. There are multiple steps involved in the photolithography process: wafer dehydration, photoresist spincoat, pre-bake, exposure, post-exposure bake, development, inspection, (optional: processing, e.g. etching), and postprocessing (typically hard-bake and fluorination, or resist stripping). Each procedure will be discussed in the following sections. Optional processing steps are addressed in separate SOPs.

3. LOCATION OF EQUIPMENT, ACCESSORIES, TOOLS, AND SUPPLIES

The Photoresist Spin Coater (Laurell WS-650MZ-23NPP), UV exposure unit (UV-KUB), hot plates (PMC Dataplate 720 and 732), and wafer inspection stereomicroscope (Zeiss Stemi 2000) are located in room 0122 of the BME MicroFabrication Laboratory (BME-MFL) in the Gateway Park I building at WPI, 60 Prescott St. Currently, the large 10" hotplate for dehydration bake (120 ºC) and the UV exposure unit are location in a Labconco cleanhood. The two smaller 7" hotplates for pre- and post-bakes (65 ºC and 95 ºC) and the photoresist spinner are located opposite in the fume hood. The stereo microscope is located on the stainless steel bench in the rear of the room.

4. PERSONAL SAFETY AND CLEANROOM ATTIRE

Personal protective equipment including a disposable cleanroom coat and nitrile gloves are required for routine operation in this facility. Shoe-covers must be worn before entering the room 0122 and are available in the adjacent room 0123. Step on the sticky mat before entering 0122 only with shoe-covers on, not regular shoes. Avoid stepping on the sticky mat upon exit, to avoid unnecessary soiling.

5. MATERIAL COMPATIBILITIES

6. PRIMARY HAZARDS AND WARNINGS

The primary hazards associated with photolithography are the chemicals, including photoresists, developer solvents, cleaning and etching solutions including acids and reactive chemicals. Therefore, safe chemical handling and storage measures must be adopted. Details on chemical storage, handling and disposal are described in the MSDS binder.
Hotplate temperatures up to 200 ºC may be required with risk of burns.
UV exposure of photoresists take place within a sealed LED illumination unit and do not pose an exposure risk. [*However, this is new technology, and be aware that most microfabrication facilities use a mercury (Hg) arc lamp a source of ultraviolet radiation during exposure that may not be fully enclosed. Ultraviolet radiation can cause burns of the skin or of the outer layers of the eye. In these systems, the user must avoid looking directly at the UV source and avoid exposure to reflected or diffused UV from the lamp. In addition, an Hg arc lamp operates at high voltage and the user should make sure that the power supply and illuminator are covered properly, and that cables are properly connected.*]

7. OPERATIONAL PROCEDURE CHECKLISTS

This Standard Operating Procedure(SOP) provides information on the photolithography process that has been developed at the MicroFabrication Laboratory. Each step involves using different equipment and it is important that user is familiar with the location of the equipment and all of its key components.

Preliminary Setup. Determine photolithography parameters

Before beginning any photolithography process, the entire procedure must be planned. The primary determinants to spin speeds and duration of baking and development steps are the photoresist material and the desired resist thickness. Refer to the photoresist spec sheets for more information, such as, for SU-8 2000 series:
http://www.microchem.com/pdf/SU-82000DataSheet2025thru2075Ver4.pdf

For example, for a 80µm thick process using SU8 2035, we find the following information from the datasheet above:

1. Spin speed: 1600 rpm (Figure 1)
2. Soft-bake times: 3 min @ 65 °C; 9 min @ 95 °C (Table 2)
3. Exposure energy: 215 mJ/cm^2 (Table 3)
4. Relative dose: 1x (Table 4)
5. Post-exposure bake: 2 min @ 65 °C; 7 min @ 95 °C (Table 5)
6. Development time: 7 min (Table 6)

The bake times directly relate to the experimental plan, but the UV exposure time must be calculated from the exposure energy, relative dose, the illumination intensity, and an empirical correction factor. The illumination intensity of the UV-KUB should be stabel at 23.4 mW/cm^2, and the correction factor is 1.5 due to the narrow spectrum of UV exposure at 365 nm. For example, from the data above, the UV exposure time should be:

$$215 \text{ mJ/cm}^2 \times 1 \text{ (multiplier)} \times 1.5 \text{ (correction factor)} / 23.4 \text{ mW/cm}^2 = 13.8 \text{ s}$$

Procedure 1. Dehydration Bake

The dehydration bake removes residual water molecules from the wafer surface by heating up the wafer on a hot plate or convection oven. Removing residual moisture increases the adhesion of the photoresist on the substrate.

This step uses the large 10" PMC Dataplate hot plate in the clean hood that can be seen in Figure 1.

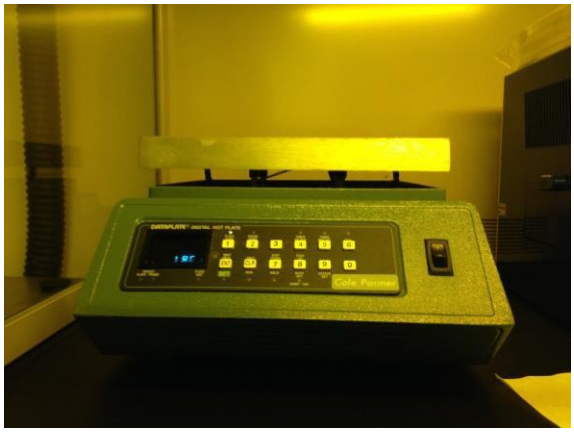

Figure 1: 10" PMC Dataplate 732

Figure 1. Hot plate front panel

1. Turn on the blower and light on the cleanhood. Let it run for a few minutes before working inside.
2. Power on the PMC Dataplate hot plate in the clean hood. Ensure the hotplate surface is clean.
3. Set the desired temperature to 120 °C. Press the following buttons in order: [**SET**], "Plate Temp" [**1**], [**1**], [**2**], [**0**], [**ENT**]. The display cycles between the set temperature and current temperature about once per second.
4. Place a clean new wafer onto the hotplate surface. The whole wafer should completely fit on the hotplate surface so that heat can conduct evenly to the wafer.

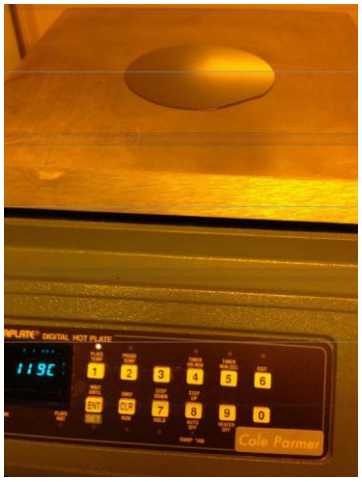

Figure 2: Hot Plate Front Panel

5. Once the plate reaches the desired temperature, heat for 5 min. To set a timer, press the following buttons in order:

[**SET**], "Timer (h:m)" [**4**], [**5**], [**ENT**].

Or: [**SET**], "Timer (m:s)" [**5**], [**5**], [**0**], [**0**], [**ENT**].

6. Carefully remove from the hotplate with wafer tweezers and allow to cool to room temperature. The wafer is now ready for the next procedure.

Procedure 2. Spin-coating

Spin-coating is a step to apply photoresist onto the wafer. This section will outline the steps of spin coating SU-8, a common type of negative photoresist that is used in the MicroFabrication Laboratory. The procedure is similar for AZ1512, a positive photoresist, except it is deposited via syringe rather than pouring due to its lower viscosity.

This step uses the Laurell spin-coater in the fume hood which can be seen in Figure 3.

Figure 3: Laurell spin-coater

Preparation stage:

Figure 4: Spin-coater Power Strip

1. Turn on the spin coater using the left power strip switch under the fume hood (Figure 4).
If the display does not light up, turn on the unit power switch at the back of the unit.

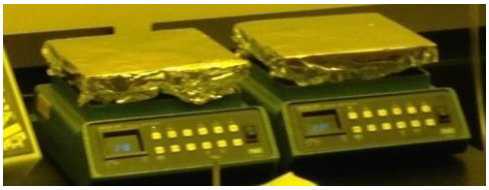

Figure 5: Dataplate Hot Plates

2. Turn on the two 7" Dataplate hotplates (Figure 5) using the right power strip switch under the fume hood (Figure 6), and set the left one to 65 °C and the right one to 95 °C as in Proc 1, Step 2 above. (Note, the "5" button sticks on one hotplate so use 96 °C if necessary).

If foil is absent, damaged, or dirty, replace with new foil.

