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Advanced Cell Culture Well for Mechanobiology

Daniela Garcia  
*Worcester Polytechnic Institute*

Jeffrey Michael Pruden  
*Worcester Polytechnic Institute*

Jeffrey Steven Kelley  
*Worcester Polytechnic Institute*

Jennifer Marie Mann  
*Worcester Polytechnic Institute*

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Advanced Cell Culture Well for Mechanobiology

A Major Qualifying Project Submitted to the Faculty of the WORCESTER POLYTECHNIC INSTITUTE

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Submitted by:

Daniela Garcia                Jeffrey Kelley
Jeffrey Pruden                Jennifer Mann

Approved by:

Prof. Kristen Billiar, Advisor
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Abstract

Cells in the human body are constantly subjected to mechanical forces. The field of Mechanobiology explores the mechanisms by which cells perceive and respond to mechanical stimuli to determine how changes in loading can alter tissue or organ physiology (Wang, 2006; Lim, 2010). Controlled \textit{in vitro} systems for stretching cells on 2D compliant substrates are generally capable of applying either uniaxial or equibiaxial loading, but cannot switch between modes of stretching. Further, the region of uniform stretch is quite small, and often not well characterized. To address these shortcomings, a computerized mechanical actuating system and flexible polydimethylsiloxane (PDMS) cell culture well optimized for uniformity of deformation were developed to facilitate multi-axial stretching of isolated cells at high strain rates on a microscope stage.
Chapter 1: Introduction

Cells in the human body are constantly subjected to mechanical forces such as compressive loads on bones and cartilage during locomotion, shear stresses on blood vessels due to blood flow, and cyclic stretching on the tissue of a beating heart (Wang, 2006). These physical loads dictate a number of cellular functions such as morphology, orientation, differentiation, gene expression, apoptosis, matrix protein secretion, and cytokine production (Lim, 2010; Wang, 2006). For example, bone will alter its shape and density in response to changes in its loading (Fung, 1990). Similarly, changes in blood pressure affect the shear stresses acting along the endothelium of blood vessels, which subsequently cause the endothelial cells to reorient (Wang, 2006).

Mechanobiology is an emerging discipline of science resulting from the realization that, in addition to biochemical and electrical signals, the cells in our body also respond to mechanical stimuli (Lim, 2010). Since mechanical loads have such a great effect on cellular function, Mechanobiology has become an essential field of research for studying how changes in loading can alter tissue or organ physiology and result in various detrimental pathologies such as fibrosis, osteoporosis, and atherosclerosis (Wang, 2006).

Generally, when cells are studied in vitro, they are grown on stiff, plastic tissue culture plates. These substrates inadequately represent the flexible and dynamic environment of the human body. When studying cellular responses in vitro, it is critical to mimic the in vivo environment to the fullest extent possible. This prompts the need for a system that is capable of replicating in vivo loads, while maintaining the proper physiological conditions necessary for cell viability.

Presently, there are a number of cell stretching devices on the market that can provide uniaxial or biaxial loading to mimic dynamic conditions. There is no device on the market designed for custom experimentation involving ranges of strain directions and strain rates. In addition, there is no integrated system that guarantees cell viability for long durations, provides a large area of uniform strain during stretching, interfaces with user-friendly software, and allows for real-time imaging.

The purpose of this Major Qualifying Project is to design a system to study the effects of multi-axial stretching at high strain rates on isolated cells under direct visualization. The device must maximize the area of uniform strain and provide the physiological conditions necessary for
sustaining cells. Lastly, the device needs to be safe, reliable, easy to use, and cost efficient. This system will be realized through extensive research of existing products, theoretical modeling using modern software tools, and lab experimentation.
Chapter 2: Background

Prior to beginning the design portion of this project, our team researched the field of Mechanobiology to develop a more complete understanding of the mechanical forces that affect cells and how cells respond to these forces. In addition, we explored current methods of how loads are applied to cells as well as the factors responsible for maintaining cell viability.

2.1 Significance of Studying Mechanobiology

The cells in our body are constantly subjected to mechanical forces, whether it be gravity pushing down on us, compressive loads acting on our bones and cartilage during locomotion, shear stresses acting on our blood vessels due to blood flow, or cyclic stretching on our heart tissue as a result of pumping blood (Wang, 2006). These physical loads affect different types of cells in different ways. For example, bone will remodel itself by altering its shape and density in response to a change in the loads acting upon it, generally referred to as Wolff’s Law (Fung, 1990). Similarly, changes in blood pressure will affect the shear stresses acting along the endothelium of blood vessels, which in turn causes the endothelial cells to reorient (Wang, 2006).

It is now well understood that mechanical loads dictate a number of cellular properties and functions such as morphology, orientation, differentiation, gene expression, apoptosis, extracellular matrix protein secretion, and cytokine production. On a larger scale, these forces are effectively responsible for maintaining tissue homeostasis (Lim, 2010; Wang, 2006). If the loading conditions on a tissue ever change, those cells will function differently, which could lead to changes in tissue or organ physiology and result in various pathologies such as fibrosis, osteoporosis, and atherosclerosis (Wang, 2006).

Mechanobiology is an emerging discipline of science, which stems from the realization that in addition to biochemical and electrical signals, the cells in our body also respond to mechanical cues (Lim, 2010). The process by which cells respond to mechanical forces is known as mechanotransduction. The principle behind mechanotransduction is that cells convert mechanical signals into a series of cellular events that eventually trigger a biological or chemical response (Ingber, 1997). However, the underlying mechanism by which cells perceive these forces and compose a molecular response is still not entirely understood.
Because mechanical loads have such a great effect on cellular function, Mechanobiology has become an essential area of research for tissue engineering, stem cell therapies, and studying disease pathology (Reilly, 2008; Choi, 2007; Lim, 2010). One way these applications are explored is through in vitro controlled, systematic studies. However, more often than not, cells in these studies are grown on stiff, plastic tissue culture plates. Problematically, these conditions are nothing like the flexible and dynamic environment of the human body, which dictate many cellular functions. When studying cellular responses in vitro, it is therefore critical to mimic the in vivo environment as much as possible. This prompts the need for a device that is capable of replicating in vivo loads, while maintaining the proper physiological conditions necessary for cell viability. To erect such a device, we need to understand the range of mechanical conditions found in vivo.

2.2 Mechanobiology in vivo

The mechanical forces continually acting on cells range from external forces like gravity to internal forces that are generated by individual cells the surrounding cells. Examples include a muscle flexing and pulling on a tendon and bone, or the heart contracting to push blood throughout the body. All of these forces create different forms of stresses and strains, namely tensile, compressive and shear (Figure 1). Tensile and compressive stresses are created by forces normal to the surface. In tension, the force is pulling outward from the surface, and in compression, the force is pushing inward on the surface. Shear stresses are caused by forces parallel to the surface (Hibbeler, 2011). Due to the dynamic nature of the in vivo environment, most stresses in the body are cyclic, which either means the stress is continuously being applied and removed or the direction of loading is alternating.

![Stress Diagrams for a) Tension, b) Compression, and c) Shear](image)

**Figure 1: Stress Diagrams for a) Tension, b) Compression, and c) Shear**
All cells in the body experience one or several of these stresses on an almost constant basis. Different cells experience different magnitudes of stresses, and more importantly, all cell types react uniquely to these stresses. Wang and Thampatty (2006) published a comprehensive review of Mechanobiology that outlines the effects of stresses and strains on numerous different cell types, including cardiac and cardiovascular tissues, musculoskeletal tissues including tendons and muscles, and endothelial tissues like skin.

The cardiovascular system is garnering a large amount of interest in the Mechanobiology field. The heart and arteries are arguably the most dynamic tissues in the body due to the pulsatile beating of the heart and blood flow. An average person’s heart beats about 100,000 times a day to move blood and nutrients around the body, resulting in constant cyclic strain to all areas of the heart as well as the arteries. The strains experienced by cardiovascular tissues are uniaxial tensile strain and biaxial tensile strain as well as shear strain (Wang and Thampatty, 2006). A study conducted to determine the mechanical properties of mitral valves in a bovine model found that the valves were repeatedly subjected to 4% strain circumferentially and 10% radially at a frequency of about 1 Hz (Krishnamurthy, 2009). Strains within arteries were found to affect the degradation, reorganization, and synthesis of the extracellular matrix, in addition to the migration, apoptosis, and proliferation of vascular cells.

A second major tissue studied in Mechanobiology is muscle tissue. Muscle cells, and in particular, skeletal muscle cells, are regularly subjected to mechanical stresses. Everyday activities such as walking put tensile stresses on muscles throughout the body. Other activities like exercising as well as injuries from trauma can place extreme stresses on muscles. The strength and size of muscle is dependent on these stresses, as well as other stimuli. When muscles are frequently subjected to high stresses, the muscle fibers undergo trauma. This causes muscle satellite cells to move to the area, proliferate, and fuse to the muscle fibers. After fusing, the fibers are repaired and new muscle protein strands are added, increasing mass and strength. In the absence of loading, muscles atrophy, resulting in the loss of mass and strength (Charge, 2004). The role of mechanical stresses in muscle strength gain and loss as well as mass gain and loss further justifies the importance of mechanically loading cells.

Mechanical stresses play an important role in many other cells throughout the human body including chondrocytes in cartilage, fibroblasts in ligaments, and tenocytes in tendons and skin. These forces regulate cellular functions and direct the many aspects of cell and tissue
homeostasis. Studying these forces and their effects is crucial to developing a comprehensive understanding of the human body. However, studying forces in vivo is infeasible in most cases due to its invasive nature, which is why the effects of these forces must be observed in the in vitro environment.

