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Bioreactor and a Novel Microengineered Vascular Network

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By

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Abstract

A major challenge in tissue engineering is overcoming the low diffusion limit of oxygen and nutrients in engineered tissue such as cardiac patches. In this study, the design team aimed to design and develop microengineered vasculature within a fibrin hydrogel capable of being perfused to maintain the viability of seeded C2C12 cells. A novel microengineered vascular network with tunable dimensions was created using a gelatin sacrificial mold that was continuously perfused at physiologic flow rates for up to twenty-four hours. Additionally, a bioreactor system with a custom microfluidic chamber was developed to provide perfused culture conditions and allow the study of small molecule diffusion and the interactions between microvasculature and adjacent tissue constructs. The system maintained cell viability and morphology of cells seeded on top of the perfused fibrin hydrogel for twenty-two hours at least 1200 μm from the edge of the perfused channel. This project suggests that microvascular networks formed using gelatin sacrificial molds and cultured in a custom microfluidic chamber have the potential to be used in the formation and culture of a tissue engineered cardiac patch.

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Chapter 1: Introduction

In the United States alone, approximately 610,000 people die every year due to heart disease, making it the leading cause of death for both men and women (CDC, 2015). Forms of heart disease include arrhythmia, congestive heart failure, and myocardial infarction. Annually, approximately 735,000 Americans suffer a myocardial infarction, commonly known as a heart attack (Mozaffarian et al., 2014). During such an event, the cardiac muscle stops receiving oxygen and nutrients due to a blockage in an artery. If blood flow is not restored quickly, a section of the heart tissue begins to die. Possible causes of a blockage in the coronary artery are an embolism or coronary heart disease, both of which cause blockages. Such a blockage in the coronary artery can cause a myocardial infarction (Vlaar, 2008).

The main goals of immediate treatments are to restore blood flow to the damaged area, to minimize the damage on the myocardium, and reduce the risk of having another myocardial infarction. Unfortunately, the current methods do not regenerate the patient's tissue or regain functionality of the starved myocardium. After a myocardial infarction, the myocardium is replaced by scar tissue and no myocardial regeneration occurs (Poss, Wilson, & Keating, 2002). Currently, the only long term treatments for myocardial infarctions are whole heart transplants. However, this procedure has associated risks which include failure of the donor heart, complications from medication, infection and negative immune response (Lindenfeld et. al, 2004). During the transplant operation, patients are given immunosuppressant drugs that reduce the activity of the immune system. This leaves the body prone to disease, cancer, other malignancies, and infection (Lindenfeld et al., 2004). Because the proposed methods do not restore the patient's heart functionality, a method of regenerating the patient's native tissue must be developed.

Researchers have begun to develop methods aimed at regenerating damaged myocardial tissue. Scientists have been researching and testing the use of cells on a scaffold to grow tissue patches. In humans, the size needed to repair an average myocardial infarction is at least one cm thick. However, oxygen and nutrients can only diffuse about 200 μm (Carmeliet & Jain, 2000; Novosel, Kleinhans, & Kluger, 2011). These issues have sparked a need for engineers to develop a microvascular layer of myocardial tissue which can be incorporated into a larger cardiac patch in order to replace all the damaged tissue. The vascular layer is needed to provide nutrients to a thick layer of tissue or many layers of stacked tissue. The patch could be used to replace the scar tissue formed after a myocardial infarction and regenerate the structure and functionality of cardiac tissue at the site of injury. The vascularization of the patch would promote angiogenesis and assist with the integration of the construct into the surrounding native tissue.

The need for tissue regeneration to restore damaged tissues can only be accomplished with the use of bioreactors. They are research and development tools that allow the study of interactions between all components within the system. Bioreactors facilitate the growth of cells and tissue constructs for biological applications that may be clinically effective and safe while maintaining tissue viability in addition to culturing cells for months through continuous perfusion of fresh medium. The bioreactor must maintain an environment with very specific parameters that promote the growth of tissue. These parameters include flow rate, temperature, sterility, and specific sizing that accommodates the needs of the client. The ultimate goal is that bioreactors can contain scaffolds within them and can monitor and control the conditions of the tissue to ensure they are suitable for long term implantation with minimal rejections.

For the completion of the Major Qualifying Project (MQP) at Worcester Polytechnic Institute (WPI), the team was tasked with creating a perfusion bioreactor and a microvascular

network within a hydrogel. After interviewing clients Dr. George Pins, Megan O'Brien, and Meagan Carnes, specific needs and constraints were identified and the team began working towards completing the desired objectives and functions. The construct had to maintain cells in a layer with dimensions of 1 cm wide by 1 cm long by 300 μm thick for up to three weeks in vitro in addition to supporting a layer composed of fibrin threads and cardiomyocytes. The bioreactor needed to allow for viewing the sample at all times in addition to maintaining a sterile environment and continuous perfusion throughout the system.

To ensure the completion of this project, a project strategy was created with detailed milestones and deadlines. The first step of the project strategy was to design and develop a bioreactor. Research was conducted, design ideas were brainstormed, suitable materials were explored, computer-aided design (CAD) designs were fabricated, and feasibility testing was conducted. Next, methods to create a single channel microvascular system were explored and tested to ensure that they were reproducible in addition to methods for creating bifurcations within the hydrogel. Continuous perfusion was accomplished first through the use of a peristaltic pump and then improved with the use of a syringe pump. Diffusion rates within the microvascular network were characterized through biochemical and biological assays and compared with physiologically relevant values. Next, a cell viability assay was developed and performed to test the survival of cells on top of the microvascular layer.

The first step in the project strategy was to select a method to fabricate the proposed microvascular network with physiologically relevant dimensions. To create microengineered vasculature with sizes on the order of arterioles, the final method selected for this was using a gelatin hydrogel as a sacrificial material. This method employed gelatin molds as temporary molds, which were cast into fibrin gels, then melted and flushed out. This left behind an empty

network of channels matching the initial mold geometry. Once a microvascular network fabrication method was selected, the team moved onto designing a bioreactor and a microfluidic chamber, which would assist in the microvascularization formation and hold the developed tissue construct. In order to meet the clients' and users' requirements for the functions of the bioreactor, the team selected a syringe pump to achieve physiologically relevant flow rates that match those of arterioles in vivo. The team then moved to design a microfluidic chamber, which would be compatible with the microvascular network fabrication method, while allowing for imaging, sustaining a cell culture over an extended period of time, and maintaining a sterile environment.

In order to quantify the success of the selected final design the team performed three main validations. The first test was establishing continuous perfusion through the fabricated microvascular network. The second was to study the diffusion of nutrients and metabolites through the gel construct. The third validation was to determine the microvascular and bioreactor system's ability to sustain cell viability. Each one of these major validation steps study the efficiency of a number of elements of the final design. The purpose of these assays and experiments was to determine the feasibility of applying the microvascular network and bioreactor system to assist in the fabrication of a multilayered cardiac patch.

In the following chapters, the team summarizes past research in the field of tissue engineered vasculature and bioreactor systems from available literature, describes the project approach with goals and objectives set by the clients, and proposes a functional final design select from various alternatives.

The team developed a protocol for creating a microvascular network that mimics native blood vessel size and designed a bioreactor system to sustain cell viability in a 3D gel construct.

This 3D cell culture creates an environment that more closely resembles that in vivo than a 2D cell culture and would allow for improved study of cell interactions. In this project, the focus was to create a microvascular network specifically for a fibrin cardiac patch. However, the fabrication approach could be adjusted and reapplied to vascular network fabrication for other organs as well.

Chapter 2: Literature Review

Chapter 2: Literature Review provides an overview of heart disease, including its associated types and current treatments. With an understanding of the current treatments, the need for a vascularized cardiac tissue replacement construct becomes clear. This chapter reviews current strategies within cardiac tissue engineering and microvasculature fabrication as well as the advantages and disadvantages to current approaches. It provides the necessary background for the development of a microvascular network and the need for an endothelial layer. Lastly this chapter defines bioreactors, explains the need for them, and what can be found on the market.

2.1 Heart Disease

Heart disease is the leading cause of death in the United States. Each year, about 610,000 people succumb to the illness, making up 25% of all deaths in the United States (Lloyd-Jones, 2010). Heart disease includes arrhythmia, congestive heart failure, and myocardial infarction. Myocardial infarction, also known as a heart attack, is the most common form of heart disease. (Lloyd-Jones, 2010).

2.1.1 Myocardial Infarction

Myocardial infarctions occur when the flow of blood to the heart suddenly becomes blocked. Coronary arteries deliver oxygenated blood to the heart muscles and if one of these arteries become blocked, the corresponding portion of the heart is deprived of oxygen. If the muscle is starved for too long due to the high metabolic demand, myocardial infarction occurs, which is death of heart muscle. The major cause of myocardial infarctions are blood clots, also known as thrombi. Over time, the arteries can narrow from a buildup of plaque. When the plaque in arteries rupture, the inflammatory response creates a blood clot which can partially or completely block flow of blood through the coronary artery (Figure 1).

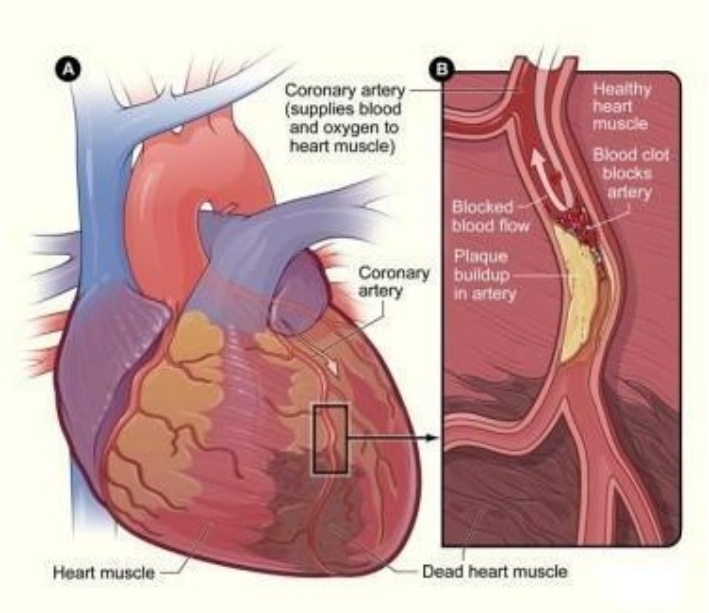


Figure 1: Heart with arterial plaque build-up. (A) Shows the dead heart muscle (darker area) caused by a blockage (highlighted area) in the coronary artery. (B) A cross-sectional image of a coronary artery showing a blood clot caused by plaque buildup (White & Chew, 2013).

The severity of a myocardial infarction is judged by the volume of heart muscle damaged, known as the infarct size. In Figure 1 above, the infarct is labeled as the 'dead heart muscle'. An average infarct size is about 8 cm^3, which is approximately 12% of the left ventricle muscle (Kawel et al., 2013, Miller et al., 1995). Depending on the infarct size and the condition of the patient, different treatments might be suggested by the doctor (Zafari, 2015). The most common medical treatments are percutaneous coronary intervention and coronary artery bypass. These treatments aim to restore the blood flow to the damaged heart section. This enables the tissue to regain some structural morphology, but not functionally, since it only reforms as scar tissue. This creates the need to develop a treatment that addresses the restoration of myocardium function. Current treatments only recover blood flow to the damaged areas created by the infarction and prevent recurrent myocardial infarctions from happening. The reduction in the myocardial function, however, leads to further complication to the patient's health.

2.1.2 Current Treatments

Currently, immediate treatments for a myocardial infarction include clot-busting medications and percutaneous coronary intervention (PCI). These are immediate treatments because their purpose is only to reestablish blood flow to the heart. Long-term treatments are coronary artery bypass surgery and whole heart transplant. The goals of these treatments are to prevent the occurrence of another myocardial infarction. Except for whole heart transplant, none of the other three treatments address the reduction in functionality of the damaged heart region (Bauer et al., 2006).

Clot busting drugs have been approved to treat coronary artery blockages and limit the damage by breaking up and dissolving clots in the arteries that have restricted the blood flow to the heart. The most prevalent risk is excessive bleeding which can lead to death, since the clot busting drugs also act as blood thinners (Antman, 2011). Percutaneous Coronary Intervention (PCI) is a minimally invasive procedure that uses a catheter to place a stent into a blocked blood vessel and widen it creating a passage. Fifteen to forty percent of the time following surgery, the body considers the stent to be a foreign object, causing an inflammatory reaction and resulting in restenosis of the artery, possibly leading to another heart attack (Sakr, 2015).

Bypass surgery is performed by taking a section of a blood vessel, typically from the leg or chest, to graft around the affected artery, bypassing the clot. This lowers the chance of having recurring heart attacks by improving blood flow. Limitations include stroke, excessive bleeding, infection, and death (Serruys et al., 2009). A heart transplant is the only method that replaces damaged and diseased heart muscle and is only used when all other treatments have failed. There are many complications and risks associated with this procedure which include a long waitlist,

infection, organ rejection, immunosuppressive medication response, long recovery period, and high cost (Lindenfeld et al., 2004).

Most of the treatments currently available do not repair damaged heart muscle or recover functionality. In addition, they have significant drawbacks. Whole heart transplant is not a reliable treatment due to the extremely long wait list. As such, there is a need for an off-the-shelf treatment to restore functionality which addresses these challenges. The most promising approach is through cardiac tissue engineering applications that have the ability to replace and regenerate damaged heart muscle, restoring the myocardium function while being readily available.

2.2 Cardiac Tissue Engineering

The purpose of cardiac tissue engineering is to create and develop a viable and efficient approach to treating heart failure. The field addresses the need through the *de novo* fabrication of implantable biocompatible scaffolds with cardiomyocytes and a functional vasculature, which can metabolically support the construct.

2.2.1 Clinical Need

The limited ability of cardiac muscle to regenerate after a myocardial infarction and the difficulty to find heart donors clearly suggests a need for new methods of restoring the damaged myocardium (Emmert, 2013). Cardiac tissue engineering is a field of engineering that has been heavily researched in recent years, specifically focusing on creating biocompatible scaffolds, finding abundant sources of cardiomyocytes, and fabricating functional microvascular systems (Dilley, 2014). None of the current treatments regenerate the damaged myocardium. As mentioned in Section 2.1.2 Current Treatments, whole heart transplant is the only treatment that can restore functionality; the process for this treatment, however, is difficult due to the long wait

list, risk of rejection, and complications following the initial procedure. Cardiac tissue engineering is a favorable alternative to current treatments; as such constructs would be more readily available, have minimal chance of rejection, and could potentially restore the structure and functionality of the patient's damaged tissue (Radisic, 2013).

2.2.2 Current Approaches

Within the field of cardiac tissue regeneration there are a few different approaches. Cell therapy, in which cells are injected into the damaged site of the heart, is a possible option. Studies done in the field have shown minimal functional improvement with little to no phenotypic differentiation into myocardial cell lines. Researchers have attributed this to the poor targeting and survival rate of the injected cells (Emmert, 2013; Dilley, 2014). Implantable 3D scaffolds have shown more promising results, so the majority of current research in the field focuses on studying possible materials for the construct fabrication, cardiomyocyte sourcing, and microvasculature fabrication (Dilley, 2014).

Currently there are two main approaches to introducing the scaffold to the damaged cardiac tissue for therapeutic use. The first is injectable hydrogel constructs. This approach allows for the materials to be introduced through a catheter and the 3D system to be built *in vivo*. This removes the need for surgery and general anesthesia (Radistic, 2013). The drawback of this approach is that there is no control over the cell and biomaterial organization, which has a significant effect on the tissue properties and function.

The second approach is to fabricate the scaffold *in vitro* and implant it at a later time. This requires an invasive surgical procedure, as the 3D construct needs to be placed on the heart. This approach to cardiac tissue engineering gives the researcher a greater variety of possible base materials and cells to use for scaffold fabrication. Researchers have used alginate, collagen, and

fibrin hydrogel scaffolds as the base material to support the seeded cardiomyocytes. (Emmert, 2013). Along with more freedom to choose different combinations, the *in vitro* scaffold fabrication approach allows for more control over the structure and properties of the scaffold and seeded cells, which can potentially improve the material's regenerative capabilities. This approach, however, also requires a bioreactor system to sustain and grow the tissue construct *in vitro* (Emmert, 2013).

2.2.3 Limitations

Although there are many advantages to various myocardial tissue engineering approaches, some limitations exist. Use of a hydrogel scaffold ensures that the desired cells reach the infarct area in addition to providing support mechanically. A disadvantage includes that the implantation procedure would be invasive and requires open chest surgery. A significant amount of research must be done to ensure the most appropriate cell type and biomaterial is chosen, in order to match the chemical and mechanical properties of the native tissue. Another major limitation is the perfusion and diffusion of nutrients through the 3D construct's microvascular networks, a vital part of maintaining life of any tissue (Ikada, 2006).

There have been major advances in the field of cardiac tissue engineering over the past ten years. These advances have been made in the fields of cardiac cell biology, stem cell, and biomaterials. The main reason tissue engineering in this field has not been able to advance as rapidly is due to the current vascularization techniques, which have lagged behind significantly. Due to the current vascularization methods and the diffusion limit of oxygen, which is approximately 200 μm, there is a limit on the volume of tissue created *in vitro.* This makes it challenging to apply the advances of cardiac tissue engineering into scaffolds of clinically relevant sizes (Dilley, 2014; Novosel, Kleinhans, & Kluger, 2011).

2.2.4 Cardiac Tissue Engineering in Pins Lab

In order to overcome the current challenge microvascularization is presenting, Pins Lab is designing a novel fabrication method for cardiac tissue constructs that allow for myocardial regeneration. The approach involves a layer-by-layer fabrication process. By alternating myocardial tissue layers, approximately 300 μm, with microvascular network layers, theoretically, a patch of clinically relevant thickness, approximately 1 cm, can be created.

The layer-by-layer approach allows for precision and accuracy in the fabrication of each layer, as well as for multi-functionality incorporation. Pins Lab has identified the microvascular network and the myocardial layers as critical for a functional cardiac tissue patch (Bornstein, Gagnon, Moutinho, & Reyer, 2015). The myocardial layer is loaded with fibrin microthreads and seeded with cardiomyocytes. This layer provides the contractile functionality and the mechanical support of the cardiac patch. The microvascular network layer, on the other hand, provides the nutrients and gasses necessary for the metabolic function of a whole patch.

This MQP focuses on developing a functional microvascular layer and creating a bioreactor to maintain whole tissue constructs. The microvascular network layer and bioreactor designs would play a vital role in the development and sustainability of a finalized cardiac patch with a clinically relevant size.

2.3 Vascular Tissue Engineering

2.3.1 Need for Vascular Engineering

Vascularization is required for the delivery of oxygen, nutrients, and clinically, the delivery of drugs, as well as the removal of carbon dioxide and other waste products as depicted in Figure 2. One cause of the low amount of clinical applications is the diffusion limit of oxygen and nutrients through the bulk of a material (Rouwkema, Rivron, & Van Blitterswijk, 2008). The

diffusion limit of oxygen is 200 µm and due to this reason, mammalian cells are typically located within 200 µm of blood vessels *in vivo* (Carmeliet & Jain, 2000; Novosel, Kleinhans, & Kluger, 2011). As a result, the size of engineered tissue is limited to approximately 400 µm. Without a method to provide cells with the required oxygen and nutrients past 200 µm, there is a need to research the creation of microvascular networks within 3D tissue constructs (Laschke et al., 2006). For tissues to be created of clinically relevant sizes, vascularization must be achieved.

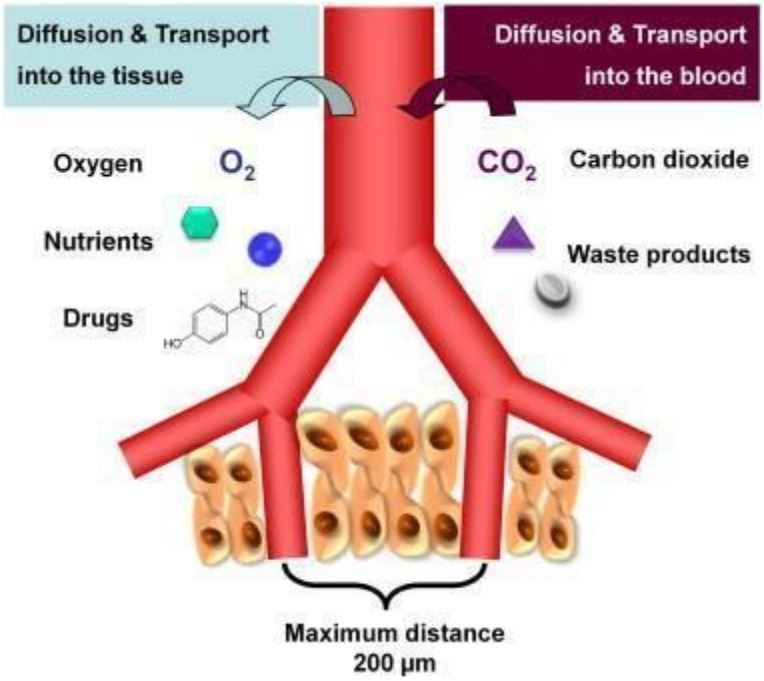


Figure 2: Schematic description of diffusion and transport processes in vascularized tissues in vivo (Novosel, Kleinhans, & Kluger, 2011)

The need for vascularization in engineered cardiac tissue is particularly strong as seen with the highest ratio of blood vessels to cells and due to the high metabolic demands of heart tissue (Post, Rahimi, & Caolo, 2013). It has been found that the physiological density of cells in engineered cardiac tissue can only survive 128 µm from a static oxygen source and that media flow significantly increased oxygen concentration within the tissue engineered construct (Radisic et al, 2006).

2.3.2 Microvasculature Design Parameters

When designing a microvascular network there are many parameters that must be assessed. Studies have shown channels ranging from 10 μm to 1000 μm in diameter using different methods (Bertassoni et al., 2014). Most channels however, were not endothelialized. Endothelialized channels, although more accurately depict *in vivo* condition, proved more difficult to produce at small diameters in recent studies. The most successfully endothelialized channels were 250 μm, 500 μm and 1000 μm. The studies with the smaller diameter channels encountered issues when perfusing because the needles used were difficult to insert correctly into the channels to facilitate flow (Bertassoni et al., 2014).

Among the different size of channels there are a few different geometries that have been tested. As previously stated, studies using photolithography mostly fabricated rectangular channels. These rectangular channels were endothelialized with some difficulty. The corners of the channels produce flow stagnation which lowers the shear stress acting on the endothelial cells, which prevents them from adhering and producing extracellular matrix (ECM) proteins (Abdelgawad et al., 2011). Studies have used sacrificial layers, such as gelatin, to create rounded channels with diameters as small as six micrometers. Even though these channels were not endothelialized they still demonstrated a more physiologically relevant shape than the square channels (Golden & Tien, 2007).

Once channels are formed in hydrogels perfusion can then be performed. The most common method is continuous perfusion (Dagenais, Rousselle, Pollack, & Scherrmann, 1999). Two metrics used to characterize perfusion are volumetric flow rate as well as pressure drops across a vascular network. The normal pressure of capillaries in the body between 10.5 and 22.5 mmHg (Shore, 2000). In order to characterize the transport rates of perfusate through a hydrogel

using channels, a study was conducted in 2007 using a variety of pressures and flow rates. The first rate was lower than physiological conditions at 5.88 mmHg and 0.0008 mL/min. The second flow rate was at the end of the range at 22 mmHg and 0.0024 mL/min. Their final rate was 36.7 mmHg and 0.005 mL/min. These flow rates gave the authors a clear view of how the rate of perfusion affects the rate of transport of rhodamine and bovine serum albumen (BSA) throughout the hydrogel. As the flow rates increased so did the rate of transport of these molecules (Golden & Tien, 2007).

2.3.3 Vascularization Methods

Vascularization can be achieved in engineered tissues through the use of microfluidic technologies which are systems with geometries on the scale of tens to hundreds of microns involving transport of fluids. There are additional advantages of microfluidics in tissue engineering beyond delivery of oxygen and nutrients and removal of waste including promoting flow-induced cell signaling, controlling spatial relationships between cells, and introducing chemical gradients, all of which help to better reproduce the *in vivo* microenvironment (Inamdar & Borenstein, 2011).

There are two main methods utilized for developing vascularization in engineered tissues, the first of which is angiogenesis or vasculogenesis, the ability of endothelial cells to form new blood vessels from existing ones or the process by which cells form new blood vessels during embryonic development (Hasan et al., 2015). A second method which will be discussed is engineering microvascular networks which involves the formation of pre-vascularized scaffolds (Hasan et al., 2015; Novosel, Kleinhans, & Kluger, 2011).

2.3.3.1 Angiogenesis and Vasculogenesis

Angiogenesis and vasculogenesis in engineered tissues are processes promoted by seeding endothelial cells as well as other cell types such as myoblasts or fibroblasts and adding angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) (Rivron et al., 2006). Some examples of applications of this technique include directing angiogenesis and endothelial cell growth along micropatterned hydrogels (Koroleva et al., 2012; Nikkhah, Edalat, Manoucheri, & Khademhosseini, 2012). Additionally angiogenesis has been induced by functionalizing biomaterials with angiogenic biomolecular cues such as VEGF and angiopoietin-1 (Chiu & Radisic, 2010; Ito & Tada, 2013). A third method, and perhaps the most common, is the use of a growth factor gradient within a hydrogel, as endothelial cells will form vascular structures from regions of low to high growth factor concentrations (Hao et al., 2007, Kaully, Kaufman-Francis, Lesman, & Levenberg, 2009; Silva & Mooney, 2010). However, two main drawbacks of these techniques are that they are not rapid enough to immediately supply oxygen to tissue scaffolds developed *in vitro* and there is somewhat limited control of the resulting vascularization (Laschke et al., 2006).

2.3.3.2 Microengineered Vascular Networks

A second approach to microvascular engineering is the formation a microvascular network. The goal of such a microengineered vascular network is to provide nutrients and waste removal to cells in an engineered construct with precise geometry by artificially, pre-developing the microvascular network. One common strategy of designing engineered microvascular networks is to mimic the vessel structure of the cardiovascular system (Prabhakarpandian et al., 2013). *In vivo*, almost all tissues are supplied with oxygen and nutrients via a highly branched

network of blood vessels. The greatest distance between capillaries is typically 200 μm which correlates to the previously mentioned diffusion limit of oxygen (Kannan, Salacinski, Sales, Butler, & Seifalian, 2005).

It should be noted that though the scope of this project has been narrowed and focused on cardiac applications, the development of a microvascular layer could be applied to many different tissue types. Currently, many methods exist that are under investigation for the creation of microvascular networks. Each method will be presented with highlighted advantages and limitations.

2.3.3.2.1 Cylindrical Rod Extraction

One simple method of creating engineered microvasculature is by encasing a cylindrical rod such as a needle or wire in a hydrogel (Chrobak, Potter, & Tien, 2006). A needle was encased in a collagen or fibrin hydrogel and after polymerization was complete, the needle was drawn back leaving a channel with a diameter of 120 μm (Chrobak, Potter, & Tien, 2006). A cell suspension of both human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HDMECs) was adhered and grown to a confluent monolayer in the channel (Chrobak, Potter, & Tien, 2006). An advantage of the cylindrical rod extraction method is the simple, low-cost procedure. A limitation of this method is the limitation of straight, single channels in addition to possible adherence between the hydrogel and the rod.

2.3.3.2.2 Laser Created Channels

A parallel array of channels were created in poly(glycerol sebacate) (PGS) using a laser cutting/engraving system. These elastomer scaffolds contained channels that mimic a vascular network. The scaffolds were seeded with neonatal rat ventricular cardiomyocytes (nRVCMs) which formed constructs that synchronously contracted upon electrical stimulation (Radisic,

Marsano, Maidhof, Wang, & Vunjak-Novakovic, 2008). Advantages of this method include channels with well-defined geometry and the ability to create multiple channels. The main limitation of this method is being limited to straight, non-branching channels.

2.3.3.2.3 Silicon Micromachining/Soft Lithography

A series of trenches that mimic the architecture of small blood vessels was created on silicon wafers and after rat lung microvascular endothelial cells were seeded directly onto the wafer, they formed a monolayer. This monolayer was able to be lifted off the wafer and then folded into a compact three-dimensional (3D) scaffold (Kaihara et al., 2000).

Researchers describe the development of a microvascular network created via silicon wafer micromachining. Multiple layers of tissue formed from the network are created to stack and provide filtration function similar to that of liver/kidney via a semipermeable membrane (Borenstein et al., 2013).

Soft lithography, refers to the technique of creating a soft mold out of a material such as polydimethylsiloxane (PDMS) from the hard photolithographed master (Kim et al., 2008). A group from Tokyo Women's Medical University used soft lithographic techniques to create a hyaluronic acid hydrogel. Cell sheets, one being HUVECs, were then placed on either side of the gel and over time formed capillary-like structures via cell movement around the gel (Sugibayashi, Kumashiro, Shimizu, Kobayashi, & Okano, 2013).

The advantages of microvascular networks created via photolithography or soft lithography allow for finer control over the geometry and structures of the network, which can include branches and bifurcations. One limitation of most microvascular networks formed via photolithography is non-circular channels. A method was developed to create cylindrical microfluidic channels using soft lithography. Two PDMS molds with semi-circular cross

sections were created and could be stacked together to form cylindrical molds which overcome the limitation of photolithographic techniques of only being able to create rectangular features. In addition, computational models were used to calculate shear stress and pressure distributions within the microvascular network (Huang, Li, Martins-Green, & Liu, 2012).

Soft lithography has also been used to form microchannels in silk scaffolds. One silk scaffold was molded with the PDMS reverse mold and a second, flat silk scaffold was adhered to the first to form the rectangular or circular cross-section channels. Human microvascular endothelial cells (hMVECs) were grown to confluence within the channels (Wray, Konstantinos, Gi, Omenetto, & Kaplan, 2013).

2.3.3.2.4 Sacrificial Molds

Another heavily researched method of microvascular network formation is through the use of sacrificial molds. Sacrificial molds use a material that can be dissolved, or melted after a hydrogel is formed around them. The removal of the material leaves a negative space used as channels of the microvascular network. Many materials have been used as sacrificial molds for the formation of microvascular networks and a review is described here.

2.3.3.2.4.1 Gelatin

Using gelatin as a sacrificial element, researchers created microfluidic networks in both collagen and fibrin hydrogels with channels as narrow as six micrometers. One advantage to using gelatin is that it is able to be melted at 37°C which avoids denaturation of protein scaffolds such as fibrin or collagen. It was also shown that human microvascular endothelial cells could attach, spread, and proliferate in microfluidic channels (Golden & Tien, 2007).

2.3.3.2.4.2 Carbohydrate glass

Researchers developed perfusable engineered microvascular networks using a sacrificial mold made of carbohydrate glass, a material made of sucrose, glucose, and dextrans. A 3D filament network was created that resulted in cylindrical microvascular networks that could be lined with endothelial cells. Perfusion was successful with high-pressure pulsatile flow and the method was compatible with multiple cell types (Miller et al., 2012). One downside to this method is a relatively complex sacrificial element fabrication process that must be completed at high temperatures.

Another group further developed carbohydrate glass as a sacrificial molding technique using 3D-printed isomalt, a sugar alcohol. Like other methods, this procedure creates a dissolvable sacrificial mold that creates a network of cylindrical channels. Improvements minimized bleeding of the carbohydrate and casting at low temperatures. This group also showed that carbohydrate glass can be printed from existing filaments or can end in free space and that the diameter can be controlled. The glass is able to be dissolved in a few different ways, via still water immersion for thirty-six hours, sonication in 41°C water for 16 hours, or immersion in boiling water for three hours (Gelber & Bhargava, 2015).

2.3.3.2.4.3 Pluronic F127

Pluronic F127, a triblock copolymer of polyethylene oxide and polyphenylene oxide was used as a sacrificial mold because of its lower critical solution temperature (LCST) in that it is a liquid below the LCST but forms a gel above the LCST (Müller, Becher, Schnabelrauch, & Zenobi-Wong, 2013). At a proper concentration, around 20 wt%, Pluronic F127 is a gel at room temperature and can be removed by cooling in 4°C water. Microvascular networks were 3D

printed in 1-, 2-, and 3-dimensions and were endothelialized with HUVECs (Kolesky et al., 2014).

2.3.3.2.4.4 Polyvinyl Alcohol (PVA)

Polyvinyl alcohol (PVA) is a water soluble, hydrophilic polymer and these properties have led PVA to be used in many different applications. One study used PVA to create micromolds. The authors made a PVA and water solution and spin coated a plate used to create a PDMS mold. When the PVA was dissolved it left grooves in the PDMS (Ferrell, 2007). Another study used PVA to create a film on a glass plate. The study used the plate to create 3D microstructures. When these structures were formed they needed to be detached from the plate so the PVA layer was dissolved in water and the microstructures were released (Guan, 2005).

Additionally, once a scaffold with a microvascular layer is implanted, it must continue to receive oxygen and nutrients and thus must connect with systemic circulation *in* vivo (Lokmic & Mitchell, 2008). It has been shown that microvascular networks developed *in vitro* can be implanted and maintained *in vivo* via spontaneous connection of endothelialized channels to systemic circulation (Koike et al., 2004, Levenberg et al., 2005)

2.4 Hydrogels for Engineered Microvascular Networks

Within our project, we will be creating a thin hydrogel scaffold containing a microengineered vascular network. The purpose of this hydrogel is to facilitate the perfusion and diffusion of necessary gases and nutrients into the surrounding scaffold. The microvascular hydrogel will be alternating with multiple fibrin layers to create a scaffold which could serve as a patch to replace damaged myocardial tissue. In order to ensure that our hydrogel will support additional layers above and below it, we must consider carefully what material we choose to use by analyzing its mechanical and bioactive properties.

Hydrogels are important for tissue engineering applications as scaffolds for cells to adhere, proliferate, and differentiate. In tissue regeneration when a wound is present, a scaffold must be created to allow cells to develop new tissue. Cells would be taken from the damaged tissue surface and placed onto the hydrogel so that they can be supported and create a viable layer of new tissue.

2.4.1 Hydrogel Properties

There are many relevant properties to be considered in selection of a hydrogel in tissue engineering applications. When selecting a hydrogel material for the microvascular layer, we must select one that encompasses the following qualities: biocompatible, allow for cell attachment, differentiation, proliferation, and should degrade appropriately with the rate of tissue regeneration *in vivo*. It should provide a scaffold until the cells can repair the damaged area and create a new extracellular matrix. It should also allow for nutrients to be delivered to the cells within the scaffold. The hydrogel material must allow for integration with the host tissue *in vivo* and degrade at an applicable rate that allows for tissue to regenerate as the scaffold is degrading (Dumitriu, 2013). Additionally, the creation of the hydrogel scaffold should be reproducible (Chen et al., 2008).

2.4.2 Natural vs. Synthetic Hydrogels

Both natural and synthetic hydrogels have advantages and drawbacks. It is important to consider the specific application of the scaffold. Some advantages of natural hydrogel materials include that they are typically biocompatible, biodegradable, and have fewer immunogenic issues. Some disadvantages include that they can have poor mechanical properties, poor processability, and there is the possibility of disease transfection. Synthetic materials can be advantageous in that they can have good processing characteristics, are more readily available

and predictable, and can be more easily manufactured. However, some disadvantages of synthetic materials include that inflammatory reactions and erosion can occur in addition to there being potential difficulty with tissue integration. The clients' constraints typically help determine which qualities to keep in mind when selecting a material to use. For example, a main constraint for this project is that it that all materials that come into contact with the scaffold or microvascular layer must be completely biocompatible. This would narrow down possible material options.

In addition to characterizing natural and synthetic materials, it is necessary to research the qualities of various hydrogel materials, as many differ. Below, Table 1 presents the properties of various materials that could be used when creating a hydrogel. The following information was gathered from various articles cited below.

Table 1: Characteristics of some natural and synthetic hydrogel materials (Chen et al., 2008; Hasan et al., 2013).

Natural	
Fibrin	Origin: Plasma Pro-angiogenic Serves as a provisional scaffold in the coagulation cascade and wound healing Good cell adhesion properties
Collagen	Origin: Tendons, ligaments, ECM of skin, bone, and cartilage Good biocompatibility Strong cellular activities Thermal reversibility Possible immunogenic response
Alginate	Origin: Cell walls of brown algae Originally developed for drug delivery Controlled release of basic fibroblast growth factor microspheres enhance vascularization of the scaffold High biocompatibility Low cost Limited long term stability Non-thrombogenic properties
Gelatin	Origin: derivative of collagen Good fabrication control High biocompatibility Promotes vascularization through growth factor release
Synthetic	
Polyethylene Glycol (PEG)	Biocompatible Hydrophilic FDA approved for multiple biomedical applications Does not cause immune response Low toxicity
Polyvinyl Alcohol (PVA)	Produced through hydrolysis of polyvinyl acetate Elasticity allows it to be used as a matrix Can connect to biological molecules Minimal adhesion properties Strong mechanical properties
Polyphosphazene	Controlled biodegradability Can be modified to be thermoresponsive Useful for drug delivery

2.5 Endothelialization

The endothelium, a thin layer of epithelial cells, lines the interior of blood vessels and lymph vessels. The purpose of the endothelium is to act as a boundary between the flowing fluids and the surrounding tissues. As a barrier, the layer controls diffusion of nutrients, oxygen, and wastes. Along with that, the endothelium plays a vital role in regulating the state of the blood. The endothelial cells control clotting and the dissolution in the blood vessels (Aird, 2007). Creating a functional endothelial layer on the interior of our designed channel would help ensure that perfusion of blood is possible, because it would provide the necessary surface and molecules to keep blood flowing and prevent clotting inside the designed vascular network.