Figure 6: Hot Plate Power Strip

3. Press **[Select Process]** and choose the appropriate spin program according to your desired parameters. If none exist yet, you must enter a new spin program. Refer to the User Manual or Appendix 1 for programming. ***If you make any changers or additions, note your changes in the MFL logbook***.

```
    Edit Program 10

Step:001/002    Vac↓req          Step:002/002    Vac↓req
Time:00:10.0    Cpm:00           Time:00:30.0    Cpm:00
Rpm : +00500 Loop:000            Rpm : +01600 Loop:000
Acel:    0100 Goto:001           Acel:    0300 Goto:001
Valv:                            Valv:
Sens:                            Sens:
```

The first step is a slow ramp to 500 rpm at 100 rpm/s and is designed to slowly spread the resist across the wafer. The second step spins faster to determine the final resist film thickness. Only the spin speed (in rpm) needs to be changed for different resist thicknesses; all other parameters should remain unchanged.

4. Remove the spin-coater lid and verify the presence of a foil liner. If the foil is not present, line the bowl with foil to catch photoresist that is removed from the wafer during spinning. Ensure that the bowl periphery is covered above the height of the chuck and wafer, and also completely covering the bottom to the chuck. Rotate the chuck and ensure that the foil does not touch the chuck or impede rotation.

5. Select [**Run Mode**].

6. Turn on the N_2 supply, seen in Figure 7, by opening the main tank valve (marked in red). Ensure an output pressure of 60-70 psi. If the display reads "Need CDA," open the round valve attached to the pressure regulator (outlined in green). Open the vacuum valve by aligning the black handle with the tubing (outlined in yellow).

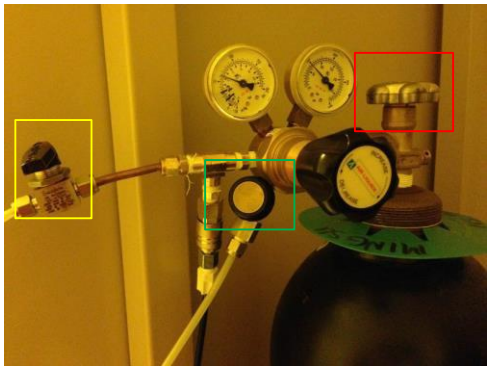

Figure 7: N2 Supply Tank Valves

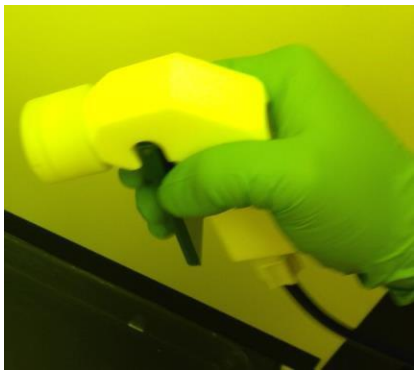

Figure 8: N2 Gun

7. Make sure that the wafer is clean and dry. Visible dust on the wafer can be removed by gently blowing the wafer using the nitrogen gun (Figure 8), which is located on the right side of the fume hood.

8. Position the 4" wafer alignment tool against the chuck, and using wafer tweezers or your gloved hand, *touching only the edge*, place the wafer on the chuck aligning to the marks on the alignment tool (Figure 9).

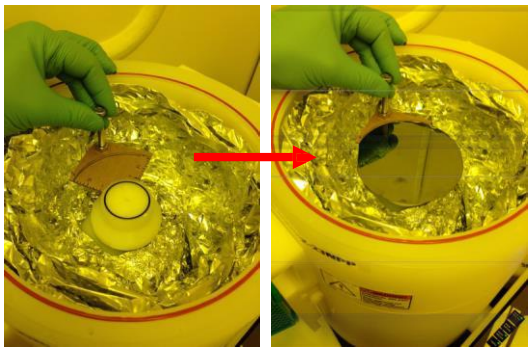

Figure 9: Spin coating set-up

9. Before removing the alignment tool, press the [**Vacuum**] button. A hiss should be audible, and the display should change from "Need vacuum" to "Ready". The wafer should now be held down on the chuck.

10. Test your alignment by beginning the spin program. Press [**START**] and observe the edge of the wafer as it turns. It should wobble less than 5 mm. If not, press [**STOP**], then [**Vacuum**] to release the vacuum, realign, and return to step 8. Reset the spin program if necessary by pressing [**Edit Mode**] then [**Run Mode**] and ensuring the display reads "Ready".

<u>Coating Stage:</u>

1. Ensure the wafer is centered and the spin-coater is programmed and ready to spin.

2. For SU8 2035 photoresists and similar high-viscosity materials, pour the resist directly from a 50 mL conical tube. It will flow very slowly. Pour approximately 8-10 mL of resist onto the wafer in one continuous motion, with the tube far enough to avoid contact with the wafer but

close enough to prevent thin filaments of resist from forming: about 1 cm. Once the resist blob covers about 5cm diameter, quickly move the tube toward the edge while tilting the tube upwards and twisting to prevent drips on the outside of the tube. See Figure 10.

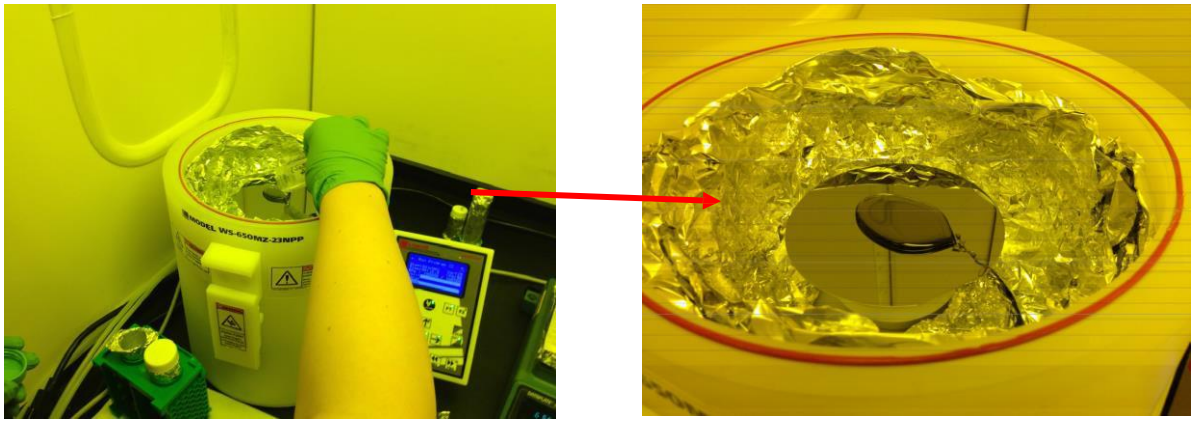

Figure 10: Resist pouring onto wafer

3. Press the [**START**] button of the spinner to start spin coating. The spin coating process takes about 1 minute, depending on the program. *[OPTIONAL:] Near the end of the second spin step, use a piece of Al foil, rolled into a rod to collect resist streams that fly off of the wafer. Do not touch the edge, but bring the rod close. This will clean up the resist at the edge and somewhat reduce the edge bead, or thicker later at the edge due that forms due to surface tension.*

4. The spinner will stop automatically when spin coating is completed.

5. Verify that the photoresist has been uniformly coated. If striations and streaks are observed, the spin coating was not successful. Some causes may include:

- dust particles on the surface (clean it better),
- bubbles in the photoresist (heat the resist tube to 40-50 °C in a water bath to remove them; see resist datasheet for more information)
- insufficient resist volume applied

6. Press [**Vacuum**] to release the chuck vacuum.

7. When the last wafer has been coated, close the vacuum and CDA valves at the N_2 tank.

Procedure 3. Prebake (Soft Bake)

The prebake (Soft Bake) procedure is required to densify the photoresist following spin coating and evaporate the solvent. In order to reduce thermal stresses due to the substantial difference in coefficient of thermal expansion between Si and resist, the temperature should be raised and lowered gradually in a 2-step process, first at 65 °C, then at 95 °C, then back to 65 °C.

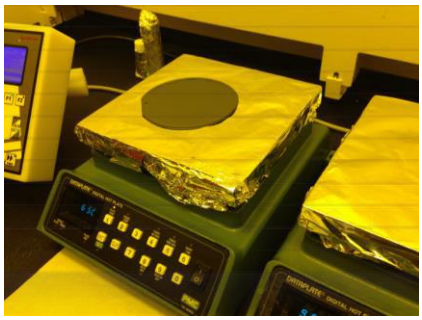

Figure 11: 65° Hotplate

This step uses the two 7" Dataplate hotplates in the fume hood.

1. Use the "removal tool" to transfer the wafer from the spinner chuck to the 65 °C hotplate (See Figure 11). Set the timer for the desired time at this temperature, and cover the wafer with a foil tent (Figure 13).