2.3 Mechanobiology in vitro

There are a number of studies that have attempted to recreate in vivo mechanical loads on cells in vitro. These studies provide us with a typical range of strain magnitudes and rates used. A superior cell-stretching device should be able to replicate this entire range as well as produce never before tested in vivo loading scenarios.

2.3.1 Effects of Mechanical Stimulation on Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are important for medical applications because under specific culture conditions they can give rise to various cell lineages such as chondrocytes, osteoblasts, and fibroblasts. In order to use MSCs for tissue engineering applications, they must be properly stimulated. A study done by S (2007) reported that mechanical forces play a central role in the stimulation and proliferation of these cells. They found that the proliferation of MSCs increased when exposed to 5% strain as compared to 0%. Ghazanfari (2009) found analogous results when they strained MSCs up to 10%. According to Rangappa (2004), mechanical loads also affect the differentiation patterns of MSCs. In this study, MSCs were cyclically stretched at 1 Hz with a Flexercell system at 4% and 8% strain for 12 hours. The researchers reported an increased production of cardiac muscle markers proteins. Another study cyclically stretched MSCs at strains of 5% and 10% for 1,2, and 4 hours durations. They reported a significant increase in the expression of α-smooth muscle actin (Ghazanfari, 2009).

2.3.2 Effects of Mechanical Stimulation on Musculoskeletal Cells

Musculoskeletal cells, including fibroblasts and chondrocytes, are used very often for in vitro studies in Mechanobiology since they are constantly subjected to cyclic loading in the body. Studies show that musculoskeletal cells are affected by the magnitude of strain, the duration of the applied strain, as well as the type of loading applied. Once such study applied 10% strain from 0.01-1 Hz to adherent cells for up to 10 hours and observed that the cells’ actin
fibers reoriented perpendicular to the stretch direction and that the extent of reorientation was dependent on the strain frequency (Tondon, 2012). A second study applied uniaxial strains of 4% and 8% at 0.5 Hz to human fibroblasts and found that the magnitude of strain affected the amount of cell proliferation, collagen I gene expression, and collagen I protein (Yang, 2004). A third study cyclically stretched the same type of cell at 5% and 1 Hz and varied the duration of stretching. For periods of 6, 12, and 24 hours, an increase in cell proliferation was observed. At 48 hours, however, cellular proliferation began to decrease, demonstrating a stretch time-dependent response (Barkhausen, 2003). A study done by Hsieh (2000) compared the results of equibiaxial strains of 5% at 1 Hz on ACL and MCL fibroblasts. In the ACL fibroblasts, collagen I mRNA increased, but collagen III mRNA did not. In the MCL fibroblasts, the opposite trend occurred. These results demonstrate the dependence of response on the source of cells.

Throughout these studies, a common range of strains, strain rates, and experimental durations are presented. Most were performed using strains between 5% and 10%, with some studies attaining strains up to 24%. In terms of strain rates, most studies were conducted between 0.1 Hz and 1 Hz. Lastly, almost all studies stretched the cells for durations of at least 6 hours (Wang and Thampatty, 2006). Being aware of other scientists’ experimental procedures will allow us to determine what specifications our device will need to meet.

2.4 Methods of Stretching Cells

There are various means of inducing strain to cells. Common methods include: (1) compressive loading, (2) electro-deformation, (3) electromotors, and (4) optical manipulation (Brown 2000; Kim 2009). An understanding of these mechanisms is critical to designing an effective cell-stretching device.

The most common means of cell stretching, compressive loading, regulates pressure to induce forces to cells either by vacuum or hydrostatically. Hydrostatic pressurization utilizes fluid dynamics by modifying the pressure within the device above and below atmospheric pressure (Drumm, 2009). Regulated, the substrate is placed on a loading dock, and when the vacuum is turned on, the substrate is forced to hang over the loading dock as it is stretched. To allow for cyclic straining, the vacuum can be turned on and off (Flexcell International Corporation). The vacuum pump is capable of static or cyclic cell loading. While compressive
loading is the most common means on the market for cell stretching devices, a notable drawback is that it does not allow for equibiaxial strains (Rana, 2007).

Electro-deformation creates strain by subjecting cells to an electric field. AC voltage is first applied to suspended cells and voltages and frequencies are later manipulated to test cellular mechanical properties. When voltage is applied to cells, elastic and plastic deformation can be measured. When the electric field is turned off, the relaxation of cells can be measured (Kim, 2009). Advantages of electro-deformation are that there is no need for moving parts or microbeads in the cell suspension, contact between the cells and device is minimized, and multiple measurements may be taken simultaneously (MacQueen, 2010).

Electromotor cellular stretching devices convert energy to physically pull on a cell culture well on which the cells have been cultured. Electromotor devices are advantageous in that they can be programed to allow for custom experimentation. These devices are capable of bidirectional stretch or compression. These devices are microscope-mountable and allow for real-time observation.

Finally, optical manipulation attaches two micro beads opposite to one another using ligands or antibodies as holders on the cell membrane. One bead is fixed to the glass surface and the other is stimulated with a laser, causing it to move away and stretch the cell. This process is called laser trapping. This method is more for single cell manipulation rather than multiple cells (Wang, 2012).

During research, the team found an interesting and unique device capable of creating multi-axial mechanical strains on a culture membrane. The device titled MechanoCulture, developed by CellScale from Ontario Canada, uses a single linear actuator connected to a series of linkages with hooks. The direction(s) of strain applied can be adjusted by connecting the substrate to different combinations of hooks, creating 1:0, 1:1, or 2:1 x:y strain ratios. Within these ratios, the device can create a maximum strain of 15% at 2 Hz. The device and culture substrate is housed in a large central well into which culture media can be placed, surrounding the entire substrate and reducing the need for media replenishments mid-test. Test parameters are uploaded to the device prior to running through a PC based computer program without a run-time required control computer.
2.5 Methods of Incubation

Another critical aspect in designing a device to better mimic an *in vivo* environment is creating the physiologic conditions necessary for cell viability to ensure meaningful results in laboratory experiments. Without a carefully controlled environment, cell cultures can behave inconstantly or expire. Factors such as the growth medium type, gas content, temperature, and humidity can be controlled to emulate an *in vivo* environment. The requirements for some of these parameters can be generalized, while others are dependent on the specific cell type. Understanding the requirements and methods for cell viability is important when working with any cell cultures, including those involved in Mechanobiology experiments.

One of the most important decisions when working with cell cultures is choosing a suitable culture medium. The medium provides the cells with nutrients, vitamins, cofactors, metabolic substrates, amino acids, inorganic ions, and trace elements that are essential for cellular function (Davis, 2011). In addition, it also regulates the pH levels and the osmotic pressure of the culture (Life Technologies). In the past, cells were almost exclusively cultured in natural media obtained from tissue extracts and body fluids (Davis, 2011). Since then, extensive research has been performed to develop improved media. Primarily, the need for media alternatives arose from issues such as high costs, problems with standardization, specificity, and variability (Life Technologies). The state of the field is now far more advanced and has many options for culture media.

All of these media options fall within three classes: basal media, reduced-serum media, and serum-free media. Basal media generally contains the components mentioned above which are required for cellular function. The majority of cell lines grow well in this type of media, but typically it must be supplemented with serum to be effective for more than a few hours (Davis, 2011). The serum supplement then becomes the source of growth, adhesion factors, hormones, lipids, and minerals (Life Technologies). Some media are designed to require minimal serum, thus reducing the associated risks. Serum-free media avoids animal sera completely by replacing serum with appropriate nutritional and hormonal formulations. Serum from reputable sources can minimize undesired effects, but serum free options are available for certain cell types (Life Technologies).
Table 1: Advantages and disadvantages of serum-free media (Life Technologies)

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Increased definition</td>
<td>• Requirement for cell type-specific media</td>
</tr>
<tr>
<td>• More consistent performance</td>
<td>• formulations</td>
</tr>
<tr>
<td>• Easier purification and downstream processing</td>
<td>• Need for higher degree of reagent purity</td>
</tr>
<tr>
<td>• Precise evaluation of cellular functions</td>
<td>• Slower growth</td>
</tr>
<tr>
<td>• Increased productivity</td>
<td></td>
</tr>
<tr>
<td>• Better control over physiological response</td>
<td></td>
</tr>
<tr>
<td>• Enhanced detection of cellular mediators</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 contains some of the advantages and disadvantages of using serum-free media. One of the key disadvantages of this media type is that the formulations are cell-specific. Overall, it is essential to understand what medium type is best for the particular cell type being studied.

An important factor for cell viability that directly relates to the cell medium is acidity. Many media even provide visual cues of the pH levels through the use of non-essential additives such as Phenol Red (Davis, 2011). This allows simple qualitative measures of whether the pH of the solution is changing or remaining constant during an experiment. The recommended acidity for mammalian cell strains is from 7.0 to 7.4 on the pH scale (Davis, 2011). This varies little for different cell strains. Some transformed cell lines have shown improved growth in slightly more acidic conditions while some fibroblast strains thrived in slightly more basic conditions (Life Technologies). Overall, the requirements for this parameter are again dictated by the cell type.