2.5.1 Endothelial Structure

Blood vessels consist of three different layers. The outermost layer is called ‘tunica externa,’ the middle layer is ‘tunica media,’ and the innermost layer is known as ‘tunica interna.’ The endothelial cells are directly anchored to the basement membrane. The basement membrane is made of connective tissue and smooth muscle cells, more specifically pericytes (Félétou, 2011). The different extracellular matrices of the layers enhance the mechanical strength and elastic properties of the channel. For our project, the goal is to create a microvascular network with properties similar to the coronary arteries, so that we can promote angiogenesis and native tissue integration. By mimicking coronary arteries, the vascular network assist in the differentiation and proper alignment of the surrounding tissues by providing mechanical and chemical signals.

There are three main types of endothelial linings depending on their structure (Félétou, 2011). The classes of endothelial linings are continuous endothelium, fenestrated endothelium,

and discontinuous endothelium. The different endothelial linings are determined by the vessel's location and its function. The continuous endothelium, shown in Figure 3, can be found in most large arteries, veins, and capillaries in the brain, skin, heart, and muscle. In the fenestrated endothelium, the cells are characterized by the presence of wide transcellular pores with diaphragms which can seal the pore. These cells are often found in sites of increased exchange between the tissues and the blood, or locations of elevated filtration. The discontinuous endothelium is characterized by the presence of large fenestrations with no diaphragms and this layer can be found in sinusoidal vasculature that can be seen in the liver, spleen, and bone marrow (Aird, 2007). Since the project is focusing on the myocardial vasculature, an ideal lining would be a continuous endothelium.

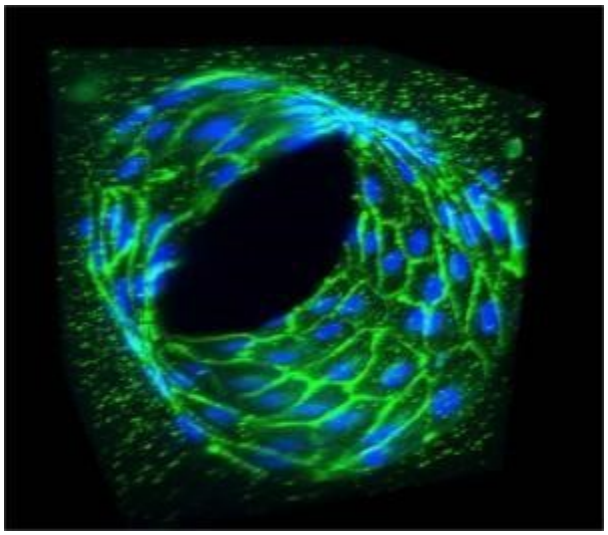


Figure 3: Endothelial cells of a blood vessel. 3D projection of a confocal Z-stack of vessel lining, fluorescent stained with PECAM-1 (green) and DAPI (blue) (Wong, Searson 2014)

In capillaries, the endothelial cells are generally about 0.1 μm thick, and they are large and elliptical in shape. Endothelial cells express a large number of clathrin-coated vesicles, multivesicular bodies, and lysosomes and these components point to the endothelial cells' endocytotic function. Other function specific components seen in endothelial cells are caveolae and vesiculo-vacuolar organelles. The transcytotic function of the organelles, the small thickness

of the cells, and the cellular structure of endothelium support the idea that a major function of the endothelial layer is to facilitate the molecular exchange between the blood and the surrounding tissues.

2.5.2 Endothelial Function

Endothelial cells have several primary functions that they perform. The functional heterogeneity is expressed by the difference in dominance between functions. A primary function of the endothelium is to control the state of the blood, as all endothelial cells regulate hemostasis, the process by which the body stops bleeding and keeps the blood within the damaged vessel. The endothelium maintains blood in fluid form, but also promotes localized blood clotting if there is breach in the vessel. Endothelial cells work closely with the platelets to control the state of the blood. The endothelium releases specific signaling proteins which act as anticoagulants and coagulants depending on their interaction with the platelets (Aird, 2007). Additionally, endothelial cells are also responsible for the process of fibrinolysis which is the opposite process to hemostasis, the dissolving of clots. Along with controlling the state of blood, endothelial cells act as the permeable barrier to the interstitial tissues.

The two major functions of interest for the project are the endothelium's controlled permeability and its control over the hemostasis. Controlled diffusion is crucial for the network to function properly, so that it can accomplish its primary purpose as a vascular system, which is to facilitate the delivery of nutrients and oxygen and the extraction and removal of wastes. The endothelial layer would also ensure that the perfusion of blood through the fabricated channels is possible. The endothelial layer is responsible for the anti-coagulant molecules which reduce the viscosity of blood, preventing it from clotting.

Understanding the structure and functions of endothelium would be beneficial to our project because a microvascular network is being constructed, which will sustain blood flow, facilitate perfusion of nutrients and gasses to the surrounding cells and tissues, and promote angiogenesis *in vivo*.

2.5.3 Engineered Vascularization

Currently, in the field of biomedical engineering, there is a need to develop an effective way of vascularizing 3D scaffolds and tissues *in vitro* and *in vivo*. Major challenges arise from the difficulty of properly forming the network, perfusion through capillaries, diffusion limitations, and ineffective integration *in vivo* (Lovett et al., 2009). Past studies have focused on two main aspects of vascularization. Some researchers focused on studying what factors affect vascularization while others are developing methods of integrating vascularization into 3D scaffolds. Endothelialization plays a crucial role in the vascularization process, as the proteins released from endothelial cells dictate the growth and development of vessels and channels. In past studies, a variety of endothelial cells have been used to test the different scaffold designs. The most commonly used types of primary line endothelial cells are human umbilical vein endothelial cells (HUVEC), human pulmonary artery endothelial cells (HPAEC), and human dermal microvascular endothelial cells (HDMEC) (Alberts, 2002).

Primary cell lines generally have an average lifespan of ten cell culture passages and can be kept for up to five months (Bouis et al., 2001). Over that time limit, cells enter senescence and soon after undergo cell death. This means that long term experiments are difficult to be done with primary endothelial cells. Depending on the source site of the cells, their behavior differs significantly. Most studies use HUVECs because of their proliferation potential; however, when doing regeneration studies targeted at specific organs and tissues, it is recommended to use

endothelial cells from blood vessels similar to the ones found in the original tissue. This is because these endothelial cells will function optimally in that tissue environment (Alberts, 2002). Primary endothelial cell cultures grow slowly *in vitro* compared to immortalized cells, but the benefit of using a primary cell line is that their behavior *in vitro* is similar to their behavior *in vivo*.

2.6 Bioreactor for Microvascular Network Perfusion

A bioreactor is a research tool to observe and characterize interactions of cells and tissues throughout experimentations. The bioreactor consists of three major parts: a pump, a microfluidics chamber, and a collection reservoir. The pump provides the perfusion of media and nutrients to the microfluidic chamber. The microfluidic chamber is the part of the bioreactor that holds and interacts with the tissue construct being studied. The collection reservoir is used to collect the media that has passed through the system for disposal.

2.6.1 Clinical Need

There is limited capacity for self-repair and restoration of original tissue once damaged. Most common treatments involve implantable medical devices with many associated limitations such as time-span and implant failure. Today, there is a large demand for providing an alternative to these previous means of restoring severely damaged tissues when needed and bioreactors are vital in the development of such treatment. Bioreactors allow cells and tissue constructs to be used towards biological applications that are clinically effective and safe. A traditional bioreactor can be defined as “a device that uses mechanical means to influence biological processes” (Darling & Athanasiou, 2003). Bioreactors maintain tissue viability by providing physical or biochemical signals and can culture cells for months by continuously feeding the cells with fresh media. A perfusion system delivers the fresh media through a porous

scaffold to maintain cell viability while removing waste media. Bioreactors allow for a safe, reproducible, and controlled environment for the creation of a tissue construct. A bioreactor, which delivers nutrients and oxygen and removes wastes, should allow for biological processes, such as cell proliferation, growth, and differentiation, while maintaining sterility and generating reproducible results (Zhao & Griffin, 2016). The bioreactor must maintain an environment with very specific parameters that promote the growth of tissue. These parameters include flow rate, temperature, and specific sizing that accommodates our clients' needs. The ultimate goal is that bioreactors can contain scaffolds within them and can monitor and control the conditions of the tissue to ensure they are suitable for long term implantation with minimal rejections. Like most bioreactors, the design should be based on a comprehensive understanding of the biological and engineering aspects of the application.

2.6.2 Microfluidic Chamber Models

Microfluidic chambers are diverse as there are many types that can be used and each is tailored to a specific need. An example microfluidic chamber from a study from 2008 was a cylinder that consisted of three chambers with a single perfusion channel in each one, as seen in Figure 4 (Chang, 2008).

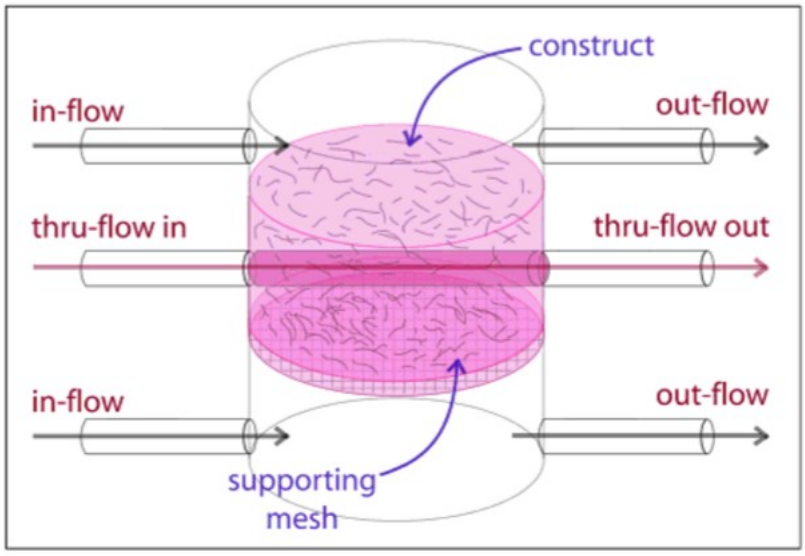


Figure 4: Simple three chambered bioreactor with hydrogel (Chang, 2008)

The center chamber contained a hydrogel seeded with rat fat microvessel fragments (RFMF). The system was perfused through each of the three chambers. This allowed the RFMF's to align into a microvascular network (Chang, 2008). This microfluidic chamber, although simple, maintained the viability of the construct while allowing the fragments to align.

C. Williams, Ph.D. and T. Wick, Ph.D. designed a *Perfusion Bioreactor for Small Diameter Tissue-Engineered Arteries* in 2004. Their microfluidic chamber allows for dynamic sequential seeding of smooth muscle and endothelial cells and monitoring of tissue growth. Cells are seeded onto porous scaffolds and cultured in the bioreactor under pulsatile flow. The bioreactor is cylindrical, flanked by two head plates made from hand-blown glass. Scaffolds were mounted on the two glass tubes within the chamber where perfusion is performed. The head plates, which can be seen in Figure 5 below, have a precision formed groove that allows the plate to form a tight and secure seal by compressing a gasket with clamps.

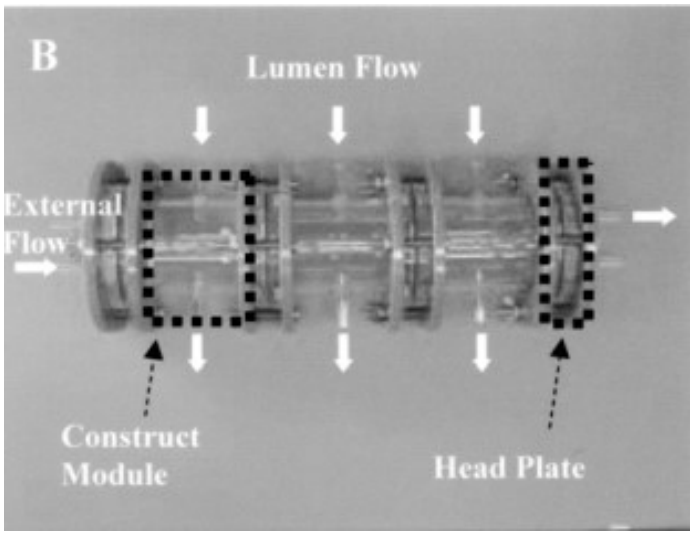


Figure 5: Cylindrical three-module bioreactor. The image shows the inlet and outlet, as wells as some of the components of the bioreactor chamber (Williams & Wick, 2004)

The head plates are closed off at one end and the other end as a small port to allow flow through the construct for nutrient delivery. This study also designed a bioreactor that allowed simultaneous perfusion of multiple constructs (Williams & Wick, 2004).

Another study from 2009 designed and developed a bioreactor system for tissue engineering of bone that enables cultivation of up to six tissue constructs simultaneously. The microfluidic chamber addressed several key design aspects including the use of multiple scaffolds, imaging capabilities, and direct perfusion. The chamber has the dimensions of a ten centimeter glass Petri dish. Below in Figure 6, the bioreactor system as well as schematic of the microfluidic chamber can be seen.

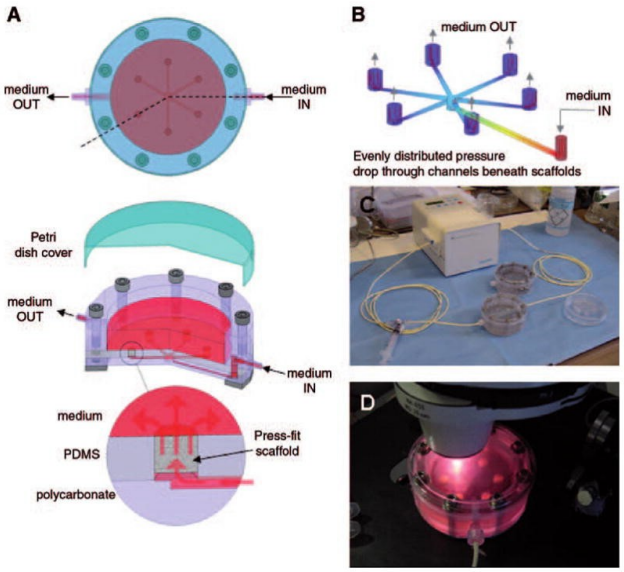


Figure 6: Bioreactor system. a) Schematic of the microfluidic chamber, b) Schematic of the bioreactor system setup; c-d) Compete bioreactor setup (Grayson et al., 2008)

The glass cover of this dish serves as the removable cover enabling access to scaffolds as well as the use of traditional cell culture techniques for media changes. The microfluidic chamber was machined from polycarbonate plastic with silicone gaskets separating the individual parts. Scaffolds were placed into the six wells which are arranged radially within the microfluidic chamber. Media entered the chamber through a central port and flowed to the center of the chamber and evenly distributed to the six channels. A peristaltic pump was used to perfuse the media through the system (Grayson et al., 2008).

A study from 2010 took a different approach from the previous bioreactor explained. This microfluidic chamber perfused a small set of scaffolds. It utilized a glass coverslip in a steel casing to maintain sterility. The microfluidic chamber utilized an insert with grooves to facilitate perfusion across the scaffolds with a gasket to prevent leakage, seen in Figure 7.

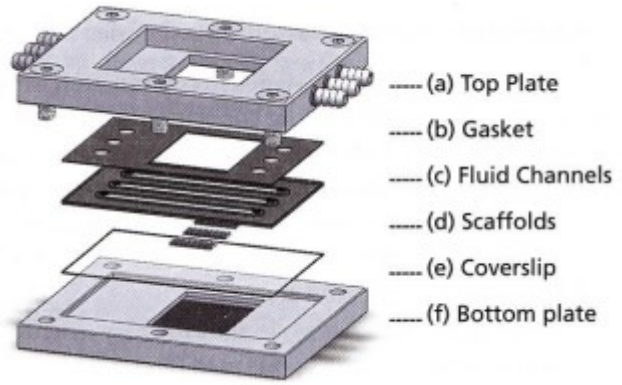


Figure 7: Microfluidic chamber components. Disassembled view of the microfluidic chamber showing of all chamber components (Fröhlich et al., 2010)

The top and bottom plates provide support and use screws to compress the assembly enough to ensure there were no fluid leaks and sterility was maintained. A low profile was maintained so that the microfluidic chamber fit under a microscope. A gasket was used to ensure a tight seal between the plates. Holes on each end of the gasket allowed fluid to flow from channels in the top plate into the perfusion chamber. The channels were made from medical grade clear silicone and UV-transparent polycarbonate plastic. Lastly, the coverslip was clear providing optical efficiency when being imaged (Fröhlich et al., 2010).

2.6.3 Microfluidic Chamber Fabrication

The microfluidic chamber of the bioreactor system contained small features that can be sometimes hard to fabricate. There are many ways to fabricate chambers with these small features such as 3D printing, milling, injection molding, and soft lithography.

2.6.3.1 3D Printing

3D printing, also known as rapid prototyping, can be used to quickly create models designed to test and validate features and means. This can be done to allow testing of a single feature or to validate a means quickly and cost effectively. One of the two most common materials used is polylactic acid (PLA). The melting temperature of PLA is 160-220°C and can

be extruded at, or higher than melting temperature (Nampoothiri, Nair, & John, 2010). A common method of printing is Fused Deposition Modeling (FDM). This method heats, melts plastic, and lays down the melted plastic with each pass of the extruder. The next layer of plastic fuses to the layer below. This creates the model layer by layer fusing each layer to the next. There are a few limitations with FDM printing. The resolution on this type of printer settles around 0.1 mm. This means the printer can print layers as small as 0.1 mm accurately. Another limitation is the tolerances of the printer. The tolerances on the FDM printers are difficult to adjust, therefore some dimensions of prints were not as tight fitting as needed (Hossain, Espalin, Ramos, Perez, & Wicker, 2014).

2.6.3.2 Milling

Milling machines are used with metals, woods, and other solid materials. Mills remove material bit by bit from a large piece of material to leave the desired part. Mills such as the Bridgeport 3 axis can be accurate within the 1/1000 of an inch (Shah, 1998). This means mills can produce much smaller features than FDM printing. This is because mills remove small amounts of material while FDM deposits material based on the size of the extruder size. Because of the better resolution, the small scale of a microfluidics device could be fabricated more easily than with FDM. The tool heads used to cut the material come in a range of sizes and although small features are possible, they can be difficult to achieve.

2.6.3.3 Injection Molding

Plastic injection molding is the analog to die casting for metals. Injection molding has a high production rate and large control over dimensions. This method uses molds to cast melted plastic into complex patterns. A granulated plastic is heated and forced, at a high pressure, through and nozzle into a mold (Oktem, Erzurumlu, & Uzman, 2007). However, small

imperfections in the plastic or changes in the manufacturing process can have undesired results. These adverse effects include warping and feature shrinking of the cooled plastic (Jain, Kumar, & Kumawat, 2012).

2.6.3.4 Soft Lithography

Soft lithography is a fabrication technique that can be used to form microfluidic chambers from soft materials such as PDMS. Lithography uses a mask to cover the desired areas of materials. UV light or chemicals are used to remove the material not covered by the mask. This method is used to quickly create chambers and networks in materials that can be used in a microfluidic chamber. However, soft lithography requires a pre-made mold and a clean room to operate in. Also the cost of obtaining small features is variable, ranging from $40-1500 (Mandenius, 2016). This makes fabricating intricate features needed in microfluidic chambers time consuming and expensive.

2.7 Summary

A myocardial infarctions results in death of a patient's myocardium which is replaced with scar tissue, leading to a decrease in heart function. Current treatments either are not readily available or only serve to prevent further damage. Importantly, there is no current clinical treatment to regenerate a patient's myocardium. Cardiac tissue engineering has the potential to fulfill this need but is limited by its inability of oxygen to passively diffuse greater than 200 μm into tissue. To develop a cardiac tissue construct of clinically relevant thickness, microvascularization is needed. There exist many methods to develop vascularization *in vitro* each with advantages and limitations.

To develop vascularized tissue *in vitro*, a bioreactor is needed. A bioreactor is a research and development tool that used to study of interactions between tissue components. There is a

need for bioreactors that facilitate the growth of cells and tissue constructs which allow for biological applications that are clinically effective and safe. The bioreactor must maintain tissue viability and culture cells for months by continuously perfusing with fresh media. The field of cardiac tissue engineering could be greatly accelerated with the development of a clinically relevant microvascular construct.

Chapter 3: Project Strategy

This chapter encompasses the project strategy the team used to drive the design process. The team followed an approach for the engineering design process described by Clive Dym and Patrick Little (Dym, Little, Orwin, & Spjut, 2009). The ultimate goals of the project were to design a microengineered vascular network and a bioreactor system with a microfluidic chamber, in which the microvascular network and a cardiomyocyte seeded fibrin layer could be sustained. This chapter presents an overview of the project strategy from the initial client statement and client interviews to the project objectives, constraints, revised client statements, and overall project approach.

3.1 Stakeholders

Dym and Little specify that keeping the stakeholders in mind at all times is a must for a successful design (Dym, Little, Orwin & Spjut, 2009). For this project, stakeholders include the client and users. Their opinions should be highly regarded as they have funded the project and will ultimately be using the device. The design team is the third party involved in the design process. It is imperative that the design team considers the constraints of the project and designs with the clients' and users' desired objectives in mind.

As shown in Figure 8 one can see the designer-client-user triangle is a cyclical process demonstrating that the client, users, and design team are all integral parts of the design process (Dym, Little, Orwin, & Spjut, 2009).

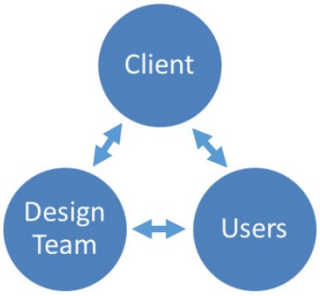


Figure 8: Dym & Little designer-client-user triangle

For this project, the clients and users overlap. The client was Dr. George D. Pins, while Megan O'Brien and Meagan Carnes acted as both clients and users. The design team consisted of Kevin S. Ackerman, Jamal J. Akid, Amanda M. Baltazar, Do M. Duc, and Stephanie R. Fariello. Dr. George D. Pins approached the team with the objective of engineering a microvascular network within a microfluidic chamber, capable of keeping multiple scaffold layers viable for a two to three week time period. The microfluidic chamber is part of a larger bioreactor system that contains a pump and collection flask as well. Megan O'Brien and Meagan Carnes are two WPI graduate students working in Dr. Pins' lab. Megan O'Brien is currently working on the development of a cardiac patch and the team's device would assist her with stacking and sustaining of multiple scaffold layers. Since the vascular network the team created can be used with multiple tissue types, it could also aid Meagan Carnes in the future with her research that is currently focused on skeletal muscle. The goal of the design team is to provide the users with a reproducible, functional bioreactor system and a microvascular network that could assist them in their research.

3.2 Initial Client Statement and Clarification

The initial client statement was presented as follows:

Design, develop, and characterize a bioreactor that provides continuous flow to a thin fibrin scaffold containing a microengineered vascular network.

Upon the initial analysis of the client statement the design team determined there were two main aspects to the project: a microengineered vascular network and a bioreactor system. This meant the team needed to assess objectives and constraints for each design. The team first focused on the microengineered vascular network. The interpretation of the client statement indicated that the vascular network must sustain the viability of cells surrounding the vasculature by providing continuous access to nutrients and oxygen.

Next, the team turned to the bioreactor system. After an initial review of the client statement, the team determined there were two elements that needed to be covered: a bioreactor system and a microfluidic chamber. The microfluidic device is necessary to contain and maintain the entire hydrogel scaffold, consisting of the microvascular network and hydrogel layers seeded with cardiomyocytes. This meant the microfluidic chamber needed to facilitate the perfusion of the microvascular network and to maintain the viability of the cells in the entire scaffold while being inside an incubator.

In order for the team to better understand the clients' needs and wants, in addition to conducting preliminary literature research, the team interviewed the clients. Many design attributes were ascertained and were recorded without being sorted by importance. The attributes, or needs and wants, from the initial client meetings for the microvascular network and the bioreactor are listed and organized below separately:

Microvascular network:

1) Mimics physiological vascular network
    a) Physiological dimensions
        i) Channel diameter
        ii) Cross-sectional geometry
    b) Physiological flow rates
    c) Physiological shear stress

2) Allow for perfusion
3) Diffusion of gases
    a) Diffusion of nutrients
4) Easy to manufacture
    a) Reproducible
        i) Accuracy and precision
5) Aid in patch formation
    a) Stackable
        i) Layers of cell / hydrogel scaffold
        ii) Layers of microvascular network hydrogel
6) Size
    a) 1 cm x 2 cm x 300 μm

Bioreactor:

7) Maintain cell viability
    a) Sustain endothelial cells in microvascular network
    b) Sustain cardiomyocytes in adjacent layer
8) Sterilizable
9) Cost effective
    a) Reusable
    b) Easy to manufacture
    c) Cheap to manufacture
    d) Minimize waste
10) Easy to use
    a) Set up
    b) Transport
11) Microscopy compatibility
    a) Transparent
        i) Allows visual monitoring
        ii) Allows imaging
    b) Size
12) Incubator compatibility

a) Size
b) Compatible with conditions inside the incubator

Through the meetings with the clients the team expanded the attributes to encompass the full project. The design team used these attributes to establish the objectives, what the design needed to achieve. In addition the team established the constraints, what limits the design space within which the team can work. The objectives of the two components of the project and the constraints are listed below in Table 2. Once all objectives and constraints were compiled, the team ranked the objectives and identified sub-objectives.

Table 2: Objectives and Constraints

Objectives	
Bioreactor	Vascular Network
Control Gas Permeability	Physiologically Relevant Dimensions
Ease of Use	Physiologically Relevant Flow
Cost Effective	Stackable
Reproducible	Reproducible
Allow for Multiple Samples	Easily Produced
	Endothelializable
Constraints	
Sterilizable Biocompatible Size of Bioreactor Size of Microfluidic Chamber Timeline Budget Incubator Conditions Microscope Compatibility	

3.3 Objectives and Sub-Objectives

To organize the design process, the project was structured into two major parts. As seen in Figure 9 and Figure 10 respectively, the first component was to produce a microvascular network within a thin layer able to be integrated into a larger construct to support a cardiac patch.

The second component was to create a bioreactor to support and maintain a thin microvascular hydrogel layer with an adjacent cardiomyocyte layer *in vitro*. The design team, in conjunction with the clients, developed objectives and sub-objectives for each component.

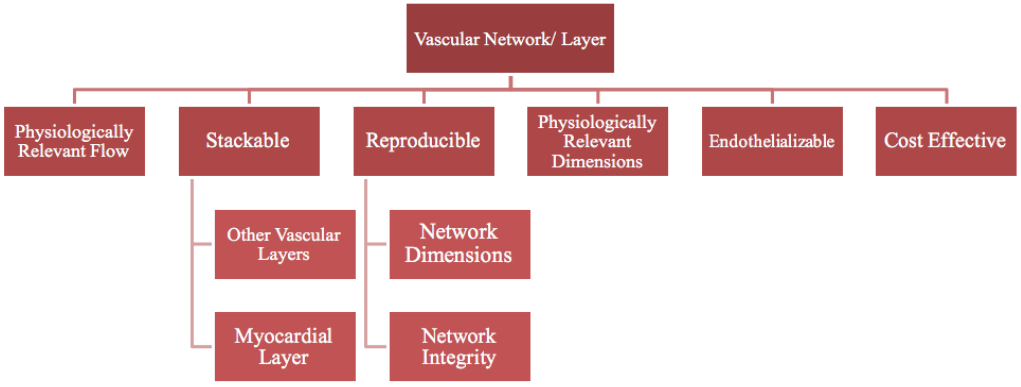


Figure 9: Vascular network layer objectives and sub-objectives

As seen in Figure 9, the first portion of the project focused on the formation of the microvascular network. The first objective was that the microvascular network should have physiologically relevant flow that provides shear forces that mimic those found *in vivo*. Next, the network layers needed to be stackable with others to increase the thickness of a patch. Another objective was to be reproducible both in terms of the network dimensions and integrity of the channels. The network needed to be formed with physiologically relevant dimensions, so the seeded cells were in an environment that closely resembled *in vivo* conditions. Another objective for the vascular network was to be endothelialized. The channels needed to create capillary channels inside the hydrogel scaffold, in order to allow for blood perfusion and protect the surrounding cells and ECM from the shear forces from the flowing fluid. Finally, the production of the network needed to be cost effective with as little wasted material as possible.

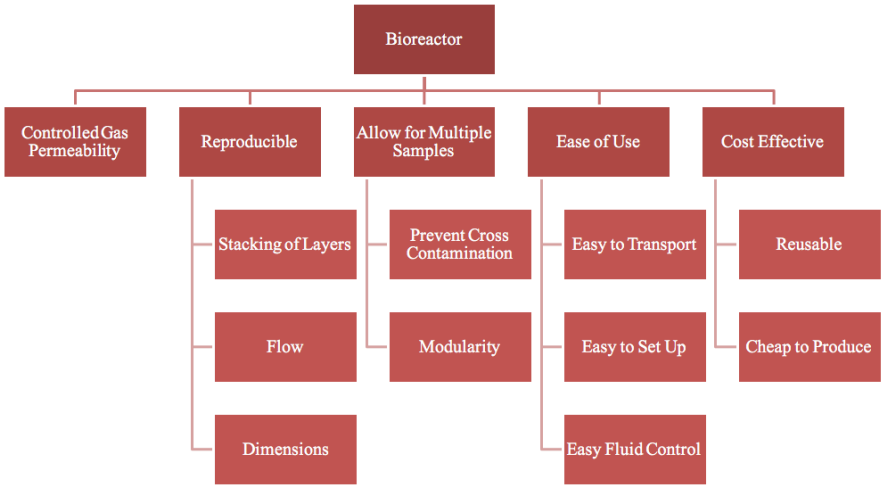


Figure 10: Bioreactor objectives and sub-objectives

The second portion of the project was to design and develop the bioreactor system. The objectives for the bioreactor can be seen in Figure 10 above. The first objective of the bioreactor was the ability to control gas permeability. Under most circumstances the bioreactor had to be gas permeable while keeping the tissue sterile and allowing diffusion of oxygen through the walls of the microfluidic chamber. However, the client also wanted the bioreactor to be gas impermeable to assess the effectiveness of the vascular system in delivering nutrients and oxygen. Therefore, the bioreactor needed an element to control the gas permeability. Another objective for the bioreactor was that it needed to be reproducible. The bioreactor needed to minimize variation between systems in production and the functions during experiments. It needed to allow for scaffold stacking and flow that could be replicated, while allowing the user to control the flow rate. The next objective for the bioreactor was to allow for multiple samples. Multiple samples needed to be perfused simultaneously in separate environments preventing cross contamination. Another objective of the bioreactor was ease of use. Sub-objectives for ease of use included that the system needed to be easy to transport, easy to set up, and allow for easy fluid control. In order for the system to be effective for our clients, it needed to work properly

while being assembled in the shortest amount of time and simplest way possible. Finally, the last objective was for the system to be cost effective. It needed to be reusable and cheap to produce, so that the overall cost of fabrication was manageable for users.

3.4 Quantitative Analysis of Objectives

To quantitatively analyze and order the objectives in terms of importance, the team created pairwise comparison charts (PCC) for the bioreactor and the microvascular network as seen in Table 28and Table 29 in Appendix B: Decision Matrices. A PCC is a method used to compare design objectives in pairs and to determine their relative importance. Each pair of objectives was discussed in detail, and the team came to agreement regarding the order of importance of the objectives for the success of this project. In the PCC, the objectives are listed as both rows and columns. The objectives in the first column are the initial objectives matched against each column. Moving across the row, if the objective in the row was more important than the objective in the column, the objective receives one point. If the team decided two objectives matched importance, the objective would get 0.5 points. The objective with the most points was ranked as the most important objective. This method allowed the team to properly allocate the necessary resources and effort to objectives that ranked highest.

Appendix B: Decision Matrices shows all the decision matrices the team used. The team asked all users and clients to create a PCC. The client PCCs can be seen in Table 30 and Table 31 in Appendix B: Decision Matrices. Each user PCC can be seen in Table 32 through Table 35 also in below. A finalized PCC was created using the input from all parties. The team created a weight averaged PCC based on the clients', users', and the team's original PCC. This was done by weighing the client and users' scores as twice as heavily as the teams' scores, therefore putting more weight in the opinions of the clients and users. Table 3 shows the final scores of the

microvascular network and Table 4 shows the final scores of the bioreactor. The PCCs were created after the discussion with the three clients. Initially, the team and clients had different understandings of what each objective entailed but once all parties agreed on the significance and meaning of each, the team was able to complete the following PCCs.

Table 3: Final pairwise comparison chart of high-level project objectives for microvascular network

Objectives: Microvascular Network							
Goal	**Reproducible**	**Cost Effective**	**Physiologically Relevant Dimensions**	**Endothelializable**	**Physiologically Relevant Flow**	**Stackable**	**Total**
Reproducible		1	1	1	0	0	3
Cost Effective	0		0	0	0	0	0
Physiologically Relevant Dimensions	0	1		1	0	0	2
Endothelializable	0	1	0		0	0	1
Physiologically Relevant Flow	1	1	1	1		.5	4.5
Stackable	1	1	1	1	.5		4.5

Table 3 shows the PCC results for the microvascular network. For the microvascular network, physiologically relevant flow and stackable ranked as the two most important objectives. The microvascular network needed to sustain continuous perfusion at a physiological flow rate and allow for diffusion through the hydrogel. This objective is important, because in order for the finalized scaffold to be implantable, the microvascular network needed to withstand the *in vivo* conditions. The cardiomyocyte and microvascular layers needed to be stackable in alternating sequence for the microvascular network layers to provide nutrients and oxygen in an efficient manner to the cell seeded adjacent layers. The next objective was reproducibility of the system which included the output results and the network fabrication. Obtaining physiologically relevant dimensions was ranked next to allow future implantation and angiogenesis around the

cardiac patch. Next, the microvascular network needed to allow for endothelialization to occur and maintain the viability of the seeded endothelial cells. Cost effectiveness was ranked last because it was much more important for the project to be successful rather than reducing the cost.

Table 4: Final pairwise comparison chart of high-level project objectives for bioreactor

Objectives: Bioreactor						
Goal	**Ease of Use**	**Cost Effective**	**Controlled Gas Permeability**	**Reproducible**	**Multiple Samples**	**Total**
Ease of Use		1	0	0	0	1
Cost Effective	0		0	0	0	0
Controlled Gas Permeability	1	1		1	1	4
Reproducible	1	1	0		1	3
Multiple Samples	1	1	0	0		2

Table 4 shows the PCC results for the bioreactor. The most important objective of the bioreactor, specifically the microfluidic chamber, was its ability for gas permeability to be controlled. This was most important because it allowed the team to understand how the perfusion system was contributing to the cell viability, independently of external environmental factors. Without proper perfusion from the microvascular channels, the cells would not be viable. The second most important objective was reproducibility. Reproducibility has two meanings in this context. First, the environment the microfluidic chamber creates needed to be the same between experiments. Along with that, all fabricated microfluidic chambers needed to have the same dimensions. The ability to accommodate multiple samples in the bioreactor was ranked as the third objective. Having multiple samples to be tested and studied simultaneously was crucial for experimental reproducibility and experimental control. Ease of use was identified as the fourth most important objective. As such, later iterations of the project could address and improve the

assembly method and ease of use over the system. Finally, cost effective was last on the ranking of importance because, although it was important to keep price in mind, it was determined the price should not impair the effective performance of the system.

3.5 Quantitative Analysis of Sub-Objectives

After completing our analysis of the primary objectives the team began to analyze our sub-objectives through background research and client discussions. This analysis allowed the design team to quantitatively rank which sub-objectives were most important. When three or more sub-objectives were identified, a PCC was used to rank the relative importance of each. When there were only two sub-objectives for a given objective, no PCC was required and the design team decided which sub-objective had the greatest importance. The sub-objectives for the microvascular network were assessed first.

3.5.1 Microvascular Network

The design team analyzed the sub-objectives for the microvascular network component of this project, the objectives of which are presented in Figure 9.

3.5.1.1 Reproducible

Two sub-objectives were determined for the objective reproducible: network dimensions and network patency. The design team decided that reproducible network patency was the most important sub-objective. A reproducible and consistent network patency integrity would ensure the pressure in the microvascular network remain at the intended level. If one portion of the microvascular network were to collapse, the pressure in the other portions would increase, which would create an inconsistency in the network. Allowing for reproducible network dimensions was ranked second. This would ensure that the seeded cells are exposed to the same environment across samples.

3.5.1.2 Stackable

Two sub-objectives were identified for the stackable objective. The first is the stackability of multiple microvascular layers and the second is the stackability of a microvascular layer and a cardiomyocyte layer being developed by Megan O'Brien. The design team determined that the stacking of a microvascular layer and a myocardial layer was most important because the purpose of the microvascular layer is to maintain cell viability of the adjacent layer.

3.5.2 Bioreactor

The design team analyzed the sub-objectives for the bioreactor component of this project, the objectives of which are presented in Figure 10.