Figure 12: 95° Hotplate

2. Transfer the wafer from the 65 °C hotplate to the 95 °C hotplate (Figure 12). Set the timer for the desired time at this temperature and cover with a foil tent (Figure 13). Use wafer tweezers to lift up the edge, but don't grab the wafer edge, since the resist is still very soft. Instead, slide the "removal tool" underneath and lift.

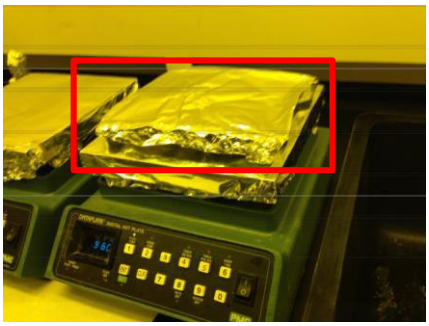

Figure 13: Foil Tent

3. Return the wafer to the 65 °C hotplate for 3 minutes, covered, then transfer it to the clean hood to cool to room temperature. Be sure to place your hand underneath as you move the wafer from the fume hood to clean hood: if you drop it, it'll shatter.

Procedure 4. UV exposure

The UV exposure procedure exposes the photoresist layer to collimated 365 nm UV light via an LED source through a photomask. A negative resist becomes crosslinked and insoluble in developer when exposed, whereas a positive photoresist becomes soluble in developer when exposed. This procedure assumes that a transparency photomask will be used in direct contact with the resist layer.

This step uses the UV-KUB exposure system in the clean hood (Figure 14).

<u>Preparation stage:</u> *(this can be done during the prebake procedure 3)*

1. Turn on the UV-KUB via the power switch at the back left, just above the power cord. Press the silver power button on the front panel, lower right. The touchscreen should light up and display "UV-KUB"

Figure 14: UV KUB

2. Touch the screen to reach the main menu. Touch [**Settings**] and [**Drawer**] to unlock the drawer. Wave your hand near the door sensor at the lower left to open the drawer. If there is a wafer or mask present, remove them. Place the 4"x 5" glass slide on the tray and wave near the door sensor to close it.

3. Return to the [**Settings**] menu (touch the [**X**] in the upper right of the screen). Touch [**Illumination**] to calibrate the UV intensity. It should display about 23.4 mW/cm^2 through the glass plate. If not, adjust your exposure time calculations in "Preliminary Setup". See Figure 15.

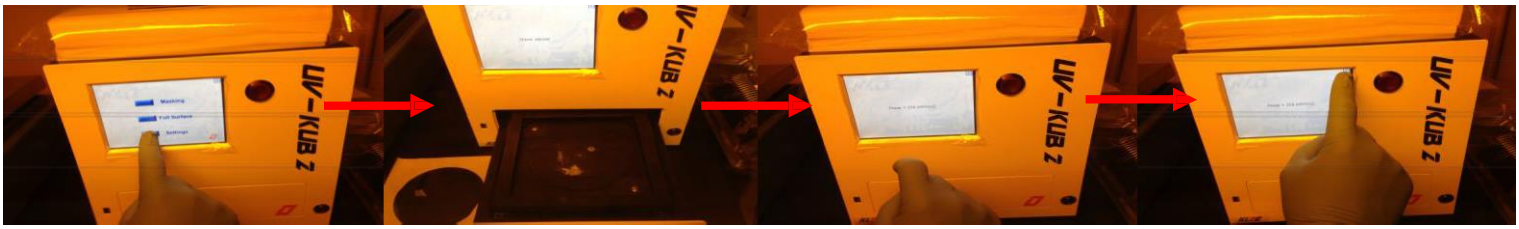

Figure 15: UV Intensity Calibration

4. Return to the main menu and select [**Full Surface**] then [**New cycle**] then [**Continuous**]

Figure 16: Set-up for exposure

(Figure 16).

5. Program the desired exposure duration and intensity. Enter the time using the touchscreen numbers, then a unit ([**h**], [**m**], [**s**] for hours, minutes, seconds), then [**v**] to confirm. Note that decimal values are not permitted, so round to the nearest second. Next enter the intensity in %, usually 100%, and [**v**] to confirm (Figure 17).

Figure 17: Duration and Intensity

6. Test the exposure by touching [**Insolate**]. The drawer will open (Figure 18). Wave it closed. The display should read "Loading in Progress". Touch the screen to start the exposure. Verify that the countdown timer begins at the proper duration.

Figure 18: Insolate

7. The exposure will end automatically and alert with a loud beep (silence by touching the screen). The drawer will open automatically. Remove the glass slide if present.

Mask alignment stage:

1. Transfer the room temperature, resist-coated wafer to the UV-KUB tray, centering it in the circular pattern (Figure 19).

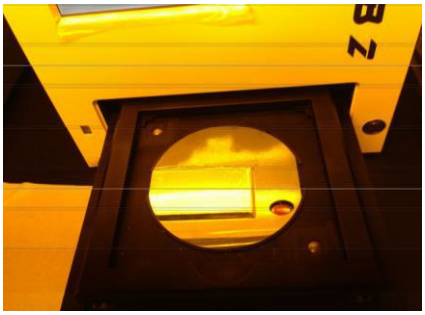

Figure 19: Loaded Wafer

2. Observe the position of any defects in the resist layer. You will try to rotate your photomask such that these defects are removed during development; i.e. they are covered with black mask regions if a negative resist, or are covered with clear mask region if a positive resist.

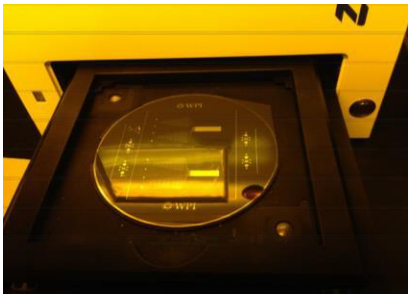

Figure 20: Photomask placed on top of wafer

3. Cut out the photomask circle using scissors, taking care not to kink the transparency film. Ensure it is free of dust, and gently wipe with a lint-free cleanroom wipe or blow with the N_2 gun if necessary.

4. Place the photomask over the resist-coated wafer and orient it such that any defects will be removed during development (Figure 20).

5. Place the 4" x 5" glass slide over the wafer and mask to keep it flat and in direct contact. First tilt the 5" side to the back corner supports, then gently move it toward you so it rests on the bottom tray surface. Finally, gently lower the glass plate onto the wafer, ensuring it is fully covering the mask and wafer, and that it did not move the mask while lowering (Figure 21).

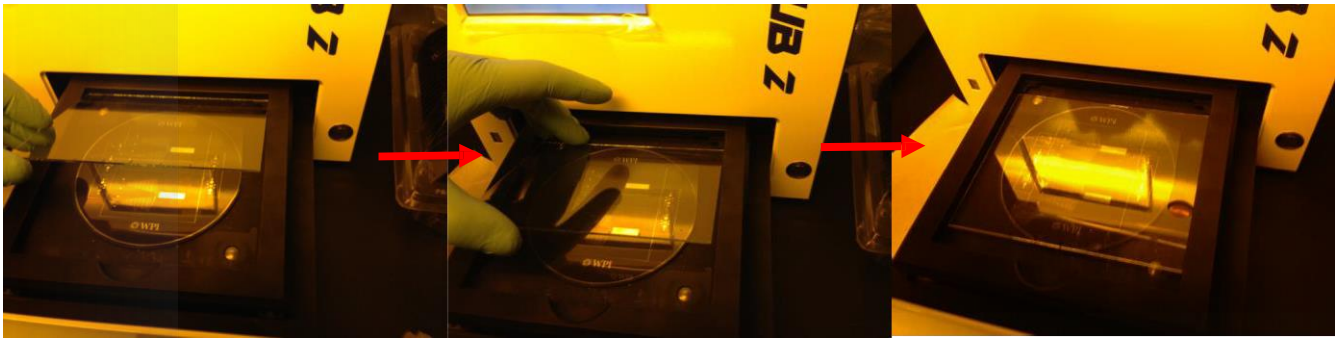

Figure 21: Slowly lower glass on top of photomask and wafer combination

Exposure stage:

1. When you are satisfied with the mask orientation and glass plate placement, wave the door closed (Figure 22). Touch the screen.