Furthermore, the acidity can be affected by the level of carbon dioxide. This is due to the fact that the many media types protect the cells from drastic fluctuations in pH by utilizing a CO₂ bicarbonate based buffer. Therefore, it is important to us exogenous CO₂ when using media utilizing this buffer type (Life Technologies). Most cell types require are four to ten percent CO₂, although five to seven percent is the typical range. It is important to follow the recommendations for the particular medium at hand however. Table 2 provides a summary of recommended CO₂ levels for some common media.
Table 2: Summary of recommended CO₂ levels for common media (Davis, 2011)

<table>
<thead>
<tr>
<th>Basal Medium</th>
<th>NaHCO₃ concentration (mM)</th>
<th>Percentage CO₂ in gas phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eagle’s MEM (Hanks’ salts)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Ham’s F12</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>TC199</td>
<td>26</td>
<td>5</td>
</tr>
<tr>
<td>DMEM/Ham’s F12</td>
<td>29</td>
<td>5</td>
</tr>
<tr>
<td>IMDM</td>
<td>36</td>
<td>5</td>
</tr>
<tr>
<td>DMEM</td>
<td>44</td>
<td>10%</td>
</tr>
</tbody>
</table>

*Although DMEM was designed for use with 10% CO₂, many methods are published that, for whatever reason, employ DMEM with 5% CO₂. It should be recognized that this will lead to the initial pH being a little more alkaline.

The CO₂ concentration becomes less important if the cell media does not use these methods to control pH levels. An alternative method is to use other buffers such as HEPES. This has a pKa of 7.3 at 37°C, and thus buffers very effectively without CO₂ (Davis, 2011). When it is necessary to control CO₂ levels, a CO₂ incubator is usually utilized. The methods employed by these CO₂ incubators will be discussed more in depth in Chapter 4.

Temperature is the next physiologic condition that must be carefully controlled. The temperature requirements for cell cultures vary based on cell type. This project focuses on human and mammalian cells that experience optimal growth conditions at 36 to 37 degrees Celsius (Life Technologies). It should also be noted that excessively high temperatures are generally more damaging to cell cultures than slightly low temperatures. Mammalian cells will die within an hour at a temperature of 45 degrees Celsius or higher (Paul, 1961). The cells’ survivability increases at slightly lower temperatures; cells can often survive for 12 to 24 hours at 42 degrees Celsius. These ranges are applicable to most fibroblasts and many epithelial types of cells arising from mammalian tissues; however it is important to research temperature requirements for the specific cells undergoing experimentation (Paul, 1961). While even slight deviations which exceed a cell’s normal temperature can cause harm, they can survive cooling to a much greater extent. In general, cells that normally require 38 degrees Celsius for rapid growth will continue to grow slowly at temperatures of 20 to 25 degrees (Paul, 1961). Even when cooled to temperatures as low as four degrees Celsius, there is no impairment to cellular function aside from delayed cell division. In general, cells must be kept above freezing to avoid the formation
of ice crystals within the cytoplasm, which will destroy the cell (Paul, 1961). Incubators are typically used to maintain the temperature in vitro. The methods, precision, and accuracy of these systems depend on the incubator type and model. In general, the temperature can be controlled within a few tenths of a degree. Temperature sensors are used to monitor the temperature of the environment and add heat as appropriate. Convection heating is the primary method of heating the cell environment. Additional methods will be discussed further in the design alternatives section.

A final key parameter is the humidity levels. For most cell types, it is essential to keep the cell culture in an environment of about 95% humidity to avoid the cells from drying out during long experiments (Life Technologies). The water used for humidification should be de-ionized or RO water to avoid contamination (Davis, 2011). Also, fans can be utilized to ensure the humidity is homogeneous throughout the chamber. Once again, alternative methods for humidification will be discussed as design alternatives.

Overall, there are several factors that must be combined to keep cells viable for the duration of an experiment. These factors which include growth medium, gas content, temperature, and humidity are all important in forming the cells’ environment. Incubators are usually used to control the physiologic conditions discussed above. When real time imaging is desired as is the case for this project, two primary incubator types are available. The two types are cage incubators, which enclose the majority of the microscope, and stage incubators which fit directly on the microscope stage. Both allow for temperature, gas content, and humidity control and will be considered as potential designs for our application.
Chapter 3: Strategy

After becoming more educated about the various aspects of this project, the team focused on outlining the scope of the project by developing objectives and constraints. Through several team-client meetings, the scope of the project was narrowed down, and a more complete and specific client statement was developed.

3.1 Initial Client Statement

To begin the process of designing a device to fulfill the needs of our client, an initial meeting was held where the goals of the project were established. From this discussion, we created our initial client statement seen below. This statement was later revised based on further interaction with the client: “Improve the existing mechanical cell culture well’s functionality and ease of use and validate the device’s effectiveness using living cells.”

3.2 Objectives

To fully understand what our client’s visions for this project, we classified features of the design as either objectives or constraints. While constraints are features that must be met in order to create a successful product, objectives are the desired attributes that the client would like to see in the designed system. The objectives and sub-objectives of our design are depicted in our Objectives Tree (see Appendix). All of our objectives fall under four overlying objectives: functional, reliable, user friendly, and cost-efficient. In regards to functionality, the ideal device will provide a complete range of uniaxial to equibiaxial strains at a range of strain rates that will allow users to specifically define how they want cells to be stretched. The device will also provide a relatively large area of uniform strain where the cells will be cultured and will be capable of running for long periods of time as dictated by the experiment requirements. In regards to reliability, we are concerned with durability and providing consistent strain fields. Thus, the device should not have many delicate components and should be able to repeatedly provide identical strain fields. To create a user-friendly system, the ideal device will be easy to use, set-up, and maintain. Lastly, the designed system will be as cost-efficient as possible.

Using a pair-wise comparison chart, we had our client rank these objectives from most important to least important. Table 3 shows how our client ranked our four overlying objectives. Functionality was determined to be the most critical objective, reliability and user-friendliness
received the next priority, while cost-efficiency ranked last. To further understand how our client ranks the sub-objectives under functionality, we created another pair-wise comparison chart (Table 4). From this analysis, we concluded that creating uniaxial to equibiaxial strains was the most important objective, providing a relatively large uniform strain area ranked second, providing a range of strain rates ranked third, and long operation time ranked last. Going forward, we will focus the majority of our efforts on our top-ranked objectives. At the same time, however, we will keep our lower-priority objectives in mind throughout the design process.

Table 3: Objectives Pairwise Comparison

<table>
<thead>
<tr>
<th></th>
<th>Functional</th>
<th>Reliable</th>
<th>User Friendly</th>
<th>Cost Efficient</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Functional</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Reliable</td>
<td>0</td>
<td></td>
<td>0.5</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>User Friendly</td>
<td>0</td>
<td>0.5</td>
<td></td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>Cost Efficient</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4: Functional Objectives Pairwise Comparison

<table>
<thead>
<tr>
<th></th>
<th>Uniaxial to equibiaxial strain</th>
<th>Range of strain rates</th>
<th>Large uniform strain area</th>
<th>Long operating time</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uniaxial to equibiaxial strain</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Range of strain rates</td>
<td>0</td>
<td></td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Large uniform strain area</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Long operating time</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
3.3 Constraints

There are certain requirements that the final design must meet to be successful. The first constraint is that the device must fit on a microscope stage. A specific microscope was chosen by the client in order to facilitate compliance to this constraint. The chosen model, an inverted Zeiss microscope with a motorized stage (see Appendix), can be found on location at Gateway Park at WPI. Dimensions of the motorized stage were measured to determine that the entire device must not exceed 152 x 103 mm. There are also slight protrusions on the stage, which further constrain the shape of the device.

In addition, there are constraints on the functionality of the device. The device must allow for incubation of the cell cultures for the duration of an experiment. The cells undergoing strain testing must be kept in homeostatic conditions, and the device must have the ability to track multiple cells throughout an experiment. While fulfilling these constraints, the device must not cause damage to any lab equipment including the microscope. The device itself must be able to withstand environmental conditions as well, which may require shielding certain components from humidity or temperature effects. The device must be safe for the user and the cells. Lastly, the device must be fully developed in 8 months’ time and for a maximum cost of $508.

3.4 Revised Client Statement

After further research and additional meetings with our client, the needs of the project were further defined and our client statement was revised. The primary change that took place between the initial client statement and this iteration is the goals of the project were generalized. In other words, instead of stating that we are improving an existing device, the implied bias of using the previous year’s device was removed. It was replaced with the primary function of the device which is to stretch cells. In addition to focusing on the functions of the device, we made it a point to include the unique aspect of our project, which is the integration of cell viability methods with the mechanical cell stretching device. Validation of the device was removed from the revised client statement because it is an implied part of the design process. These changes are reflected in the revised client statement below.

“Design a system to study the effects of multi-axial stretching at high strain rates on isolated cells under direct visualization. The device must maximize the area of uniform strain
and provide the physiological conditions necessary for sustaining cells. Lastly the device should be safe, reliable, easy to use, and cost efficient.”

3.5 Project Approach

This project can be broken down into two major design components, each with their own challenges. The first of these considerations is the task of stretching cells. The various devices available now, as well as the design resulting from a previous project (see Appendix), have the same set of challenges associated with applying strains to cultured cells. In a theoretical, one-dimensional world, the creation of a uniform strain is relatively simple. A uniform strain is defined as a strain of the same magnitude across a specified distance or area. In a one-dimensional problem, this means the entire length of an element with an applied force must have the same strain along it. When additional dimensions are added, creating a uniform strain becomes increasingly difficult. Various material properties and the physical shape of the element both affect the created strains. In addition, the method used to apply the strain to the element will affect the strain pattern. As such, current devices and culture wells can only create a uniform strain over a small area. The remaining area of the wells tends to contain irregular and uncontrollable strain patterns (B-Bridge International; Cell Lines Service; Flexcell International Corporation). A second challenge related to stretching the cells is the viewing and tracking of individual cells. With current devices, the cells must be removed from the device in order to view them under a microscope. Removing the well from the device is cumbersome and time consuming.