3.5.2.1 Reproducible

Three sub-objectives were evaluated under the reproducible objective: reproducibility of dimensions, flow, and stacking of multiple layers with both accuracy and precision, seen in Table 37, Appendix B: Decision Matrices. The team determined that the highest priority sub-objective was the reproducibility of flow. The client needed to be able to achieve the same flow rate conditions throughout the bioreactor system over multiple iterations of the experiments. The next most important sub-objective was that the bioreactor, specifically the microfluidic chamber, needed to be reproducible in dimensions. The fabrication process of the microfluidic chamber needed to produce devices with the same dimensions and characteristics. Lowest priority was given to the stacking of multiple layers because the team determined that at the current stage of the project, the device needed to be able to support two layers. At later stages of the project, this sub-objective has the potential to rise in importance, once the multilayer system becomes scalable to larger sizes.

3.5.2.2 Allow for Multiple Samples

Two sub-objectives were identified for the allowance of multiple samples: prevent cross-contamination and modularity. Prevention of cross-contamination was determined to be more important by the design team. It was crucial for the device to prevent issues of contamination and cross-talk between samples in experiments, because that could cause skewing of the collected data or failure of the experiment. Modularity would allow the number of samples to be adjusted depending on the experiment's need as opposed to allowing for a single pre-set number of samples.

3.5.2.3 Ease of Use

The team determined three sub-objectives of the higher level objective ease of use, which were as follows: ease of transport, ease of set-up, and ease of flow rate control. Using the PCC seen as Table 36, Appendix B: Decision Matrices, the design team decided that ease of transport was the most important sub-objective. Since the client had specified the device should be able to be moved from its location in an incubator to a microscope for visualization and imaging during the courses of a two to three week experiment, it was decided that ease of transport should be the first priority. Because the clients desired to use the device over multiple weeks, it was decided that the second most important sub-objective was the ease of fluid flow control. Since the device needed to be set up only once per experiment, this sub-objective had the lowest priority.

3.5.2.4 Cost Effectiveness

Two sub-objectives were identified under the cost effectiveness objective. The first sub-objective was for the bioreactor to be reusable and the second sub-objective was to be inexpensive to produce. Upon analysis, it was decided among the design team and clients that the sub-objectives would have equal weight since if only one sub-objective could be achieved, either

would be sufficient. If the device was reusable, the cost would be limited to a one-time purchase or infrequent purchases over extended periods of time. On the other hand, if the device was cheap to produce, purchases could be made more frequently, and the user could save on time and resources trying to maintain the device.

3.6 Constraints

The team along with our clients formulated a list of constraints as seen in Table 2, in Section 3.2 Initial Client Statement and Clarification, for the microvascular network and bioreactor, as well as a list of constraints that are applied to the project as a whole. The constraints that applied to the microvascular network were biocompatibility, sterility, and size. The materials used for the microvascular network had to be biocompatible, since the microvascular network needed to sustain cells seeded around the channel. Along with that, the materials used for the microvascular network needed to be sterilizable in order to provide the seeded cells with a clean and safe environment. The clients and users set another constraint of the material chosen for the microvascular network. The fabrication material needed to be compatible with fibrin, since that would be the material used to make the cardiomyocyte seeded layers, which would be stacked on the engineered microvasculature. The microvascular network layer needed to be 1 cm by 1 cm in area, while being less than 1 cm thick, because that is the desired thickness of the final scaffold.

The constraints for the bioreactor system were size, biocompatibility, sterility, transparency, and integrity in the incubator environment. Size wise, the whole bioreactor system needed to be compact enough to fit inside the incubator and the biosafety cabinet. More specifically for the microfluidic chamber, the size needed to be small enough to allow for imaging with a microscope, while having enough internal space to fit both the microvascular

network and a cardiomyocyte seeded gel layer, which is approximately 1 cm x 1 cm x 300 μm. The whole bioreactor system needed to allow for sterilization. This was important to protect the seeded cell culture from possible contamination, which would cause failure of the experiment. It was determined that the microfluidic chamber needed to have a transparent element, which would allow for microscope imaging. This was important for the users, so that the condition of the seeded cells could be observed, and adjustments to the experiment could be made if necessary. The last constraint for the whole bioreactor system was that it needed to sustain its structural and chemical integrity at 37°C temperature, 5% CO_2 concentration, and 95% humidity, since this is the environment inside the incubator.

Constraints that were placed on the project as a whole were time and budget. The first constraint was the timeline. The team was given twenty-eight weeks to complete the assigned project. In order to address this, the team planned guided timelines, which are described in more detail in Section 3.11.2 Project Management. The team followed the Gantt chart, as seen in Figure 111, Appendix A: Timelines, as a model. The second project constraint was the budget imposed through the official MQP guidelines. Each member of the team was given a budget of $156 adding up to a total of $780 for the entire project.

3.7 Functions

Based on the ranked objectives set by the design team, the users, and the clients, functions for the engineered microvascular network and the bioreactor were determined. The functions of the fabricated microvascular network were to imitate native vascular tissue, in order to create an environment that resembles *in vivo* conditions for cells to be cultured in. This could be achieved by recreating physiologically relevant dimensions of the channels and a physiologically relevant flow through the network, both of which are described in more detail in

Section 3.8 Specifications. The second function of the microvascular network was to sustain the cell viability of the cells seeded in the whole stacked system. This implied that the diffusion of the perfused nutrients and oxygen needed to reach and be sufficient for the cells seeded in the adjacent layer, representing the cardiomyocyte layer of the cardiac patch.

Once the functions of the microvascular network were defined, the team moved onto the bioreactor. A large focus of the whole bioreactor system was the microfluidic chamber, since the team needed to design the device to be compatible with the fabrication method of the microvascular network. The first function of the bioreactor system was to sustain continuous perfusion at a physiologically relevant flow rate, which could be adjusted based on the user's need. The second function of the bioreactor system was to maintain a sealed system that would limit the gas permeability, while maintaining a sterile environment. A specific function for the microfluidic chamber was that it needed to mold the microvascular network. Along with that the device needed to hold the multilayer construct, consisting of the cell seeded and microvascular network layers. All of these functions lead to the higher level function of the bioreactor system, which was to sustain the cell viability of a 3D cell culture construct.

After defining the functions of the microvascular network and the bioreactor system, the team needed to define specifications in order to achieve the set objectives and functions, while remaining within the constraints.

3.8 Specifications

Specifications are specific metrics that assist and guide the design process. These statements and ranges limit the design space and help set a bar through which the team can measure the success of alternative designs and assist with selection of a final design.

A major aspect of the microvascular network design was that it needed to resemble the native tissue. From literature, the team determined the range of diameters of blood vessel types and their corresponding flow rates, as shown in Table 5 below. For the project, the team aimed to imitate arterioles, which range from 11 μm to 1000 μm, as the focus of the design was to create a fabrication method, rather than refine a pre-existing protocol. In order for the microvascular network design to be successful, the team needed to be able to fabricate channels within the set diameter range. These diameters and flow rates are seen in Table 5.

Table 5: Average diameter ranges and flow rates in human blood vessels (Freitas, 1999)

Blood Vessel Type	Diameter Range (μm)	Flow Rate (mL/min)
Arteries	1000 - 6000	96.0 - 342.0
Arterioles	11 - 1000	0.0015 - 0.21
Capillaries	8 - 11	$4.5 * 10^{-6}$
Veins	5000 - 10,000	240.0 - 720.0

Another specification that was defined based on the objectives for the microvascular network was the diffusion distance. The microvascular network needed to promote diffusion to a distance sufficient to sustain an adjacent 300 μm thick layer. This sets the lower limit the of diffusion distance for the engineered microvascular network.

Specifications for the bioreactor are specific to each component. The main purpose of the bioreactor system was to sustain a cell culture, and in order to do this efficiently, the microfluidic chamber, which holds the sample, needed a favorable environment for cell culture. The generally accepted environment for cell culture is 37°C, 5% CO_2, and 95% humidity, since this closely resembles the *in vivo* conditions in the human body. These conditions are recreated inside incubators, so it was decided that the whole bioreactor system needed to fit inside the incubator, so that there was a minimized chance of contamination. The incubator at Pins Lab is 0.47 m x

0.47 m x 0.83 m, which means the whole assembled bioreactor system needed to be smaller than that. Since one of the objectives was for the system to recreate physiologically relevant flow, the pump needed to produce a flow in the range of 0.0015 - 0.21 mL/min, since that is the average range of the flow rate found in arterioles as described in Table 5. The users wanted to be able to observe the cell culture in the microfluidic chamber so the chamber needed to fit on a microscope stage. Based on the measurements from the microscopes available to Pins Lab, it was determined that the microfluidic chamber profile needed to be lower than 19.5 mm. The user desired to use the created bioreactor system for an extended period of time. The necessary time for the cell culture to proliferate and differentiate is two to three weeks, so the bioreactor system needed to be able to sustain the tissue construct for up to three weeks.

3.9 Standards

For any commercial product, especially when being used for medical purposes, strict standards must be followed. ISO International Standards ensure that products are safe, reliable and of good quality. The most important standard for the team's application is ISO 10993: Assessing the biocompatibility of medical devices and materials. This is necessary for any medical devices that come in contact with the human body and is in place to determine potential toxicity that could result from contact of the device's material with the body (ISO 10993, 2009). This is examined by testing the biocompatibility of the material used, in the team's case, Polytetrafluoroethylene (PTFE) and polyoxymethylene (POM). An agar diffusion assay can be used to determine the biocompatibility of each material by placing the test material on top of an agar layer, followed by incubation of the cells. Cytotoxicity of the material is indicated by formation of zones containing degenerative or lysed cells under and around the test material (Nemani, Moodie, Brennick, Su, & Gimi, 2013). Next is ISO 11737: Sterilization of Medical

Devices. This standard is in place to determine the amount of microorganisms that might be on the product, test the sterility, and to validate/maintain the sterilization process (ISO 11737, 2006). ISO 13022: Medical products containing viable human cells are the next standard that the team must adhere to. This standard has been put into place as an application of risk management on the manufacture of medical products utilizing viable human material (ISO 13022, 2012). To test sterility, the device could be sterilized and filled with media. The device is left for five days in an incubator, and then examined under a microscope to determine microbial growth. The final standard that applies is ISO 16792: Technical product documentation using CAD. CAD is being used to design the bioreactor. Any devices that are created through CAD must adhere to this standard which outlines specific methods on documenting design drawings (ISO 16792, 2015).

3.10 Revised Client Statement

By meeting with the clients frequently, the team was able to keep them updated regarding the progress of the project and continually receive their feedback. By clarifying the client statement, quantitatively analyzing and ranking our objectives, and defining constraints and functions, the team developed a more detailed, revised client statement:

> *Design, develop, and characterize a microengineered vascular network as well as a bioreactor that provides continuous perfusion through a thin hydrogel scaffold containing the microengineered vascular network. The bioreactor must allow for imaging under a microscope, be easy to use and cost effective, allow for reproducible stacking and flow, be sterilizable, and maintain an environment at 37°C, 5% CO_2, and 95% humidity because these are the widely accepted environmental conditions for cell culture in vitro. The microvascular network must*

mimic physiologically relevant dimensions and sustain physiologically relevant flow. The microvascular network layer must also be endothelializable, stackable with other hydrogel layers, and cost effective. The entire system must maintain cell viability of an anisotropic, contractile myocardial tissue layer of 1 cm x 1 cm x 300 μm in vitro for two to three weeks.

The revised client statement contained more details and helped clarify the ultimate goal of the project. By incorporating significant objectives and specifications, project details were highlighted. The client statement was referenced by the design team during the entirety of the engineering design process.

3.11 Project Approach

3.11.1 Technical

In order to keep the project on track, the team created five project strategy steps that were wanted to ensure were accomplished. The order in which they were placed follows the order in which the team strived to fulfill the milestones and keep up with the design strategy. The project strategy is shown below in Figure 11:

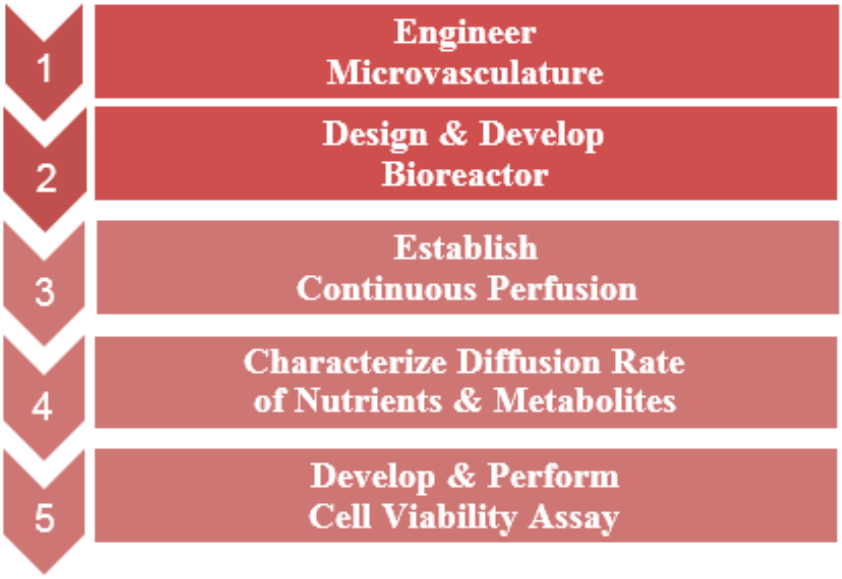


Figure 11: Project strategy steps

The first step was to design and develop the microvascular network. Research of current approaches and techniques employed for channel formation was complete and based on that an original protocol for the vascular network fabrication was developed. Modifications to the protocol were made in order to match the objectives, constraints, and functions defined for the project. Once a single channel system was fabricated, the team moved on to form a bifurcated system. Once both processes were successful, the team moved onto designing and developing the bioreactor.

A major element of the bioreactor system design process was the selection of components. Since specifications were defined, elements for the bioreactor were selected so that they could match the set operational ranges. Special attention was paid to the design of the microfluidic chamber, since this was the component that directly molded the microvascular network and held the tissue construct. After preliminary research was completed, the team created a concept design. After performance testing, modifications were made to the design to ensure the bioreactor system achieved the determined objectives.

The next step in the project strategy is to establish continuous perfusion through the fabricated microvascular network. This meant that the bioreactor pump component needed to provide a continuous flow through the microfluidic chamber, and the vascular network needed to sustain its patency and the provided flow. Once continuous flow was established, it was necessary to determine the performance of the microvascular network.

The primary function of the vascular network was to sustain the viability of cells. The first characteristic that needed to be determined was the diffusion distance of nutrients. This was necessary to ensure that the fabricated microvascular network was feasible to provide nutrients to the adjacent layer. The last step of the project was to test the viability of these cells. Based on the proposed project strategy, a timeline with deadlines was created to keep the team on track and finish with the given time period.

3.11.2 Project Management

The team created a Gantt chart to highlight deadlines for various portions of the project beginning in A term and ending in D term. Every aspect from report writing to prototype production can be found with a deadline of completion. The yearlong Gantt chart can be seen in in Appendix A: Timelines. As the project progressed and tasks were completed, the team marked them off as complete, allowing the team's efforts and time to focus on the next tasks. If the team experienced problems or difficulty completing a set task during the project, the Gantt chart at times would be modified and adjusted appropriately to allow for enough time to complete the task thoroughly. Figure 111 in Appendix A: Timelines displayed a Gantt chart for A term and Figure 112, Figure 113, and Figure 114, and show Gantt charts for B, C, and D Term, respectively. These contain more details on the task deadlines than Figure 111. These Gantt charts can be seen in Appendix A: Timelines.

Each week, the team met with the advisors and clients to update them on the progress and direction of the project. During these meetings, the team gave presentations that highlighted the milestones completed the previous week, the goals of the coming week, and any problems the team might have encountered. Our advisor and clients challenged the team each week to think differently about certain tasks to ensure that alternative designs were considered and the best final design could be chosen.

3.11.3 Financial

A constraint of the project was the team's budget. Each member was allotted $156.00 giving the team an accumulative budget of $780.00. The significant portion of the team’s budget went to purchasing materials for the microvascular network media, and growth factors. Before the end of A term, about 70% of our budget had been spent on those materials. The budget that was left went towards having allocated lab space in Goddard Hall 006, and materials necessary to run the proposed validation experiments.

Chapter 4: Design Process

In this chapter, different design alternatives for the microvascular network and the bioreactor are described and evaluated to understand how well each would accomplish the project's objectives, functions, and specifications. The team specifically designed the microfluidic chamber component of the bioreactor system. This chamber is the interface between the bioreactor and the microvascular network. The team started by brainstorming and creating simplified conceptual designs. Based on the ideas proposed during these sessions, multiple possible alternative designs were created. The potential performance of each design was analyzed. Based on how well the design matched the set objectives, functions, and specifications, a final design was chosen. In Sections 4.2 Microengineered Vascular Network and 4.3 Bioreactor and Microfluidic Chamber, the design considerations and process are described respectively for the microvascular network and the bioreactor system.

4.1 Design Concept

After formulating the project approach, the team created a conceptual design of the project including the microvascular network, bioreactor system, and more specifically, the microfluidic chamber. With an understanding of the functions, objectives, constraints, and specifications, the following design concept was made in Figure 12. A design concept is the underlying basis for creating alternative designs for the entire project.

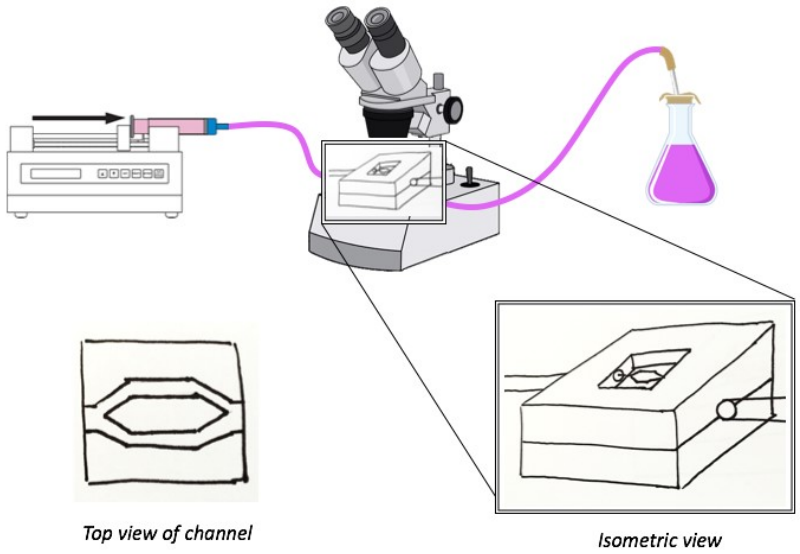


Figure 12: Design concept of the project. The design shows the completely assembled bioreactor system with the microvascular network

The design concept shows a crude representation of what the final project setup will look like with general components. This figure shows a pump that provides continuous perfusion at physiologically relevant flow through a microfluidic chamber which contains a microengineered vascular network in a sterile environment. The microengineered vascular network replicates physiologically relevant dimensions and sustains cell viability through diffusion of perfused nutrients. The perfused media then flows through the outlet into a collection flask. The microfluidic chamber needs to allow for imaging, so it was designed to fit on the microscope stage with a viewing window.

4.2 Microengineered Vascular Network

4.2.1 Needs analysis

Based on the objectives, described in detail in Chapter 3: Project Strategy, the team analyzed which elements of our project are needs and which are wants, based on our clients and users' input. For the microvascular network it was determined that the system needed to sustain a physiologically relevant flow ranging between 0.0015 - 0.21 mL/min and the fabricated channels

needed to have a diameter within the physiologically relevant range of 11 - 1000 μm. Along with that the microvascular network needed to maintain its patency. In order for statistically relevant studies performed on the system, the microvascular network fabrication needed to be reproducible and give reproducible results when tested under the same conditions.

Wants defined for the microvascular system were that it should be endothelializable and cost effective. In order to be endothelializable, the network needed to be made from a material that promotes cell adhesion to the inner wall of the channel and angiogenesis. In order to be cost effective, the microvascular network fabrication method should minimize waste, and allow for tools to be reused for the process after being washed thoroughly.

4.2.2 Feasibility studies

This section contains the feasibility studies for methods of creating the microengineered vascular network. Each feasibility study was conducted to determine whether the method could result in the formation of a single channel.

4.2.2.1 Cylindrical Rod Extraction

In order to determine the feasibility of the cylindrical rod extraction method as a technique for microvascular network fabrication, three factors were evaluated. The first aspect was the rod's geometry, which needed to be uniform without damaging the channel. The second factor taken into account was the mobility and assembly of all the tools necessary to fabricate the channel. The third aspect evaluated was the ability to produce an empty perfusable channel.

The idea of this method came from previous work of an earlier MQP project: "Fibrin-based Microvascular Network for the Modular Design of a Cardiac Patch." Small rectangular frames glued to glass slides were used as wells for the fibrin gel, as seen in Figure 13.

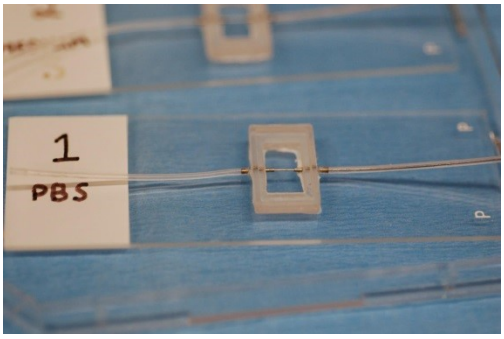


Figure 13: The well frames with attached tubing for channel fabrication

The frames had two openings on opposite walls, which acted as the inlet and outlet for fluid perfusion. Initially, the method employed a thin 30G needle, which was inserted through the openings. Once the needle was in place, fibrinogen and thrombin solutions were cast into the well to produce the fibrin gel. Once the gel was fully polymerized, the needle was extracted, which left behind a small cylindrical channel, which can be seen in Figure 14. Using bright field microscopy, the channels were characterized.

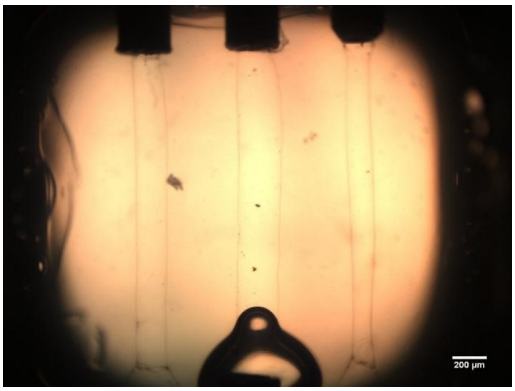


Figure 14: Channels fabricated using cylindrical rod extraction method. Three straight single channels fabricated using needles in the same gel. The channels were not structural uniformity both along the same channel and between different channels.

Even though the channel produced had imperfections and defects, such as some deformation along the walls, it clearly connected the two ends of the gel, allowing for perfusion to occur. This simple method allowed for the fabrication of single straight channels in a fibrin gel. Since the technique successfully covered all aspects of the feasibility test, it was determined that this was an adequate method to produce the microvascular network.

4.2.2.2 Sacrificial Mold

To assess the feasibility of a sacrificial material to ultimately be used to develop a microvascular network, the team determined the ability of a sacrificial mold to produce a single channel. Three criteria were assessed: the production of a sacrificial mold with adequate geometry, the ability to transfer the mold, and the ability to form an empty channel.

4.2.2.2.1 Pluronic F127

The feasibility of Pluronic F127 as a sacrificial material was assessed. A 24.5% solution of Pluronic F127 in phosphate-buffered saline was prepared due to its unusual thermoresponsive properties. The material exists as a gel at room temperature and liquid at 4°C and it is this property that allows the Pluronic to be used as a sacrificial material. The phase diagram showing these properties is shown in Figure 15.

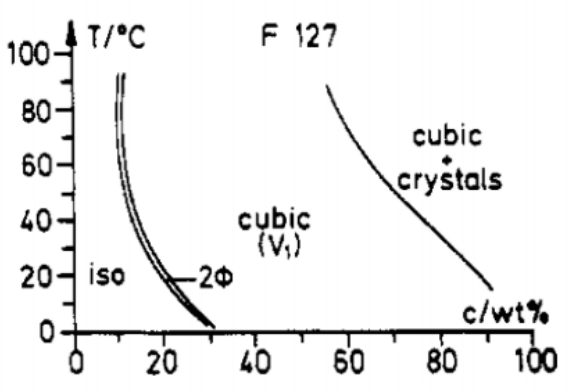


Figure 15: Phase diagram of Pluronic F127 (Müller, Becher, Schnabelrauch, & Zenobi-Wong, 2013)

Extrusion of Pluronic Cylinders

Pluronic sacrificial molds were produced by extruding the gel at room temperature to form cylinders. Various sized cylinders were produced by extruding out of a 1 mL syringe using various sized needles as seen in Figure 16.

Figure 16: Multiple sizes of Pluronic cylinders extruded from 1 mL syringe (left) and 23G needle (right).

Mold Transfer

As a gel, the Pluronic cylinders are not structurally sound and are unable to be transferred to another location. To overcome this property, the cylinders were dehydrated on the bench top for at least one hour. The dehydrated Pluronic cylinders were then stiff enough to be picked up and moved into place in a mold, as can be seen in Figure 17.

Figure 17: Dehydrated Pluronic cylinder a) resting against acetyl slide to show stiffness and b) placed in PDMS mold.

Channel Formation

To form a channel in fibrin, a dehydrated Pluronic cylinder was placed in a PDMS mold and secured with laboratory tape. After the addition of fibrin components in a method as found in Appendix D: Protocols: Fibrin Gel Preparation Protocol, the cylinder rehydrated, as seen in Figure 18.

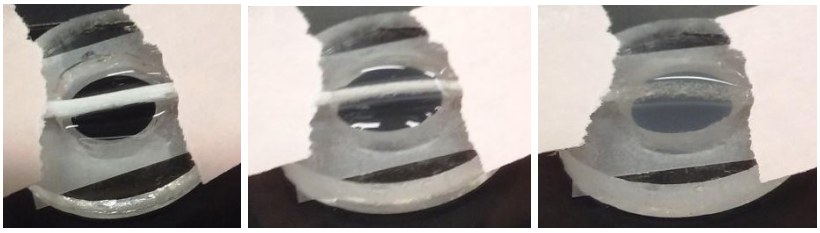


Figure 18: Observed Pluronic cylinder rehydration (passage of time left to right) during fibrin polymerization.

After fibrin polymerization was complete, the scaffold was placed in water at 4°C to liquefy the Pluronic sacrificial mold. After the sacrificial material was fully removed, blue food-

colored water was perfused into the channel to show successful channel formation, as seen in Figure 19.

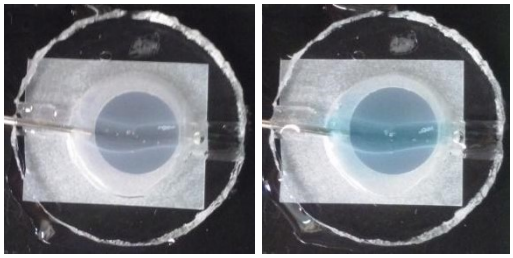


Figure 19: Channel formation from Pluronic sacrificial mold. Empty channel after Pluronic liquefying (left) and after brief filling with blue food-colored water (right).

Through this feasibility study, it was shown that sacrificial molds could be created with Pluronic F127 which could be transported to a PDMS mold, liquefied, and forms a channel that could be filled.

4.2.2.2.2 Gelatin

The feasibility of gelatin as a sacrificial material was assessed. A 10% w/v solution of gelatin was created using a protocol originally adapted from Golden & Tien, found in Appendix C: Client Meetings. The crucial sacrificial material property for gelatin is its gel transition temperature, between 20°C and 35°C depending on concentration, as seen in Figure 20. This sacrificial material is heated above the melting temperature, formed in a mold, and cooled below its gelation temperature. Once in place in a fibrin gel, created using the protocol found in Appendix D: Protocols: Fibrin Gel Preparation Protocol, the material can again be heated above its melting temperature and flushed out, leaving behind a channel.

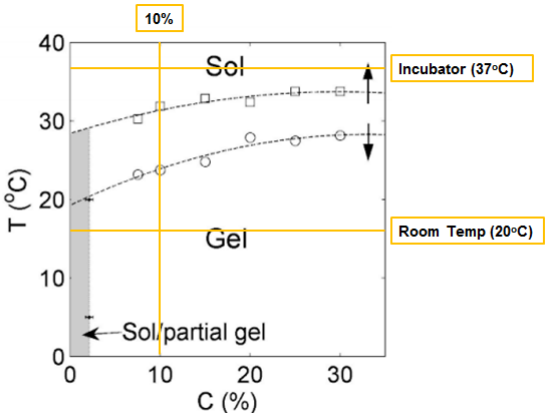


Figure 20: Concentration-temperature phase diagram of aqueous gelatin sample showing the transition temperatures for melting (upper) and gelation (lower). The gelation line depends on cooling rate, which was 0.5 K min-1 here (Parker & Povey, 2012). At a concentration of 10%, gelatin exists as a gel at room temperature and a liquid at 37°C.

Gelatin sacrificial molds were fabricated by melting the gelatin solution and then filling PDMS molds, both cylindrical and a half bifurcation, as seen in Figure 21.

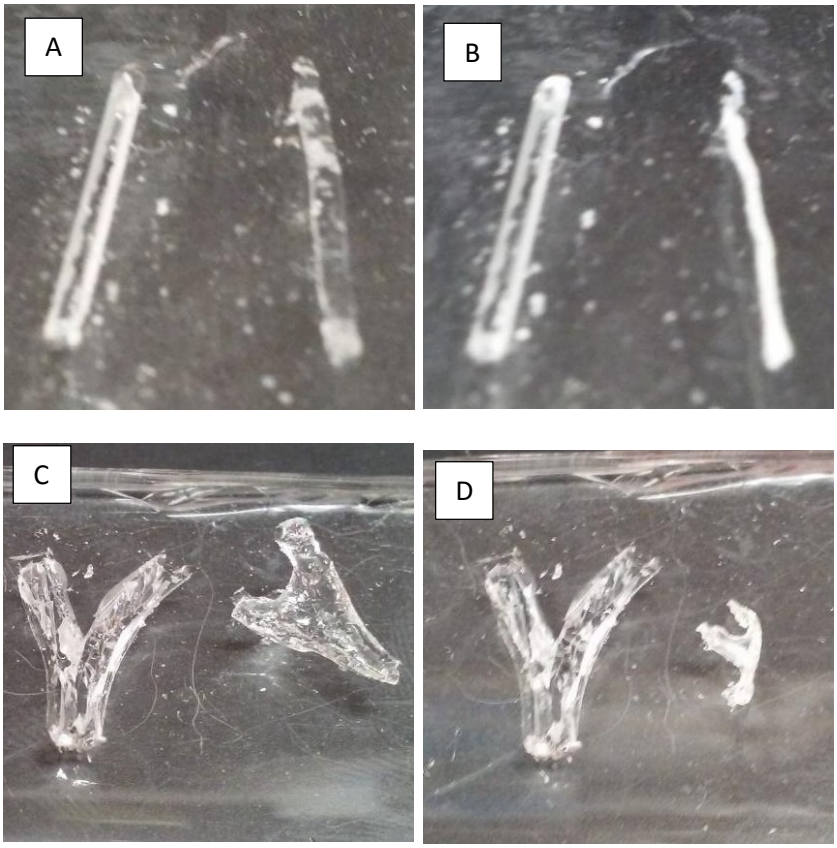


Figure 21: Straight and y-shaped gelatin molds. Straight gelatin channel molds and y-shaped structure formed in PDMS mold after gelation (A & C) and after drying overnight (B & D).

Gelatin sacrificial molds were also fabricated via extrusion of gelatin in its gel state. Gelatin was heated while submerged in water on a hot plate to melt. Liquid gelatin was then pulled into a one milliliter syringe and let cool for approximately 15 - 20 minutes. The gel was then extruded out of the syringe directly or through a 20G needle. The result was successful fabrication of sacrificial mold cylinders via extrusion of solid gelatin, as seen in Figure 22.

Figure 22: Fabrication of gelatin sacrificial mold cylinders via extrusion of solid gelatin.

After gelation, the sacrificial materials were able to be transported as a gel. It was noted that upon dehydration of the gelatin sacrificial molds, a qualitative decrease in size was observed. In addition to a decrease in diameter, this property would allow for easy transport of the sacrificial mold to another location.

A channel was formed, as seen in Figure 23, using a dehydrated gelatin cylinder which was encased in fibrin and heated at 37°C. This whole process was completed in a *Box Mold*

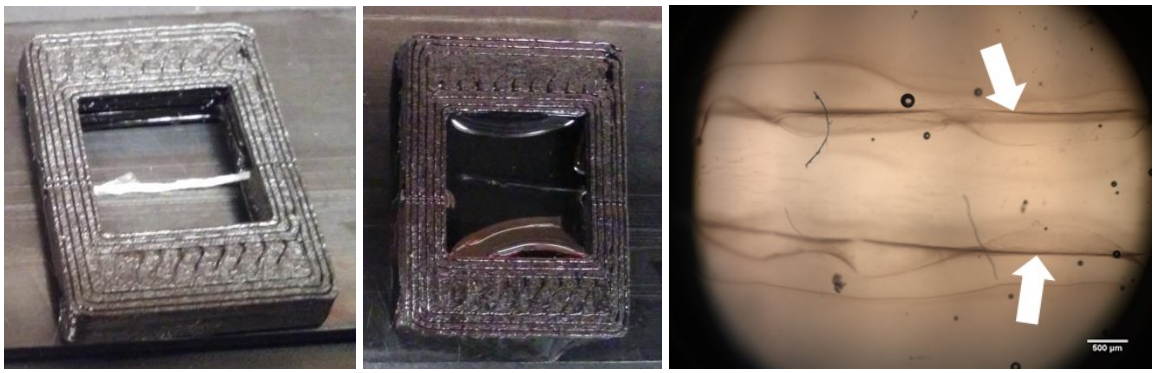


Figure 23: Channel formed from gelatin sacrificial material. a) gelatin cylinder in Box Mold, b) gelatin after filling mold with fibrin, and c) channel after gelatin flushing, viewed under 20X magnification. Arrows indicate channel edges.

This feasibility study showed that sacrificial molds could be created from gelatin that could be transported to a mold, liquefied, and formed into a channel.

4.2.2.2.3 Polyvinyl Alcohol

The feasibility of polyvinyl alcohol (PVA) as a sacrificial material was assessed. PVA is a water soluble synthetic polymer with high dependency on the humidity of its surroundings. Higher humidity means that more water is absorbed. A potential method of channel formation included placing a PVA cylinder inside the mold, casting the fibrin layer, and seeing if the PVA would dissolve, thus creating a channel. To determine the feasibility of PVA as a sacrificial material for vascularization, a 0.4 mm PVA cylinder was 3D printed. Next, the PVA was carefully placed into the indentations on the base of the two piece mold. Then, the top piece was placed onto the sacrificial material, making sure the pieces were aligned carefully and that the material was secured. A binder clip was used to keep the two pieces together. This would ensure the top and bottom piece would not separate during the experiment and can be seen in Figure 24 and Figure 25 below.

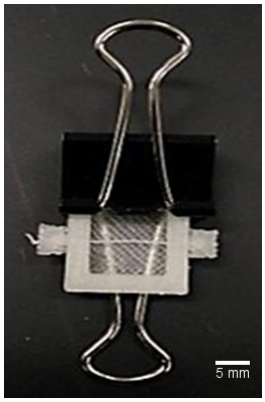


Figure 24: Two Piece Mold with Base with PVA channel before adding fibrin

Next, the fibrin gel, prepared using the protocol found in Appendix D: Protocols: Fibrin Gel Preparation Protocol, was cast within the mold. This was done by adding a mixture of

fibrinogen, thrombin, $CaCl_2$, and PBS. After about ten minutes, the PVA began to dissolve into a viscous substance as seen below in Figure 25.

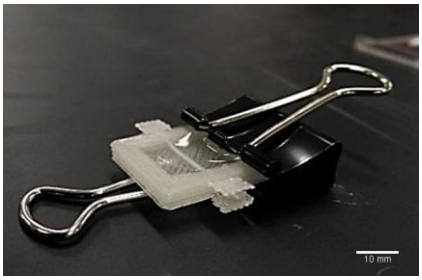


Figure 25: Two Piece Mold with PVA channel and fibrin scaffold forming

After waiting fifteen more minutes, the team attached tubing and tried perfusing water through the system. However, the PVA was too thick and the water could not dissolve thoroughly or push the PVA through the channel. Another issue that arose included that the PVA began to sink to the bottom of the mold. This could be a result of the weight of the PVA, in addition to the possibility of the indentations of the mold being too small. It is possible this did not allow the PVA to expand as necessary within the water. As a result of this experiment, the possibility of using PVA as a sacrificial material to create a channel within the fibrin layer was eliminated.

4.2.2 Microengineered Vascular Network Verification Tests

Once the feasibility studies for cylindrical rod extraction and sacrificial molds were complete, verification tests were performed. These tests were completed to determine any key limitations or additional factors to consider before choosing the method of forming the microengineered vascular network for the final design.