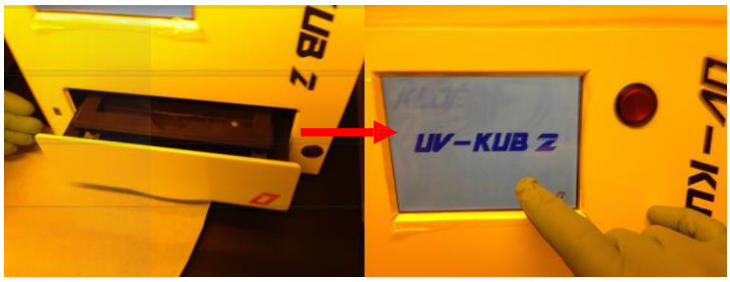

Figure 22: Close the drawer and tap screen to begin

2. When is asks: "What do you want to do?", touch [**Continue**] on the screen.

The last used program will begin *automatically* after 1-2s. Verify the correct exposure. If anything is awry, immediately press the large red button to abort and retry.

3. The exposure will end automatically and alert with a loud beep (silence by touching the screen). The drawer opens automatically.

4. Gently lift the glass slide with wafer tweezers and set aside. Gently lift the photomask with wafer tweezers and set aside. See Figure 23.

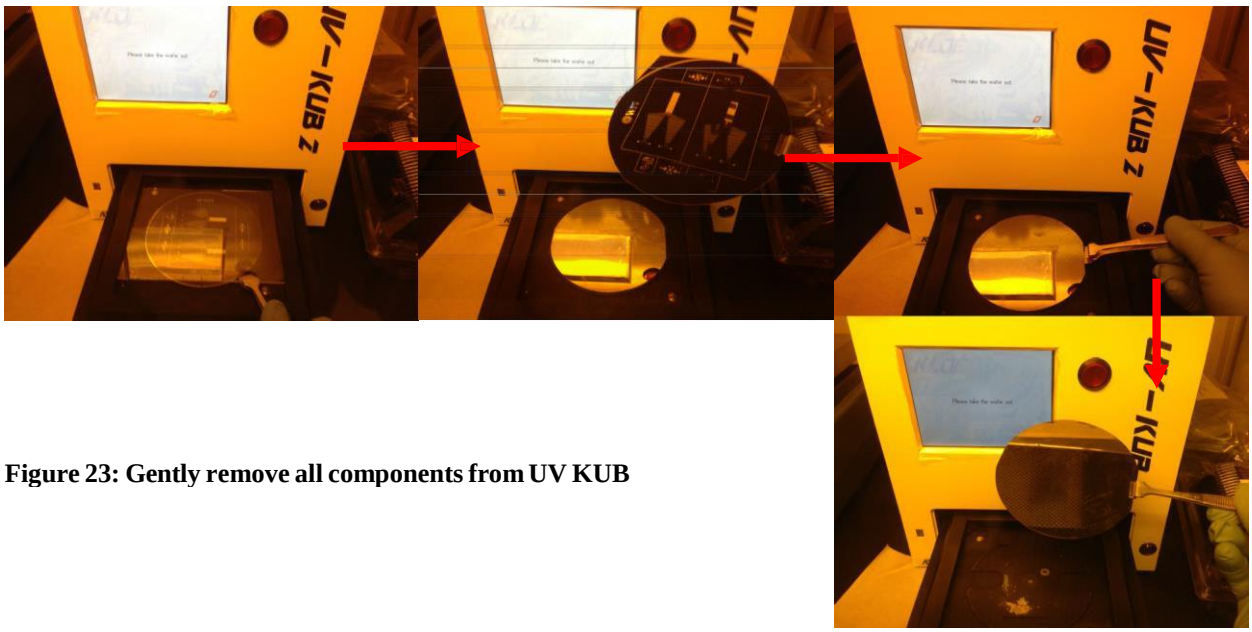

Figure 23: Gently remove all components from UV KUB

5. Observe the resist surface. At this point, no pattern should be easily visible. If it is, the exposure time was too long.

6. Wave the drawer closed when done exposing, then touch the screen and select [**Cancel**].

Procedure 5. Post-Exposure Bake (PEB)

The post-exposure bake completes the process of crosslinking a negative resist or solubilizing a positive resist. As in the prebake, a two-step heating and cooling is required to minimize resist layer thermal stresses.

This step uses the two 7" Dataplate hotplates in the fume hood.

1. Transfer the wafer from the UV-KUB to the 65 °C hotplate in the fume hood. Be sure to place your hand underneath as you move the wafer so it doesn't drop. Set the timer for the desired time at this PEB temperature.

2. Observe the resist surface. With ideal exposure, the mask pattern will become slightly visible in 5-30 s (See Figure 24). Cover with a foil tent.

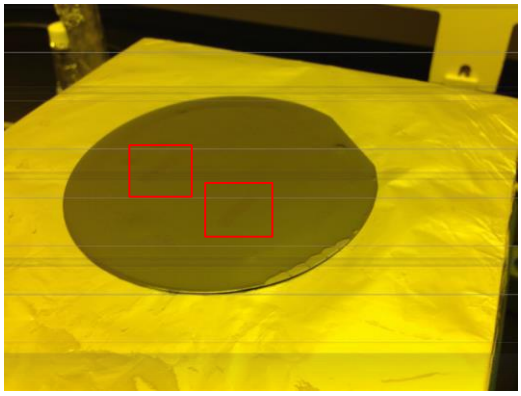

Figure 24: Slightly Visible mask pattern

3. Transfer the wafer from the 65 °C hotplate to the 95 °C hotplate and cover. Set the timer for the desired time at this temperature.

4. Return the wafer to the 65 °C hotplate for 3 minutes, then transfer it to a cleanroom wipe on the work surface to cool to room temperature. At this point, the mask pattern should be clearly visible. If not, exposure and/or baking times were too short.

Procedure 6. Development

The development step dissolves away the unexposed negative photoresist (or exposed positive photoresist). It is performed by immersing the wafer in developer liquid and agitating until the resist is dissolved and only the insoluble pattern remains.

This procedure uses a glass dish and developer chemical in the fume hood. Developers are located in the flammable cabinet below the fume hood, left side.

1. Ensure the glass dish is clean. Clean and dry with a cleanroom wipe if necessary. Pour developer in the dish to about 0.5-1 cm depth.

2. Immerse the wafer in developer and gently slosh/agitate, taking care not to splash developer out of the dish (See Figure 25). Start a timer on the hotplate with the desired development time.

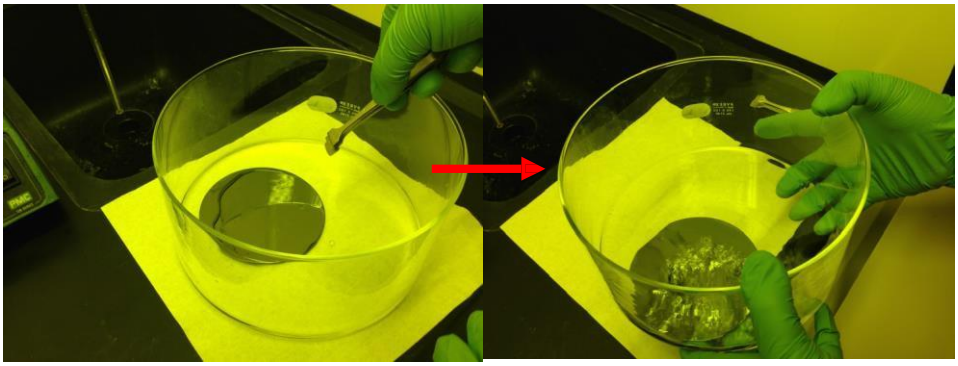

Figure 25: Development Process

3. Observe the wafer periodically. Bare Si regions will become visible after ~30s - 1 min. The resist at the edge is thicker than in the center, and therefore tends to be the last part to dissolve away. See Figure 26.

4. When all resist appears dissolved, remove it from the developer bath with wafer tweezers and run under a gentle stream of water in the hood sink. Grasp the wafer in your hands at the edges to ensure it doesn't fall and break! See Figure 27.

Note the time of development in your lab notebook.