The second major component of this design is the ability to create the proper physiological conditions required for sustaining cells during testing. The environmental conditions needed for cultured cells to survive are very specific and allow for very little variation. PH, temperature, and humidity must be constantly controlled and kept within very small ranges (Chen, 2005). Typical devices for stretching cells do not include means of controlling the culture wells’ environments, requiring users to either provide their own means or conduct the stretching over a short period without environmental control (B-Bridge International; Cell Lines Service; Flexcell International Corporation). To add to the complexity of maintaining physiological conditions, the means for maintaining them must work in conjunction with the
stretching device. Not only does this create a challenge with size and shape, but conditions must be maintained while cells are actively being stretched.

To address the aforementioned complexities, we have set specific, system-level constraints. Where other devices cover only a few specific variations of Mechanobiology experiments, we aim to address all aspects of an experiment from cell straining to viability. To achieve each individual constraint, numerous tools and resources will be utilized. For example, we will begin by researching existing designs and methods to understand how other devices meet our client’s needs. In addition, we will use high-density mapping and finite element analysis to facilitate the design of an improved well and stretching method. Periods of experimentation, iteration, and validation will follow to ensure a successful design.
Chapter 4: Design Alternatives

Presently, there are a number of cell stretching devices on the market that can provide uniaxial or biaxial loading to mimic dynamic conditions. However, there is no device on the market designed for custom experimentation involving ranges of strain directions and strain rates. In addition, there is no integrated system that guarantees cell viability for long durations, provides a large area of uniform strain during stretching, interfaces with user-friendly software, and allows for real-time imaging. The purpose of this Major Qualifying Project is to fulfill this gap by fabricating a cell stretching device and incubation system. This section details the functions and specifications of our system and summarizes the various design alternatives we considered.

4.2 Functions and Specifications

The two functions of our project are to cyclically stretch cells and maintain physiological conditions. These functions are both essential for emulating an in vivo environment. In regards to the first function, the device must be capable of straining cells in the uniaxial and equibiaxial directions, allow for various strain rates between 0.01 Hz and 1 Hz, and provide strain from 0.05 to 0.3 within 10% accuracy. These specifications were chosen because they represent the range of strains that act on cells in vivo. In order to study the effects of mechanical stimulation on as many cell types as possible, the device must be able to replicate as many body environments as possible. Additionally, this device must maximize the area of uniform strain to at least 1 cm² and be capable of operating for a minimum of 6 hours without overheating. This is to ensure that any experiments performed on the device will contain enough cells and run long enough to provide useful data. In regards to the second function, the system will need to generate and maintain the physiological conditions necessary for cell viability, which entails a temperature of 37 °C ± 1°, a CO₂ level between 5% and 10%, a pH within the range of 7.0 - 7.4, and a humidity of at least 90%.

4.3 Design Alternatives: Cell Stretching Device

Due to the nature of our project, we have divided our design alternatives into two components: (1) stretch the cells and (2) provide physiological conditions. As such, we have created eight distinct design alternatives, four for each component. The culture substrate that will
translate the mechanical forces to the cells will be discussed in later sections after the cell stretching device is finalized.

4.3.1 Four Motor Design with 2 stacked motors

The first design we considered for stretching cells comes from the previous MQP. The design consists of four linear actuator motors aligned to provide the full range of motion from pure uniaxial to equibiaxial. The design achieves the range of motions through a stacked motor configuration, as seen in Figure 2. A flexible cell culture well is attached to four hooks; one is fixed and the other three connected to a motor stage. To provide uniaxial motion, two linear actuators pull the corners adjacent to the fixed corner. The third corner, diagonal from the fixed corner, is moved by a stage with stacked motors. On top of the stage is a linear actuator aligned to provide motion in the x direction. The stage then has a second motor attached to it to provide motion in the y direction. This stage can provide uniaxial motion in conjunction with one of the other corner stages by utilizing only one of its motors. If biaxial motion is desired, all four motors can be used simultaneously. In addition, by using four motors, the device can produce a full range of modes of stretching from uniaxial to biaxial.

Figure 2: Previous MQP Design (Duoba, 2012)
4.3.2 Butterfly Screw

The second option for stretching cells is what we call the butterfly screw method, which was developed by Rana (2007) and can be adapted for our project. The design consists of two parallel plates connected at the corners by screws and a third culture plate sandwiched in the middle, shown in Figure 3. The culture plate consists of flexible wells in which cells can be placed. Using butterfly nuts, the top plate forces the middle plate against the bottom plate, which has PVC discs aligned with the culture wells. By pushing the middle plate against these discs, equibiaxial strain can be achieved. The magnitude of the strain is controlled by how the number of turns of the butterfly screw. To adapt the design for our requirements, several changes can be made. The manually turned butterfly screws can be replaced with motors to provide cyclic stretching. Secondly, the shape of the PVC discs pushing on the wells and the wells themselves can be changed to provide different strain patterns. Interchangeable plates could provide the various strain patterns necessary.

![Figure 3: Butterfly Nut Design](image)

4.3.3 Dielectric Elements

The third design alternative considered was to use dielectric electrodes to stretch the cells (Figure 4). The system consists of two electrodes on opposing sides of a flexible Polydimethylsiloxane (PDMS) membrane. The bottom electrode is a 100 µm wide strip of gold through which current is run. The top electrode is a blanket electrode covering the entire membrane. These electrodes are placed onto a pyrex plate with a channel perpendicular to the
gold electrode. When a substantial voltage is applied to the gold electrode, the top electrode is attracted towards it, squeezing the PDMS membrane into the channel. This creates a uniaxial strain on the cells in the direction of the channel. Similar to the butterfly nut method, changing the shape of the channel can produce different strains (Akbari, 2012).

Figure 4: Dielectric Electric Cell Stretching Method

4.3.4 Vacuum

A final method of generating strain fields is utilization of a vacuum in which a flexible cell culture substrate is situated on a post over a solenoid-regulated vacuum source (Figure 5). To produce the cyclic stretching, the solenoid opens and closes, creating and removing negative pressure to the edges of the substrate. While under the pressure, the substrate stretches across the post, creating a strain for the cells. With two sides opposite each other open to the vacuum, uniaxial strain will be created. If a circular post with open sides all around is used, equibiaxial strain is created. This method can generate rapid strain changes via opening and closing the solenoid valve (Flexcell International Corporation, 2011).
4.3.5 Conceptual Tentative Final Design (Cell Stretching)

To determine the feasibility and effectiveness of each of our cell stretching design alternatives, we created a selection matrix, where we evaluated each design’s ability to meet the project’s constraints and rated each design’s ability to achieve the project’s objectives. We assigned each objective a multiplier based on the score it received in our pairwise comparison chart – an objective that ranked higher in our comparison chart received a larger multiplier in our selection matrix. Table 5 displays our selection matrix for means of stretching cells. As you can see, all four design alternatives met our two constraints; however, design 1 – using the previously fabricated device (Duoba, 2012), ranked highest in terms of meeting the project’s objectives. Design 1 was chosen over the other options because it was built with our same objectives and constraints in mind. It was designed with a double-stacked motor configuration to allow for both uniaxial and equibiaxial strain, and it uses four Hayden-Kerk Size 14 linear actuators to provide enough power to provide up to 30% strain within 10% accuracy at a rate up to 1 Hz. Moving forward, however, an improved version of Douba (2012) cell culture well will need to be develop to translate the motors’ loads to cells. The well created by (2012) had a number of shortcomings such as its opaque membrane and small area of uniform strain.
Table 5: Selection Matrix for Methods of Stretching

<table>
<thead>
<tr>
<th>Objectives and Constraints</th>
<th>Design 1: Old Device</th>
<th>Design 2: Butterfly Screw</th>
<th>Design 3: Electric Stimulation</th>
<th>Design 4: Pressure Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscope Compatible</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Safe for User</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Uniaxial to equibiaxial strain (x3)</td>
<td>(8 × 3) 24</td>
<td>(8 × 3) 24</td>
<td>(3 × 3) 9</td>
<td>(3 × 3) 9</td>
</tr>
<tr>
<td>Larger uniform strain area (x2.5)</td>
<td>(7 × 2.5) 17.5</td>
<td>(6 × 2.5) 15</td>
<td>(5 × 2.5) 12.5</td>
<td>(7 × 2.5) 17.5</td>
</tr>
<tr>
<td>Range of strain rates (x2)</td>
<td>(5 x 2) 10</td>
<td>(2 x 2) 4</td>
<td>(5 x 2) 10</td>
<td>(7 x 2) 14</td>
</tr>
<tr>
<td>Reliable (x1.5)</td>
<td>(9 x 1.5) 13.5</td>
<td>(9 x 1.5) 13.5</td>
<td>(8 x 1.5) 8</td>
<td>(7 x 1.5) 10.5</td>
</tr>
<tr>
<td>User Friendly (x1.5)</td>
<td>(9 x 1.5) 13.5</td>
<td>(9 x 1.5) 13.5</td>
<td>(9 x 1.5) 13.5</td>
<td>(9 x 1.5) 13.5</td>
</tr>
<tr>
<td>Cost Efficient (x1)</td>
<td>(10 x1) 10</td>
<td>(9 x1) 9</td>
<td>(2 x1) 2</td>
<td>(7 x 1) 7</td>
</tr>
<tr>
<td>Totals</td>
<td><strong>88.5</strong></td>
<td><strong>79</strong></td>
<td><strong>55</strong></td>
<td><strong>71.5</strong></td>
</tr>
</tbody>
</table>

4.4 Design Alternatives: Incubation Chamber

The second main function that our device has to perform is to keep the cells alive during testing by providing and maintaining physiological conditions. It is important for our device to mimic *in vivo* conditions as much as possible in order to create accurate results and sustain the
cells for the duration of the experiments. In this section, we discuss our four design alternatives based on incubator shape.