4.2.2.1 Cylindrical Rod Extraction

As described earlier in the chapter, this method initially employed a thin 30G needle as the channel molding tool. The needle was placed in the gel mold through the inlet and outlet needles, and fibrin gel was cast around it, using the protocol in Appendix D: Protocols: Fibrin

Gel Preparation Protocol. Once the polymerization process was complete, the needle was removed leaving behind a channel inside the gel. In order to perfuse through the system, polyethylene tubing was connected to the inlet and outlet needles. This attachment of the perfusion tubing often caused damage to the fibrin gel. The inlet and outlet needles were not glued so that they could be cleaned and replaced periodically. An external force was exerted on the outside of the mold needles while trying to connect the tubing, causing the needles to dislocate inwards. This was an undesirable result, since by dislocating inwards, the mold needles made it difficult to connect the perfusion tubing and damaged both the gel and the channel. In order to prevent this dislocation of the inlet and outlet needles, the polyethylene tubing needed to be attached to the needles prior to the fibrin gel casting. This allowed for the tubing to be secured properly and the inlet and outlet needles to be readjusted as needed.

By pre-attaching the polyethylene tubing, it became impossible to use the 30G needles, since they were too short. In order to overcome this obstacle, thin steel wires were employed. The wires were cut to a length that would allow them to be threaded into the perfusion tubing and through both the inlet and outlet needles, as seen in Figure 26.

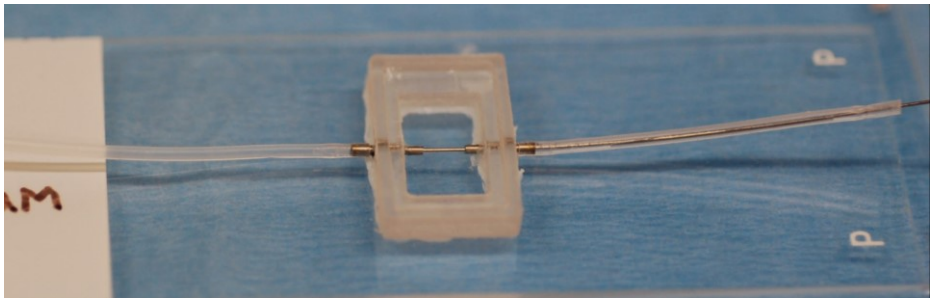


Figure 26: Cylindrical rod extraction method setup

The tubes are attached prior to the wire being introduced through the inlet and outlet. Once in place, the fibrin gel is cast. In the same manner as in the needle extraction method, the wires were removed once the fibrin gel polymerized leaving behind a single channel.

Unfortunately, due to the adhesive nature of fibrin, a problem occurred during the extraction process. The fibrin adhered to the wire, which caused channel to lose uniformity, and in more serious cases, the whole gel to be malformed, as shown in Figure 27.

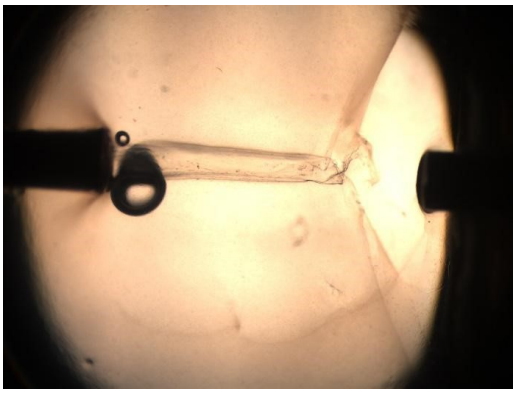


Figure 27: Channel damage in cylindrical rod extraction. Gel damage occurring due to the adhesion of the fibrin to the steel wire during extraction

The method needed to be adapted in order to reduce the adhesion of the fibrin gel to the steel wire. In order to do so, the steel wires were coated with Pluronic F127. With the Pluronic coating, the adhesion of fibrin to the wire was significantly reduced allowing for channels to be properly formed.

In some cases, even after applying the Pluronic coating, serious tears along the walls of the channels were observed. Due to the manner of cutting, defects formed on the tips of the wire. These defects acted as “hooks,” which caused tears and damage along the wall of the channels. In more serious cases, the damage would be enough to compromise the integrity of the channel and then pierce the gel. In order to prevent these tears, the tips of the wires were filed using fine diamond files. The smoothened tips are shown in Figure 28. Using the filed wires, while coated with Pluronic, straight single channels were fabricated uniformly and with minimal damage as seen in Figure 29.

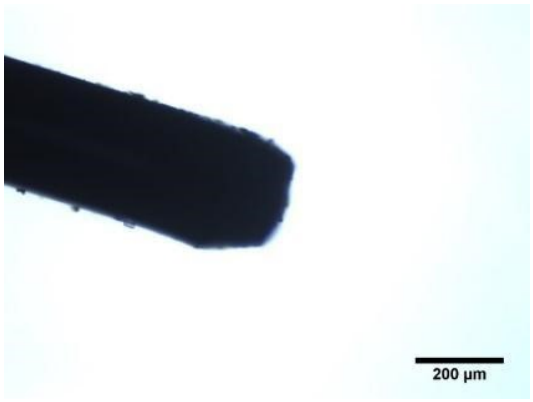


Figure 28: Filed tip of the steel wire. As it can be seen, the cutting artefacts were removed, as the edges were filed with a diamond file.

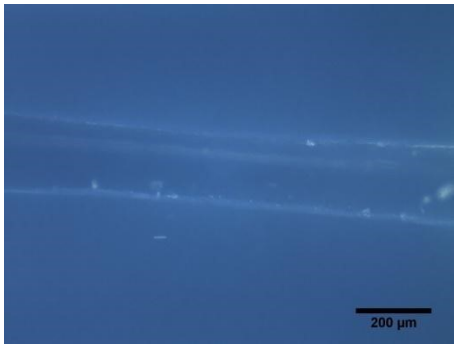


Figure 29: Cylindrical rod extraction channel. Channel in fibrin gel fabricated through cylindrical rod extraction method, using filed and Pluronic coated wire

One of the major challenges the microvascular network faced was perfusion. Even though the channel was properly formed, perfusion had been temporarily unsuccessful because of a blockage which formed from trapped air as fluid was being pumped into the channel. The blockage formation is shown in Figure 30.

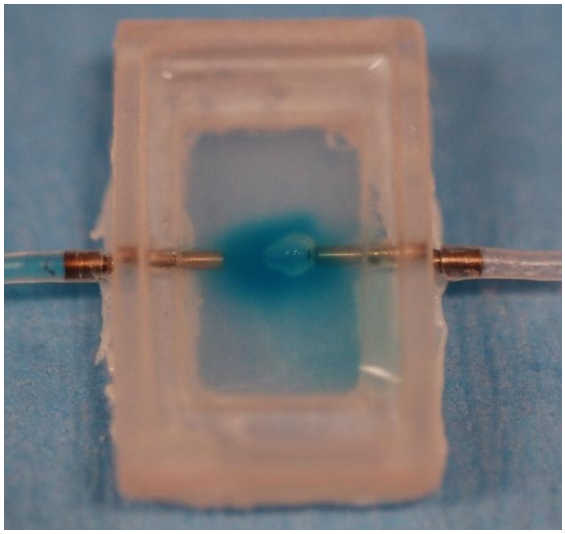


Figure 30: Air bubble blockage. Image of the air bubble blockage that forms when using the cylindrical rod extraction method, obstructing perfusion

One procedure that proved to be successful was air perfusion. Once the needle was extracted, leaving behind a channel in the gel, air was perfused through at a low rate. This initial perfusion indicated possible blockages in the channel, which could be opened by reinsertion of the wire and removed. This step ensured that the channel is empty, and allowed for fluid flow.

4.2.2.2 Sacrificial mold – Pluronic F127

After the Pluronic feasibility study was complete, Pluronic bifurcated channels were created on acetyl plastic using the hand-printed extrusion production method as seen in Figure 31.

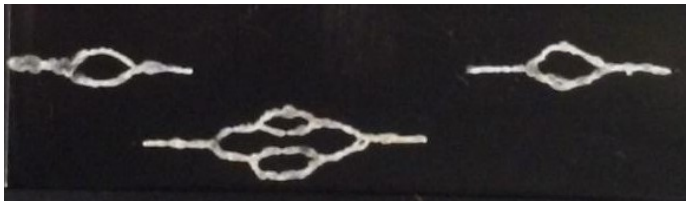


Figure 31: Pluronic bifurcations via hand-drawn extrusion

Though bifurcated sacrificial materials were created, the Pluronic adhered to the surface of the acetyl plastic. Multiple surfaces were tested for Pluronic adherence. Pluronic printing on polystyrene, glass, and vellum paper all resulted in adhered Pluronic material.

However, Pluronic was found to not stick on PDMS surfaces. A bifurcated Pluronic material was printed on PDMS and shown to be non-adhered as seen in Figure 32.

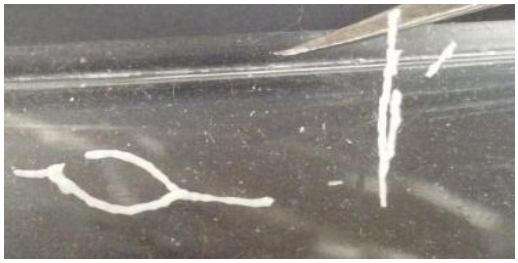


Figure 32: Pluronic bifurcation printed on PDMS and lifted to show non-adherence.

A Pluronic cylinder was placed and enclosed in a two-part PLA mold to test perfusion. It was observed however that after fibrin components were added, the cylinder malformed over time as seen in Figure 33. Fibrin gel was formed via the protocol found in Appendix D: Protocols: Fibrin Gel Preparation Protocol. After fibrin polymerization was complete, the sacrificial material was removed and a channel formed.

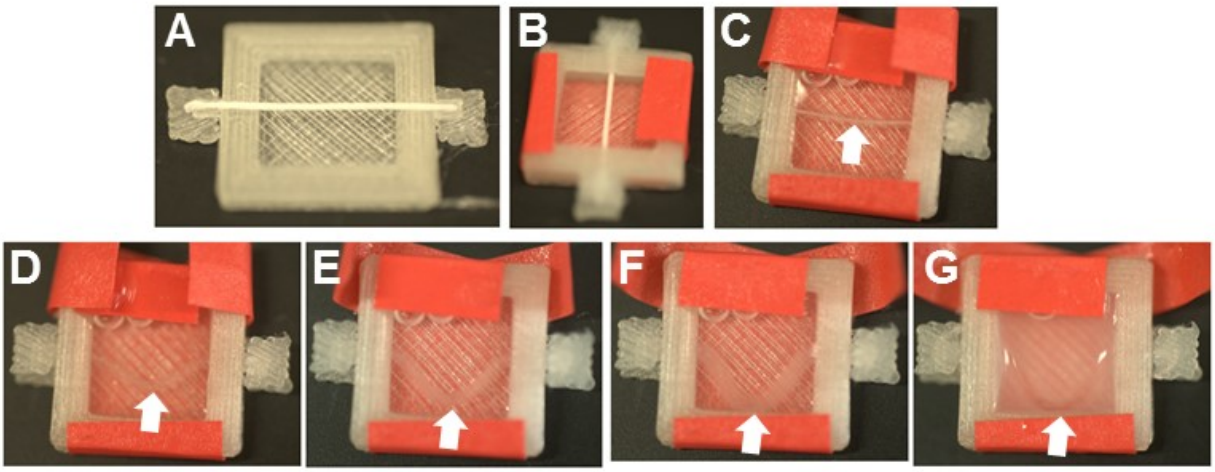


Figure 33: Pluronic mold channel fabrication. a) Pluronic cylinder across lower PLA mold, b) with top mold attached, c-f) channel malformation during fibrin polymerization, g) channel formation after sitting in 4°C water.

It was hypothesized that the Pluronic cylinder swelled during rehydration after the fibrin precursors were added to the mold. Since the cylinder was actually clamped on either side by misalignment of the top and bottom molds, the middle portion of the cylinder likely swelled but had no room to expand in the inlet and outlet holes. To test if a Pluronic mold resting in an open

mold would exhibit the same effects; a bifurcated Pluronic sacrificial mold was created and placed in an open PDMS mold. Swelling and malformation were again observed, as seen in Figure 34. It was observed that the ends of the Pluronic did not rehydrate. The ends did not shift as the Pluronic malformed and they appeared to stick to the PDMS, causing malformation of the Pluronic within the mold.

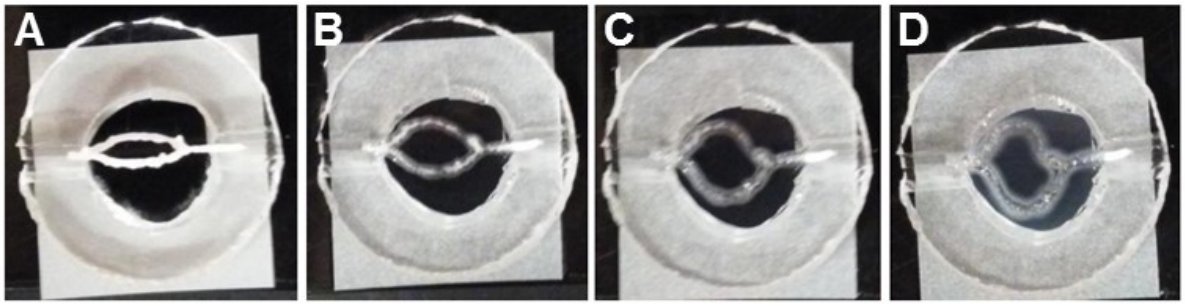


Figure 34: Pluronic bifurcation channel formation. a) Pluronic bifurcation in PDMS mold, b-d) Pluronic bifurcation over time (~15 minutes) after addition of polymerizing fibrin.

To model the change in size of the Pluronic the diameter of cylinders (n=4) were measured during dehydration at time points of 5, 30, and 240 minutes, as seen in Figure 35. The results were inconclusive as some samples had increases in diameter at early time points between five and thirty minutes after extrusion, while there was little change in diameter after thirty minutes. There was some time after extrusion before images were first taken so it is possible that changes in diameter went unrecorded. If Pluronic was chosen as a sacrificial material, further tests could have focused on the change in diameter in this initial time point that was not captured.

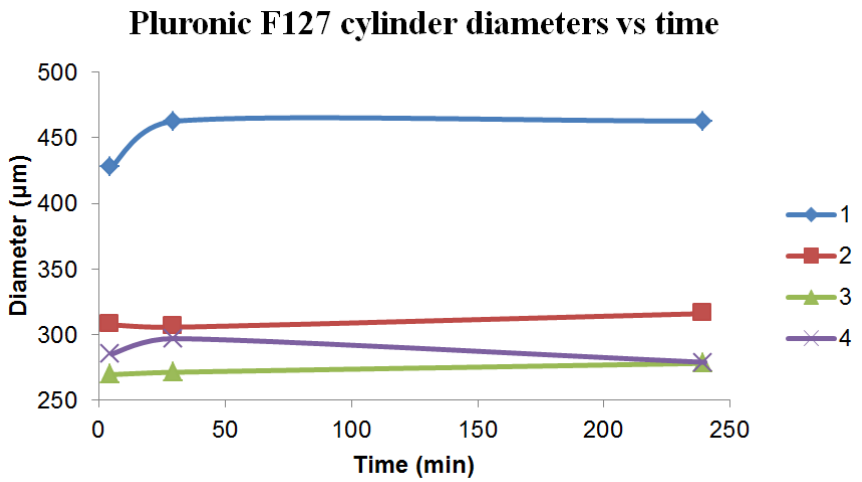


Figure 35: Change in diameter of Pluronic F127 in PBS over time. Note the initial increase in diameter of samples 1 and 4 from 5 to 30 minutes post-extrusion.

One hypothesis for the swelling as well as observed brittleness of the Pluronic was salt crystallization from the phosphate-buffered saline (PBS) in which the Pluronic polymers were dissolved. A new Pluronic solution was created using the same protocol, replacing PBS deionized water. It was hypothesized that though there may not have been a change in diameter, a change in length during dehydration may have been observed. As seen in Figure 36, a decrease in length was observed for all cylindrical (n=5) and bifurcated (n=2) samples at time points of 5, 10, 20, 55, 70, and 78 minutes. The average decrease in length was 9% and 10% for cylindrical and bifurcated samples respectively. However 5 minutes passed after fabrication of the sacrificial molds before images were taken. Since there was an observed sharp decrease in length in the first ten minutes recorded, it is possible the actual decrease in length was much greater than 9%.

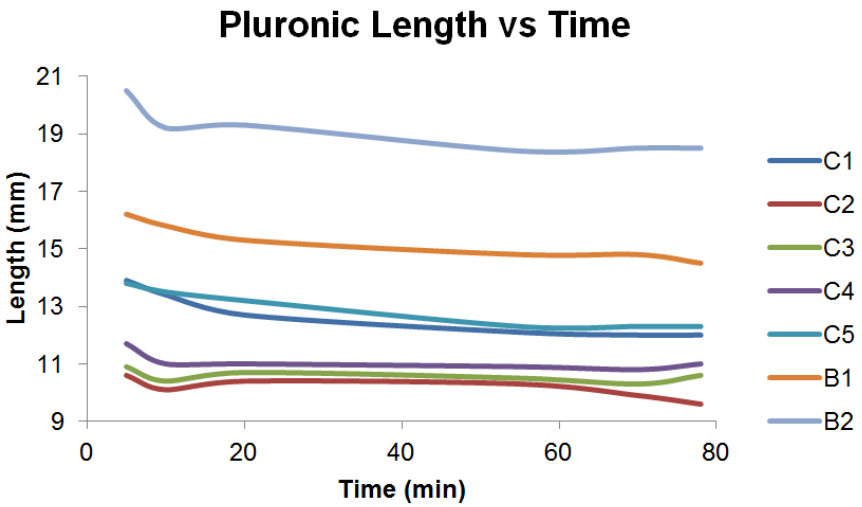


Figure 36: Change in length of Pluronic F127 in deionized water over time. Note the overall and immediate decrease in length. C = cylinder, B = bifurcation

4.2.3 Alternative Designs

This section contains alternative designs pertaining to the microengineered vascular network. Presented are methods of forming a microengineered vascular network followed by sacrificial mold fabrication methods, and sacrificial mold loading methods.

4.2.3.1 Microvascularization Strategies

Table 6: Means chart for the creation of a microvascular network

Function – Create microvascular network of physiological dimensions		
Means	**Advantages**	**Limitations**
Angiogenesis / Vasculogenesis	Cells create own vasculature Only 1 hydrogel required Physiologically relevant size	Time consuming Cannot perfuse immediately Difficult channel formation at input / output of microfluidic chamber
Bonding of Pre-Vascularized Hydrogels	Well-defined geometry Complex geometry capabilities (bifurcations possible) Fills effectively via capillary action	Difficult sealing backflow of fluid around inserted needle 2 Hydrogels required - leads to problems adhering multiple gels Some difficulty aligning channel w/ inlet & outlet
Cylindrical Rod Extraction	Simple set-up, channel formation Channels always line up w/ input & output Multiple channels possible	Limited to straight, non-bifurcated channels Non-uniform geometry Hydrogel damage due to shear upon extraction
Sacrificial Mold	Only 1 hydrogel required No attachment nor adhesion problems Bifurcations possible No damage due to shear Proper sealing at input & output	Potential for unwanted residue along the channel

Table 6 shows the advantages and disadvantages of methods to create the microvascular network. One commonly used method of creating a microvascular network is through the natural processes of angiogenesis or vasculogenesis. The advantages of this method are that endothelial cells produce the microvasculature either *de novo* or from existing vessels. As a result, the

vasculature formed is of physiologically relevant dimensions. Only one hydrogel would be required which avoids problems adhering multiple gels. Limitations of this method include the process being time consuming, on the order of days, and as a result the vasculature cannot be perfused immediately. Since cells are required to create the vasculature, they themselves need vascularization to remain viable if the thickness of the construct is greater than 400 μm. Finally it was difficult to direct the formation of blood vessels to and have proper sealing at the input and output of a microfluidic chamber.

An additional means of creating a microvascular network was via the bonding of pre-vascularized hydrogels. Through the use of precise manufacturing processes such as silicon wafer photolithography, well-defined geometry within the hydrogel after network fabrication was maintained. Complex, small-scale geometries could be formed and these channels could be filled well via capillary action. A major drawback of this method was that it only allows for half-channel formation. In order to complete the channel, an additional layer needs to be applied on top of the molded fibrin layer. This produces a number of issues to the method. Adhesion between two fully polymerized fibrin layers is a difficult process and often results in weak attachment. This means that the seals at the inlet, outlet, and around the channel walls were compromised and may have led to leakage. An additional complication arose from the need to align the inlet and outlet of the network with the bioreactor.

Another method of creating a microvascular network was via cylindrical rod extraction in which a wire or needle is suspended and fibrin is cast around it. Once the fibrin polymerizes, the wire was then extracted, leaving behind a cylindrical channel. Of the means described here, this method had the simplest set-up and procedure for channel formation. Finally, it was possible to create multiple, parallel channels with an associated mold. Some limitations of the cylindrical

rod extraction method included being limited to applications of straight, non-bifurcated channels and non-uniform geometry or gel tearing due to adhesive interactions between the wire and hydrogel.

A final method considered for creating a microvascular network involved making use of sacrificial materials. In this method, the channel was produced by a material, which was dissolved and flushed out, leaving a channel behind. One advantage over the photolithographic method was that the fibrin gel can be formed in one piece as opposed to two, which eliminated the problem of gel adhesion. Similar to photolithography, but contrary to the cylindrical rod extraction method, sacrificial molding could create more complex geometries than the cylindrical rod extraction method. One limitation, similar to photolithography, was the difficulty of aligning of the network with inlet and outlet of the bioreactor. Another drawback of this method was that it might leave unwanted residue along the channel.

4.2.3.2 Sacrificial Mold Fabrication Methods

Four sacrificial mold production methods were determined and advantages, disadvantages, were compiled as seen in Table 7.

Table 7: Analysis of sacrificial mold production method

Production Method	Advantages	Disadvantages
Free Extrusion	Reproducible geometry Extrusion rate independent Cost effective	Limited to single channel geometry
Hand-Printed Extrusion	Bifurcations possible Cost effective	Less reproducible geometry Less reproducible extrusion rate Difficulty forming solid connections in bifurcations
Cast in Mold	Bifurcations possible, geometries only limited by reverse mold Reproducible geometry Can be cost effective	Problems removing sacrificial material from mold
3D Printed	Complex possible Reproducible geometry Reproducible extrusion rate	Most expensive Extrusion size constraint Limited material options for sacrificial mold method

The first sacrificial mold production method was free extrusion in which a sacrificial material was extruded out of a syringe and needle. The advantages of this method were its reproducible geometry, process that was independent of extrusion rate, and cost effectiveness. The main disadvantage of extrusion was the limitation to production of single channel geometry.

A similar production method was hand-printed extrusion. In this method, the user extrudes the sacrificial mold out of a syringe and lays down cylindrical material to build a more complicated structure such as a bifurcation. The main advantages of this method were being able to create materials with more complicated geometries than cylindrical extrusion and its cost effectiveness. Disadvantages of this method included difficulty maintaining regular thickness, forming solid connections in bifurcations, less reproducibility over the geometry, extrusion rate, and lateral printing speed.

Another production method of sacrificial materials was to make a mold with a material such as PDMS in which the sacrificial material could be added and solidified. The advantages of

this method were reproducibility, vast number of geometries possible, limited only by what can be produced as a reverse mold on which to cast the PDMS, and potential cost effectiveness depending on the mold fabrication method.

3D printing was another possible method to produce sacrificial materials. The advantages of this method were the potential for printing bifurcations and increased reproducibility in extrusion rate and lateral printing speed. The disadvantages of this method were the large extrusion size, limited sacrificial materials available and considerably greater expense when compared to other methods.

4.2.3.2.1 Sacrificial Mold – Photolithography Feasibility Study

Another feasibility of gelatin as a sacrificial material was assessed, this time using a PDMS mold created from a photolithographic silicon wafer. Liquid gelatin was added to the top of the PDMS mold and excess liquid was scraped off. The gelatin was cooled and allowed to gel at 4°C. The solidified this sacrificial material film was then peeled off the PDMS as seen in Figure 37, but the gelatin was somewhat damaged when removed with forceps. In the future, 1% bovine serum albumin (BSA) would be added in an attempt to help remove the film without causing damage.

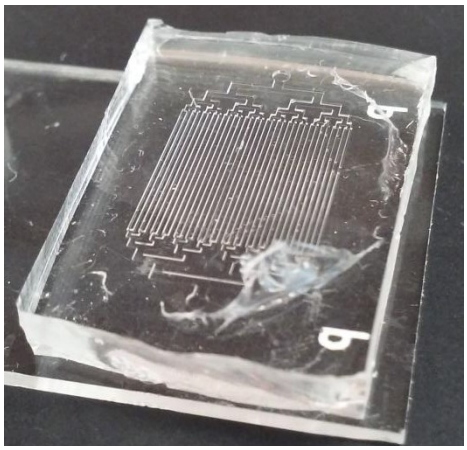


Figure 37: Gelatin on PDMS stamp with microvascular network pattern formed via photolithography.

After successful formation and transport of the sacrificial material, it was placed in a PDMS mold and fibrin was cast around it. After polymerization, the mold in fibrin was imaged, the gelatin was melted at 37°C, and the resulting structure was imaged again, as seen in Figure 38.

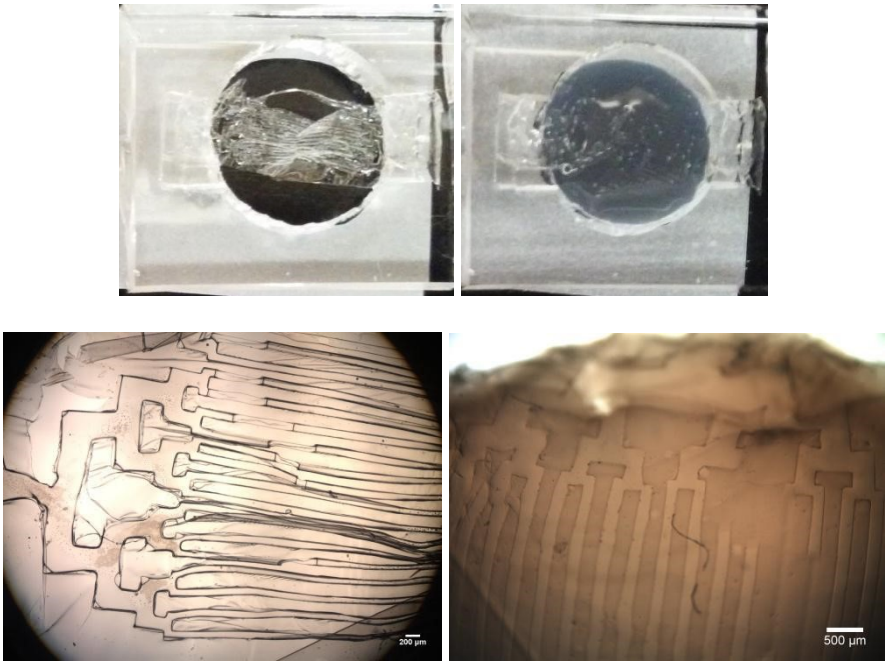


Figure 38: Sacrificial mold - photolithography feasibility study: gelatin sacrificial material formed and placed on fibrin. Top: Sacrificial material in PDMS mold before (left) and after (right) fibrin addition. Bottom: Gelatin sacrificial mold on top of fibrin (left) and pattern left on top of fibrin after gelatin melted. Note: microvascular network is on top of fibrin gel.

This feasibility study showed gelatin could be patterned on a PDMS surface, and could leave a pattern on fibrin after melting and removal. A limitation of this study was that the sacrificial material floated to the top of the fibrin so the pattern formed on the top of the fibrin gel.

4.2.3.3 Sacrificial Mold Loading Mechanisms

Part of the design of the microvascular network using a sacrificial mold included the loading mechanism of the mold into a microfluidic chamber. The loading mechanism refers to

the way the sacrificial mold is placed into the microfluidic chamber after production. Mechanisms were brainstormed and analyzed, as seen in Table 8.

Table 8: Analysis of sacrificial mold production method

Loading Method	Advantages	Disadvantages
Semi-circle laying	Allows for complex geometries Simple to "drop" sacrificial mold in place Mold does not need to be transferred once in place	Limits design of bioreactor inlet and outlet
Lateral tube-loading	Allows for complex geometries	Mold needs to be placed in tube laterally Difficult to move if loaded outside of bioreactor
Threading	Mold does not need to be transferred once in place	Limited to straight, cylindrical sacrificial molds

The semi-circle laying mechanism consisted of placing the sacrificial mold in a semi-circular portion of a mold. One design, as seen in Figure 39, was to design the inlet and outlets as semi-circles on the top and bottom of the microfluidic chamber.

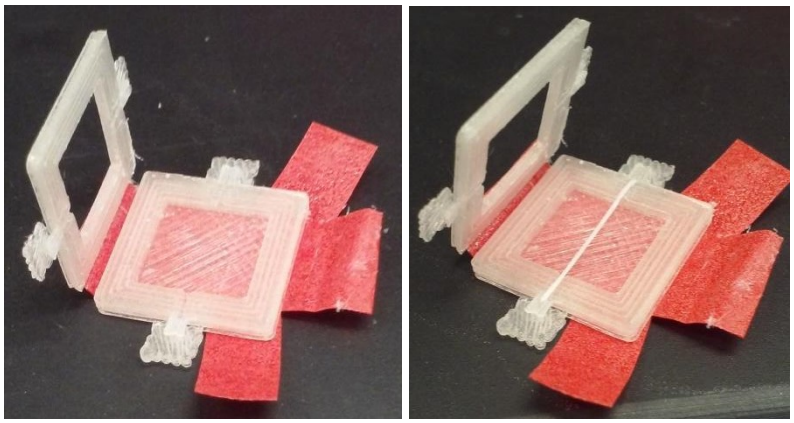


Figure 39: "Semicircle laying" loading mechanism: sacrificial material is placed into semicircular grooves in the bottom portion of a mold before the top of the mold is dropped into place.

The lateral tube-loading method consisted of placing a mold into a tube and then sliding the second tube into place over the other end of the mold, as seen in Figure 40. The main

advantage of this method was that molds with more complex geometries could be loaded into the bioreactor. Disadvantages included difficulty in loading the mold laterally and difficulty moving the tubes once the mold was in place.

Figure 40: "Lateral tube-loading" loading mechanism: a) Sacrificial material is inserted into one tube and then b) the other tube is moved laterally into place over the other end of the cylinder.

The threading method consisted of pushing a cylindrical mold through the inlet, though the volume of the mold, and through the outlet, as seen in Figure 41. The advantage of this method was that the mold did not need to be transferred once it is put in place. The main disadvantage was that the mold must be cylindrical.

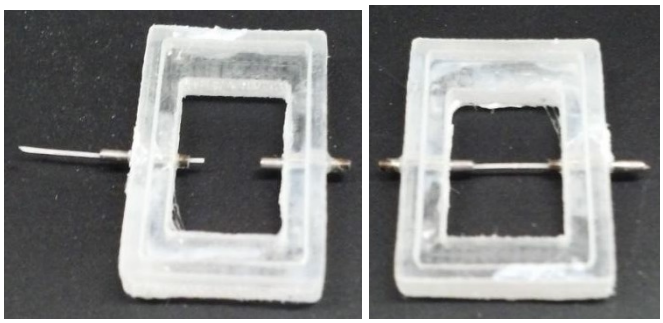


Figure 41: "Thread" loading mechanism: a) Pluronic cylinder is inserted into the inlet needle and then b) pushed and inserted through the outlet needle.

4.2.4 Final Design Selection

To select a final design for the microengineered vascular network, the team assessed vascularization methods, materials and fabrication methods.

4.2.4.1 Vascularization Method

To select a vascularization method, a Pugh Method decision matrix was created as seen in Table 9.

Table 9: Pugh Method decision matrix for the selection of the vascularization method

					Vascularization Methods			
	Selection Criteria	Spec.	Score	Weight	Angiogenesis / Vasculogenesis	Bonding of Pre-Vascularized Hydrogels	Cylindrical Rod Extraction	Sacrificial Mold
Selection Criteria	Avoid Damage due to Shear	Yes	1	5	1	1	0	1
		No	0					
	Sealing of Multiple Hydrogel Layers Required	No	1	3	1	0	1	1
		Yes	0					
	Sample Fabrication Time	Minutes (1 Step)	1	1	0	1	1	0.5
		Minutes (2 Steps)	0.5					
		Days	0					
	Sealing at Input & Output	Yes	1	6	0	0	1	1
		No	0					
	Bifurcations Possible	Yes	1	2	0.5	1	0	1
		No	0					
	Immediate Perfusion after Addition of Cells	Yes	1	4	0	1	1	1
		No	0					
	Total Score				**9**	**12**	**14**	**20.5**

The selection criteria were avoiding damage due to fluid shear upon removal of the mold, the requirement of sealing multiple hydrogels, sample fabrication time, sealing at the fluid input and output, the possibility of creating bifurcated channels, and whether or not it was possible to begin perfusion immediately after adding cells. These criteria were created based on the previously mentioned, in Section 4.2.3.1 Microvascularization Strategies.

The first step in the selection of the final vascularization method was to assign weights to the selection criteria with 6, sealing at input and output being the most important and 1, sample fabrication time, being the sixth most important. It was then determined if each method met the selection criteria as stated in the selection criteria specification in the second column.

The sacrificial mold vascularization method was selected because it met five out of six criteria in full and ended with 20.5 out of a possible 21 points. The sacrificial mold method overcomes many key limitations of other methods. There is limited damage to the hydrogel due to shear since the mold is a liquid when it is removed from the mold. An entire hydrogel is cast around a pre-fabricated sacrificial mold which means that only one hydrogel would be needed and there is no need to adhere multiple gels. The sample fabrication time for the sacrificial mold

method was slightly longer than some others because there were two steps: fabricating the sacrificial mold and forming the vascularization. However this method, on the order of minutes, is still much faster than angiogenesis/vasculogenesis, on the order of days. Because the sacrificial mold could be integrated into the microfluidic chamber before hydrogel formation, there would be proper sealing at the input and output of the hydrogel. Finally, bifurcations were possible and perfusion would be possible after the addition of cells as soon as the mold was removed.

4.2.4.2 Sacrificial Mold Material

After the sacrificial mold vascularization method was chosen, the next aspect of the final design considered was the sacrificial mold material. The final material was selected after the creation of a decision matrix, seen in Table 10.

Table 10: Pugh Method decision matrix for the selection of the sacrificial mold material

					Sacrificial Mold Material			
				Weight	Gelatin	Carbohydrate Glass	Pluronic F127	Polyvinyl Alcohol
Selection Criteria	Sacrificial Material Fabrication Ease of Use	Easy	1	1	1	0	0	1
		Difficult	0					
	Residue Free Channel	Yes	1	3	1	1	1	0
		No	0					
	Sacrificial Material Ease of Removal	Easy	1	2	1	0	0.5	1
		Moderate	0.5					
		Difficult	0					
	Total Score				**6**	**3**	**4**	**3**

The selection criteria were the sacrificial material fabrication ease of use, the ability to form a residue free channel, the ease of removal of the sacrificial material, reproducibility of geometry and extrusion rate, cost effectiveness, and ease of use with respect to removal of the produced sacrificial mold. These criteria were chosen based on the previously mentioned, in Section 2.3.3.2.4 Sacrificial Molds, as well as the sacrificial mold feasibility studies in Section

4.2.2 Feasibility studies. As in the previous section, weights were assigned to the selection criteria and total scores calculated.

The sacrificial mold material chosen for the final design was gelatin since it received 6 out of a possible 6 points. The mold fabrication was easy since it was done at room temperature, no visible residue remained in the channel in the feasibility studies, and the material was easy to remove by heating to 37°C in an incubator and flushing.

4.2.4.3 Sacrificial Mold Fabrication Method

After the sacrificial mold vascularization method and material were chosen, the sacrificial mold fabrication method was the next aspect of the final design for the microengineered vascular network. The final fabrication method was chosen after the creation of a decision matrix, seen in Table 11.

Table 11: Pugh Method decision matrix for the selection of the sacrificial mold fabrication method

					Sacrificial Mold Fabrication Method			
	Selection Criteria	Spec.	Score	Weight	Free Extrusion	Hand-Printed Extrusion	Cast in Mold	3D Printed
Selection Criteria	Bifurcations Possible	Yes	1	2	0	1	1	1
		No	0					
	Reproducible Geometry	High/NA	1	3	1	0	1	1
		Lower	0					
	Reproducible Extrusion Rate	High/NA	1	1	1	0	1	1
		Lower	0					
	Cost Effective	Yes	1	4	1	1	1	0
		No	0					
	Ease of Use - Mold Removal	Yes	1	5	1	1	0	1
		No	0					
		Yes	1					
		No	0					
	Total Score				**13**	**11**	**10**	**11**

The selection criteria were the ability to form bifurcations, reproducibility of geometry and extrusion rate, cost effectiveness, and ease of use with respect to removal of the produced sacrificial mold. These criteria were chosen based on the previously mentioned, in Section

4.2.3.1 Microvascularization Strategies. As in the previous section, weights were assigned to the selection criteria and total scores were calculated.

The fabrication method chosen for the final design was free extrusion since it received 13 out of a possible 15 points. The free extrusion method allowed for reproducible geometry and extrusion rate, was cost effective, and mold removal was easy. The free extrusion method was however limited to single channel molds. The strategy of this project was to take incremental steps and for this reason initial tests were planned to be conducted on single channel systems.

The hand-printed extrusion method was selected as a secondary fabrication method for this project to form bifurcated channels. The 3D printed method, though it received the same score as the hand-printed extrusion method, was cost- and time-prohibitive for this project.