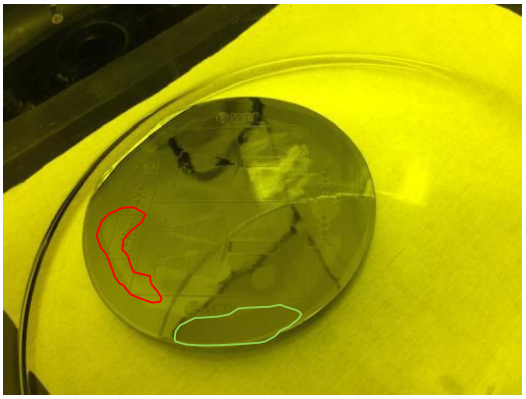

Figure 26: Lighter parts of wafer show dissolved and bare Si regions (highlighted in red) and darker spots show where dissolving has not yet taken place (highlighted in green)

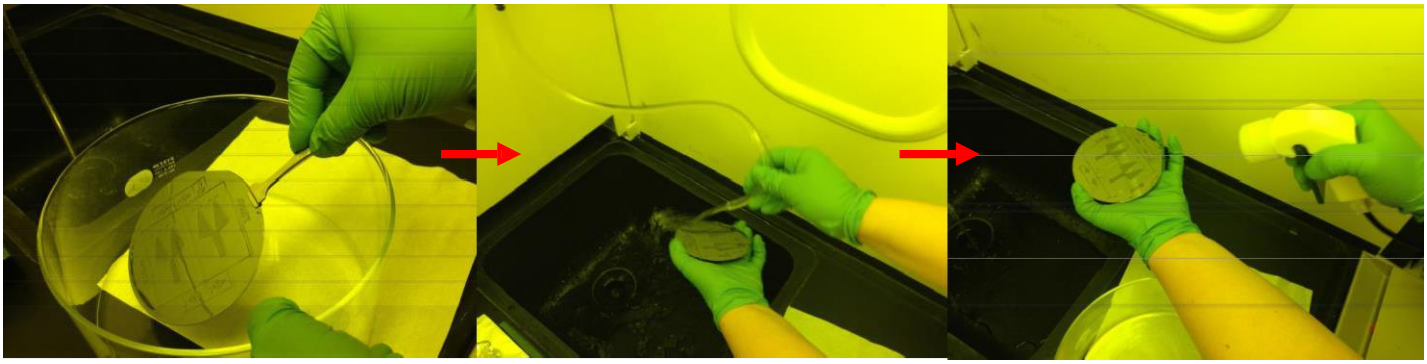

Figure 27: Wafer cleaning after development

5. After both front and back sides are rinsed in H_2O, dry both sides with the N_2 gun. Bring the nozzle close to the wafer and sweep side to side, especially in areas with small resist features (Figure 27).

6. Inspect the wafer as described in Procedure 7 below, and then perform a final cleaning development by holding the wafer with tweezers horizontally over the dish and squirting a small amount of fresh developer on the wafer (Figure 28). Gently slosh side-to-side for about 15s. Rinse with H_2O and dry with a N_2 gun.

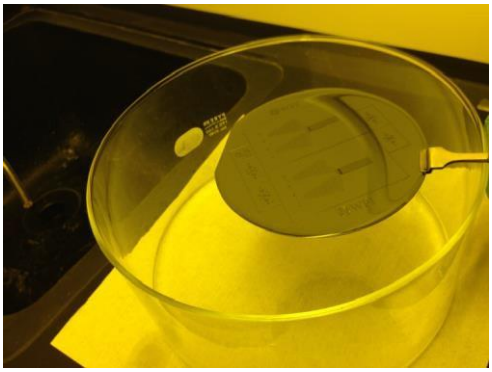

Figure 28: Slosh developer side to side on wafer for final cleaning

Procedure 7. Inspection

Inspection is a step to verify general process quality and the development process. This section will outline the main feature distortions that are encountered in photolithography process. The Zeiss Stemi-2000 stereo microscope is equipped with a fiber-optic light ring and is used to visualize the wafer in reflectance mode.

After initial development and rinsing, the wafer will appear dirty (see Figure 29). This is OK! It is due to the resist that has dissolved in the developer and will be cleaned to a shiny surface after brief wash with fresh developer. Also, sharp corners and large resist fields will likely display surface cracks. This is also OK! It is due to the thermal stresses during bakes, which were minimized by gradual heating and cooling but not fully eliminated. These cracks will be eliminated with the Post-bake, Procedure 8.

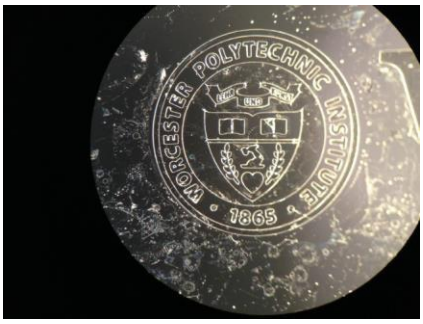

Figure 29: Dirty Wafer

1. Development time. Pay attention to the smallest features in the resist pattern. Lines should be sharp, with no evidence of resist material in regions where it should be removed. If not, development is incomplete. Return the wafer to the developer bath and repeat for ~30s, then rinse, dry, and reinspect. Instead, if the resist layer that should remain looks especially cloudy or rough, the wafer may be over-developed. Additionally, overdevelopment may narrow a resist feature or widen a resist "hole", and underdevelopment may do the opposite as in Figure 30.

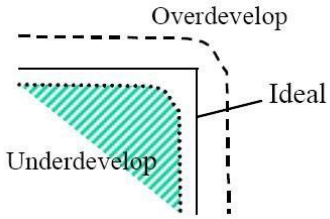

Figure 30: Under-development and Over-development.

2. Bake times and temperatures. The extent to which a feature deviates from its ideal size is a function of the exposure time, prebake temperature, prebake time, development temperature and development time. Any of these parameters could be the cause for overdevelopment or underdevelopment and it is therefore important that one understand some important troubleshooting techniques. The key idea troubleshoot the distorted feature is to observe the effect of changing a parameter while holding the other parameters at constant. The following example illustrates this idea.

Figure 31 shows the changes in feature size as the exposure time is increased, while holding the other parameters at constant. It can be observed that by changing the exposure time while holding the other parameters at constant, there is a time window where the feature size is optimal, i.e. between 15s and 25s in this example. If the changing of this parameter does not produce the desired feature size, the problems are most likely to be caused by other parameters or combinations of several parameters. Repeated troubleshooting with other parameters should be carried out.

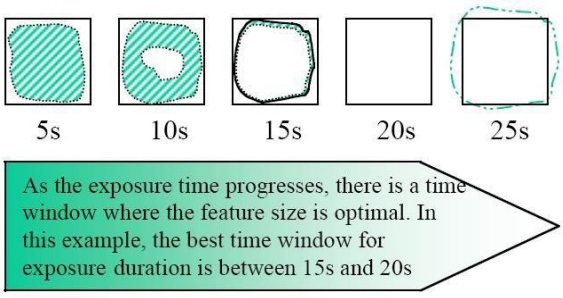

Figure 31: Changes in feature size due to increasing exposure time

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Procedure 8. Post-bake

The Postbake procedure is required to stabilize and harden the developed photoresist prior to processing steps that the resist will mask. Typical post-bake temperature is 150 °C for 30 min for SU8 (or 90-120°C for 5 min for other thin resists).

This procedure uses any of the Dataplate hotplates.

1. Place the developed wafer on a hotplate at no more than 65 °C.
2. Set the ramp rate to 6 °C/min or 360 °C/hr: [**SET**], "Ramp °C /hr" [**6**], [**3**], [**6**], [**0**], [**ENT**]. Set temperature to 150°C. Set the timer for 45 minutes. Set the hotplate to automatically turn off then the timer ends, by pressing "Auto Off" [**8**]. Cover with a foil tent.
3. The hotplate will slowly ramp up to 150°C over about 15 mins, maintain temperature for ~30mins, then turn off and slowly return to room temperature. This will take around 1 hr total.
4. After the wafer has returned to room temperature, inspect the wafer again and verify that surface cracks have disappeared. Document selected microscope fields with a camera.

Post-procedure Cleanup

Following a photolithography process, equipment must be cleaned and properly shut down. Perform the following steps:

1. ***Ensure you have logged your usage information in the MFL logbook***. This is important, as the MFL is a shared use facility. Note any consumables running low, dirty areas, and any other relevant information.
2. Clean hood. Turn off the hotplate and UV-KUB via the front panel (silver button, lower right) and back switch (rear, lower left, above the power cord). Then power off the lights and blower of the clean hood itself.
3. Microscope. Turn off the illumination system.
4. Fume hood. Dispose of photoresist devleoper into the properly-labeled waste container, stored below the fume hood on the right side. Place the waste container in the hood sink, and use a funnel while pouring from the glass dish. Wipe the dish with a cleanroom wipe. Then rinse it with water in the large sink and set to dry.
5. Power off the spin coater and hotplates if not in use.
6. Close the main N_2 tank valve and depressurize with the gun.
7. Clean up the benches, put away your photomasks, etc.
8. Dispose of any photoresist-contaminated solid (foil, gloves, etc) in the waste container labeld PHOTORESIST WASTE.

VIII. Soft Lithography Using Poly(Dimethyl Siloxane): PDMS Standard Operating Procedure (Dirk Albrecht, PhD)

INTRODUCTION

This SOP describes the steps uses to prepare devices using "soft lithography" which refers to the flexible nature of elastomeric polymer, most commonly polydimethylsiloxane, or PDMS. The general procedures are:

1. Fluorination of the micropatterned Si mold;
2. Preparing the PDMS by mixing and degassing;
3. Casting PDMS over the mold and curing;
4. Preparing the final PDMS device by trimming and punching inlets;
5. Plasma bonding to a substrate (for an irreversible bond).