4.4.1 Cage Incubator Design

A cage incubator is an incubator that encompasses a microscope. Our first incubator design alternative, shown in Figure 6 is meant to enclose the stretching device and the majority of the microscope. The easy to remove hood made of a clear plastic like acrylic or lexan could sit around the microscope with enough space for movement of the stage and the device. The incubator would need to include the appropriate cutouts for the microscope as well as access panels to get to the stretching device. To heat the enclosure, forced convection could be used across a temperature controlled heat coil, similar to that of a hair dryer. The hot air could pass through/across a wicking sponge to generate a high humidity in the incubator. Lastly, CO₂ could be pumped into the enclosure from an external tank, and DMEM with 10% fetal bovine serum could be used as our culture media to provide other essential nutrients for the cells.

![Image of Cage Incubator Design]

**Figure 6: Cage Incubator Design**

4.4.2 Stage Incubator Design

The second proposed incubator form factor is the idea of a stage incubator custom tailored for our stretching device. A stage incubator is a miniature incubator meant to sit on the microscope stage and maintain conditions for a small area. For our stage incubator, we could place a small incubator enclosure in the hole that the cell culture well sits in. The design, shown in Figure 7 incorporates all of the same environmental controls as the larger cage incubator, although the heat source would have to have lower wattage to account for the smaller volume,
thermal mass, and load. To allow stretching of the well, slots could be cut into the sides of the well for hook movement. The top of the incubator could be removable and slide off and on. Additionally, a hole could be cut into the bottom to allow the microscope optics to have a clear view of the culture well.

![Figure 7: Stage Incubator Design](image)

4.4.3 Extended Stage Incubator Design

The extended stage incubator is very similar to the stage incubator but with one relatively major difference. The stage and extended stage are both miniature incubators that sit in the well area of the stretching device. With the extended state incubator, however, the volume that the well sits in would be expanded. To allow for a larger volume and hopefully a more stable environment, the dimensions of the device would be enlarged. The motors would be moved farther away and the dimensions of the hole for the cell culture well would increased, as represented in Figure 8. While causing a change in the device, this would provide more space to work the stage incubator in as well as the aforementioned advantage of having a larger thermal mass. For the environment, the same methods would be used as the previous two design alternatives.
4.4.4 Separate Incubator Design

The last design alternative considered by the team is to use a separate, already produced incubator. Worcester Polytechnic Institute already owns several stand-alone incubators which are available for use by students and faculty projects. The incubators are dual layer insulated metal containers that maintain internal temperature and humidity, and usually also control CO₂ levels and often pressure. This design alternative will most likely be used for preliminary testing of the device because it requires no design work or fabrication by the team. Unfortunately, the use of these incubators restrains the team from access and visualization of the device during the experiment. The team would also need to remove the stretching device in order to observe the cells under microscope. This design alternative is meant to be a stepping stone and safe fallback point.

4.4.5 Conceptual Tentative Final Design (Incubation Chamber)

To evaluate the design alternatives for our second function – maintain physiological conditions – we created another selection matrix (Table 2). While our first three designs met our project constraints, our fourth alternative failed to be compatible with the microscope. Therefore, we did not feel the need to rank this design since it could not be used. As shown, our first design alternative – using a cage incubator – ranked the highest. The main reason why the cage incubator ranked higher than the two stage incubators is because there is not a lot of room between the motors, the well, and the microscope when the entire device is set up. This makes it challenging to design an incubator that can sit on the microscope stage and encase the well without interfering with the motors or the imaging capability of the microscope. Building a cage incubator that can surround the entire device is much more feasible. Since the motors will be in contact with the high temperature and humidity in a cage incubator, we spoke with a
representative from Hayden Kerk to ensure that this environment would not adversely affect the motors. According to the representative, a temperature of 37°C and humidity of 90% should not affect the motors over extended periods of operating time. However, a potential problem that could arise with the cage incubator design is that high humidity and CO₂ levels may not be easily maintained in a large enclosure. To address this issue, we conceived the idea to combine both the cage and stage incubator design alternatives. Forced air convection will be used in the outer incubator, which will eventually heat up the smaller enclosure. CO₂ from an external tank will be bubbled through sterile water and transferred in a small tube to an opening in the stage incubator – this will create an atmosphere with high humidity levels and the correct CO₂ concentration for the cells inside the small enclosure. Convection was chosen over other methods of heat transfer such as radiation and conduction because radiation would require using a bulky heat lamp, which could also interfere with the imaging and viewing capabilities of the microscope, and conduction requires direct contact for heating, which is not feasible for our system. To ensure that the temperature remains at 37°C ± 1°C, we will use a temperature sensor, such as a thermocouple, integrated with a computer system to control the range. To maintain the humidity level, a hygrometer will be used to monitor the humidity in the incubator. We will perform some tests to determine the optimal location and layout of the humidity device to maintain a minimum of 90% humidity rather than actively controlling it via a computer controller. Ideally, the final design will also monitor the CO₂ to ensure that these conditions are met as well.
### 4.5 Feasibility Study and Experimental Methodology

It is essential to complete feasibility studies for conceptual design components in order to ensure project completion within allocated schedules and budgets. Through simple tests, it is possible to discover certain components may be difficult to manufacture, do not fulfill the design’s needs, or are simply impractical.

<table>
<thead>
<tr>
<th>Objectives and Constraints</th>
<th>Design 1: Cage</th>
<th>Design 2: Extended Stage</th>
<th>Design 3: Stage</th>
<th>Design 4: Separate Incubator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscope Compatible</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Safe for User</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Maintain Temperature (x2)</td>
<td>(8 × 2) 16</td>
<td>(7 × 2) 14</td>
<td>(7 × 2) 14</td>
<td>-</td>
</tr>
<tr>
<td>Maintain Humidity (x2)</td>
<td>(7 × 2) 14</td>
<td>(7 × 2) 14</td>
<td>(7 × 2) 14</td>
<td>-</td>
</tr>
<tr>
<td>Compatible with Device (x1.5)</td>
<td>(9 × 1.5) 13.5</td>
<td>(4 × 1.5) 6</td>
<td>(5 × 1.5) 7.5</td>
<td>-</td>
</tr>
<tr>
<td>Cost Efficient (x1)</td>
<td>(7x1) 7</td>
<td>(5 x1) 5</td>
<td>(5 x1) 5</td>
<td>-</td>
</tr>
<tr>
<td>Totals</td>
<td>50.5</td>
<td>39</td>
<td>40.5</td>
<td>-</td>
</tr>
</tbody>
</table>
To begin, we need to verify that the cell stretching design chosen to fulfill our first function will adequately fill our needs. Choosing the preexisting device is advantageous because it has already been manufactured, and several parameters have already been tested. The previous project team (Duoba, 2012) completed feasibility studies to ensure the motors are capable of stretching the PDMS well at the desired strain magnitudes, directions, and frequencies. They also verified that the device interfaces properly with the client’s chosen microscope by taking the device to the lab to test the fit. In addition, they ensured that their chosen motors could run for at least six hours at room temperature without overheating. They did not, however, show that the motors could run at 37 °C (the temperature inside the incubator) for six hours. Therefore, we will need to perform an experiment to ensure that the motors can withstand these conditions. If we find the motors are close to overheating in this experiment, the cage incubator design becomes less feasible, or at least more difficult to implement since environmental shielding would need to be designed. We will also need to ensure that our incubator’s heating and humidifying components can maintain the temperature and humidity at near appropriate levels for extended periods of time. Using a simple temperature and humidity gage, we will monitor these parameters over time. We do not yet have a device to regulate CO2, but when we do, a similar test will need to be carried out. In regards to the culture well, we will perform some preliminary imaging to ensure that cells can grow on our substrate and that high-resolution images can be taken through it.

Overall, completing these studies early in the design process avoids pursuing concepts that may have fundamental flaws. In turn, performing these quick analyses can reduce the time and cost to develop a successful final product.
Chapter 5: Final Design

To address each component of our client statement, the development of our final design was divided into four parts: (1) creating a user-friendly GUI, (2) determining the optimum design for the cell culture well, (3) fabricating the well, (4) and creating a custom incubator, compatible with the whole system. This section details the methods and tests that were used for each component of the project. Together, these components form our complete final design.

5.1 Device Operation

The device shown below (Figure 9) was the chosen design alternative for applying strain to our cell cultures. The device contains four stepper motors, with two in a stacked configuration, to apply forces to three of the four corners of the well. These forces cause strain throughout the well, including the central area where the cells are located. In order to implement this concept, the user needs a way to communicate with the motors.

![Cell Stretching Device](image)

Figure 9: Cell Stretching Device

Many potential solutions were investigated as a means to communicate with the device. Since the goal was to communicate with the device through a user’s computer, different software platforms and programming languages were considered. LabVIEW from National Instruments provides a user-friendly interface between electronic devices and computer systems. It allows users to build their own control panels with simple graphics based programming. Although this software is popular at universities as well as in industry, it is high cost, and is not specifically designed to interface with the microchip and drivers of our device. Another option is to exclusively use a program called MPIDE. This stands for “Multi-Platform Integrated Development Environment”, or multi-platform IDE. This is a programming environment
designed specifically for the chip used in our device. There are many advantages to using this system because it is open source. This includes no cost and an abundance of free resources. This is a popular IDE for hobbyists and academics that need to interface with a microchip. This IDE however does not have the capability to produce a simple graphical user interface (GUI). However, another program known as Processing is commonly used in tandem with MPIDE to produce the graphical front end for communicating with a microcontroller board. Other alternatives such as programming in Java, Visual Basic, or other languages was considered, but it is much simpler to use a programming environment already designed for this type of work.