4.2.4.4 Sacrificial Mold Loading Method

After the sacrificial mold vascularization and fabrication methods were chosen, the next aspect of the final design for the microengineered vascular network was the sacrificial mold loading method. The final loading method was chosen after the creation of a decision matrix, seen in Table 12.

Table 12: Pugh Method decision matrix for the selection of the sacrificial mold loading method

					Sacrificial Mold Loading Method		
	Selection Criteria	Spec.	Score	Weight	Semi-Circle Laying	Lateral Tube-Loading	Threading
Selection Criteria	Bifurcations Possible	Yes	1	2	1	1	0
		No	0				
	Ease of Loading	Easy	1	3	1	0	0
		Difficult	0				
	Sacrificial Mold Added in Place	Yes	1	1	1	0	1
		No	0				
	Total Score				**6**	**2**	**1**

The selection criteria were the ability to form bifurcations, ease of loading, and loading the sacrificial mold in place without having to transfer to another location. These criteria were

chosen based on the previously mentioned, in Section 4.2.3.1 Microvascularization Strategies. As in the previous sections, weights were assigned to the selection criteria and total scores were calculated.

The loading method chosen for the final design was semicircle laying because it scored 6 points out of a possible 6. This method allowed for loading of both single and bifurcated channels. It was easy as the mold was simply dropped into place, and did not need to be transferred after loading. To summarize, the final design for the microengineered vascular network was a gelatin sacrificial mold fabricated via free or hand-printed extrusion and loaded by semi-circle laying method.

4.3 Bioreactor and Microfluidic Chamber

The bioreactor and more specifically, the microfluidic chamber was designed to contain the fibrin layer and the cardiac scaffold during experimentation through perfusion. There are a series of functions that needed to be addressed in each microfluidic chamber design in order for the system to be effective. The functions are discussed in detail in Section 4.3.1 Needs analysis.

4.3.1 Needs analysis

Based on the objectives of the bioreactor system, the team determined what elements were necessary for the success for the project. It was determined that for the bioreactor to be successful, it needed to control and limit the gas permeability of the system and be reproducible. The control over gas permeability was necessary, because the users needed to be able evaluate the efficiency of the microvascular network in delivering nutrients and gases to cells seeded in the tissue scaffold. Along with that, the bioreactor needed to generate reproducible results under the same conditions, in order for the system to be a successful research tool. The bioreactor needed to have a fabrication protocol that would allow it to be accurately reproducible.

Wants determined by the set objectives on the bioreactor were for the system to allow for multiple samples to be sustained simultaneously, to be easy to use, and to be cost effective. If the bioreactor system was capable of sustaining a single tissue scaffold, maintaining multiple samples simultaneously would be a question of scaling up the components. According to the clients and the users, cost effectiveness and ease of use were secondary objectives. If the bioreactor system achieved all other objectives, it would already be considered a successful system.

4.3.2 Functions & Means

Based on the needs of the clients and users, a list of functions the device needed to perform was created. The functions are in no particular order. All needs and specifications that should be achieved to result in a successful bioreactor were listed in Section 3.8 Specifications. When design functions were determined, the team brainstormed different means of accomplishing the functions. In Table 13 below, a list of the functions and means for the bioreactor can be seen. After different means were determined, each was evaluated based on its capabilities of reaching the required specifications.

Table 13: Functions means chart for bioreactor

Function	Means							
Allow for continuous perfusion	Peristaltic Pump		Syringe Pump		Gravity Fed			
Controlled gas exchange	Permeable Coverslip				Impermeable Lid of Closed Bioreactor			
Maintain sterility	Gasket Tape		Clips			Screws & Gaskets		
Allow visualization	Polyethylene		Silicone		PDMS		Glass Coverslip	
Contain Fibrin Microvascular Network	Rectangular Mold	Two Piece Peg Mold	Tongue & Groove Mold	Box Mold	Simple Mold	Hinged Mold	Insert Mold	Tetris Mold

One function of the microfluidic chamber is to allow for continuous perfusion throughout the microvascular network in the mold. Along with this function are the specifications of proving flow of 0.001 mL/min and maintaining that flow for up to three weeks. This could be achieved with the use of a peristaltic pump, syringe pump, or gravity fed system. The peristaltic pump could be beneficial because the rate of perfusion could be set prior to beginning an experiment and the pump is able to mimic pulsatile flow. A drawback to this pump is that the perfusion rate did not produce a consistent flow when the rate was at its lowest speeds around 0.14 mL/min. A specification explained in the previous chapter was that the pump must precisely reach flow rates between 0.0015 - 0.21 mL/min. Therefore, the peristaltic pump could not meet this specification. A benefit of the syringe pump was that the amount of fluid that will flow through the system could be predetermined. Additionally, the syringe pump allowed for rates of flow as low as 0.001

mL/min. Another advantage to the syringe pump was that the amount of fluid flowed through the system could be controlled with the size of the syringe used, meaning the length of the experiment can be modulated to the time needed. The gravity fed system removed the need for electricity to power the system but a drawback was the difficulty to maintain a constant flow rate. This was difficult because as the media was depleted, the force of gravity became less, decreasing the flow rate. In Table 14 below, the advantages and limitations of each method can be seen.

Table 14: Advantages & limitations for continuous perfusion methods

Function - Allow for controlled continuous perfusion		
Means	**Advantages**	**Limitations**
Peristaltic pump	Mimics *in vivo* pulsatile flow	Does not reach low flow rates well One sample
Syringe pump	Continuous flow Controlled lower flow rates Volume modulation for long experiments Multiple samples	Large size
Gravity fed	Does not require power	Hard to control flow rate

Another function was to allow for controlled gas permeability. This allowed the team to control how the system and tissue was supplied with oxygen. The system must only allow the tissue to receive oxygen from the perfusion of media. This would mean the tissue was supported only by the microvascular network. Using two methods, one impermeable and one with oxygen, would give the most strict gas monitoring and allow for controlled gas exchange. Means for this include the use of impermeable coverslips and lids as seen in Table 15.

Table 15: Advantages & limitations for gas exchange methods

Function - Controlled gas exchange		
Means	**Advantages**	**Limitations**
Impermeable coverslip	Not gas permeable	Delicate; Easily broken
Impermeable Lid of Closed Bioreactor	Not gas permeable	Difficult to find perfectly transparent material

The system must also be tightly sealed to ensure a sterile environment. This could be achieved by using screws, gaskets, or clips to provide a tight seal against gases and liquids. Each means created and ensured a tight seal from the outside environment by securing each piece of the microfluidic chamber, making the device airtight and fluid tight. The screws along with gaskets would be most efficient for ensuring an airtight seal since the screws would tighten and keep the entire system in place, also preventing air from entering the system around the gasket. This would also prevent fluid from leaking around each piece of the chamber. Clips could be applied to many of the designs which simply allow for a tight close between the lid and base. However, although this ensured the lid will remain in place on the chamber, this did not necessarily prevent air from entering the system if there were regions on the sides of the chamber where air could enter. Table 16 contains the advantages and limitations of the means to maintain sterility.

Table 16: Advantages & limitations for sterility methods

Function - Maintain sterility		
Means	**Advantages**	**Limitations**
Gasket	Airtight Allows for separation of like materials	Not always reusable
Screws	Secures layers of bioreactor Reusable	Could be difficult to fabricate threads in device
Clips	Cheap	Bulky

The fourth function was to allow for visualization. The bioreactor needed to allow for imaging with a microscope and observation throughout its use. This could be done with various materials such as glass coverslips, silicone, and polyethylene. Table 17 contains the advantages and limitations of the means to allow for visualization. In addition the height profile of the chamber has to be below 19.50 mm in order to obtain a clear image from the microscope.

Table 17: Advantages & limitations for transparent materials

Function - Allow for visualization		
Means	**Advantages**	**Limitations**
Glass coverslip	Easily accessible Rigid Gas impermeable Cheap	Difficult to clean/reuse Fragile
Silicone	Gas permeable Cheap	Flimsy/flexible
PDMS	High gas permeability Cheap	Can be difficult to fabricate such a thin sample Soft & flexible
Polyethylene	Cheap	Not very strong

The final function was to contain the microvascular network with a formation mold. This is where the fibrin is formed and what will hold the gel throughout testing. This mold needed to be easy to use for simple or complicated vascular networks and hold liquids and the hydrogel without leakage. It is the link between the microvascular network and the bioreactor as a whole. This mold could vary in design and alternatives can be found later in Table 19. The advantages and limitations for containing the microvascular network were categorized for each means of completion and can be seen in Table 18 below.

Table 18: Advantages & limitations for containing the microvascular network

Function - Contain microvascular network		
Means	**Advantages**	**Limitations**
Rectangular One Piece Mold	Easy to set up	Allows for only straight channels
Rectangular One Piece Mold without Nozzles	Allows for use of needle tips	Movement of needle tips could occur
Two Piece Peg Mold	Allows for bifurcated channels	Difficult to manufacture
Two Piece Mold w/ Base	Allows for bifurcated channels	Must be held together with clips Difficult to line up
Tongue and Groove Mold	Easier to manufacture than peg mold Allows for bifurcated channels	Top and bottom pieces could slide
Box Mold	Allows for bifurcated channels Assists in alignment	Top and bottom pieces could slide Top piece can warp, releasing the seal
Simple Mold	Allows for bifurcated channels	Poor alignment outside of bioreactor
Hinged Mold	Allows for bifurcated channels Assists in alignment Top and bottom constantly attached	Difficult to manufacture Difficult to accommodate in a chamber
Insert Mold	Allows for bifurcated channels Reduces axis of rotation during use	Fragile Difficult to fabricate
Tetris Mold	Allows for bifurcated channels Assists in alignment	Fragile Difficult to fabricate

4.3.3 Bioreactor Feasibility Studies

4.3.3.1 Glass vs Transparent Material

In order for visualization to be possible, either glass coverslips must be used or transparent materials such as polyethylene and silicone. The team spoke with professionals in the field of materials as well as manufacturing. After a few conversations, it became clear that glass would be the best option. Polyethylene and silicone are difficult to machine especially when the end result needs to be transparent and produce a clear image when viewing through a microscope. Machining any surface of these clear materials would leave microscopic scratches that leave a haze or uneven clarity on the material. This haze would be unacceptable because it would distort the image under a microscope. Polyethylene poses the highest chance of working compared to silicon because it is commonly machined for many products and can be almost 100% transparent. The team looked at microscope images of machined polyethylene from literature. One study shows the following image (Figure 42) of polyethylene after being milled.

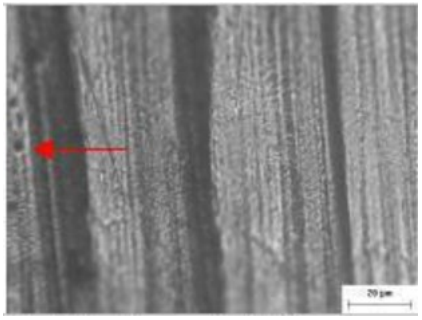


Figure 42: Microscopic image of milled polyethylene (Carr & Feger, 1993)

There are many imperfections in the material once machined. The high feed rate had great influence in the generation of the surfaces roughness. Glass coverslips and slides are used in imaging because of their transparency. Glass slides are also produced in multiple sizes and thicknesses so there is no need to machine it. Glass therefore is the best option for a viewing window.

4.3.3.2 Clips vs Screws

To determine whether clips or screws would work best in keeping the microfluidic chamber closed tightly to ensure sterility, a feasibility test was performed. Clips were used to hold down a top and bottom piece of 3D printed PLA mold and filled with water which can be seen below in Figure 43.

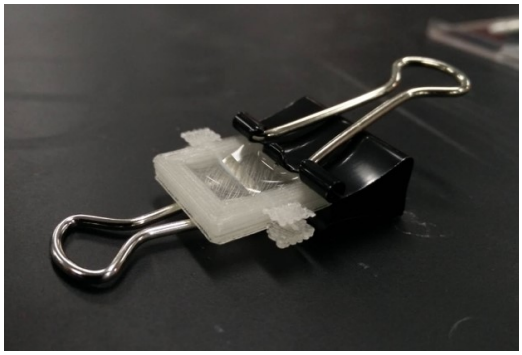


Figure 43: Using clips to hold PLA mold together

The system was placed into the incubator for twenty-four hours. After the twenty-four hours, the system still had water remaining within the top and bottom pieces with no signs of leakage. Because of the humidity of the incubator, the clips oxidized leaving rust on the PLA which can be seen in the Figure 44 below.

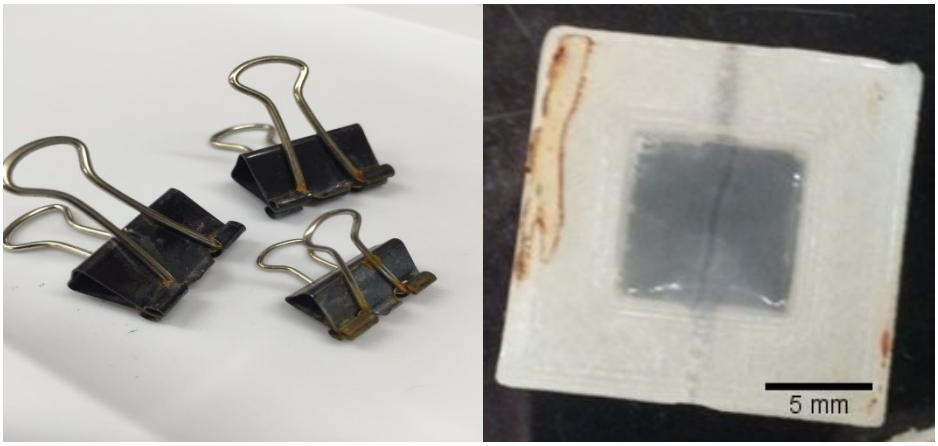


Figure 44: Corrosion buildup on binder clips. a) Rusty clips b) rust on mold

The rust left from the clip would ruin the developed microfluidic chamber so screws would be the best option at keeping the system closed while maintaining sterility.

4.3.3.3 Inlet and Outlet

Previous work in Pins Lab used needle tips as inlets and outlets that were not fused, for their mold designs. The needle tips could be pushed into or out of the system when attaching tubing, causing structural damage to the fibrin gel. Other options were looked into for inlet and outlets such as 3D printing inlet and outlet nozzles, infusing needle tips into our final design, or having only openings as opposed to extruded nozzles. Below, Figure 45 is shows a 3D printed mold made from PLA.

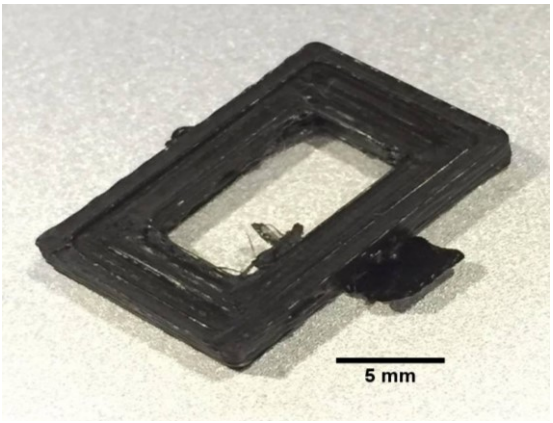


Figure 45: Attempt to 3D print nozzle

The pieces of material extruding out of the right side and bottom of the mold are the supports for the nozzles; the nozzles, however, themselves did not print, even though they were included in the CAD designs. 3D printing inlet and outlet nozzles when printing the molds proved to be extremely difficult, because the available 3D printer lacks the precision necessary to form the miniscule nozzles. In order to print the nozzles, the pieces needed structural support; however the support was still unsuccessful in allowing the nozzles to print.

An alternative method to this was to design for holes as inlets and outlets as opposed to extruding nozzles where metal needle tips could be used. To prevent movement of the needles when attaching or detaching tubing, the metal tip could be heated to a temperature higher than the glass transition temperature of PLA, 60°C, and then fused into the holes of a 3D printed

mold. This would permanently secure the tips in place and not allow them to move during experimental testing. A feasibility study was performed to see if this method would be effective. First the tip was heated through an open flame as seen in Figure 46 below.

Figure 46: Heating needle tip over flame

Once the tip turned orange (800 - 900°C), it was removed from the flame and immediately placed into the two piece mold as seen below in Figure 47. Immediately, the PLA touching the heated metal began melting around the tip. As the PLA cooled, it solidified around the metal tip, securing it into place.

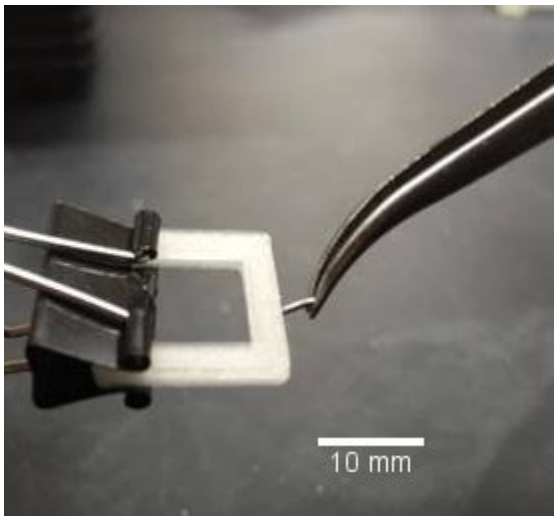


Figure 47: Inserting needle tip into inlet

After letting it cool for a few minutes, pushing and pulling the needle tip with pliers resulted in no movement, proving to be very secure. Fusing the needle into the mold was an effective method of securing the nozzles. The fused needle tips can be seen in Figure 48 below.

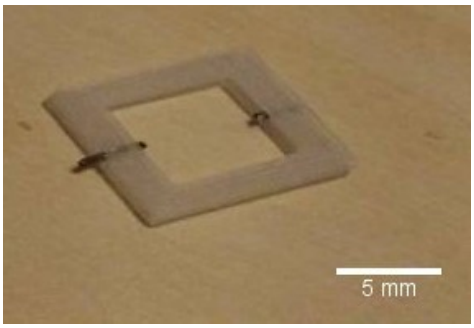


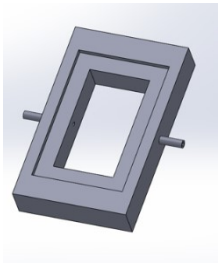
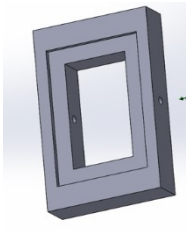
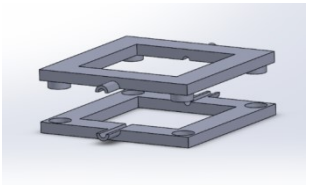
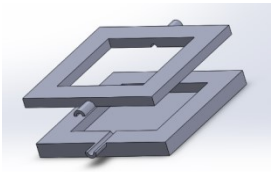
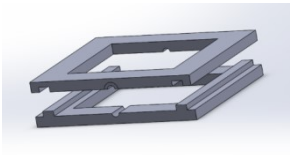
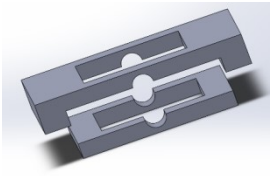
Figure 48: 3D printed mold with fused needle tips in inlets

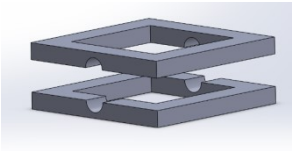
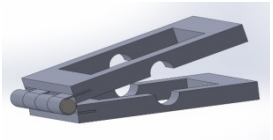
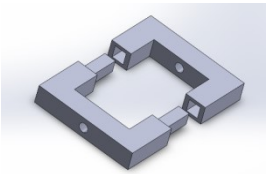
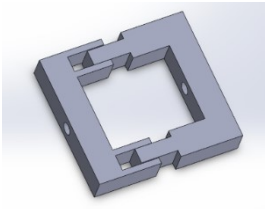
The issue with this method is that fusing both needles to be perfectly straight was extremely difficult by hand. It was very important for the needles to be straight for the creation of a linear channel through the mold. The third option was to just leave a hole for the inlet and outlet where tubing or full needles could be inserted to. As long as the tolerance between the tubing or needle and the holes were small enough, there would be enough resistance to ensure a secure fitting. Given the advantages and limitations of these methods, leaving a hole where a needle or tube could be inserted into seemed to be the most feasible option.

4.3.4 Conceptual & Alternative Designs

The team brainstormed a series of designs, both for the mold and the microfluidic chamber. A summary table of the conceptual and alternative designs for the mold piece can be seen in Table 19 below.

Table 19: Design alternatives of mold

Design Stage	Design Alternative	Model
Conceptual Designs	*Rectangular One Piece Mold*	
	Rectangular One Piece Mold without Nozzles	
Alternative Designs	*Two Piece Peg Mold*	
	Two Piece Mold with Base	
	Tongue and Groove Mold	
	Box Mold	

	Simple Mold	
	Hinged Mold	
	Insert Mold	
	Tetris Mold	

Molds

The following molds were designed to hold the team's microvascular network. The mold is placed into the final bioreactor design and connected to tubing, allowing perfusion through the mold. The mold was not designed to be airtight itself because that function would be fulfilled by the microfluidic chamber. The size of the mold was designed to fit the myocardial layer being provided to the team to ensure a secure fit and no movement of the mold upon movement of the microfluidic chamber.

Rectangular One Piece Mold

The Rectangular One Piece Mold design is the simplest of the mold designs. This mold has an inlet and outlet nozzle allowing tubing to be connected. The design is very robust because of the entirety of the mold being one piece. This contributes to why this mold is user friendly. Fabricating the nozzles could pose a challenge because of the small diameter. Defects within the inlet have potential to affect the perfusion, especially with an already very low flow rate. The external nozzles can be seen in the Figure 49 below.

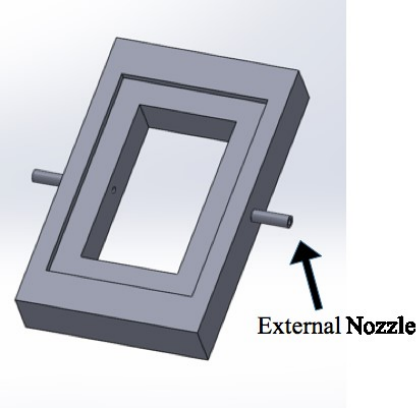


Figure 49: Rectangular One Piece Mold

Rectangular One Piece Mold without Nozzles

This design is a replica of the previous design with one modification. The nozzles were removed because of the difficulty to fabricate a nozzle with such a small diameter. Now, there is an inlet and outlet hole where tubes could be fed through or needles could be fused in. The inlets can be seen in Figure 50 below.

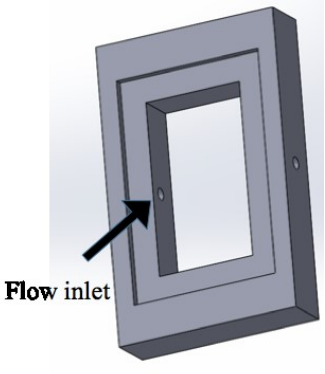


Figure 50: Rectangular One Piece Mold without Nozzles

Two Piece Peg Mold

This design uses pegs to align the top and bottom of the mold, seen in Figure 51.

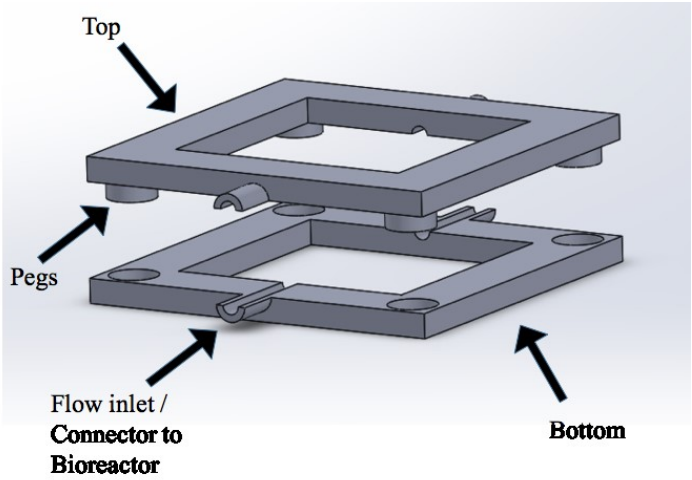


Figure 51: Two Piece Peg Mold

The pegs ensure an accurate fit when stabilizing the mold. The flow inlets were designed so that tubing could easily be attached around them. Fabricating connectors and pegs are very difficult because of the small size of the mold. Manufacturing the pegs with the correct tolerance to fit tightly into the holes is a challenge at the millimeter scale. Manufacturing small diameter nozzles precisely with no defects is also difficult. Defects within the inlet could potentially affect the perfusion, especially at low flow rate.

Two Piece Mold with Base

This mold was designed to be used only outside the bioreactor for testing. The mold has a bottom that cannot be imaged through, but the fibrin does not leak out the bottom when moved or when not in contact with a glass slide on the bench top, as seen in Figure 52. As in previous mold designs molds, the nozzles on the sides are hard to manufacture.

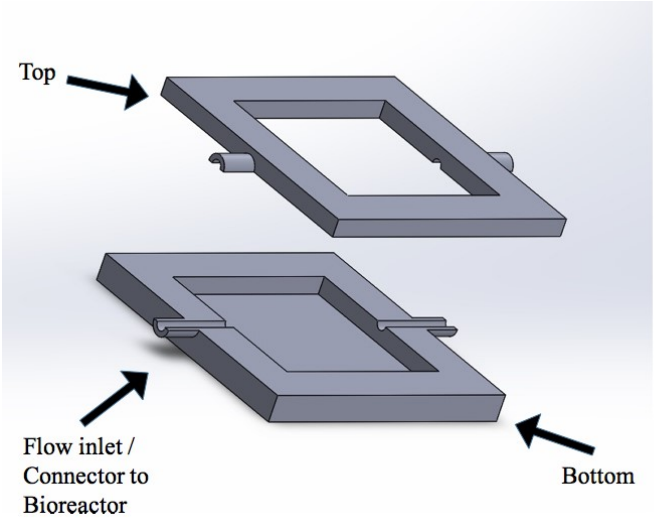


Figure 52: Two Piece Mold with Base

Tongue and Groove Mold

This design is similar to the Two Piece Peg Mold, but for ease of fabrication, the pegs have been removed. They have been replaced with grooves in the top piece that are placed around extruded material on the bottom piece. In turn, this secures movement in one direction. With this design, the top still has a degree of movement in the y direction, front and back. To further simplify the fabrication of the mold, the inlet and outlet nozzles were removed seen in Figure 53. Now, there is an inlet and outlet hole where tubes could be fed through or needles could be fused.

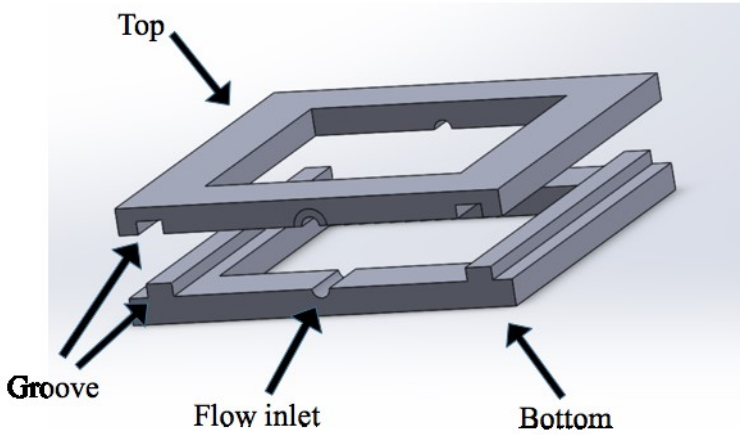


Figure 53: Tongue and Groove Mold

Box Mold

This mold has a much simpler design than the Two Piece Peg Mold. There is a top and bottom that incorporates an inlet in the middle of the two, seen in Figure 54. This design removes the extruded nozzles and pegs, both features that are difficult to fabricate. These inlets allow tubing or needles to be inserted straight through both the microfluidic chamber and the mold, up to the microvascular network. The lip of the top piece wraps around the sides of the bottom piece to stabilize the mold and provide an accurate fitting and alignment.

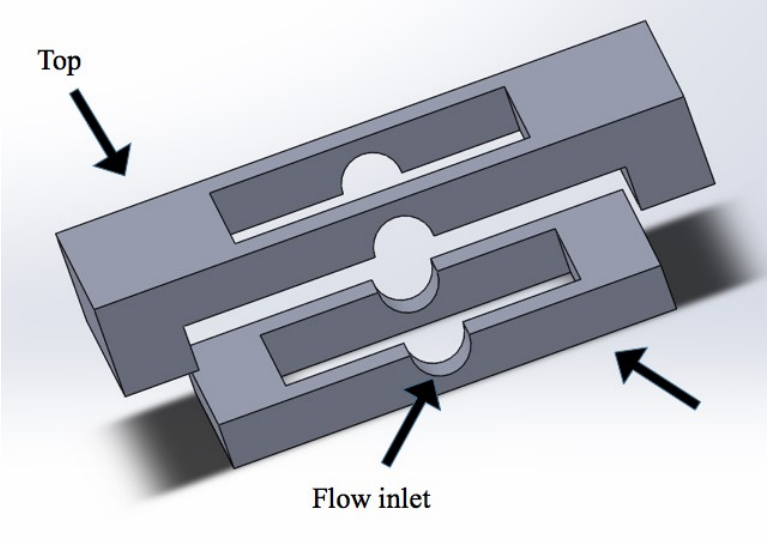


Figure 54: Box Mold

Simple Mold

This is a simple design for a mold. A slight modification was made to the previous design to remove the lip from the lid, seen in Figure 55. This is because its only use would be inside the microfluidic chamber. It is difficult to align the top and bottom pieces of the mold outside the microfluidic chamber. While in the microfluidic chamber, the mold would be secured in place, therefore eliminating this issue. Tubing or needles could be placed inside the flow inlets on each end of the mold.

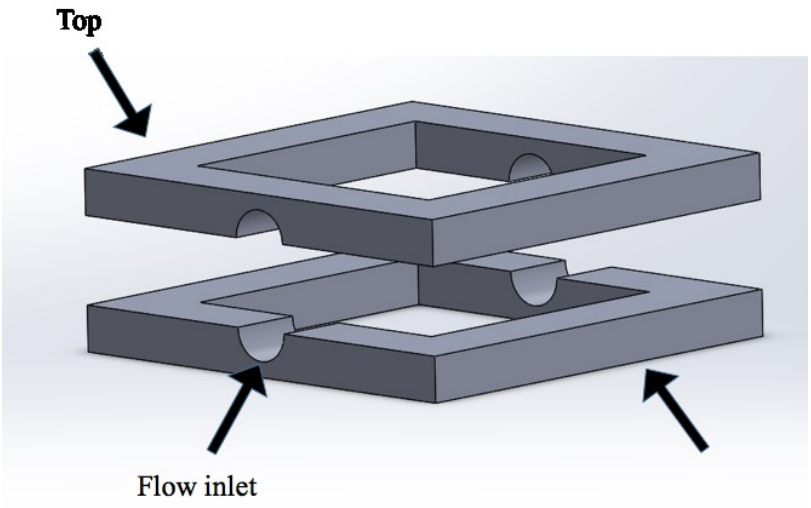


Figure 55: Simple Mold

Hinged Mold

The Hinged Mold design uses a hinge for easier handling and alignment of the mold. With this connection between the top and bottom piece, the entire mold becomes only one piece. This concept disposes the need of any pegs or lip around the top piece to stabilize the mold. This design is very user friendly and simple. Similar to the previous design, tubing or a needle will be inserted straight through both the bioreactor and the mold right up to the microvascular network,

as seen in Figure 56. Fabricating the hinge could be a challenge because of how small and thin it is. The diameter of the hinge would allow a one millimeter pin to fit through it.

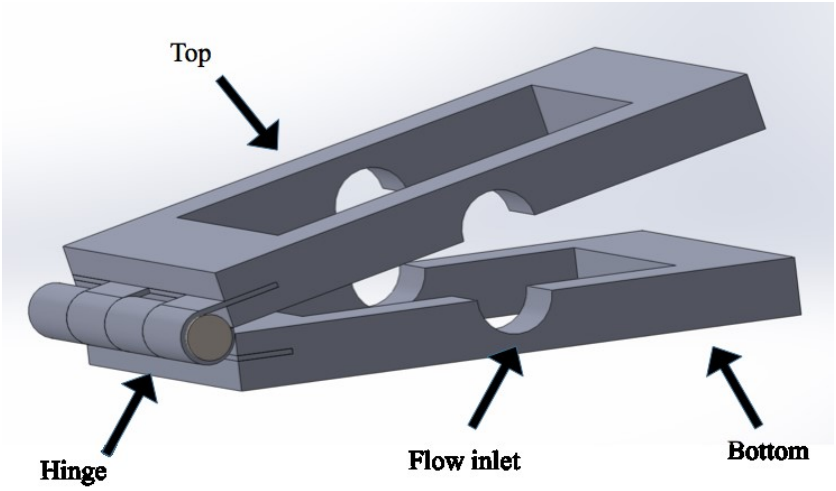


Figure 56: Hinged Mold

Insert Mold

This mold was designed with a different way of ensuring stability. Here, pegs are used that would be pushed into the insert holes, securing the two halves of the mold. As opposed to having a top and bottom, this design has a left and right piece that come together, as seen in Figure 57. The flow inlet and outlet are through the center of the mold. Similar to the past designs, tubing or a needle will be inserted straight through both the bioreactor and the mold right up to the microvascular network. The tubing or needle would be secure in these holes because they are designed to fit a very specific diameter. Needles could be fused to the inlets of the mold to ensure a lesser chance of needle movement. Keeping the mold together and sealed in the middle where the two halves meet may pose a challenge.

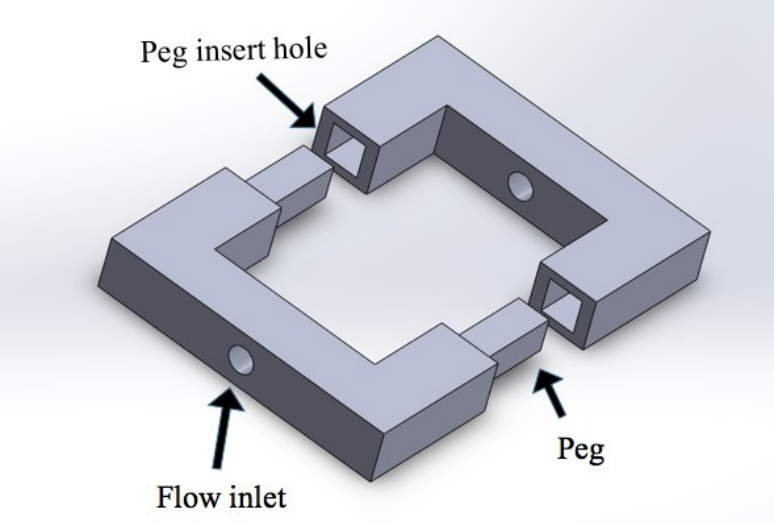


Figure 57: Hinged Mold

Tetris Mold

This mold was designed similarly to the Insert Mold, but modified to simplify its fabrication. The Insert Mold can be seen in Figure 58. Manufacturing this mold would be less complicated than creating the pegs of the previous design. Though creating the mold would be simpler, this design is not as stable. Here, there is chance of rotation. Either side of the mold could bend up or down around the point of contact, unlike the Insert Mold where the pegs ensure there cannot be any movement in the vertical plane.

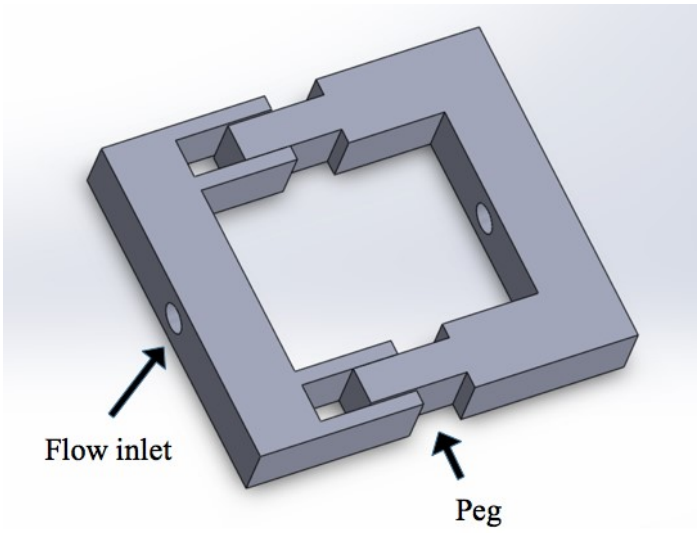
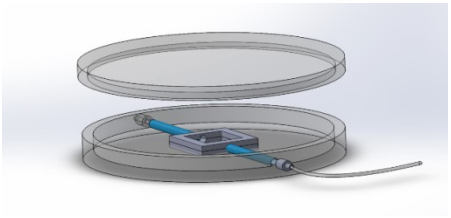
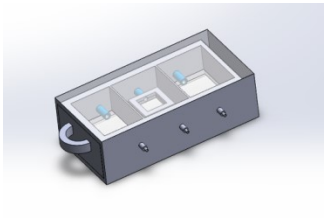
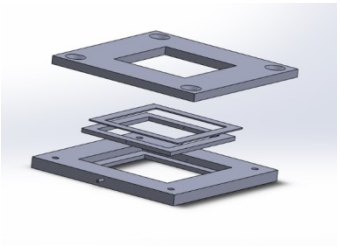
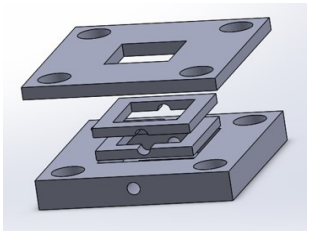
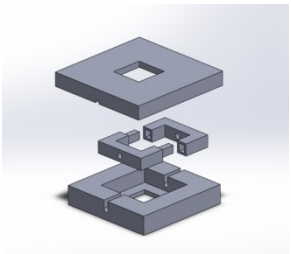


Figure 58: Tetris Mold

Microfluidic Chamber

The brainstorm process resulted in five alternative designs of a microfluidic chamber: three conceptual and two preliminary. The conceptual designs include the Petri Dish, Drawer, and Layered. The preliminary designs include the Pressure Square and the Boxed. The preliminary alternative designs can be seen in Table 20 below.