PROCEDURE 1: Fluorination of the Micropatterned Substrate

This procedure facilitates mold release by covalent treatment of Si or glass surfaces with a fluorosilane chemical by vapor deposition. The treatment renders the Si or glass hydrophobic, and maintains the micropatterned SU8 features as long as possible without delamination by reducing the forces applied during PDMS de-molding.

1. Set up the vacuum dessicator inside a fume hood. Line the bottom surface with foil if damaged, missing, or dirty. Prepare a support ring (cardboard or other material) and line up Si wafers (or glass slides) along the inner part of the ring, with the side to be treated facing inwards (Fig. 1A).
2. Make aluminum foil boat big enough to hold 40 uL (about 1 drop) and place in the center of the platform (Fig. 1A).

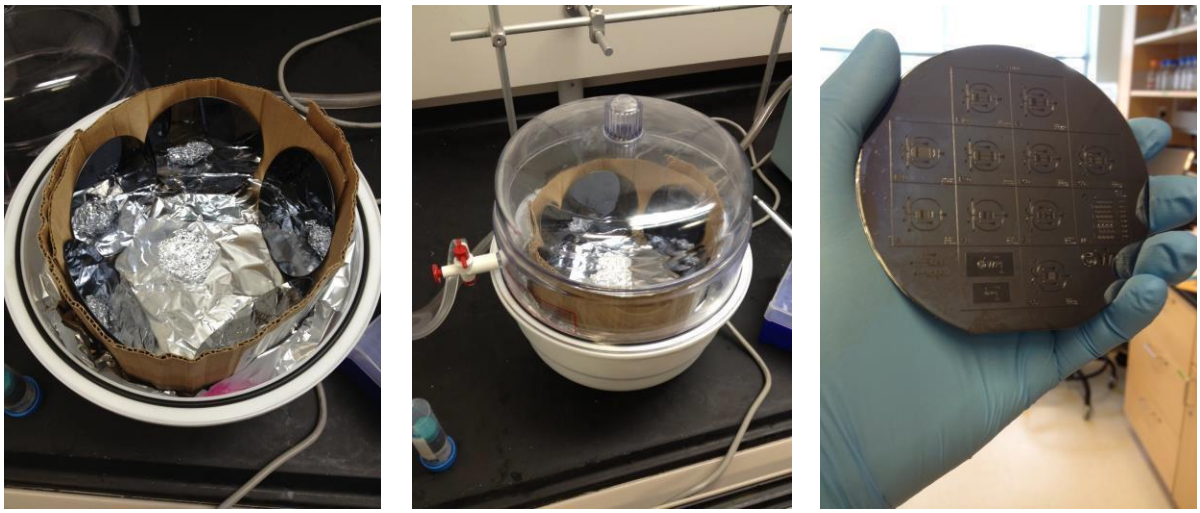


Figure 1A-C. Fluorination procedure in the vacuum chamber. (A) Cardboard ring supports five 4" Si wafers, with a foil boat containing the TFOCS treatment chemical. (B) Closed chamber. (C) After 1hr treatment, a haze may be visible on the Si surface. Clean with isopropanol for ~15-30s.

CAUTION (Tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane (TFOCS; Gelest, SIT8174.0; or United Chemical Technology, 6H-9283) is corrosive and toxic! Avoid direct contact and always handle it in the fume hood. (Figure 1D)

Figure 1D: Tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane

3. Pipette 40ul of the TFOCS chemical directly from stock bottle and place into the aluminum foil boat you just made. Remove the pipette tip by hand and gently place into the vacuum chamber (Do not eject it!)

4. Close the chamber and vacuum for 1 hour (Fig. 1B).

5. After 1 hr, remove the treated Si wafers (or glass). If any hazy film appears (Fig. 1C), remove with 15 - 30s contact with isopropanol, rinse with water, and dry in an air stream.

6. Fluorinated pieces are ready to use right away. Verify hydrophobicity by observing contact angle of water drops on the treated surface. Water drops should roll off the surface, leaving it dry.

7. After a few hours, the chemical liquid will have evaporated. Discard foil boat and pipette tip in hood waste bag.

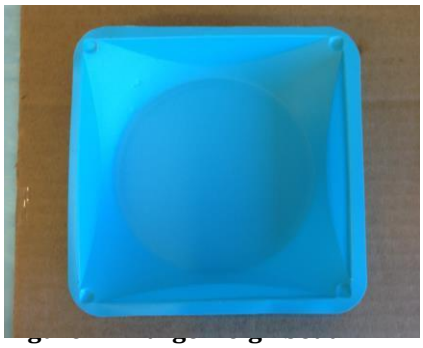
Link to ordering chemical:
http://shop.gelest.com/Product.aspx?catnum=SIT8174.0

PROCEDURE 2: Preparing the PDMS Mixture

This procedure prepares a PDMS mixture for casting. We use Sylgard 184 (Figure 2A), which comes as a kit with Part A (monomer) and Part B (cross linker). A typical ratio is 10:1 (w/w); 20:1 to 5:1 rations will cure properly, with greater cross linker amounts resulting in stiffer cured polymer. For simplicity, we typically weigh out the components into a single weigh boat on a balance.

Figure 2A: Sylgard 184

1. Set up a paper tower on the balance, ensuring it does not hang over the edges, and a large weigh boat (Figure 2B). Remove any visible dust.



2. Determine your desired PDMS volume. Each wafer requires about 50-60g PDMS. Ideally, you should make about 80-120 g PDMS per weigh boat, up to three boats at a time.

3. Tare the weigh boat (set weight to 0.0g). Pour Part A (Figure 2C) into the weigh boat until the desired weight (e.g., 91.2g). Then divide this value by 10 (for 10:1 ratio), tare again to 0.0g, and pour Part B (Figure 2D) to the desired weight (e.g. 9.1g). Within -0.2/+0.5g is ok.

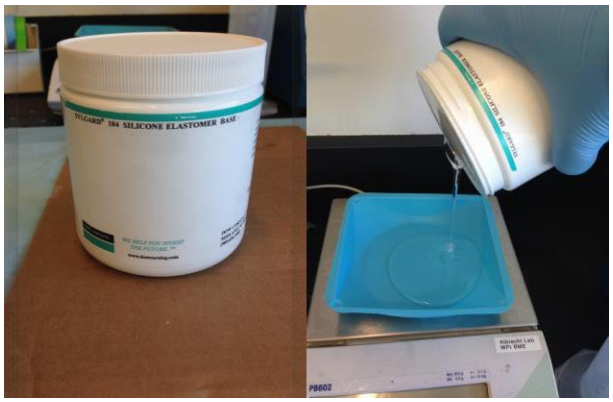


Figure 2C: Part A monomer

Figure 2D: Part B cross linker

4. Using a transfer pipette, slowly and gently fold (as in baking) the low-viscosity Part B into the high-viscosity Part A. Once Part B is no longer visible on the surface, increase your folding speed. Ensure that all edges have been mixed. Mixing should take at least 1 min, ideally >2. (Technique is more important than time here). *There should be lots of bubbles!* See Figure 2E.

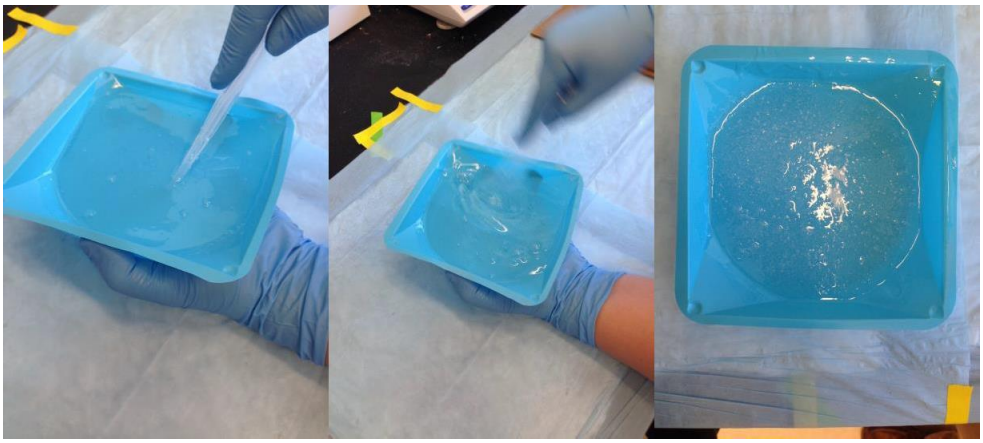


Figure 2E: Gentle folding with transfer pipette turns into high folding speed and results in bubble production

5. Place the weigh boat into the vacuum chamber (Figure 2F). If more than one is prepared, invert a second weight boat on top, rotated such that the PDMS in the lower boat is visible, and place the second PDMS boat on top. Repeat one more time for three total, as needed.