This led to the chosen solution of using MPIDE in conjunction with Processing. MPIDE is used to create the firmware, which is loaded onto the chip, and Processing is used to create a GUI. This is the optimal solution for this application because it is free and compatible with all operating systems including Windows, Mac OS, and Linux. It also has a large online community, which provides numerous resources. One type of resource is libraries, which contain predefined functions. One example is the AccelStepper Library with contains many useful functions for working with stepper motors. Using this library saves time and effort, which would otherwise need to be exhausted to write the code from the ground up. MPIDE and Processing also allow for extensive customization of their respective programs.

Using these two programs, firmware was coded in addition to a user friendly GUI. The firmware is loaded onto the chip and is never seen by the ordinary user. The graphical user interface is used by the user to set desired conditions for a test. The current GUI design (Figure 10) allows the user to specify cyclic frequency in Hz, and strain magnitude in the x or y direction. Other functionality includes the user’s ability to modify the motor behavior as defined by the waveform visuals. Outputs of the system include the number of cycles and the calculated duty cycle.
5.2 Finite Element Analysis of PDMS Well

Application of a strain field onto cultured cells was achieved through the use of a flexible PDMS substrate. Forces applied to this substrate cause the substrate to deform, and consequently the cells cultured on it are subjected to the desired strain. In order to design a suitable substrate, a working understanding of the strain fields in the substrate was required. The shape of the substrate and applied points of force directly affect the strain pattern in the well, and in turn, the area of uniform strain in the center of the well. For our team to design a well to the required specifications, the strain fields in various well designs were calculated and compared. To gain a preliminary understanding of the ideal strain fields generated in different substrate designs, we conducted a finite element analysis (FEA) using the ANSYS computer software package. The use of FEA provided the team with a method for designing the optimal well as well as a means for characterizing the chosen well design for experimental purposes.

Using the CAD software SolidWorks, the team created 3D solid computer models of the Strex® wells and our well design alternatives, an example of which is shown in Figure 11.
Each well model was imported into an ANSYS Workbench project, where each well could be analyzed and the results could be easily compared. For all analyses, the properties of PDMS used were 0.540 MPa for the Young’s Modulus and 0.45 for the Poisson’s Ratio. All analyses were conducted in the same manner with identical settings. Meshes were created with 0.00075 m element size, medium relevance center, medium smoothing, and medium span angle center, with all other settings default. Thermal strain effects were turned off and large deformations were turned on. The analysis was done with program controlled stepping off and number of substeps set to 6, allowing for the same time spacing between displacements and results for each well.

To determine the best shape for our well design, a series of CAD models with varying sizes were created. In particular, the team was curious of the effects of membrane thickness and sidewall thickness on the size of the area of uniform strain. CAD models were created with a constant membrane thickness of 400 microns and sidewalls ranging from 1 mm thick to 5 mm thick. Secondly, models were created with membranes ranging from 100 microns to 500 microns thick, keeping the sidewalls constant at 2 mm thick.

5.2.1 Uniaxial

For uniaxial stretching, each model was fixed in the bottom right and top right corners and ramped displacements of 0 mm to 7 mm (0 to 0.233 engineering strain) were applied to the bottom left and top left corners in the X direction. By solving with ANSYS, the normal strains
over the area of the well can be graphed in the x and z direction in terms of the displacement of the well corners. For each well, a graph of the displacement to center strain in the X direction was created and a linear regression performed to get an equation. An example of one of these graphs is shown below in Figure 12. Using this equation, each well was then stretched to 0.1 center strain.

![Graph of Displacement vs. Center Strain](image)

Figure 12: Linear regression for 200 micron membrane well

\[ y = 19.1x - 0.003 \]

\[ R^2 = 0.9978 \]

ANSYS was then used to graph each well’s area of uniform strain in the center. To do this, the area of the well with strain of ±10% of the center strain was graphed as green, while the area with strains higher were graphed as red and the area with strains lower were graphed as blue. This gave a clear depiction of the area of uniform strain as defined by our client. Figure 5 shows an example of the results for a well with 2 mm sidewalls and a 150 micron-thick membrane.
Once all of the wells were analyzed, an open source computer program called ImageJ was used to measure the usable area of the well. In ImageJ, the user can set a scale for pixels per unit using a known length in an image. A screen shot of each set of results displayed in true deformed scale was taken with the ANSYS scale displayed. Using the scale at the bottom of the image, each screenshot was calibrated and ImageJ’s rectangle tool was used to determine to scale area. These areas were then graphed versus sidewall thickness as well as membrane thickness. The resulting curves are shown Figure 14 and Figure 15.

\[ y = 438.9x^{0.344} \]
\[ R^2 = 0.9798 \]
The best fitting trendline for the membrane thickness relation is a power function with a decay as the membrane thickness increase. There is no large increase in uniform area until 200 µm and above 300 µm, the area decreases, but only slightly. This suggests that if a well with a membrane thickness smaller than 300 µm is too difficult to reproducibly manufacture, there is little added benefit from going below 500 µm.

![Graph](image)

**Figure 15: Uniform Strain Area versus Wall Thickness**

The wall thickness appeared to follow a polynomial curve with the uniform area increasing as the sidewall thickness increased. This is due to the thicker sidewalls, which are in line with the applied forces, resisting more of the contraction of the well from the Poisson ratio created strains effects in the membrane. This curve suggests that the well chosen by the team should have the thickest walls possible. The thickness of the actual chosen walls does however depend on the strength of the linear actuators being used in the device. At a certain wall width, the force required to stretch the well to the desired strain at the desired rate will exceed the abilities of the motors. As seen on the graphs, the well with the largest area will need to have the thinnest feasible membrane and the thickest sidewalls that the motors are capable of stretching.

Additionally, in order to create pure uniaxial strain, the strain in the Z direction created in reaction to the X direction strain must be compensated for. Contrary to what would be expected, a positive Z normal strain is shown to be in the center of the well. This is possibly due to the
nature of the well shape. As the well is stretched, the sidewalls contract in toward the center, creating negative strain, or compression. Because the well is experiencing 0 engineering strain in the Z direction, areas of the well must be positive, or in tension, in the Z direction in order to average to 0 strain across the entire cross section. In Figure 16 below, the green represents -0.01 to 0.01 normal strain in the Z direction.

![Figure 16: Z direction 0.0 strain for 200 micron well](image)

In order to create an approximate Z normal strain in the center of the well of 0, the device will need to compress the well in the Z direction by about 0.02, a result seen for most well shapes.

### 5.2.2. Equibiaxial

For equibiaxial stretching, the bottom right corner of each model was fixed. The top right corner was then stretched from 0 mm to 6 mm in the Z direction and the bottom left corner from 0 mm to 6mm in the X direction. The top left corner of the well was stretched from 0mm to 6mm in both the X and Z direction. The uniform strain area for both the X and Z direction were virtually identical, with the areas of higher and lower strain switched. Additionally, similar relations between uniform area versus sidewall thickness and uniform area versus membrane thickness were observed. Because of this, the same conclusion is reached stating that the chosen well should have the thinnest feasible membrane and thickest sidewalls capable of being stretched by the device’s motors. Figure 17 shows the uniform areas in the X direction (Figure 17a) and Z direction (Figure 17b) for one of the well sizes.
The user can specify anywhere from pure uniaxial strain to any type of biaxial strain. The current limits on the strain magnitude are anywhere from 0 to 30 percent, and the frequency ranges from 0 to 1 Hz. The user can adjust these settings at any time during an experiment without stopping or resetting. The user also has buttons to set the home position of the well, stop the experiment, and run at the given settings. Additional features are explained in the program’s user manual (still in development).

5.3 PDMS Well Fabrication

The next major aspect of this project was creating the substrate on which to stretch the cells. For their substrate, Duoba (2012) fabricated an elastic cell culture well made of Polydimethylsiloxane (PDMS) as shown in Figure 18.
To create their well, Douba et al. used a custom-designed acrylic mold (Figure 19) to form the walls and then pre-tensioned and glued a commercially-available, ultrathin PDMS sheet to the bottom of the well.

After performing a HDM analysis of their well being stretched, the team realized that the well was not achieving the same area of uniform strain as was predicted by their FEA. This points to a manufacturing problem. We hypothesized that by molding the bottom membrane of the well directly to the walls of the well, rather than gluing on a sheet, the uniform strain in the well would improve and more closely match the FEA results. Therefore, to improve upon Duoba et al.’s design, we created a new acrylic mold that forms the entire PDMS well, including the
thin bottom membrane. In addition to potentially increasing the area of uniform strain, this approach streamlines the well fabrication process and saves users money since the PDMS sheets will no longer have to be purchased. Figure 20 shows the differences between the previous group’s acrylic mold and our design concept. The two major differences are that (1) the center piece in our design is attached to a top layer rather than the bottom layer of acrylic, and (2) a thin metal sheet is used as a spacer between the first and second layers so that a thin sheet of PDMS forms underneath the center piece. Theoretically, the PDMS sheet that forms should have the same thickness as the metal spacer, which is approximately 150 um. Since the current gold-standard on the market is the Strex well, which has a membrane thickness of 200 um (Strex Devices, 2012).