Table 20: Preliminary design alternatives of microfluidic chamber

Design Stage	Design Alternative	Model
Conceptual Designs	*Petri Dish*	
	Drawer	
	Layered	
Alternative Designs	*Pressure Square*	
	Boxed	

Petri Dish

This design was created for its simplicity. Because Petri dishes are common items in laboratories, this design could be created by modifying an existing product. Holes where the nozzles would enter would be drilled into the sides of the base of the Petri dish as seen in Figure 59. The external and internal nozzle is one piece that would be placed through the drilled hole on either side of the dish. The lid is a typical Petri dish hood which is transparent.

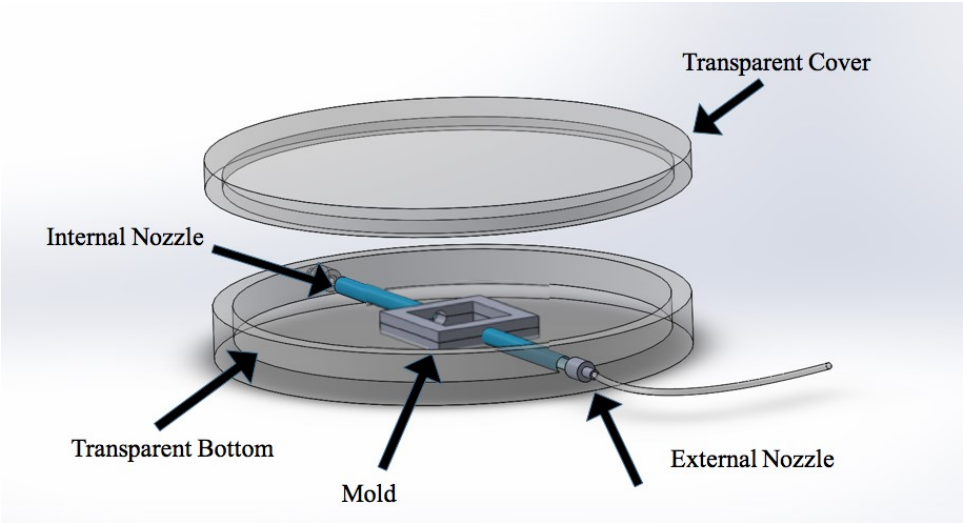


Figure 59: Petri Dish Design

The transparent cover and base allow for imaging with a microscope. The cover is easily removable if necessary. Each nozzle design involves an external and internal portion that both make one piece. The nozzle would be inserted into the separate mold containing the microvascular network to ensure that there is no gap where fluid flowing through the created channels could leak through. This ensures consistent input and output through the network. This design would be most convenient to manufacture, as it would simply involve drilling holes into the dish and inserting nozzles. Though it would be simple to manufacture, drilling holes into a Petri dish would not be ideal because the drilling process will most likely shred the material of the dish and make it difficult to re-seal, causing it to lose its sterility. Another problem with this

design is that it can only accommodate one of the molds which carry the microvascular network since there are no divisions or walls within the Petri Dish design.

Drawer

This concept was created to mimic a drawer with a removable container to allow for an easy and efficient means of mounting the mold, as shown in Figure 61. The outside of the drawer contains three external nozzles matching up with the three internal nozzles of the removable container, labeled in the Figure 60. This design can accommodate up to three molds and can be modified to allow for more or less samples. The mold designed to hold our samples would connect to the internal nozzles of the microfluidic chamber to allow for continuous isolated flow. Each sample is enclosed by a wall to ensure cross contamination does not occur. The top and bottom of both pieces are transparent to allow for visualization of the samples using a microscope. With proper insulation on all edges of the drawer, this design would be airtight, maintaining the sterility of the samples.

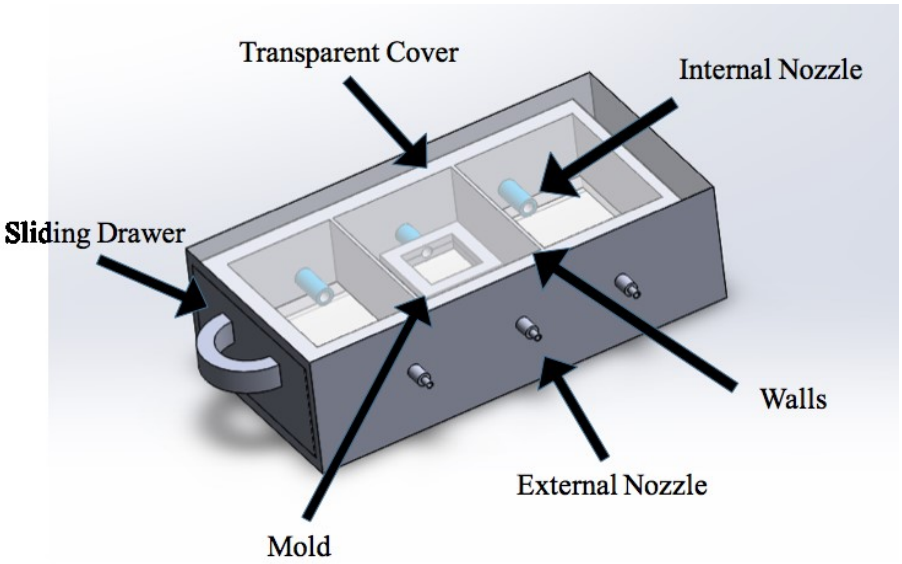


Figure 60: Drawer design

This alternative design is beneficial because it is robust and sturdy. Accessing the samples would be simple and quick. Simple modifications to the CAD design would allow for as many samples as the user would like this microfluidic chamber to accommodate. This design could be difficult to manufacture in order to align the holes of the inside and outside compartments. The diameters of the nozzles are so small that aligning them precisely could be difficult. If not aligned, media could leak between the two parts during perfusion. In order to assure an airtight seal, the tolerance must be very small and manufacturing such a small product so accurately could be difficult.

Layered Design

This design was created utilizing screws to keep the system together and maintain sterility. The center opening in the top and bottom pieces each hold a coverslip to allow for visualization. The bottom piece has a cavity where the inner layers fit into. This allows the top and the bottom pieces to sit on each other and create a closed system when the screws are in place. This design also has a spacer for the myocardial layer to fit into allowing the top and bottom to sit flush. There are external nozzles for tubing to be connected allowing media to be perfused through the microvascular network. Each layer of the microfluidic chamber can be seen in Figure 61.

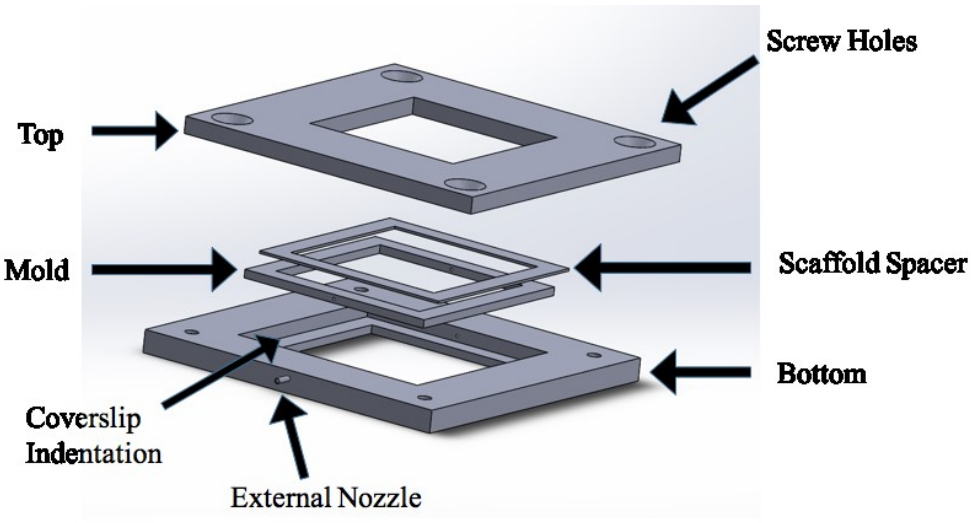


Figure 61: Layered design

This design is airtight once sealed, maintaining the sterility of the microvascular network. Unlike the other conceptual designs, this design has many components that are all independent of each other. This makes it less user friendly because the user would need to manually place more parts into the chamber, leaving more room for human error. Another issue is the difficulty in fabricating the external nozzles. The nozzles are very small and finding a machine that could manufacture such a small diameter precisely has proven to be difficult.

Pressure Square Design

This design simplifies the previous Layered design by removing the scaffold spacer and using a different mold, as shown in Figure 62. Instead of using the scaffold spacer to create more space for the microvascular network, the client would use vellum paper surrounding the myocardial layer. This design also uses screws to tighten the top and bottom, creating an airtight, sterile environment. The holes on the sides of the bottom piece of the microfluidic chamber line up with the holes of the mold to allow for perfusion.

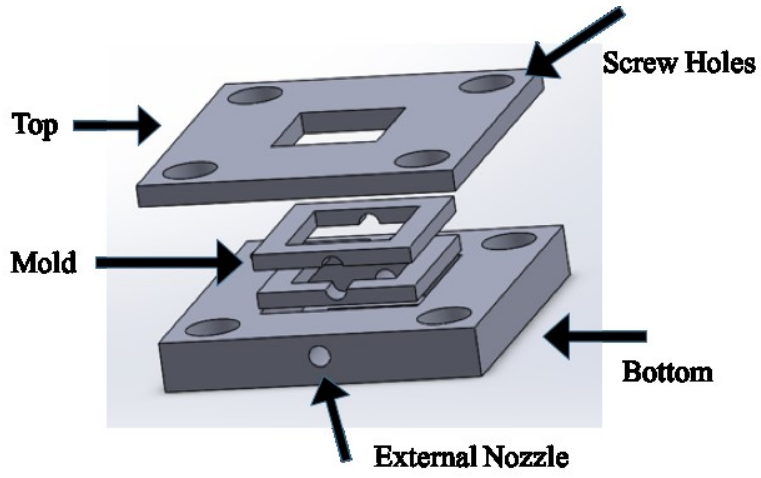


Figure 62: Pressure Square design

This design is not very user friendly because it could be difficult to insert a sacrificial material into the mold when it is in the microfluidic chamber. Although this is the case, with use of the cylindrical rod extraction method for creating a channel, the setup could be completed in very few, simple steps. With this specific design, the user is restricted to only one sample though it can be easily modified to accommodate more samples. The inlet diameters have been increased in order to fabricate them more easily and accurately.

Boxed Design

This design seen in Figure 63 incorporates a lid with a lip which helps secure the entire microfluidic chamber. The setup is very similar to the Pressure Square design. What has been changed is that the top now has a lip that will wrap around the bottom piece; this can be better seen in Figure 64. Also, a different mold is used in this version. The sides have been extended to allow for easier handling of the microfluidic chamber.

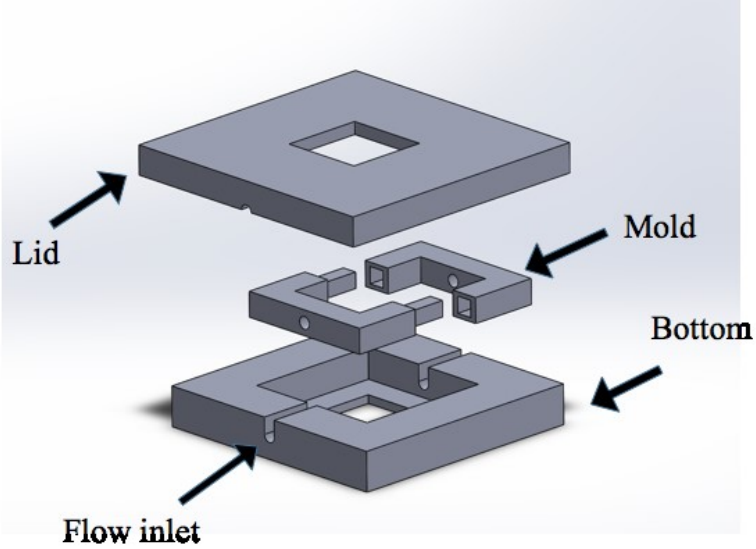


Figure 63: Boxed microfluidic chamber top view

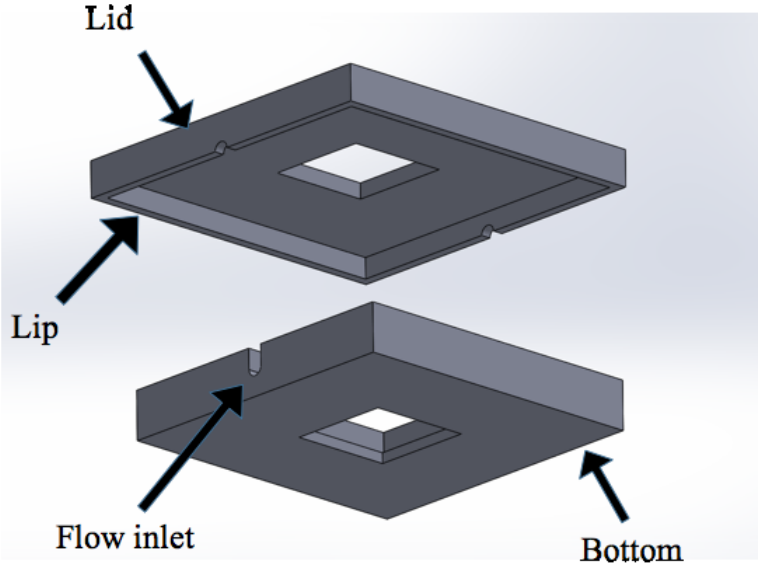


Figure 64: Boxed microfluidic chamber bottom view

This design incorporates all the aspects of the Pressure Square design. The addition of the lip is to provide increased stability and an accurate fitting. Also, the half circle inlet on the bottom piece is not a full circle to allow for tubing or a fused needle connected to the mold to easily fit into place by just lowering the entire part into the microfluidic chamber. The other half of the inlet was incorporated into the lid which would be lowered onto the bottom.

4.3.5 Final Bioreactor Selection

4.3.5.1 Final Mold

To select a final mold and microfluidic chamber design, a Pugh Method decision matrix was created as seen in Table 21 and Table 22. The Pugh Method is a decision-matrix that incorporates end-use applications of design objectives as the initial product specifications. This technique establishes a set of criteria options which are scored and summed to gain a total score. The higher the score, the better that design satisfies that selection criteria. This method is not weighted to provide a quick selection process, each design was discussed and analyzed prior to creating this matrix in order to properly score each (Thakker, Jarvis, Buggy, & Sahed, 2009).

Table 21: Pugh Method decision matrix for the selection of the mold

	Selection Criteria	Spec.	Score	Weight	Two Piece Peg	Two Piece with Base	Tongue and Groove	Box	Simple	Hinged	Insert	Tetris
Selection Criteria	Ease of Use	High	1	2	1	1	1	1	1	1	1	1
		Low	0									
	Secure Sample	Yes	1	5	1	1	1	1	1	1	1	1
		No	0									
	Visualization	Yes	1	6	1	0	1	1	1	1	1	1
		No	0									
	Stability	High	1	1	1	0	0	0	0	1	1	1
		Low	0									
	Ease of Sacrificial Mold Loading	High	1	4	1	1	0	1	1	1	0	0
		Low	0									
	Ease of Manufacturability	High	1	3	0	0	0	1	1	0	0	0
		Low	0									
	Total Score				**18**	**11**	**13**	**20**	**20**	**18**	**14**	**14**

The selection criteria were ease of use, being able to secure the sample, allowing for visualization under a microscope, stability of the entire mold, allowing for ease of sacrificial mold loading, and ease of manufacturability. These criteria were created based on the advantages and limitations of the different molds presented in Section 4.3.4 Conceptual & Alternative Designs. In order to select a final mold design, each of the selection criteria were assigned weights 1 - 6. 6, being the most important, was assigned to visualization and 1, being the least important, was assigned to stability. It was then determined whether or not each mold design could meet the selection criteria.

The Box and Simple mold ranked highest, both with 20 out of 21 points. Both of these designs satisfied all of the selection criteria except for stability. The other molds have a mechanism that secures the top to the bottom whether a hinge or pegs. The Box and Simple molds do not provide any similar mechanism for stability but this was ranked lowest because a function of the microfluidic chamber is to secure the mold, providing the necessary stability. For further analysis, both of these molds were 3D printed and tested. After testing, it was determined the simple mold would satisfy the selection criteria best. Both are easy to use, but setup and cleanup of the simple mold was easier than the box mold because there were no edges or pockets to clean. Finally, the simple mold was much easier to manufacture solely because of its simplistic design.

4.3.5.2 Final Microfluidic Chamber

The selection criteria for the microfluidic chamber are shown in Table 22.

Table 22: Pugh Method decision matrix for the selection of the microfluidic chamber

	Selection Criteria	Spec.	Score	Weight	Petri Dish	Drawer	Layered	Pressure Square	Boxed
Selection Criteria	Ease of Use	High	1	2	1	1	1	1	1
		Low	0						
	Secure Mold	Yes	1	5	0	1	1	1	1
		No	0						
	Visualization	Yes	1	6	1	1	1	1	1
		No	0						
	Stability	High	1	1	0	1	1	1	0
		Low	0						
	Ease of Manufacturability	High	1	4	0	0	0	1	1
		Low	0						
	Maintain Sterility	High	1	3	1	0	1	1	1
		Low	0						
	Total Score				**11**	**14**	**17**	**21**	**20**

The selection criteria included ease of use, being able to secure the mold, allowing for visualization under a microscope, stability of the entire system, ease of manufacturability, and being able to maintain a sterile environment. These criteria were created based on the advantages and limitations of the various microfluidic chamber designs as discussed in 4.3.4 Conceptual & Alternative Designs.

Weights were assigned to the selection criteria with 6 assigned to visualization of the sample to allow for imaging with a microscope being the most important and 1, stability of the system, being the least important. It was then determined if each method met the selection criteria as stated in the selection criteria specification in the second column.

After completing the matrix, the Boxed chamber received a total score of 20 out of 21 points and the Pressure Square chamber received a total of 21 out of 21. Both chambers and their respective molds were fabricated through 3D printing to help determine which design would prove to be most effective at completing the objectives and functions of the project.

Pressure Square Design

The 3D printed Pressure Square prototype made of PLA can be seen below in Figure 65.

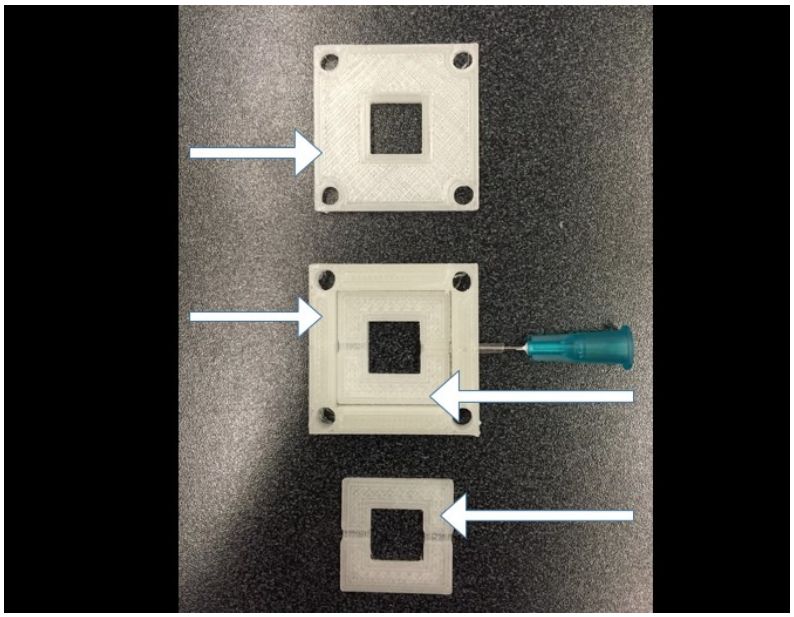


Figure 65: Pressure Square parts separated

This design uses tubing that is fed through the inlet of the microfluidic chamber and sits on top of the mold inlet. The figure above shows the bottom piece of the mold already placed inside the microfluidic chamber and the tubing sitting above the bottom mold. A closer view of this setup can be seen below in Figure 66.

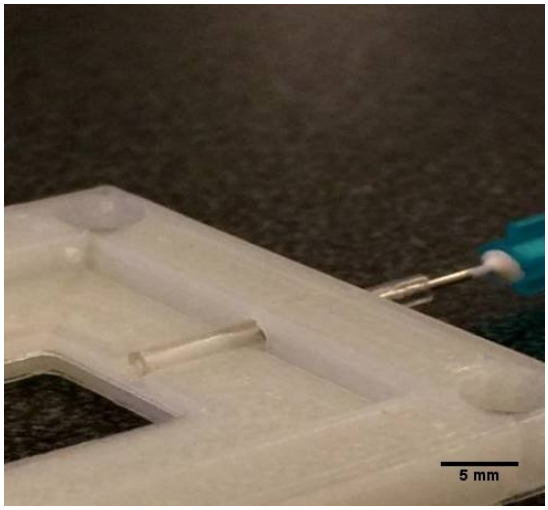


Figure 66: Pressure Square internal tubing

Figure 67 below shows the top piece of the mold placed on top of the bottom piece, securing the tubing. Figure 68 shows the complete setup of the microfluidic chamber with the cover on top as well as the 23G needle inside of the tubing.

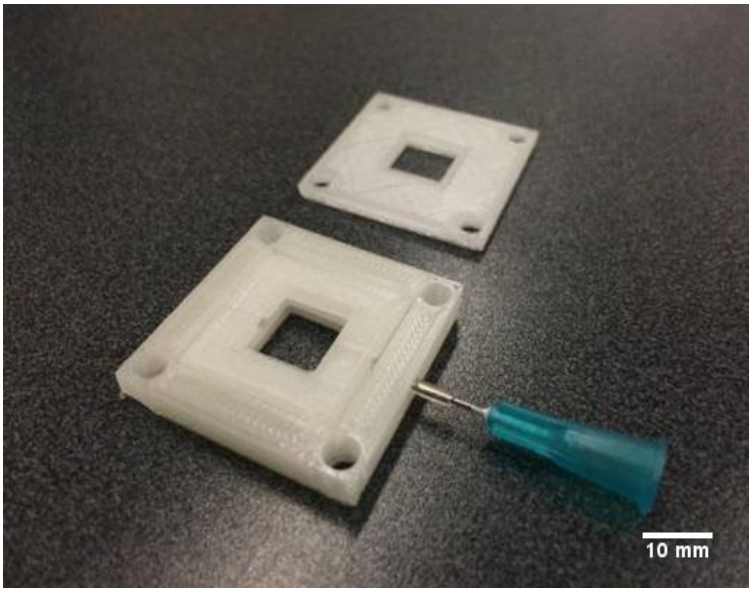


Figure 67: Pressure Square with mold inside

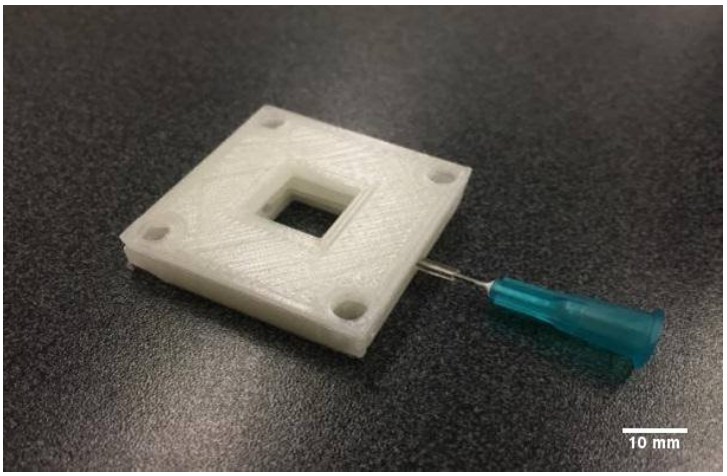


Figure 68: Pressure Square with cover

Boxed Design

As opposed to the last microfluidic chamber, this design does not insert tubing directly into the inlets. Instead, 20G needles are fused into the mold. The mold with the attached needles is then placed into the microfluidic chamber. Below, in Figure 69, all pieces separated can be seen individually.

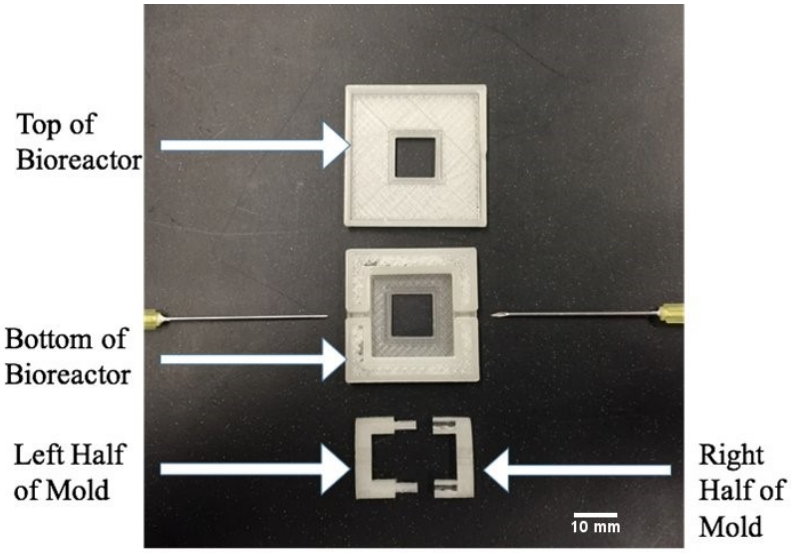


Figure 69: Boxed Design parts separated

Figure 70 below on the left shows the 20G needles fused into the mold and placed into the microfluidic chamber. Figure 71 on the right shows the mold with the fused needles after it

was placed into the microfluidic chamber. Instead of the chamber having a hole where the needle would go into, there are openings where the needles can be lowered into.

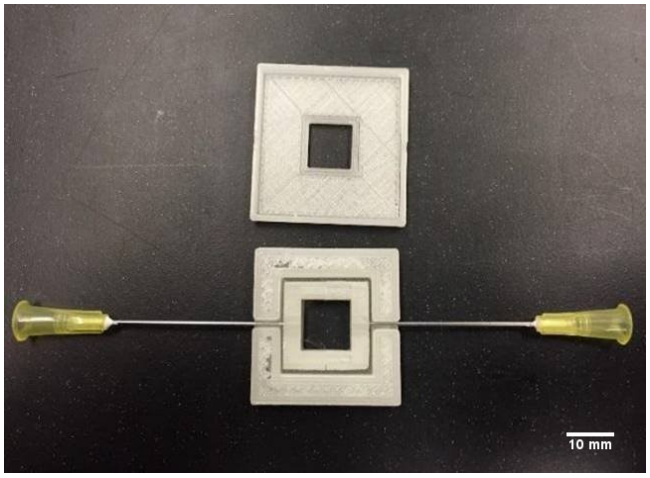


Figure 70: Boxed with mold inside

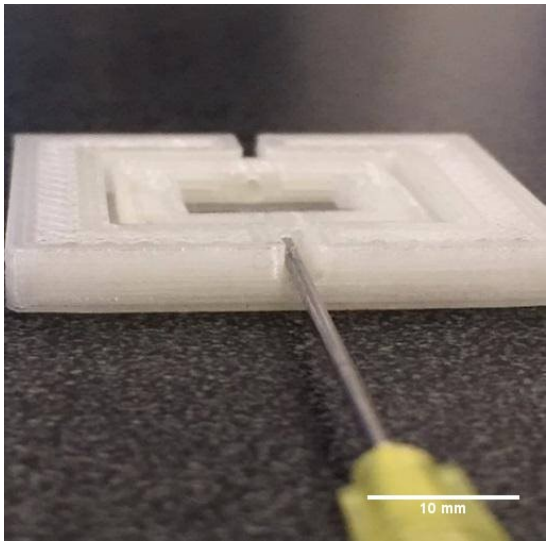


Figure 71: Needle in mold

Figure 72 below shows the shows the complete setup of the microfluidic chamber with the cover on top as well as the 20G needle fused into the mold.

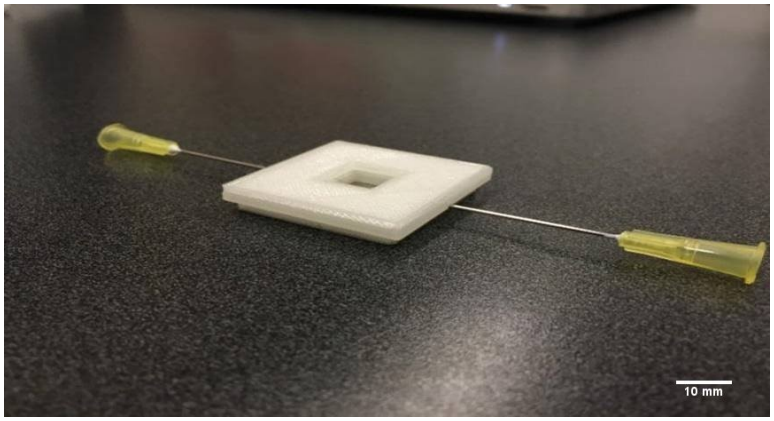


Figure 72: Boxed chamber with cover

The Pressure Square chamber was selected because it met all six of the design criteria best. The Pressure Square chamber does not face the limitations of the other chamber designs. It is extremely easy to use with its intuitive design. It allows the mold to be secure and remain in place once the system is assembled. It allows for visualization so the sample can be imaged with a microscope. The system also has high stability because of the use of screws that ensure the system is secure. The Pressure Square chamber is easy to manufacture as it does not have nozzles or extensions that would be difficult to fabricate. Additionally, it is able to keep the entire system sterile as there is no way for contaminants to enter the closed system.

It was chosen over the Boxed design, even though they ranked the same, partly because it is not as stable of a system when compared to the Pressure Square chamber. The method of placing the needle in the Boxed design and placing a cover over the top does not provide any means of sealing the needle nor does it provide a reliable way to close the system tightly. If you refer to Figure 71, the extra space above the needle can be seen. This extra space could potentially hinder the sterility of the microfluidic chamber. In addition, the lid of the Boxed design did not supply a proper seal of the microfluidic chamber, once again limiting the sterility of the system and allowing for possible leakage. It was decided regardless of the final design that it would need to incorporate screws to ensure a proper seal and maintain sterility during testing.

The Pressure Square design was selected as the final design for the microfluidic chamber and was then professionally fabricated because 3D printing was only for preliminary tests. 3D printing results in poor quality and would not suffice as a reliable device. Thomas Partington, the Lab Manager in Goddard Hall within the Chemical Engineering Department at Worcester Polytechnic Institute, fabricated the Pressure Square microfluidic chamber. The fabrication is further discussed in Chapter 6: Final Design and Validation. Below in Figure 73 is an image of the setup of the machined microfluidic chamber.

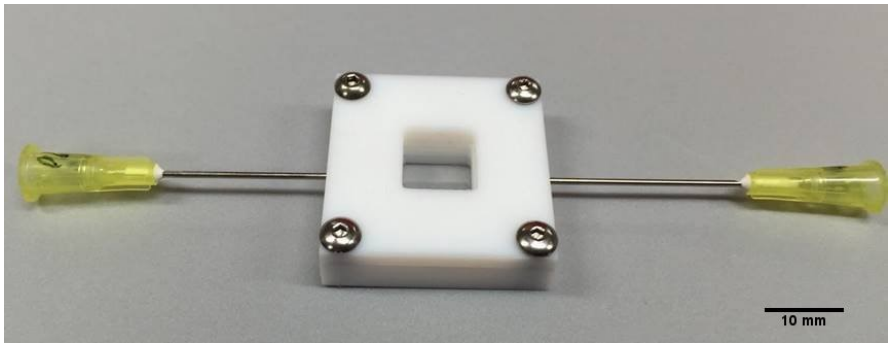


Figure 73: Machined microfluidic chamber

The chamber was made from PTFE. The PTFE was chosen because of its biocompatibility in addition to its hydrophobicity, which minimize resistance of flow through the system. Initial material options were PTFE, POM, and stainless steel. POM and stainless steel were both ruled out because of their rigidness and poor malleability. PTFE is more easily malleable which allows for a tighter seal between layers once the chamber is fully screwed and tightened into place. This is because the layers slightly adjust shape to fill in any micro gaps which reduces the chance fluid leakage.

Figure 73 shows two 20G needles inside the input and output holes of the microfluidic chamber. The preliminary design used silicon tubing to flow media in and out of the chamber. Feeding the tubing through the inlet and outlet holes proved to be extremely difficult because the tubing and hole must be very similar in diameter to ensure a tight seal, preventing leakage and

maintaining sterility. To ensure an equally tight seal, the tubing was removed and only the needles were used as inlets and outlets. There is a very small tolerance between the diameters of the 20G needle and inlet and outlet holes. There is a lot of resistance between the holes and needles, exactly what was needed to prevent any leakage. The preliminary design used 23G needles, but the 20G were used in the final design because the sacrificial mold that created the channels through fibrin fit easier into the larger diameter. The diameter of the 23G needle was too small to allow the mold to sit within it.

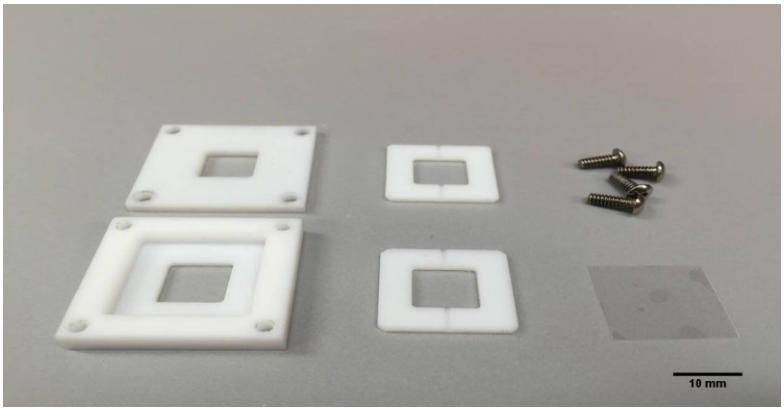


Figure 74: Microfluidic chamber components

In Figure 74, the top and base of the chamber, two part simple mold, glass coverslip and screws are shown. The 4 - 40 screws, the 4 indicates the standard number of the screw and 40 indicates the number of turns per inch, were used to ensure a tight seal between the top and bottom of the microfluidic chamber in order to maintain sterility of the system and prevent any leaking. The glass coverslips were placed above and below the simple mold which allowed for visualization and prevented contamination of the components within the microfluidic chamber. The depth of the pocket in the middle of the chamber accounts for the thickness of the two coverslips, the two part mold, and the 300 μm scaffold. After the initial fabrication of the microfluidic chamber, modifications and additions were made to better improve the functionality of the system.

To further prevent leakage and maintain sterility from occurring between the top and bottom of the microfluidic chamber, a groove was machined into the bottom face of the top piece of the microfluidic chamber. It was machined around the viewing window as seen below in Figure 75. However, adding a gasket to the PTFE top proved to be ineffective, as the PTFE was too smooth and the gasket would not remain in place. POM was then selected as an alternate material to make the top of the chamber. POM, as stated before, is not as malleable but having bottom of the chamber remain PTFE still ensures a tight seal because the PTFE slightly adjusts its shape when pressed against the POM. The groove was machined around the viewing window and the gasket remained in place. It can be seen in Figure 75 below.

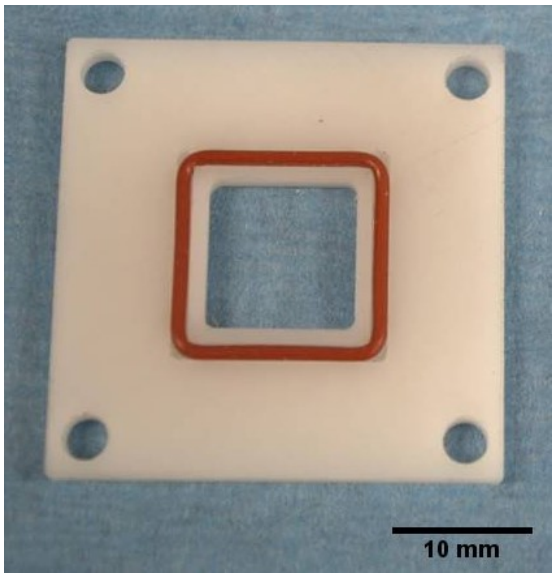


Figure 75: POM top with Silicone gasket in groove

Although the silicone gasket helped eliminate leakage occurring from the sides of the microfluidic chamber, leakage still occurred around the needles at the inlet and outlet ports. During perfusion, it was noticed that if pressure was applied to the top of the microfluidic chamber above the inlet and outlet ports, leakage did not occur and fluid flowed completely through the chamber and into the reservoir. In order to apply uniform pressure to the system to

ensure a tight seal, the next alteration was the addition of stainless steel plates. Below in Figure 76, the microfluidic chamber is seen with stainless steel plates in place.