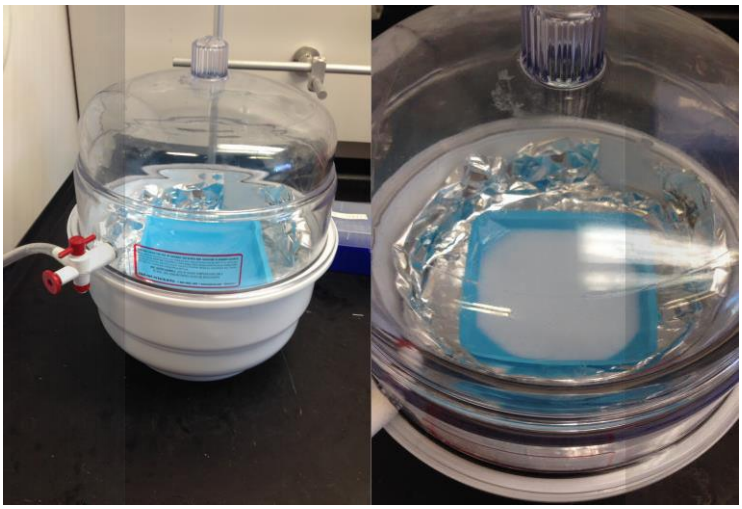


Figure 2F: Put weigh boat in vacuum chamber and start vacuum. Bubbles will appear making the PDMS appear

6. Apply a vacuum and observe bubble enlargement (Figure 2F). Release the vacuum after 1 min as necessary if bubbles appear as though they may overflow. This pops many of them, and reduces the likelihood of spillage.

7. Degas for 1 hr. At this point, all bubbles should be gone (Figure 2G) and PDMS is ready to pour in Procedure 3. **Be careful when releasing vacuum!** Air rushing in could knock over the PDMS boats.

Figure 2G: Bubbles are gone from the PDMS

PROCEDURE 3: Casting and Curing PDMS

During this procedure, mixed PDMS is poured over the Si/SU8 mold master in a dish or foil vessel, bubbles and/or dust particles are removed, and the PDMS is cured by baking at 65C for >3hrs.

1. Prepare casting vessels by bending a foil sheet over the bottom of a 500 mL Erlenmeyer flask. Flatten the edges until they are about 10 - 15mm high. Ensure the bottom surface is flat. See Figure 3A.

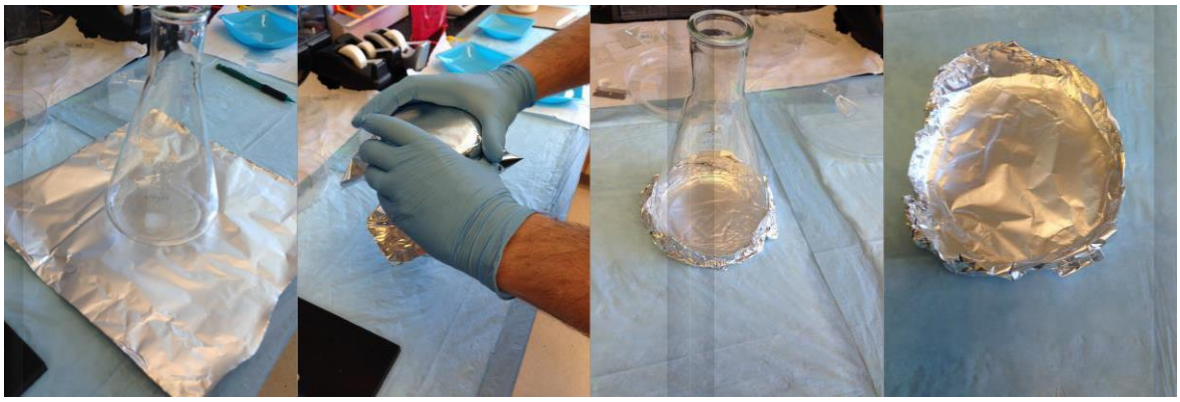


Figure 3A: Preparation of casting vessel with Erlenmeyer flask

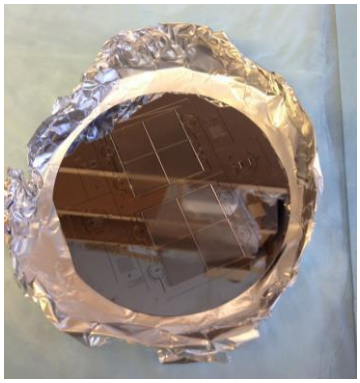


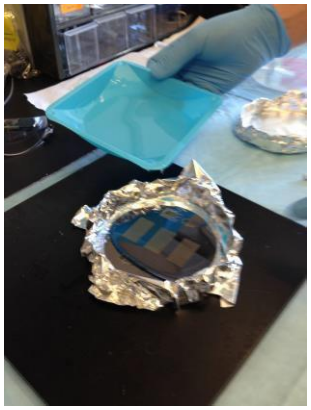
Figure 3B: Master ready to be cast in casting vessel

2. Set up the masters to be cast on the bench top (Figure 3B) covered with absorbent mats and a paper towel. If dust is visible, blow with the air gun. Weigh the master and vessel, recording the weight.

3. Once the PDMS mixture has been degassed for 1hr, and surface bubbles are gone, bring them to the masters.

4. Pour PDMS mixture across the wafer, from one side to the other, in a continuous movement. This reduces the number of bubbles formed. At this stage only the wafer needs to be covered. See Figure 3C.

5. Weigh the PDMS+master+vessel and subtract the master_vessel weight. About 60g PDMS is the target. If more is needed, bring the vessel back to the absorbent pad and pour more. Repeat until the desired PDMS weight is achieved.



bubbles

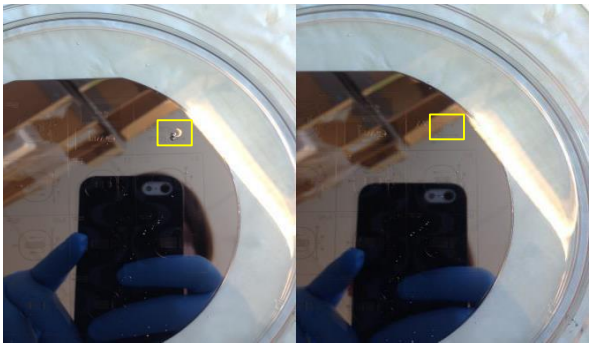


Figure 3D: Surface bubbles can be blown off and removed

6. Cover to prevent dust and observe after a few minutes any bubbles or dust remaining.

7. Surface bubbles can be removed by mouth blowing (from about 10 cm away) (See Figure 3D). Deeper bubbles can be left until they rise to the surface. Bubbles

adherent to the Si or SU8 surface can be dislodged by tilting the vessel back and forth (causing shear forces). Be careful not to spill any PDMS! It's messy, sticky, and hard to clean off

8. Large dust particles can be moved or aspirated with a disposable transfer pipette.

9. Once you are satisfied with the casting, place it onto a level shelf in the 65C oven, and bake for at least 3hrs. Leaving overnight is also OK.

PROCEDURE 4: Preparing a PDMS device

This procedure completes a PDMS device, including punching inlet and outlet holes for microfluidic devices.

1. Demold the cured PDMS from the Si master. Peel off the foil and carefully remove the Si wafer. If PDMS coated the underside of the wafer, you may need to cut it out with a scalpel or razor blade. Store the Si master in a safe place, ideally a wafer holder.

2. Set up the rubber cutting pad. Use a straight razor blade to identify the indentation line that separates adjacent devices, if present. Then, align the razor vertically and apply pressure to complete the cut. If necessary, move the razor to the next position and cut with downward pressure. *Do not slide the razor through the PDMS! It will deform as you cut.*

3. Once your device has been trimmed, determine the size of any inlet and outlet holes.

4. Apply Scotch Magic tape to the micropatterned side. If desired, mark the center of each hole for easier viewing.

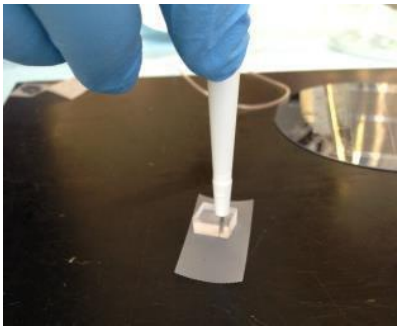


Figure 4A: Use dermal punch downward into device until contact with cutting pad

5. Flip over the device, tape and channel side on the rubber cutting pad.

6. Using a dermal punch of desired diameter, punch downward and in a straight line until contact with the rubber cutting pad. See Figure 4A.