![Figure 20: Douba el al.'s design (A) versus our final design (B).](image)

In order to design the optimum acrylic mold for fabricating the PDMS wells, a number of design iterations were performed. See the Appendix for a table that summarizes this design
process by highlighting the major problems and changes for each acrylic mold iteration. The Appendix also includes a table that summarizes the optimum method for fabricating a PDMS well.

In addition to being easy to fabricate by hand, the bottom membrane of our well needs to be as thin, optically-clear, and smooth as commercially-available wells. It is essential that our PDMS membrane meet these standards so that it allows for clear images to be taken and ensures cell adhesion and viability.

To confirm that cells can adhere and survive on our membrane and that it is optically-clear enough to take relatively high resolution images, we cultured cells directly onto the bottom membrane of the well. First, we sterilized the well by spraying it with 70% ethanol. Then, we applied a pepsin-extracted collagen coating to the membrane. After rinsing off excess coating with PBS, mouse fibroblasts cultured in DMEM + 5% FBS were seeded onto the well. The cells were incubated overnight and then imaged. As Figure 21 displays, the cells were clearly visible through the membrane and appeared adherent and viable.

![Figure 21: Bright-field image of fibroblasts on PDMS membrane, 10x magnification.](image)

To determine the thickness of self-made PDMS membrane, two different people using two different micrometers measured three different areas of two different wells. This came to a total of 24 different thickness measurements, which are summarized in a table in the appendix. These measurements ranged from 200 – 230 µm, with the average calculated to be 209 ± 9 µm. According to our FEA in the previous section, a 200 µm-thick membrane provides a relatively large area of uniform strain. In addition, Strex claims that their wells have a 200 µm thick
membrane, which means that our well fabrication process is capable of creating PDMS membranes as thin as commercially available products.

To characterize the roughness of our membrane, an Olympus LEXT confocal microscope was used to focus on its top surface. Using LEXT and Mountains® software, the surface was analyzed for its roughness (Figure 22). The maximum height difference on the surface was determined to be 0.711 µm, while the average height change was found to be 0.116 µm. Since most cells are at least 10 µm, such a small scale of roughness is unlikely to affect the adhesion or behavior of cells cultured on the membrane.

![Figure 22: Surface roughness of PDMS membrane, 20x magnification](image)

5.4 Incubation

To sustain cells for experimental durations up to 6 hours, a custom acrylic incubator (Figure 23) was designed to provide the proper physiological conditions of 37°C ± 1°C, 10% CO₂, and a relatively high humidity (over 70%). In addition, the incubator was specifically designed to not hinder the stretching device’s functionality, specifically the motor control. A preliminary acrylic incubation chamber was provided at the start of this project. To further incubator development, testing was conducted to choose the ideal means for heating, humidifying, and providing carbon dioxide. In humidity testing, evaporation of a water supply and a humidifier were unsuccessful as the humidity was either insufficient or produced an excess of condensation (undesirable for both motors and microscope). The team then established that humidity would need to be applied locally. This resulted in the combination of both a stage-type and cage-type incubator. The cage incubator houses the hot air input supplied by a standard hairdryer as well as an enclosed CO₂ bubbling chamber (2 psi was applied). This cage incubator
was designed for ease of use with the motor system as well as optimized for ideal heat source location.

![Image of stretching device and custom incubator chamber mounted on Zeiss inverted microscope](image)

**Figure 23**: Stretching Device and Custom Incubator Chamber mounted on Zeiss inverted microscope

To supply humidified CO₂ to the well, a pressurized 10% CO₂ per 90% compressed air mixture was flowed through water (2 psi), bubbled, and supplied to the well at a high humidity (73 HR achieved). A flow meter and temperature/humidity probe were used to monitor these conditions. The probe controls the hot air source on or off, dependent on temperature (within 1°C). A PDMS ‘coverslip’ was fabricated (20:1 ratio to not affect stretched well) to maintain the desired temperature and humidity at the well (excess pushed into the cage incubator due to positive pressure). The temperature and relative humidity over time of the system is depicted in Figure 24 and Figure 25, respectively. The complete experimental setup is depicted in Figure 26.
Figure 24: Incubator Temperature versus Time, n=1, SD = ±0.1 °C

Figure 25: Incubator Humidity Ratio versus Time
Figure 26: Incubation System
Chapter 6: Final Design Validation

6.1 High Density Mapping Analysis

HDM analysis was conducted on a custom well stretched to 15% uniaxial center strain at 0.5 Hz and 15% equibiaxial center strain at the same frequency. The results of the uniaxial strain test (Figure 27) showed good agreement with the FEA results (measured center strain = 0.15; frequency = 0.515 Hz). The uniform measured area from the HDM analysis, as defined by 15% ± 1.5% strain, validated the value from FEA of approximately 70 mm², indicating an improvement from the commercially available STREX Well.

![Figure 27: Uniaxial Results - A) Strain in direction of applied strain, B) Strain perpendicular to applied strain; white box shows area of uniform strain](image)

Equibiaxial results (Figure 28) similarly confirmed the FEA analysis, showing a predicted issue in which larger strains are created within the center of the well than called for using the linear regression previously determined. The error displayed by the HDM was a strain larger than called for by about 0.02 strain, and was expected from the FEA. A different curve will be implemented into the software for calculating post displacement in the future.
6.2 Incubator Validation

To ensure cell viability in our custom incubator, two PDMS wells were sterilized and coated with collagen as previously described and seeded with mouse fibroblasts. One well was placed stationary in our incubator for 3.5 hours, while the other well was placed in a standard incubator for the same duration. Both wells were later stained with an Invitrogen Live/Dead® Cell Viability Kit and imaged (Figure 29). ImageJ was then used to quantify the number of live and dead cells (Figure 30).

ImageJ analysis of the Live/Dead® assay shows that 97.4% of the cells cultured in the well kept in our incubator remained alive during the 3.5 hour experiment, while there was 96.6% cell viability in the well in the standard incubator. These results suggest that there is no statistically significant difference between the cell viability of a standard incubator and our custom incubator.
Figure 30: Control and Test Well Live/Dead Image J Analysis; live cells are green, red cells are dead
Chapter 7: Discussion

The primary objective of this Major Qualifying Project was to develop a system to overcome the shortcomings of current commercial systems for Mechanobiology experiments. Some of these shortcomings include high costs, limited operation time, and limited strain rates and profiles of the mechanical loading device. In addition, commercial substrates are a high recurring cost, and they have limited areas of uniform strain necessary for meaningful results. Other considerations include microscope compatibility and methods to maintain cell viability for the duration of experimentation. The system developed in this project focused on multi-axial stretching of a 40 mm by 40 mm PDMS well while maintaining cell viability and microscope compatibility for real time imaging. The cell viability was maintained with a custom incubator verified to have equivalent performance to commercial products through live-dead testing. The incubator and device were constructed such that they are compatible with a Zeiss microscope available on site. The table below compares the custom cell stretching device to a commercially available product, the Strex ST-190-XY.

Table 7: Our device has expanded functionality at a fraction of the cost.

<table>
<thead>
<tr>
<th>Comparable Parameter</th>
<th>Our Custom Device</th>
<th>B-Bridge International ST-190-XY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Device Cost</td>
<td>~ $2,000</td>
<td>$20,000</td>
</tr>
<tr>
<td>Modes of Stretching</td>
<td>Uniaxial and Biaxial</td>
<td>Uniaxial and Biaxial</td>
</tr>
<tr>
<td>Stretch Magnitudes</td>
<td>0% to 25% at any value</td>
<td>0% to 20% in predefined intervals</td>
</tr>
<tr>
<td>Frequency</td>
<td>0.6-60 cycles per minute at any value (0.01-1.00 Hz)</td>
<td>1-60 cycles per minutes at predefined intervals (0.017-1.00 Hz)</td>
</tr>
<tr>
<td>Waveform types</td>
<td>Any</td>
<td>Two (square and sine)</td>
</tr>
<tr>
<td>Operating time</td>
<td>In excess of 6 hours</td>
<td>20 minutes</td>
</tr>
</tbody>
</table>
As demonstrated above, the commercial device’s capabilities are matched or exceeded by this project’s device whose cost is tenth of the commercial device’s price. The custom device can handle strain magnitudes up to 10% at 1.00 Hz and any specifiable strain magnitude at 0.5 Hz. These values are approximated based on rough video analysis. Encoder implementation or more thorough video analysis would be required to better characterize the motors’ speed capabilities. The commercial device simply states that its motors cannot retract or extend faster than 0.5 seconds, however it does not specify accuracy over any range of rates or magnitudes. In addition, the mechanical user interface of the commercial device which consists of physical buttons is replaced with user friendly software for our device. The graphical user interface helps experimenters to visualize their inputs and track cycles of stretch throughout the experiment.

The table below compares a commercial cell straining substrate to the custom culture well developed for this project.

<table>
<thead>
<tr>
<th>Comparable Parameter</th>
<th>Our PDMS Well</th>
<th>Strex Well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cost</td>
<td>$0.50</td>
<td>$50</td>
</tr>
<tr>
<td>Uniaxial and Biaxial?</td>
<td>yes</td>
<td>No, one or the other</td>
</tr>
<tr>
<td>Uniform Strain Area</td>
<td>68 mm²</td>
<td>60 mm²</td>
</tr>
<tr>
<td>Optically-clear</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>
This project’s well matches or exceeds the commercial product’s parameters at a cost one hundredth of the commercial well’s price. Our well is equally effective for all strain profiles and has a larger area of uniform strain.