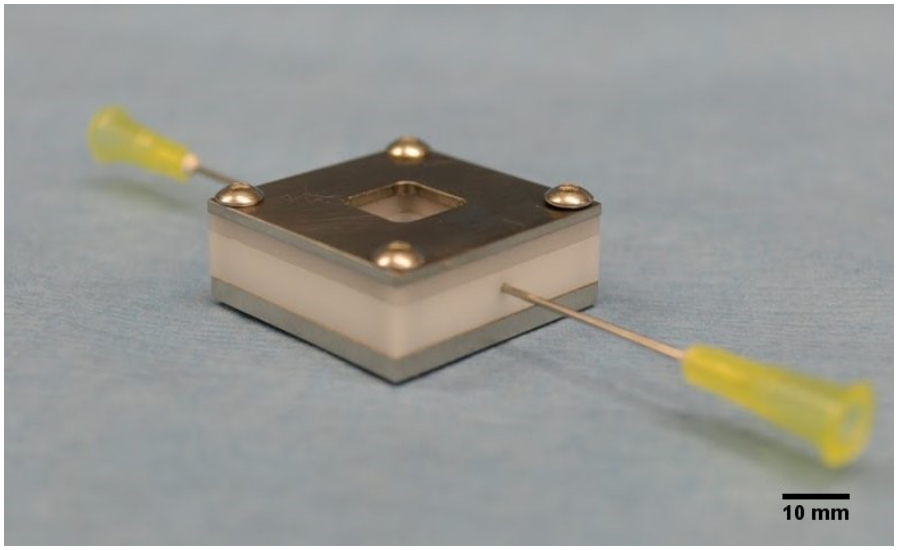


Figure 76: Microfluidic chamber with Stainless Steel plates in place

The stainless steel plates proved to be effective as they eliminate all leakage and prevent bowing within the layers of the chamber, by distributing the pressure created by the screws in the corners. Below in Figure 77, a side view of the chamber can be seen which shows the elimination of bowing within the component layers.

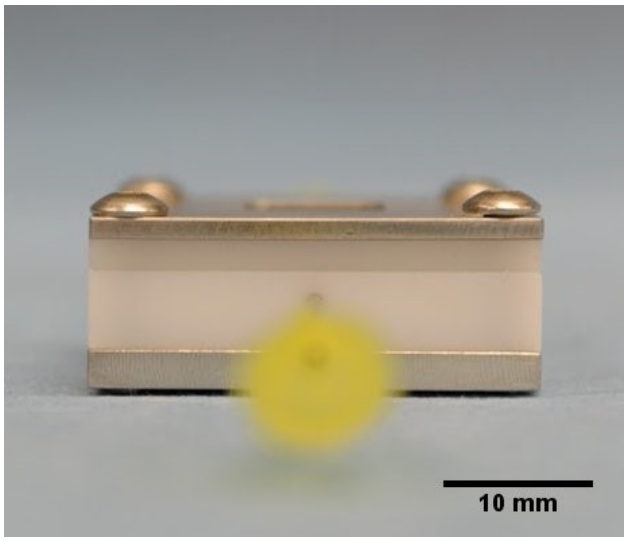


Figure 77: Microfluidic chamber Stainless Steel plates prevent bowing

All components of the finalized microfluidic chamber are seen in Figure 78 below.

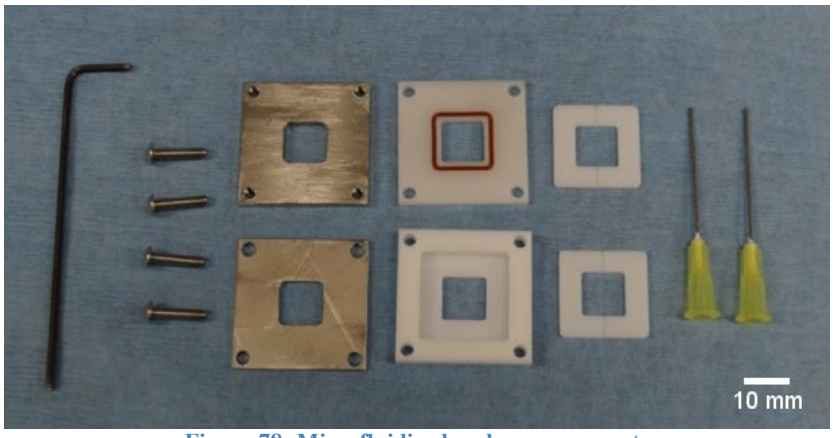


Figure 78: Microfluidic chamber components

An additional microfluidic chamber was fabricated to allow for testing multiple samples simultaneously.

4.3.5.3 Pump Selection

The last major component of the bioreactor system is the pump. The pump is used to perfuse media and nutrients through the microfluidic chamber. The gravity-fed pump, peristaltic pump, and syringe pump. After testing each of the options, it was determined that the syringe pump would be most suitable because it can precisely reach very low flow rates, especially flow rates of physiological relevance like 0.003 mL/min. The gravity fed pump was able to reach very low flow rates, but it was all dependent on the amount of media in the bag acting against gravity. Because of this, accuracy and precision were poor. The peristaltic pump would lose functionality when flow rates close to 0.003 mL/min were reached, making it unfeasible for this project. The syringe pump also has the ability to perfuse more than one sample at a time because multiple syringes can be connected unlike the other two options. The syringe pump was determined to be best suited because of its ability to precisely reach physiological flow rates and perfuse multiple samples simultaneously.

4.3.5.4 Complete Bioreactor

Below in Figure 79, the entire bioreactor setup can be seen including the pump (left), microfluidic chamber (middle), and collection flask (right) with tubing that connects the syringe to the chamber and the chamber to the collection flask.

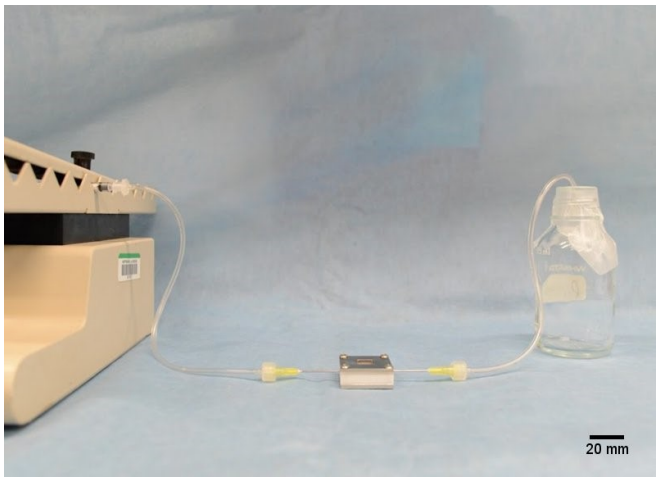


Figure 79: Bioreactor setup with pump, microfluidic chamber, and reservoir

The final components of the bioreactor system include a syringe pump, microfluidic chamber based off the Pressure Square alternative design, and a collection flask. The syringe pump was chosen because of its precision when operating at physiologically relevant flow rates such as 0.003 mL/min and its ability to perfuse multiple samples simultaneously. The microfluidic chamber was based off the Pressure Square design. This design was chosen because it is extremely easy to use with its intuitive nature. It allowed the mold to be secure and for visualization with a microscope. The Pressure Square design also allowed for the system to have high stability due to the screws which ensure a secure system. This design is easy to manufacture and is able to maintain a sterile environment through the use of a gasket, specific materials with low tolerances, and screws.

Chapter 5: Design Verification

This chapter contains the results of this project which include all steps of the development of the microengineered vascular network protocol, establishment of continuous perfusion, and the findings of the diffusion assay. A cell viability assay was also designed and performed to verify the components of the bioreactor met the objectives and functions.

5.1 Microengineered Vascular Network Development and Analysis

A protocol was developed to fabricate the microengineered vascular network using a gelatin sacrificial mold. Steps of the protocol included production of both single cylinder and bifurcated gelatin sacrificial molds, transfer of the mold to a microfluidic chamber, addition and polymerization of fibrin, melting of the mold at 37°C, and flushing of the liquid gelatin.

To validate the microengineered vascular network, continuous perfusion was shown to be established. Additionally, a diffusion assay was conducted to determine the diffusion rate of fluorescein isothiocyanate (FITC) from a single channel through fibrin.

5.1.1 Gelatin Sacrificial Mold Method Development

A summary of the process of forming microvasculature from a gelatin sacrificial mold is depicted in the Figure 80. The completed protocol is found in Appendix D: Protocols: Formation of Channels from Gelatin Sacrificial Materials.

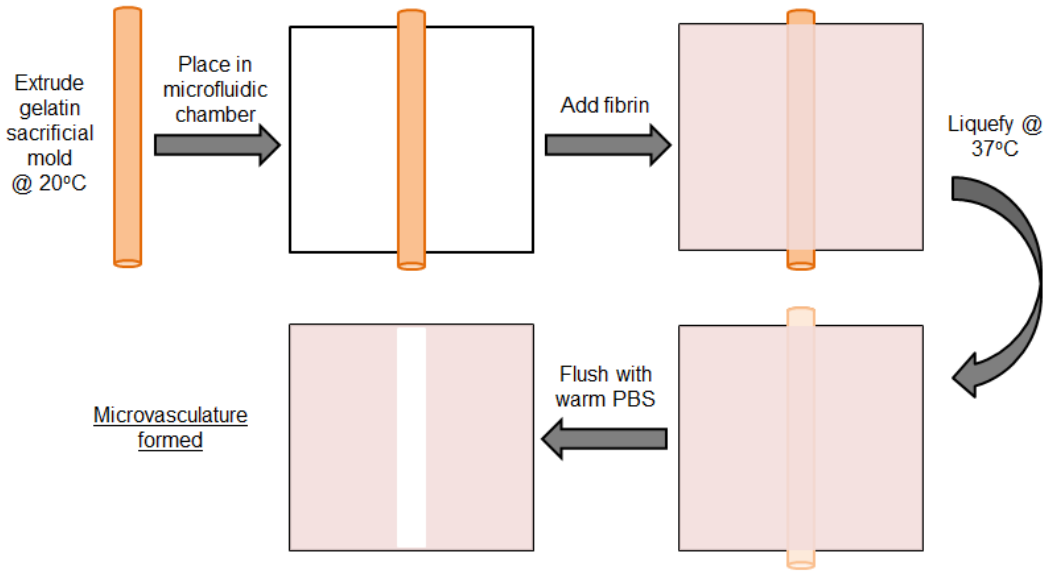


Figure 80: Gelatin sacrificial mold to formation of microvasculature schematic. Extrude gelatin at 20°C (A), transfer to microfluidic chamber (B), add fibrin (C), liquefy gelatin at 37°C (D), flush gelatin out with warm PBS (E).

5.1.1.1 Production

The production of the 10% w/v gelatin sacrificial molds was initially adapted from a method from Golden & Tien (2007). For this project, free and hand-printed extrusion would be used to create single cylinders and bifurcated molds respectively. For both methods, gelatin was melted in a hot water bath before being drawn into a one milliliter syringe. In addition, for both methods, gelatin was extruded onto a non-coated PDMS surface which was found to be non-stick, allowing for easy removal of the molds. To fit in the microfluidic chamber, the overall length of the channel had to be greater than 10 mm.

5.1.1.1.1 Single Cylinder

It was found that extruding the gelatin once it had been cooled for ten to fifteen minutes and had reformed as a gel resulted in successful production of single gelatin cylinders. Extruded single cylinder molds are later seen in the top of Figure 82.

5.1.1.1.2 Bifurcation

When extruding gelatin cylinders, an interesting observation was made. Due to heat transferred to the solid gelatin from the extruder's hand, the gelatin entered a liquid-like, gel-like state in its gel transition temperature, as seen in Figure 81.

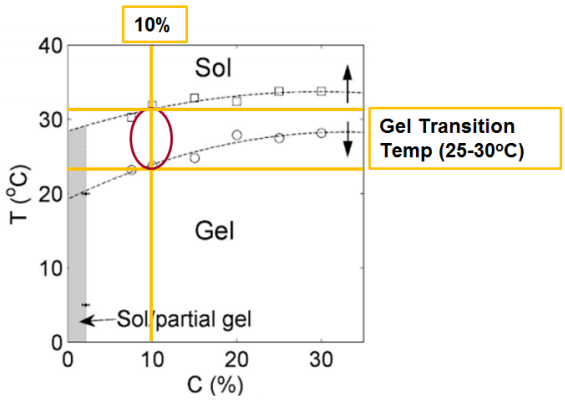


Figure 81: Gelatin phase diagram with 10 and w/v concentration used in this project and gel transition temperature range. Diagram adapted from Parker & Povey (2012).

This gelatin flowed out of the needle without pressure being applied to the syringe. This property was taken advantage of to draw bifurcations. One of the problems of drawing bifurcations with Pluronic was connecting sides of the bifurcation. The Pluronic material would temporarily connect but as it dried would pull apart. Contrary to this, the gelatin in its gel transition state fully connected due to its liquid-like properties but still maintained shape due to its gel-like properties. Single cylinder, hand-printed single bifurcations, as well as double bifurcations, can be seen in below in Figure 82.

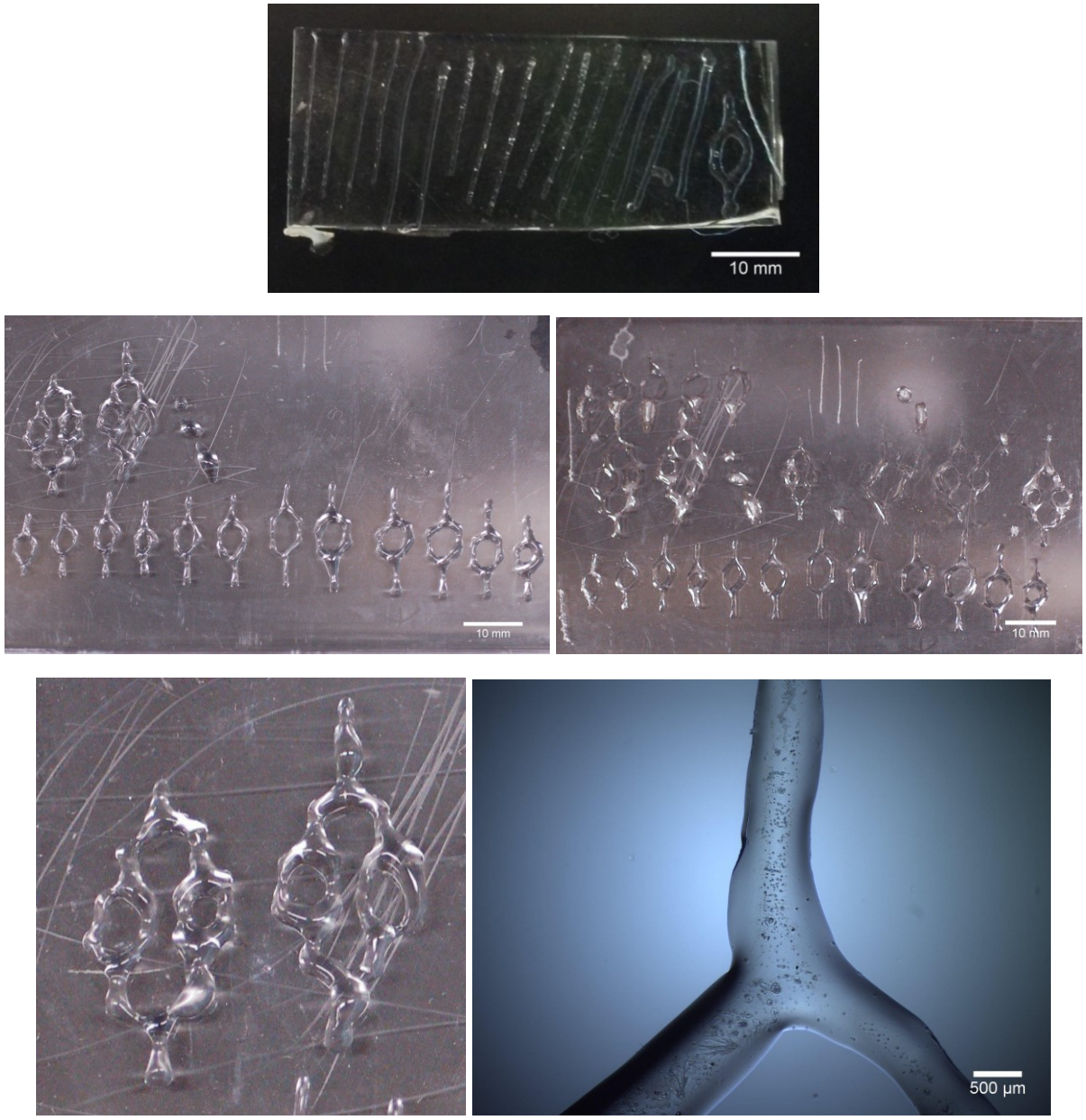


Figure 82: Different gelatin geometries. Top: Single cylinder gelatin sacrificial molds. Middle: Hand-printed single- and double-bifurcations before (left) and after (right) dehydration. Bottom: Magnified image of hand-printed double-bifurcations (left) and 20X magnification image of bifurcation

5.1.1.2 Transfer and Placement in Microfluidic Chamber

Once the sacrificial mold was formed, the next problem to overcome was transferring it to the microfluidic chamber. Immediately after extrusion, the gelatin exists as a gel and cannot be easily lifted with forceps without inflicting damage. It was found that dehydration on the

benchtop at room temperature (20°C) for approximately thirty minutes resulted in sacrificial molds that could be handled with forceps without causing damage. Dehydration occurred automatically after extrusion.

It was found that during dehydration, the channels decreased in diameter. This change in diameter was studied by extruding ten gelatin cylinders through a 20G needle which has an inner diameter of 603 µm so it is expected that the cylinders have an initial outer diameter of around 600 µm. The cylinders were then dehydrated over multiple days then measured and an average diameter was calculated (three measurements per sample). Results of these measurements can be seen in Figure 83. The average diameter was determined to be 260 ± 32 µm, a 57% reduction in size.

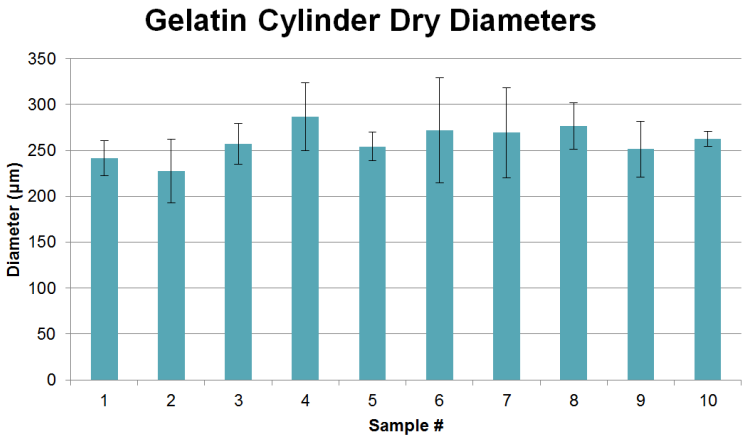


Figure 83: Measurements of the dry diameters of gelatin cylinders after dehydration over multiple days

The next important design challenge was to overcome the low density of the gelatin cylinder. When fibrin components were added into the microfluidic chamber, the gelatin cylinder would rise to the top of the fibrin gel instead of remaining in the center. To overcome this problem, the ends of the sacrificial mold were inserted into the inlet or outlet needles as seen in Figure 84. This was sufficient to hold the mold in place.

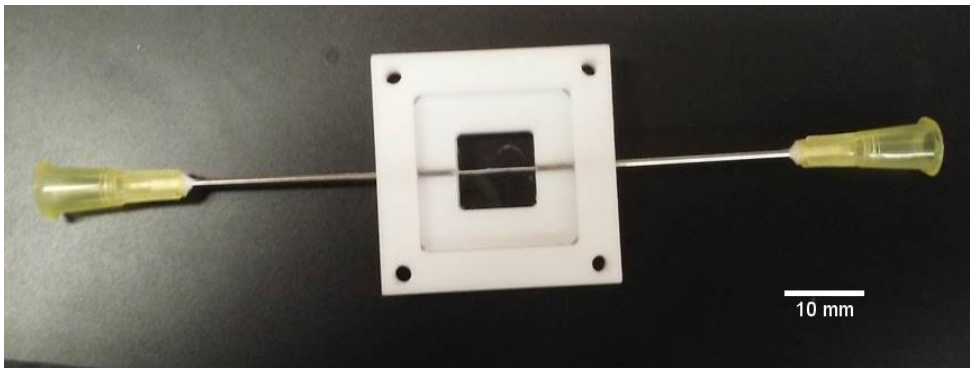


Figure 84: Gelatin channel mold stabilization. Gelatin cylinder transferred to microfluidic chamber, inserted into both inlet and outlet needles to keep cylinder from rising to the top of the fibrin gel.

5.1.1.3 Channel Formation

To form a channel in a fibrin hydrogel, a sacrificial mold must maintain its shape during the process of fibrin polymerization. A gelatin cylinder was placed in a prototype microfluidic chamber, one insert mold and one simple mold, to attempt channel formation. After fibrin polymerization was complete, the samples were placed in the incubator to melt the gelatin and images were taken. As seen in Figure 85, the gelatin cylinders maintained their cylindrical shape during fibrin polymerization. Furthermore, to develop a relatively straight channel as opposed to one that ends up with a greater curvature, it was determined that adding fibrin slowly or dropwise on either side of the sacrificial mold prevented the curvature.

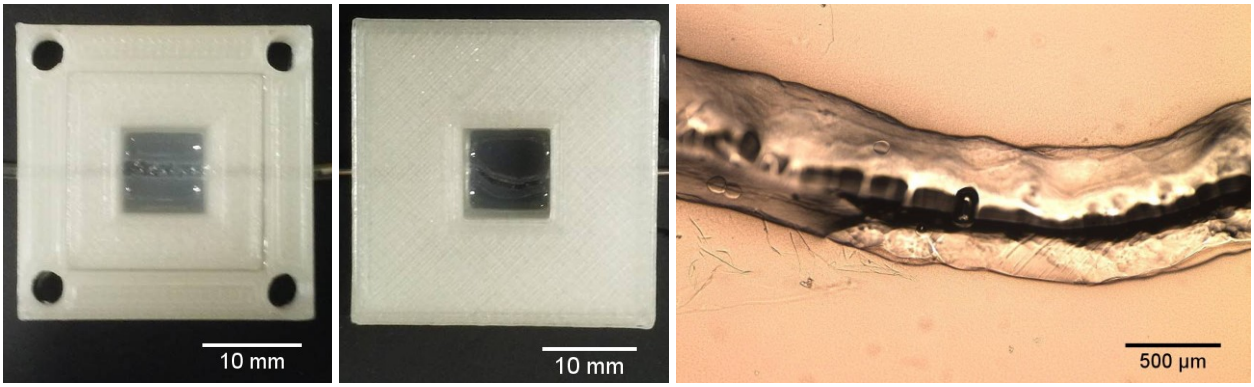


Figure 85: Gelatin cylinders maintain geometry during fibrin polymerization. Microfluidic chamber with gelatin sacrificial mold immediately after fibrin component addition (left), after fibrin polymerization (middle), and microscope image of gelatin in fibrin (right)

Next, removal of the gelatin was attempted. The sample and a syringe with PBS were placed in the incubator to warm and then brought to the benchtop in an attempt to clear the gelatin. However, the flushing of the gelatin was unsuccessful as seen in the left part of Figure 86. PBS with blue food coloring as a contrast agent was used to attempt to visualize the channel clearance of gelatin. The PBS did not flow through the channel and instead flowed into the fibrin and up through the top. When removing the fibrin, it was found that the gelatin cylinder was still in place, even though it had been melted in the incubator, as seen in the right image of Figure 86. It was determined that though the gelatin melted in the incubator, it must be flushed out in the incubator as well to ensure that the gelatin melts, and stays in liquid form until it is been completely removed.

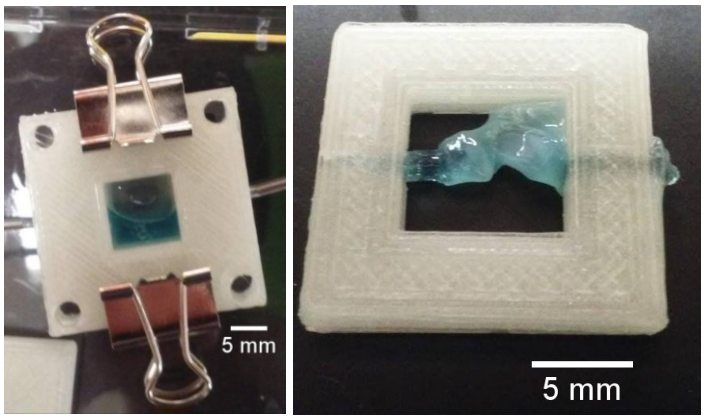


Figure 86: Gelatin solidification during channel fabrications. Perfusion unsuccessful (left) because the gelatin cylinders resolidified after melting as seen when the fibrin was being removed (right).

After establishing the strategy of flushing the liquid gelatin out of the fibrin in the incubator, channel formation and gelatin flushing were successful, as seen in Figure 87. The left images show the gelatin sacrificial mold surrounded by the fibrin hydrogel. The right images show the channel formed after the gelatin was flushed out with PBS with a blue dye. To clear the gelatin, around 2.5 mL of PBS was perfused with a syringe pump at a flow rate of 0.1 mL/min.

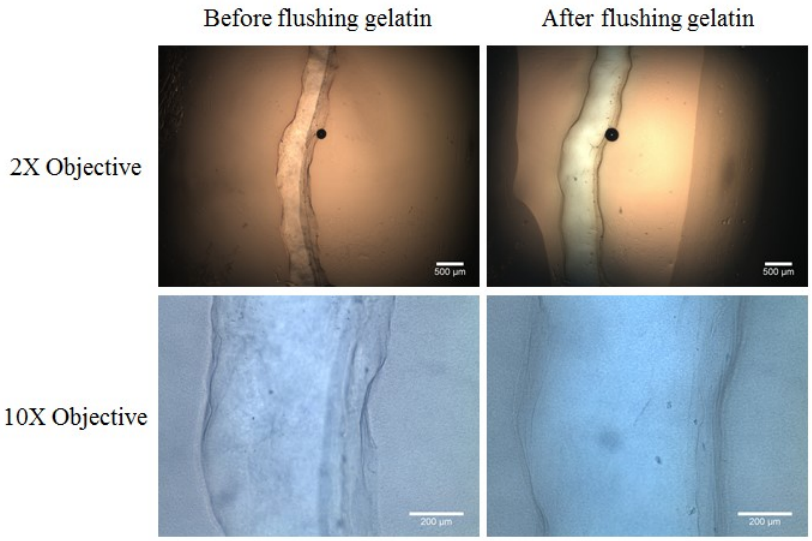


Figure 87: Images of gelatin channel in fibrin gel before (left) and after (right) flushing with warm, blue, PBS with 2X (top) and 10X (bottom) objectives.

5.1.1.4 Tunability of Channel Diameter

The team's goal was to be able to tune the dimensions of the microengineered vascular network based on initial sacrificial material dimensions during fabrication.

To accomplish this goal and to characterize the change in size of the gelatin cylinders, measurements of gelatin sacrificial mold diameter immediately after extrusion, after 25 minutes of dehydration, after 3 days of dehydration, after encasing in fibrin, and final channel diameter were measured, as seen in Figure 88.

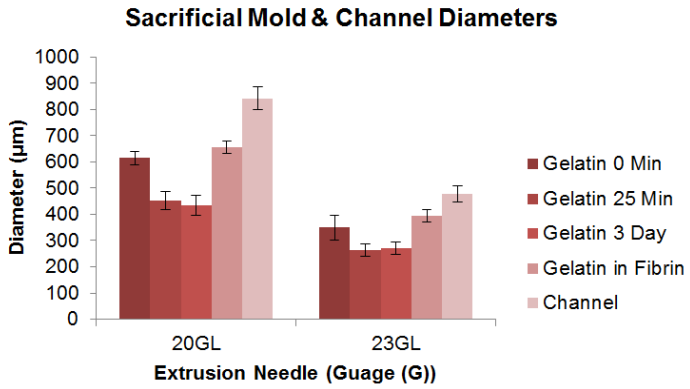


Figure 88: Measurements of sacrificial mold diameters, extruded from 20G and 23G needles and final channel diameter at different stages of the protocol. From left to right: gelatin sacrificial mold diameter immediately after extrusion, after 25 minutes of dehydration, after 3 days of dehydration, after encasing in fibrin, and final channel diameter.

The initial sacrificial mold diameter relative to the inner diameter of the extrusion needle was first assessed as seen in Figure 89.

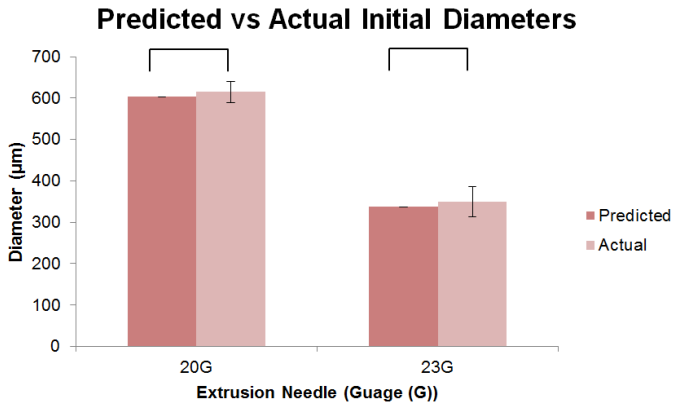


Figure 89: Comparison of predicted vs actual initial diameters of gelatin cylinders.

To determine if there was a significant difference between the predicted and actual initial diameters of the sacrificial molds, a two-sample t-test was run for each. It was found that there was no significant difference between the predicted diameter (the inner diameter of the extrusion needle) and the actual outer diameter of sacrificial molds extruded through 20G ($n = 3$, $p = 0.2929$) nor 23G ($n = 3$, $p = 0.1409$) needles.

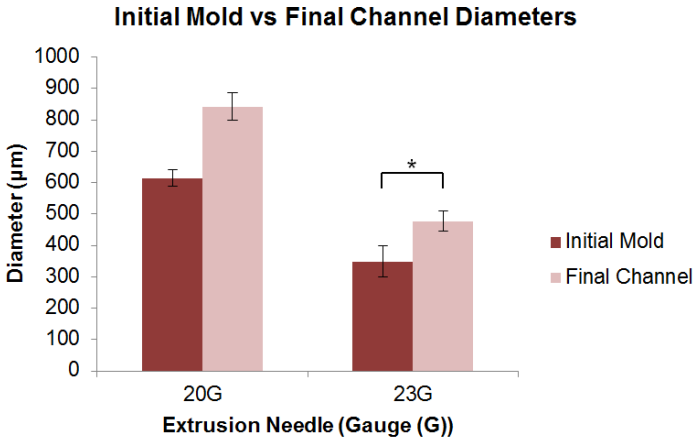


Figure 90: Comparison of initial diameter of gelatin cylinder and final channel diameter.

To determine if there was a significant difference between the initial diameter of gelatin cylinders and final channel diameters, a t-test was run. There was found to a significant

difference between the initial mold extruded out of 23G needles and final channel diameter (n = 3, p = 4.23E-9, *), seen in Figure 90. A limitation of this experiment is that only one 20G sample of the original three successfully formed a channel, but the data does suggest that there is a difference between the initial mold formed from a 20G needle and the final channel diameter.

The goal was to predict the ultimate channel diameter and the initial diameter of the sacrificial material was found to be equal to the inner diameter of the extrusion needle. A relationship between the initial sacrificial mold diameter and ultimate channel diameter was sought.

To determine the tunability of the microengineered vascular network, diameter measurements were first made of gelatin cylinders immediately after extrusion through 20G and 23G needles. It was previously shown that the initial diameters were not significantly different than the inner diameter of the extrusion needle. Diameter measurements were also taken of the final channel formed from each of the cylinders. These relationship between these measurements was modeled linearly and the slope was determined to be 1.41 μm/μm. As a result, the dimensions of the microengineered vascular network were determined to be tunable based on the initial sacrificial mold dimensions during fabrication. By varying the inner diameter of the extrusion needle, the initial sacrificial mold diameter (d_{sm}) and subsequently final channel diameter (d_c) could be controlled. The final channel diameter was modeled by the linear relationship

$$d_c = (1.41)\, d_{sm} \tag{1}$$

which was used to predict the ultimate channel diameter based on various extrusion needles, as seen in Table 23.

Table 23: Needle gauge and inner diameter with associated predicted channel diameter based on the channel diameter equation (Sigma-Aldrich, 2016).

Needle Gauge	Needle Inner Diameter	Predicted Channel Diameter
20G	603 µm	850 µm
22G	410 µm	578 µm
24G	310 µm	437 µm
26G	260 µm	367 µm
28G	180 µm	254 µm
30G	160 µm	226 µm

5.1.1.5 Reproducibility of Sacrificial Mold Production

Finally, the reproducibility of the production of gelatin sacrificial molds was assessed. Using the same data above, the average diameter of the molds immediately after extrusion (0 min), twenty-five minutes after extrusion, and seventy-five hours after extrusion was measured for molds extruded through 20G and 23G needles, as seen in Table 24.

Table 24: Reproducibility of gelatin sacrificial mold production at various time points in the dehydration process

	0 minutes	25 minutes	75 hours	
20G	615 µm ± 4%	453 µm ± 8%	435 µm ± 9%	n=9
23G	350 µm ± 10%	270 µm ± 8%	265 µm ± 8%	n=18

5.2 Bioreactor Verification Studies

5.2.1 Perfusion Test

Tests were performed to verify if the amount of liquid perfused into the microfluidic chamber was equal to the amount perfused out. First, a syringe pump was set up and connected to the microfluidic chamber. A syringe filled with 1 mL of water was connected to the pump which can be seen below in Figure 91.

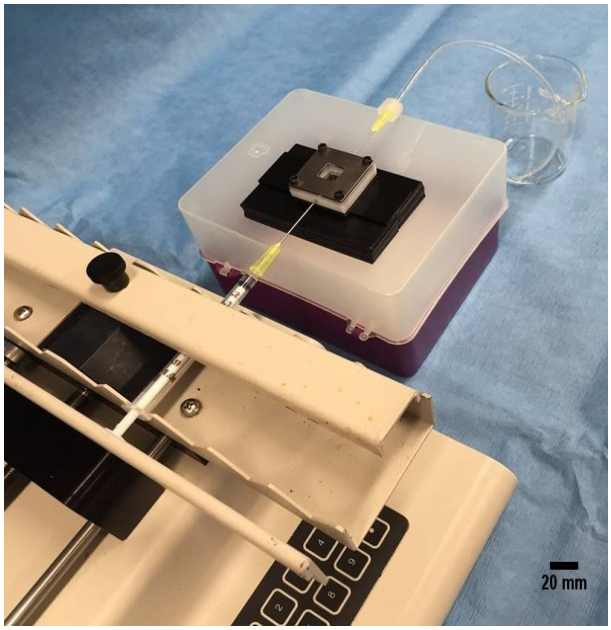


Figure 91: Setup of perfusion test

Three separate flow rates were chosen: 0.01 mL/min, 0.1 mL/min, and 1 mL/min. The inside of the microfluidic chamber was first filled with water before perfusion began. Once it was full, the syringe pump was turned on and the water perfused through, coming out the other side into a collection flask. There was no difference between the input and output amounts of water. 1 mL was perfused into the chamber and 1 mL was collected once the syringe had fully emptied all water into the chamber. All water perfused into the microfluidic chamber flowed into the collection flask. To further test this observation, a sealing test with constant perfusion for twenty-four hours was conducted which is described in the next section.

To verify the perfusion rates of the syringe pump, a syringe was loaded with 1 mL of water. The water was perfused through the microfluidic chamber at a programed rate of 0.005 mL/min. The process was timed and the end time was recorded at 200 min. Using the equation:

$$Q = C / t \quad (2)$$

In the above equation Q is the flow rate, in mL/min, C is the capacity (Volume), in mL, and t is time, in minutes. Using the equation, the perfused volume and resulting time was used to calculate a flow rate of 0.005 mL/min. Thus, verifying the programed flow rates of the syringe pump were accurate.

5.2.2 Sealing Test

This test was performed to verify that the microfluidic chamber did not leak while media was being perfused. First, a syringe pump was set up and connected to the microfluidic chamber. Next, a syringe was filled with three milliliters of C2C12 media and was connected to the pump. The setup was identical to the previous test, which can be seen in Figure 91 above. The media was perfused at a rate of 0.003 mL/min in order to mimic physiological flow for twenty-four hours. This test resulted in no leaked media and all media that perfused through the system was collected in the collection flask. This was verified by measuring the volume of media in the collection flask and comparing it to the starting volume. The measured volume of media in the collection flask was 2.7 mL. The missing media can be accounted for because the chamber of the microfluidic chamber holds 0.3 mL of media. The media inside the chamber was withdrawn using a syringe and exactly 0.3 mL was retrieved, proving all media that was perfused into the microfluidic chamber came out the outlet or remained inside the system.