7. Lift up the device, ***leaving the punch inserted***, and a cored PDMS piece should protrude from the channel/tape side. Remove it before gently removing the punch. See Figure 4B.

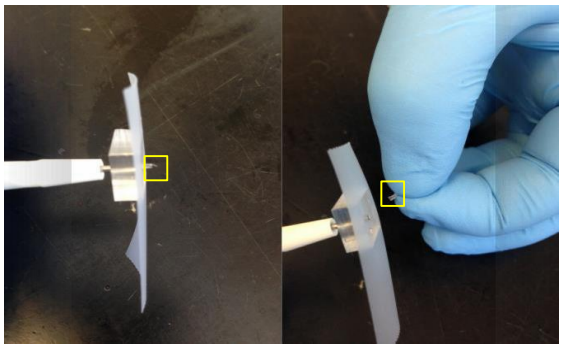


Figure 4B: Small, cored PDMS piece is removed by hand BEFORE removing dermal punch from device.

8. Repeat steps 6-7 until all holes are punched.

9. Clean the punched holes by squirting water through each hole with a wash bottle. Repeat with ethanol and water again. Then dry in an air stream. This process removes any PDMS particles that may have been left behind during punching.

PROCEDURE 5: Plasma Bonding

This procedure covalently binds PDMS to glass, Si, or PDMS by oxygen plasma treatment of clean surfaces. After plasma activation, surfaces are brought into contact, forming an instant and irreversible bond. Oxygen plasma is also useful for cleaning substrates and vaporizing organic materials. (This is a relatively slow process, and it will remove organic thin films, not clean off dust.)

Materials and equipment needed: glass tray, test slide and scrap PDMS piece, tape, plasma cleaner, vacuum pump

<u>Plasma bonder/cleaner setup:</u>
(Set-up required only if plasma system has not been used recently)

Figure 5A: Vacuum Pump Switch

1. Turn on the vacuum pump (Figure 5A) and open the "specialty vacuum" valve on the fume hood (labeled "SV"). A hissing noise should be heard in the chamber.

2. Close both valves on the round metal door. Align it to the glass vacuum chamber, and after a few seconds ensure that it is firmly held onto the chamber. *Support it and do not let it drop!* See Figure 5B.

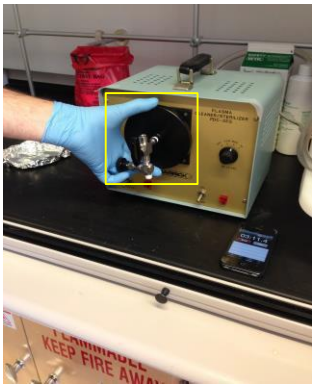


Figure 5B: Hold the round metal door to the vacuum chamber

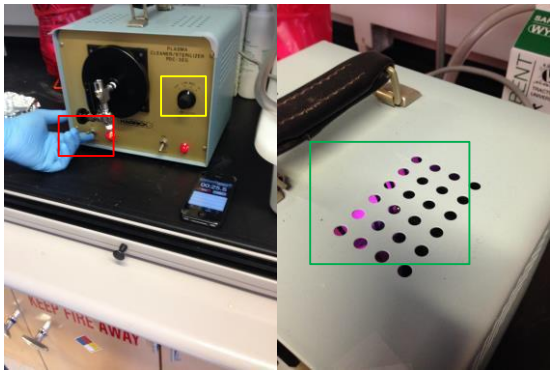


Figure 5C: Set power level to high, marked in yellow, and turn on the power with switch highlighted in red. Purple glow should be visible (highlighted in green).

3. Start a timer. After about 15s, turn on the power and set power level to [High] (Figure 5C). A purple glow should be visible through the vent holes after a few seconds.

4. Once a purple-glowing plasma is visible, ***slowly*** open the needle valve ***a very small amount*** to let in room air and oxygen. The plasma should brighten and become more orange (See Figure 5D). If it dims too much, close the needle valve slightly and observe the bright plasma return after a few seconds.

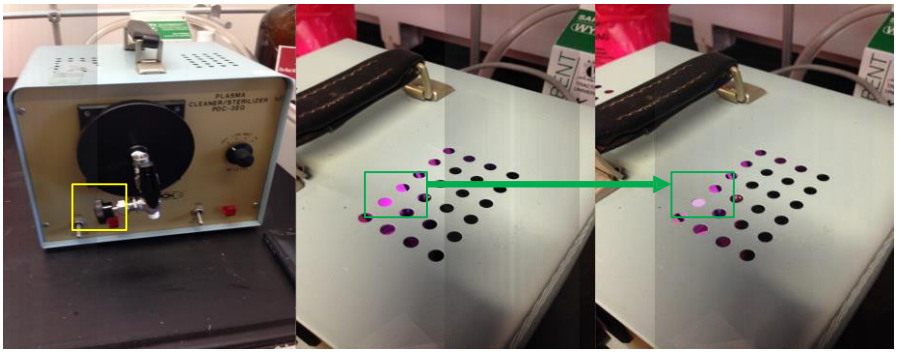


Figure 5D: Slowly open the needle valve (highlighted in yellow) to let in a little air. Purple glow should change color slightly, highlighted in green.

5. Allow the chamber to clean for 1-2 minutes.

Figure 5E: Open exhaust valve (highlighted in yellow) while holding the door to release vacuum

6. When plasma treatment is completed, turn off the unit power and the vacuum pump power. Slowly open the exhaust valve until the vacuum has been released (Figure 5E). *HOLD ONTO THE DOOR*, or it will fall!

PDMS Bonding:

1. (Optional) Prepare a test bonding sample, such as a scrap of clean PDMS and a clean glass fragment (or two PDMS scraps). Remove dust with tape. Then
   follow Steps 2-10, and if successful, repeat Steps 2-10 with the desired parts to be bonded.
2. Seal the PDMS on the tray slide with treatment side facing up. Next to it, place the glass fragment (or the second PDMS piece). See Figure 5F.
3. Insert the tray into the chamber (Figure 5F). Ensure the door valves are closed, turn on the vacuum pump, and align the door until it is held in place.
4. After ~5s, turn on unit power and wait for purple plasma as described in steps 3-4 above. Start a time when it appears and adjust needle valve to generate brighter plasma.

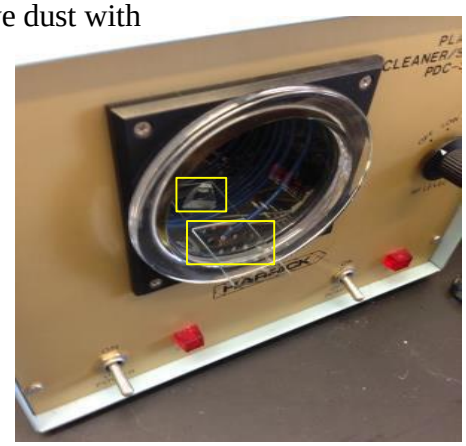
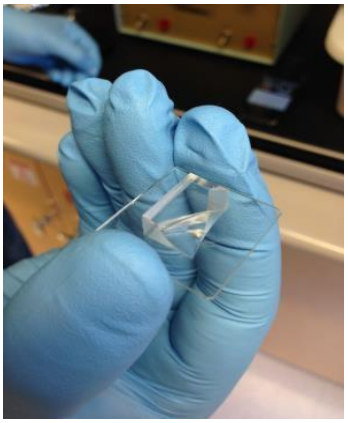


Figure 5F: PDMS and glass fragment are sealed to the tray side, treatment side up (highlighted in yellow)

5. Treat PDMS surfaces for 60s.
6. Turn off unit power and the vacuum pump power. Slowly open the exhaust valve until the vacuum has been released. As before, *HOLD ONTO THE DOOR*, or it will fall!
7. Carefully remove the plasma-activated PDMS and glass.
8. Gently invert the glass onto the activated PDMS surface. Bonding is covalent and instantaneous, so there is no opportunity to realign! Make sure you align before any contact, and be as gentle as possible. See Figure 5G.



9. Once the PDMS is sealed, apply light pressure the remove any air bubbles that may have been trapped inside.

Figure 5H: PDMS breaks after attempting to peel it away from the glass slide

10. Wait about 15 - 30s, and test an edge for bonding by ***very gently*** peeling up at the corner. A successfully bonded PDMS piece will not peel away from the substrate, and will break internally before debonding!

See Figure 5H.

Figure 5G: PDMS is sealed to glass