All of these improvements allow users to perform Mechanobiology experiments which could not be specified by previous devices. Due to the minimal cost, this system can potentially increase the availability of equipment to a greater number of interested parties if brought to market. It is an economically feasible solution to fill the needs of individuals interested in studying Mechanobiology.
Chapter 8: Conclusions and Recommendations

The mechanical actuating system described herein allows for stretching of isolated cells along two orthogonal axes, which are independently controlled by user-friendly software. Our flexible PDMS cell culture well provides a substantially larger area of uniform deformation than previous designs, while our custom incubator supports cell viability for long-term experiments. All in all, this system will facilitate future Mechanobiology studies for fraction of cost of current systems.

Recommendations

Although the system was successful in meeting the requirements set out by the client, there are a few areas which can be improved further. The mechanical loading device could benefit from the implementation of encoders which are already installed on each of the four motors. They are already wired to the plate on the device, and a one-to-one connection cable has been created to bring the encoder signals to the housing containing the controllers. In order to implement the encoders however, it is recommended to purchase a second microcontroller dedicated solely to processing encoder feedback. Attempts to utilize the microcontroller currently dedicated to processing motor signals for encoder processing as well resulted in the introduction of excessive latency to the system.

The cell culture well manufacturing process could also benefit from a few small changes. One improvement would be to manufacture the well mold from a material with a greater hardness than that of acrylic. Acrylic was chosen as the current mold material due to ease of manufacturing, but it scratches easily during cleaning, and these scratches can translate to the membrane of the final product. In addition, a mold which can create multiple wells simultaneously would increase the time efficiency of the well making process. Finally, thinner membranes can help to increase the area of uniform strain. The current thickness was chosen because it was the thinnest membrane which could be consistently manufactured. With improvements to the mold, a slightly thinner membrane (ideally 100\(\mu\)m) may be possible.
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Flexcell International Corporation. Flexcell international corporation product catalog


Lifetechologies.Cell culture basics handbook


Appendices

Appendix A: Previous MQP Device
Appendix B: Zeiss Inverted Microscope

Motorized stage where the stretching device sits:

Microscope-compatible incubator:
Appendix C: Objectives and Functions

Objectives Tree:
Function-Means Analysis:

<table>
<thead>
<tr>
<th>FUNCTION</th>
<th>MEANS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Stretch Cells</td>
<td>“Old” Device</td>
</tr>
<tr>
<td>Incubator Shape</td>
<td>Cage</td>
</tr>
<tr>
<td>Temperature</td>
<td>Radiation/Infrared Lamp</td>
</tr>
<tr>
<td>pH</td>
<td>Bicarbonate Based Buffer</td>
</tr>
<tr>
<td>Humidity</td>
<td>Humidifier</td>
</tr>
<tr>
<td>Control</td>
<td>Unmonitored &amp; Uncontrolled</td>
</tr>
</tbody>
</table>
Appendix D: Acrylic Mold Design Iterations

*Duoba’s (2012) Design*

(pictured without clamps and alignment posts)

**Problems:**
- A PDMS sheet has to be bought and glued onto the bottom of the well.
- HDM analysis revealed inconsistencies between the strain fields in the FEA and the actual well.
Problems:
• After pouring in the PDMS, it is difficult to hold all three layers in place while inserting the screws → PDMS leaks through the layers
• Since 0.25” thick acrylic was used to fabricate this mold, the walls of the well are less than 0.25” tall. These walls are not tall enough to easily contain media.
• Several of these wells were stretched on the device and the post holes eventually tore.

Changes:
• Incorporated a 3-layer design concept so that the bottom membrane could be fused with the walls of the well; the thin metal spacer dictates the thickness of this PDMS membrane.
• Aligned the screw holes closer to the center to create a stronger clamping force around the well.
• Significantly reduced the size of the acrylic mold to minimize the amount of acrylic needed.
Version 2.0

(pictured without metal spacer, mold posts, and screws)

Problems:
- PDMS still leaks through the layers. This makes it very difficult to release the well from the mold after it has hardened.
- There is no enough room between the metal spacer and the screw holes, so an opening had to be made for the screws in the spacer. This is a problem because the PDMS leaks into the screw holes and makes it difficult to untighten the screws after hardening.

Changes:
- Introduced a two-screw system that allows the two bottom layers to be screwed into place before pouring the PDMS. After the PDMS is poured, the third layer can be laid down on top and then all three layers can be screwed together. This minimizes PDMS leakage during pouring.
- Added lips onto the sides of the bottom and middle layers to make it easier to pry the mold apart after the well has formed.
- Bought 0.5” acrylic to increase the height if the well’s walls.
- Increased distance between post holes and walls to minimize tearing while stretching.
Changes:
• Bought 4 c-clamps to prevent the PDMS from leaking through the layers.
• Made the mold slightly larger to allow room for the clamps.
• Made the distance between the well and the screws holes larger so that a larger spacer could be used → prevented PDMS from leaking into screw holes.
• Added another set of lips to the top layer to make it easier to pry the mold apart.
## Appendix E: PDMS Well Fabrication Process

<table>
<thead>
<tr>
<th>Step 1: Prepare PDMS</th>
<th>Prepare Sylgard® 184 silicone elastomer kit at a 15:1 ratio – weigh out 15 grams of the base and then add 1 gram of the curing agent. Mix thoroughly.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 2: Remove air bubbles</td>
<td>Place mixed PDMS in vacuum chamber until there are no air bubbles present.</td>
</tr>
<tr>
<td>Step 3: Clean the mold</td>
<td>To remove dirt and excess PDMS, spray each layer of the mold with degreaser. Be sure to wipe off the degreaser with a material that will not scratch the acrylic (such as Kimwipes®).</td>
</tr>
<tr>
<td>Step 4: Clean the mold</td>
<td>To remove dust, blow compressed air at each piece. (It’s important for the surfaces of the mold to be dust/dirt-free so that the bottom surface of the PDMS mold is as smooth as possible.)</td>
</tr>
<tr>
<td>------------------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Step 5: Assemble the bottom layers of the mold</td>
<td>Using the 4 short screws, screw together the bottom layer, metal plate, and middle layer of the mold. Now the mold is ready for the PDMS.</td>
</tr>
<tr>
<td>Step 6: Add PDMS</td>
<td>Pour the bubble-free PDMS mixture into the mold. Fill it about a third of the way up the wall of the mold. (Pour slowly so that additional bubbles do not form.)</td>
</tr>
<tr>
<td>Step 7: Assemble top layer of mold</td>
<td>Very slowly place the top layer of the mold over the PDMS. Ensure that no air bubbles get trapped underneath the center piece of the mold. Using the 2 tall screws, screw all 3 mold layers together.</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Step 8: Insert posts</td>
<td>Slowly insert the cylinder posts into the four postholes. Again, make sure that no air gets trapped.</td>
</tr>
<tr>
<td>Step 9: Clamp the mold</td>
<td>Clamp down the four corners of the mold. This is to ensure that no PDMS leaks through the layers. NOTE: If any bubbles formed during the above steps, place this entire configuration in the vacuum chamber again – the bubbles will be able to escape through the air holes.</td>
</tr>
<tr>
<td>Step 11: Cure PDMS</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td></td>
</tr>
<tr>
<td>To assist the polymerization process, place the mold in an oven at 60°C overnight.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 12: Remove well from mold</th>
</tr>
</thead>
<tbody>
<tr>
<td>After removing the mold from the oven, let it cool. Then, remove the 4 central screws and carefully lift off the bottom layer. Next, remove the 2 outer screws and 4 attachment holes (may need pliers to remove the posts). Then, use the lips on the outer edges to remove the top layer from the middle layer.</td>
</tr>
</tbody>
</table>
## Appendix F: PDMS Membrane Thickness Measurements

<table>
<thead>
<tr>
<th></th>
<th>Micrometer 1</th>
<th>Micrometer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Well 1 (µm)</td>
<td>Well 2 (µm)</td>
</tr>
<tr>
<td>Person 1</td>
<td>210, 210, 210</td>
<td>200, 210, 210</td>
</tr>
<tr>
<td>Person 2</td>
<td>200, 200, 210</td>
<td>200, 200, 200</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>209 ± 9 µm</td>
<td></td>
</tr>
</tbody>
</table>
Appendix G: Collagen-Coating PDMS Well

1. Added 1 ml of HEPES (pH = 8.5) to an eppendorf tube.
2. Mixed in 4 ul of DMSO (4ul/ml of HEPES) to help Sulfo-SANPAH dissolve.
3. Weighed out 0.005 g of Sulfo-SANPAH and mixed it into the HEPES and DMSO solution. (The solutions should be slightly red)
4. Pipetted solution onto surface of well until the whole surface was completely covered.
5. Placed well under UV light for 6 minutes. (The solution should turn brown)
6. Removed well from UV and dumped out the solution. Rinsed well with HEPES.
7. Repeated steps 1-6.
8. Added enough pepsin (clear liquid) to coat surface of well.
9. Let pepsin sit in well for 30 mins – 2 hours, and then rinsed it out with HEPES or PBS.
Appendix H: Finite Element Analysis Results

Uniaxial Variable Wall Thickness Results
Uniaxial Variable Membrane Thickness Results
Comparison of STREX® to Our Well
Appendix I: Device Operation

To operate the device, the user must install software off the provided disk. The disk provides instruction for installing the software and drivers. General resources can be found at the following links:

Drivers: [http://www.ftdichip.com/Drivers/VCP.htm](http://www.ftdichip.com/Drivers/VCP.htm)
Processing Download: [http://processing.org/download/](http://processing.org/download/)
MPIDE Download: [https://github.com/chipKIT32/chipKIT32-MAX/downloads](https://github.com/chipKIT32/chipKIT32-MAX/downloads)