5.2.3 Setup Time of Microfluidic Chamber

For the purpose of determining the setup time of the chamber, the team determined how long it would take to setup the microfluidic chamber components. The chamber was assembled with all of its components three times and the time to complete was averaged. During each assembly, the base was first placed on the benchtop. Vacuum grease was then applied to the bottom of the mold and an 18 mm x 18 mm glass coverslip was placed onto it. The bottom piece of the simple mold was then placed into the base of the chamber and the top part of the mold was then placed on top of that, aligning the mold channels. A coverslip and the top of the chamber were placed next. Then the stainless steel plates were screwed into place. Last, the needles were inserted into the inlet and outlet ports. The duration of each setup time trial is seen below in Table 25.

Table 25: Setup time trials & durations

Trial (#)	**Duration (s)**
1	84.32
2	80.00
3	75.77
Average	80.03

As seen above in Table 25, the average of the three trials took 80.03 seconds to complete. Additionally, the duration to complete the setup of the microfluidic chamber decreased with each trial.

5.2.4 Dimension Validation

To determine the accuracy of the fabrication method of the microfluidic chamber, a dimension validation test was performed following the Dimension Validation protocol in Appendix D: Protocols. Images of components of the chamber were taken and were then analyzed using ImageJ. These measurements were averaged and compared to the desired length within the CAD drawing prior to fabrication. The mean measured length and desired length of chamber components can be found in Table 26 below.

Table 26: Microfluidic chamber component measurements

Corresponding Number	Measurement	Average Length (mm)	Desired Length (mm)	Error	% Error	SD
1	Groove in POM Chamber Top for Silicone Gasket	1.20	1.20	0	0	$1.73*10^{-3}$
2	Silicone Gasket	0.94	1.00	-0.06	-6.00	$1.62*10^{-2}$
3	PTFE Simple Mold Indentation	0.90	0.90	0	0	$1.37*10^{-2}$
4	Screw Hole	2.93	2.90	0.026	0.897	1.0010^{-2}
5	Full PTFE Chamber Base Length	30.86	30.0	0.857	2.857	$4.68*10^{-1}$
6	PTFE Chamber Base Pocket Length	19.78	20.0	-0.22	-1.10	$3.52*10^{-1}$
7	PTFE Chamber Base Viewing Window Length	9.80	10.0	-0.202	-2.02	$1.63*10^{-1}$
8	PTFE Chamber Base Height	5.28	5.20	0.075	1.44	$08.24*10^{-2}$
9	POM Chamber Top Length	30.64	30.0	0.639	2.13	$5.52*10^{-1}$
10	POM Chamber Top Viewing Window Length	10.35	10.0	0.347	3.47	$2.53*10^{-1}$
11	POM Chamber Top Height	2.10	2.08	0.015	0.721	$5.20*10^{-2}$
12	Stainless Steel Top Plate Length	29.70	30.0	-0.297	-0.990	1.15
13	Stainless Steel Top Plate Height	1.36	1.40	-0.045	-3.21	$4.70*10^{-2}$
14	Stainless Steel Bottom Plate Length	30.11	30.0	0.111	0.370	$9.66*10^{-1}$
15	Stainless Steel Bottom Plate Height	2.62	2.60	0.019	0.731	$1.28*10^{-1}$
16	PTFE Simple Mold Length	20.47	20.0	0.468	2.34	$3.39*10^{-1}$
17	PTFE Simple Mold Viewing Window Length	10.07	10.0	0.071	0.710	$1.68*10^{-1}$

In Table 26 above, the average length of similar measurements, desired length of dimensions, error, percent error, and standard deviation can be seen. The dimensions with

corresponding numbers are shown below in Figure 92 along with a clear representation of what was measured.

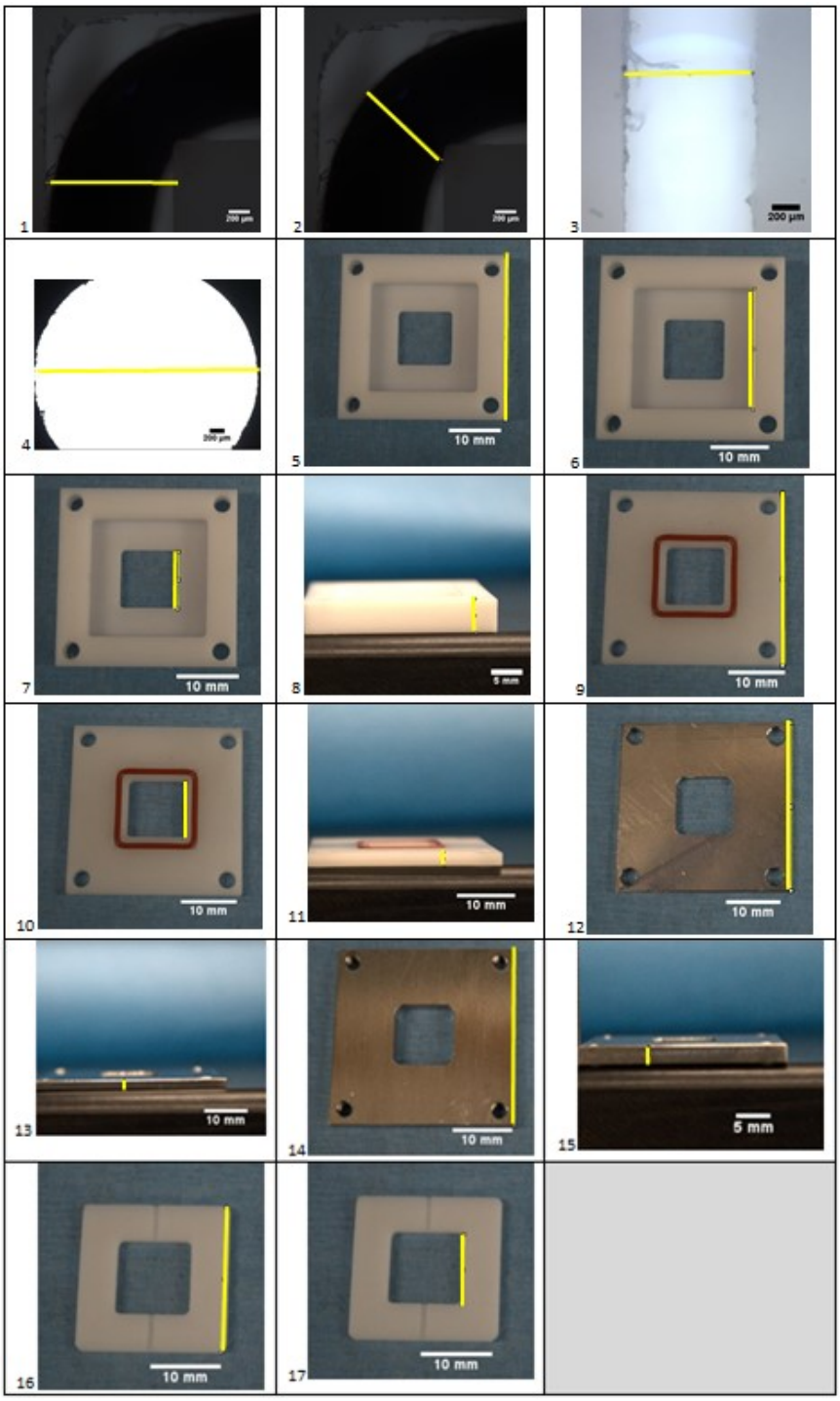


Figure 92: Corresponding numbers and measurements

The specific measurements were selected to validate as many dimensions of the microfluidic chamber pieces as possible.

5.2.5 Sterility Testing

In order to test the microfluidic chamber's ability to maintain an aseptic environment, a sterility test was completed. This test analyzed the bacterial growth on the microfluidic chamber by sterilizing it then filling it with media and letting the chamber sit in the incubator for five days. First, all components of the bioreactor were sterilized using 70% ethanol inside a biosafety cabinet (Refer to Appendix D: Protocols: Cell Viability). Once all parts were submerged in ethanol for 1.5 hours, the microfluidic chamber was rinsed with deionized water, assembled, and filled with C2C12 complete media, the protocol for making the media can be seen in Appendix C: Client Meetings. The entire system was transported in a sterile Petri dish into the incubator and left for five days. On day five, the coverslips from the chamber were taken out. Below is an image taken with an upright bright-field microscope at 10x magnification.

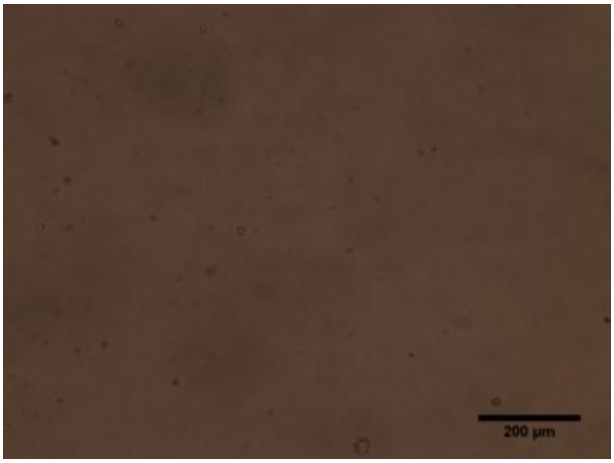


Figure 93: Top of lower coverslip from chamber prior to staining with Methylene Blue. Imaged at 10x with an upright bright field microscope.

As can be seen above in Figure 93, no bacteria were present on the coverslip. The streak marks show evidence of scratches but no bacteria. Next, each coverslip was placed in a separate well and stained with a mixture of 0.05% Methylene Blue. After one minute of staining, the

coverslips were washed with water and imaged under a microscope to look for bacterial growth. Figure 94 below shows an image of the stained coverslip.

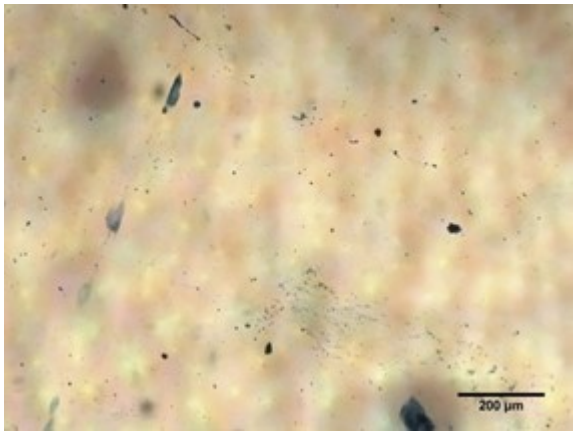


Figure 94: Top of the bottom coverslip of the chamber after staining with 0.05% Methylene Blue. Imaged at 10x with an inverted fluorescent microscope of the Rolle Lab.

No growth was found anywhere inside the chamber. All images did not detect any presence of live culture. In the image above, specs of dust can be seen as well as a smudge which can be attributed to the vacuum grease that was in between the base of the chamber and glass coverslip. The resulting media was not inspected for contamination, only the inside of the chamber was inspected. This test was done once due to time restrictions.

5.3 Continuous Perfusion

Once the sacrificial mold protocol was developed, and a microengineered vascular network could be created, the team worked to validate the process by attempting continuous perfusion through the channel.

The final design challenge to overcome before establishing continuous perfusion was to get flow out the outlet needle of the microfluidic chamber. Many attempts were performed before it was determined that the needle needed to be primed with fluid before starting perfusion to promote flow out of the microfluidic chamber. The setup can be seen in Figure 95.

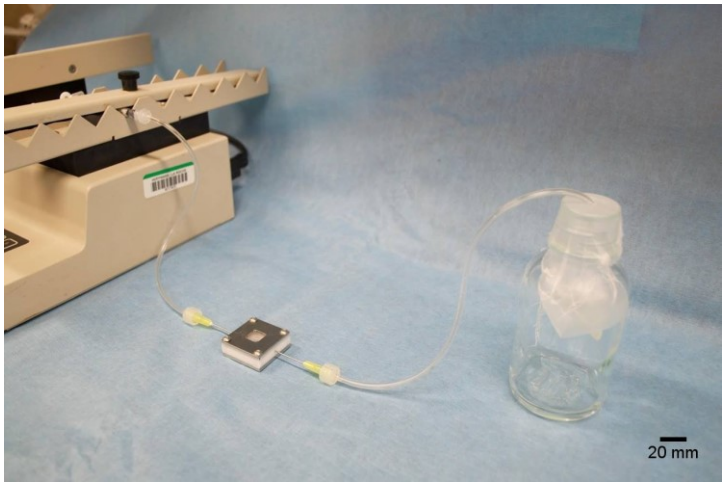


Figure 95: Continuous perfusion of a microengineered vascular network in a microfluidic chamber with a primed outlet needle, pre-filled with fluid to promote flow.

Continuous perfusion of a single channel was initially shown at a flow rate 0.01 mL/min for around 2.5 hours. The perfusion set-up and sample after the 2.5 hours of perfusion are seen in Figure 96.

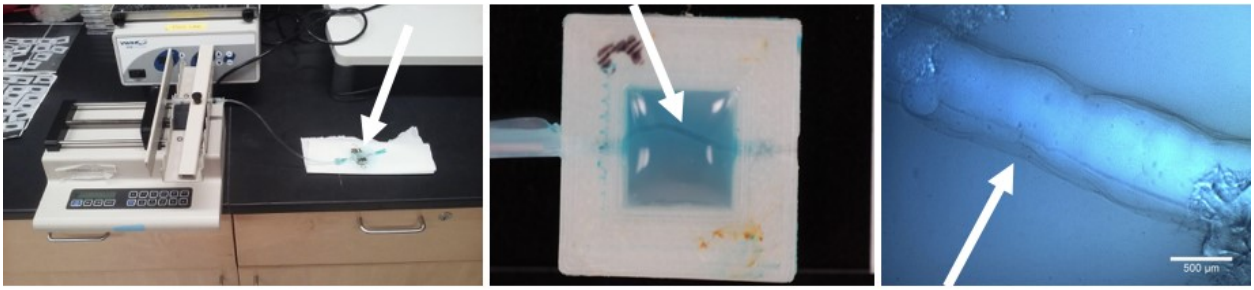


Figure 96: Microvascular network perfusion. Perfusion set-up (left) of channel perfused at a lower flow rate of 0.01 mL/min for 2.5 hours; arrow points to microfluidic chamber. The gel (middle); arrow points to the formed channel. The channel (right) was imaged at the end of the experiment; arrow points to the channel. Note in the middle image the diffusion of blue PBS throughout the fibrin.

A bifurcated channel was created to be perfused. As previously mentioned, the ends of the sacrificial molds are placed inside the inlet and outlet needles but the drawn bifurcations were too large for this to occur. For this reason and due to its low density, the bifurcation rose toward the top of the fibrin surface. The bifurcation was pushed down to try to get it below the surface. This contact damaged the gelatin since it had already begun hydrating and forming a gel again. However continuous perfusion through the bifurcation was still successful, as seen in Figure 97.

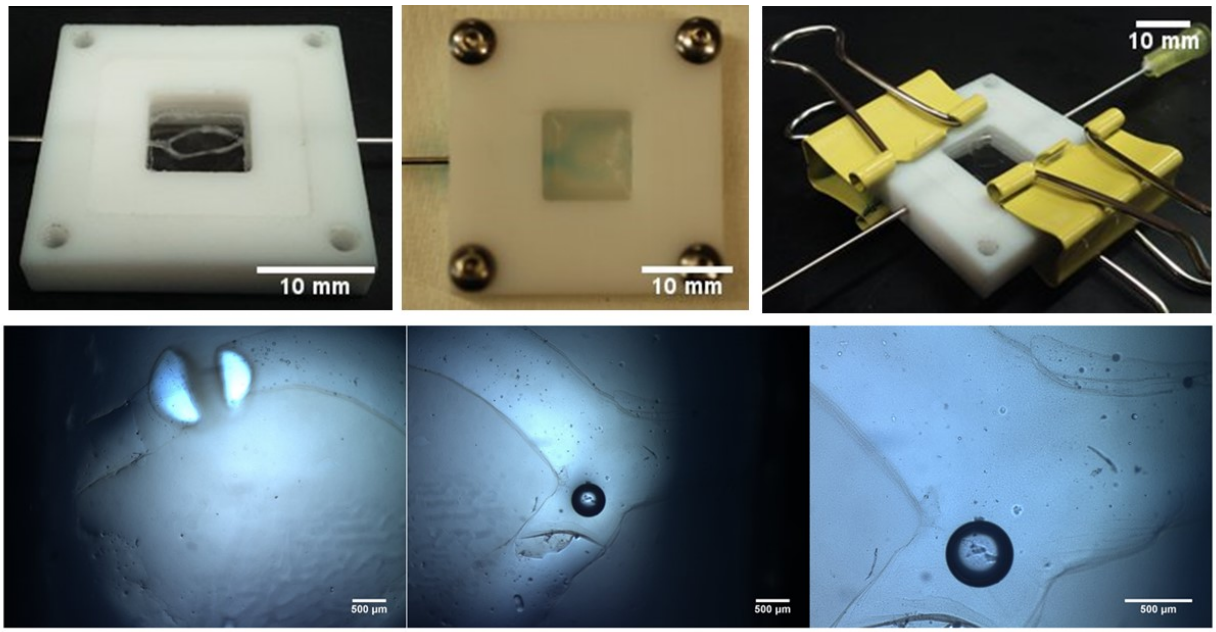


Figure 97: Bifurcated channels in fibrin gels. Top row: Bifurcated gelatin sacrificial material in PTFE bioreactor before addition of fibrin (left), after the addition of fibrin (right) and after flushing and perfusion (middle). Bottom row: Microscope images of bifurcated channel at damaged site (left) and one of the bifurcations at 20X (middle) and 40X (right) magnifications.

Continuous perfusion through the microengineered vascular network was conducted for as long as twenty-four hours at flow rates ranging from 0.001 mL/min to 0.1 mL/min. These flow rates are within the range found *in vivo* through arterioles.

5.4 Diffusion Assay

Analyzing the diffusion of molecules through the gel was the next element of the project strategy. The diffusion subsequently influences the scaffold's ability to sustain cell viability. In order to characterize the diffusion, diffusion was observed through a single channel using a fluorescent dye. This allowed the diffusion to be observed in real time, and thus gave more precise data. We were interested in what the diffusion rate and distance were, since these properties affect the nutrient delivery to the seeded cells in an adjacent layer. The diffusion distance was going to tell us if the microvascular network increased the diffusion limit enough to

reach the furthest point of the adjacent cell layer. The diffusion rate was crucial to determine the time necessary for the nutrients in the channel to reach the seeded cells.

The dye chosen for this diffusion assay was fluorescein isothiocyanate (FITC). The molecular weight of FITC is 389.4 g/mol. Even though the molecular weight (MW) of glucose is half that of a FITC molecule, the molecular weights of the two molecules would not significantly change the diffusion. At such small molecular weights, the difference between the MW of glucose and FITC would have negligible effects on the diffusion rate and diffusion coefficient, so FITC can be used as a glucose analog. FITC was chosen because it is a fluorescent dye, can be used to replace glucose, and is readily available.

For the experiment, microvascular networks were first fabricated. 0.0625% w/v FITC solution was loaded into a syringe and perfused at 0.005 mL/min through the fabricated channel system. For the first thirty seconds, using screen capture software and the live image on the computer, video of the diffusion was recorded in real time. The initial diffusion was rapid due to the large concentration gradient between the solution inside the channel and the solution in the gel around the channel. After the initial thirty seconds, images were periodically gathered for fifteen minutes because by that point the diffusion had reached the mold's wall and was reaching equilibrium. To repeat the data collection process, the assay was run two times. A detailed protocol of the experiment can be found in Appendix D: Protocols: Fibrin Gel Diffusion Assay.

Once all the images were gathered, the team determined the diffusion distance from the channel wall. First, the channel wall was defined. The second step was to set a grey scale threshold which would define the diffusion boundary. The darkest areas, where the FITC had not reached, ImageJ measured a grey scale value of 5, while the inside the channel, where FITC solution concentration was the highest, ImageJ measured an average grayscale value of 75.

Based on those grey scale values and the standard deviation, which was 3, a grey scale threshold of 10 was defined to indicate the boundary of diffusion. Using ImageJ, as demonstrated in Figure 98, the distance between the diffusion boundary and the channel wall was measured. Based on the gathered data, the team modeled the relationship between the diffusion distance and time.

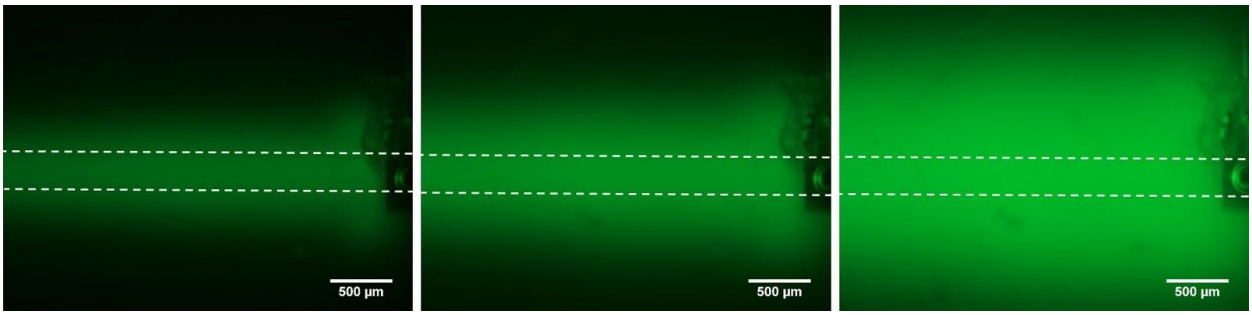


Figure 98: FITC Diffusion. Images gathered from fluorescent imaging of FITC diffusion.

The team found out that in the initial ten seconds of the diffusion, the model was logarithmic. This can be explained by the concentration gradient between the solution inside and outside of the channel. After the initial ten seconds, the slope stabilized and remained linear until the final data point is reached. This can be explained as the system had reached a steady state, where the concentration has a linear slope based on the distance from the channel. As Figure 99 demonstrates, the diffusion of FITC reached 1600 µm, while remaining at a constant rate. The equation which describes the linear relationship between diffusion distance (µm) and time (s) is:

$$d=2.31*t+235.5. \quad (3)$$

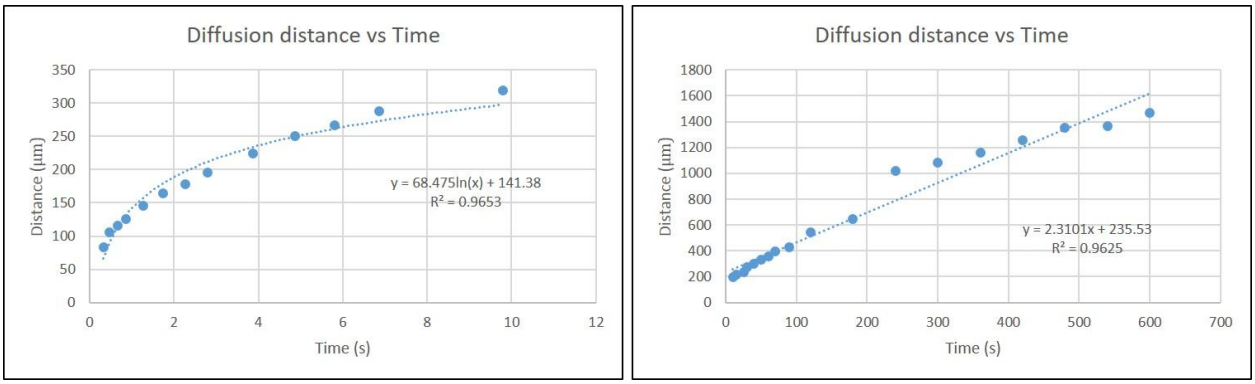


Figure 99: Model graphs of the diffusion of FITC. The left graph shows the logarithmic model for the first 10 seconds, while the right graph shows the steady state diffusion model, which matches a linear slope.

The calculated R-squared value was 96.25% for the linear curve fit, which indicated that the model is a good fit to measured data points.

5.5 Cell Viability Assays

One of the high level objectives is for the microvascular network to maintain the viability of cells. The experimental setup aims to imitate physiological conditions. The only source of oxygen, nutrients, and serum molecules was from the fluid perfusing through the channel. In order for all the seeded cells to survive, the diffusion rate of the molecules through the gel needed to be high enough to reach the furthest cells before they no longer remained viable. To develop these assays a preliminary cell survivability study was conducted. Two types of cell viability were performed, the first of which was cells seeded directly on top of a perfused microengineered vascular network and the second of which consisted of a cell-seeded fibrin hydrogel placed adjacent to a perfused microengineered vascular network.

5.5.1 Preliminary C2C12 Survivability Study

For this project, C2C12, mouse myoblasts were used for each cell viability experiment due to their skeletal muscle cell type which is one of the closest cell types to cardiac muscle, ease of culture, and availability in the lab. In order to develop cell viability assays for the microengineered vascular network, the C2C12 cell viability was initially characterized in standard culture. Unfavorable conditions in which C2C12 cells cannot survive or cell viability is not maintained were tested. In the experiment, three conditions were tested, C2C12 in media, C2C12 in PBS, and C2C12 in air. The cells were seeded in well plates and sampled at three different time points: four, eight, and twenty-four hours. At each time point, the cells were stained with a LIVE/DEAD stain, calcein and ethidium homodimer. Fluorescent images were gathered at different locations of the sample wells and qualitatively studied to determine the cell

culture survivability, which can be seen in Figure 100, Figure 101, and Figure 102. A detailed protocol of the assay can be found in Appendix D: Protocols: C2C12 Survival Assay.

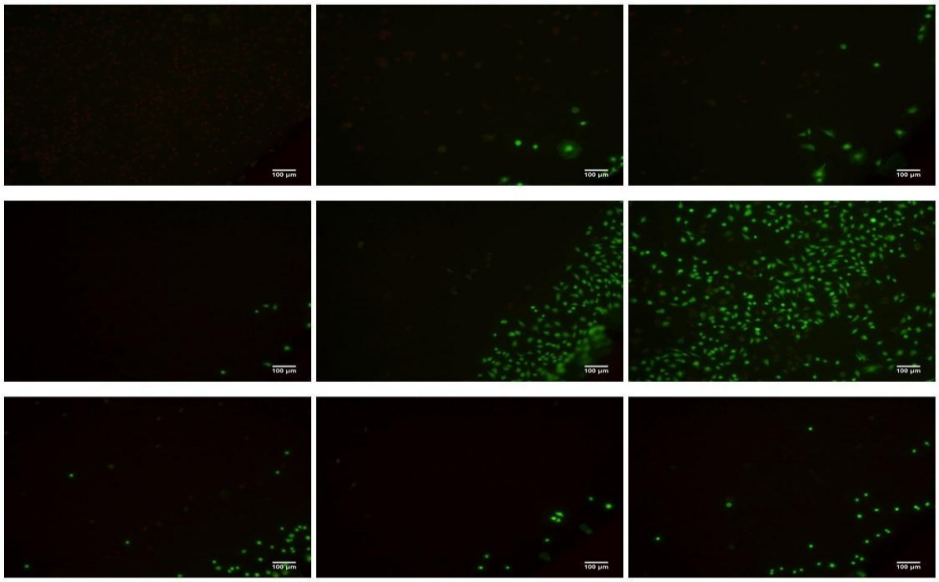


Figure 100: Fluorescent images at 4 hours. Taken from the top left corner of the well. Row 1 is C2C12 cells in air, row 2 is C2C12 in media, and row 3 is C2C12 in PBS.

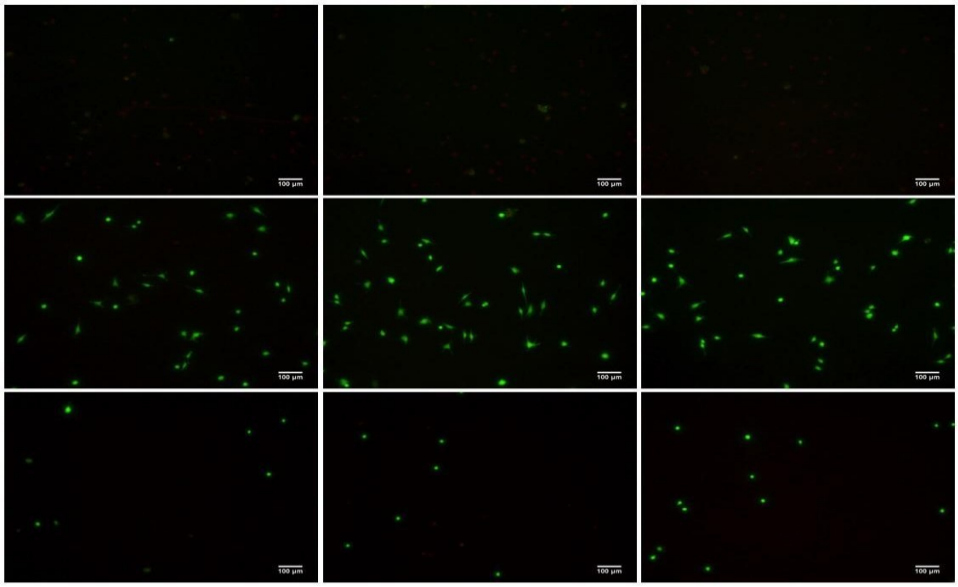


Figure 101: Fluorescent images at 8 hours. Taken from the middle of the well. Row 1 is C2C12 cells in air, row 2 is C2C12 in media, and row 3 is C2C12 in PBS.

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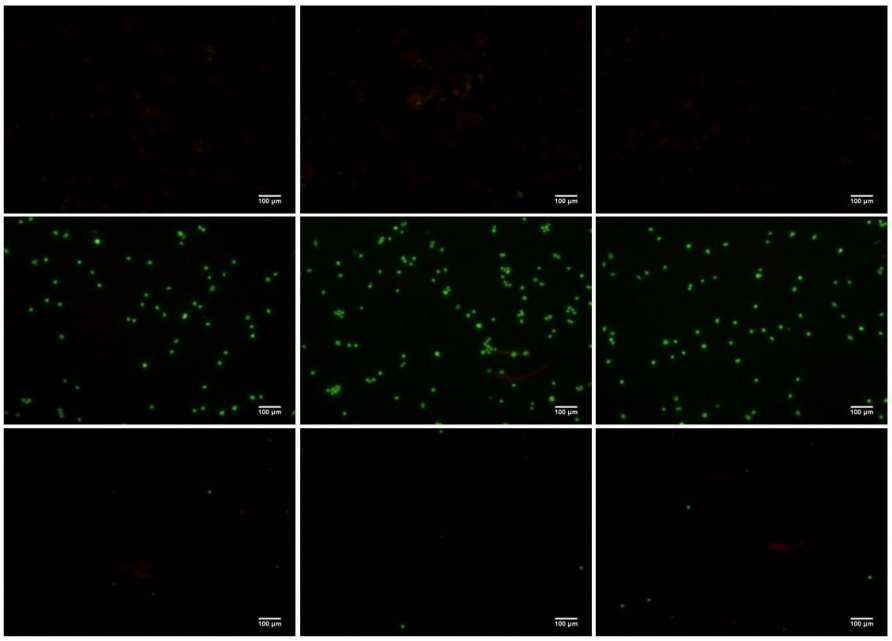

Figure 102: Fluorescent images at 24 hours. Taken from the middle of the well. Row 1 is C2C12 cells in air, row 2 is C2C12 in media, and row 3 is C2C12 in PBS.

Based on the gathered fluorescent images, the team drew conclusion about the cell viability of C2C12 in the different experimental conditions. The purpose of this preliminary study was to determine the minimal amount of time necessary to study C2C12 cell viability. The qualitative data showed after four hours, a number of cells in the PBS condition and air condition survived at the edge of the well, where there was hypothesized to be media residue. At 4 hours, C2C12 cells in the media condition remained viable. The images also demonstrated that after 8 hours, C2C12 in PBS and in air are all dead, determined by the lack of green fluorescence and rounded morphology of the cells. At eight hours, it can be seen that the C2C12 in cell media maintained their viability. At twenty-four hours, it seemed only the cells in media were remained viable.

5.5.2 Cells on Top of Perfused Single Channel Viability Assay

The objective of these experiments was to determine if perfusion through a single channel in a fibrin hydrogel could maintain cell viability of cells seeded on top of the hydrogel.

The schematic for this assay can be seen in Figure 103. C2C12 cells were seeded directly on top of the perfused microengineered vascular network fibrin hydrogel. The distance from the edge of the channel to cells directly over the channel was approximately 1200 µm and the horizontal distance to the cells furthest from the channel was approximately 4700 µm.

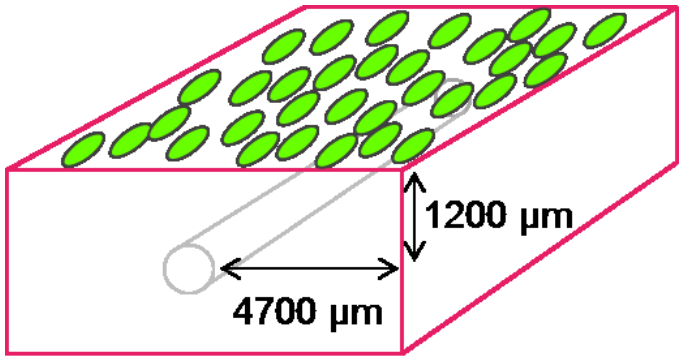

Figure 103: Cells on top of perfused single channel viability assay schematic. The distance from the edge of the channel to cells directly over the channel was ~1200 µm and to the furthest cells was a horizontal distance of 4700 µm.

5.5.2.1 Cell-Seeded Coverslip

The first experimental set-up had cells seeded on top of a coverslip and then placed upside-down on a perfused gel. This set-up was chosen because of the ease of imaging at the end of the twenty four hour experiment. The coverslip was removed from the microengineered vascular network, washed, and stained with a LIVE/DEAD stain.

However, because the top of the gel was concave, an air bubble formed between the gel and coverslip. Even though perfusion through the gel was successful, the cells did not come in contact with the media and subsequently died as seen in Figure 104. The curvature of cells seen corresponds to the edge of the air bubble under the coverslip. To adjust for this problem, the cells were seeded directly on top of the gel in the next cell viability experiment.

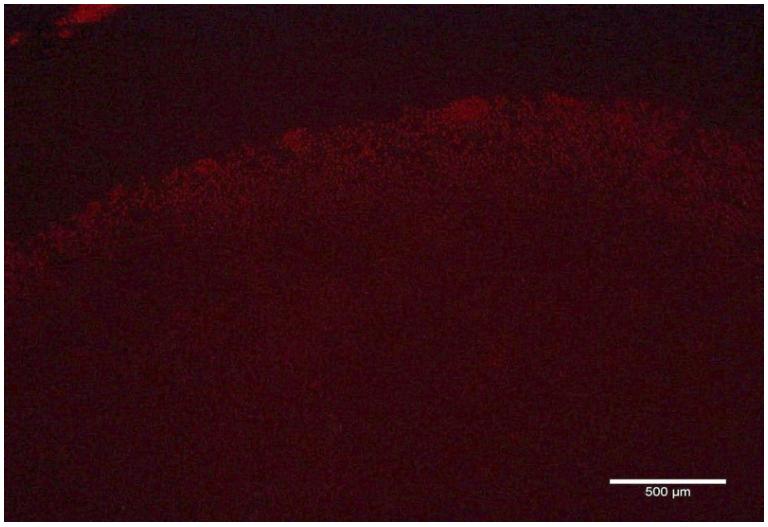

Figure 104: Coverslip fluorescent staining for cell viability assay. Image of cells stained with ethidium homodimer to show dead cells on coverslip. Note the curved line of cells which corresponds to the location of the air bubble between the coverslip and the perfused gel.

5.5.2.2 Cells Seeded Directly on Top of Gel

The next cell viability experiment set-up included a positive (+) control of cells in media (n=3), negative (-) control of cells in PBS (n=3), and (-) control of cells on a gel with a non-perfused channel (n=1). There were also two experimental groups of media perfused (n=1, PTFE bioreactor) and PBS perfused (n=2, PLA molds).

The experiment lasted twenty-two hours (Perfusion rates: two hours at 0.01 mL/min, seven hours at 0.001mL/min, eleven hours with no perfusion, 0.5 hours at 0.01 mL/min). A rate of 0.01 mL/min was initially used to ensure gelatin has been thoroughly flushed out. Because there was an extended period of time in which group members could not be in the lab to refill syringes, the flow rate was dropped to 0.001 mL/min. When one group member refilled the syringes, the "run" button was not pressed on the syringe pump. As a result the samples were not perfused for eleven hours overnight. Upon arrival in the morning the samples were perfused for 0.5 hours at 0.01 mL/min to flush out waste and supply nutrients to cells.

As seen in Figure 105, the positive control stained for living cells had healthy, elongated morphology. The sample perfused with PBS showed no living cells after twenty-two hours as

seen by the rounded morphology and solely red stain on the merged images. To note, a gel that was not perfused dried out over the course of the twenty-two hours while gels perfused with either PBS or media remained fully hydrated.

There is some background fluorescence in the perfusion with media images because all of the media in the gels was not able to be washed out. There are esterases in the serum that react with the calcein, just as the esterases in live cells do. However it is clear the gel is covered with living cells with healthy morphology like the positive control. Images are included of two regions of the gel, one at the edge of the sample and another directly over the channel. There does not seem to be a difference in cell viability between the two locations.

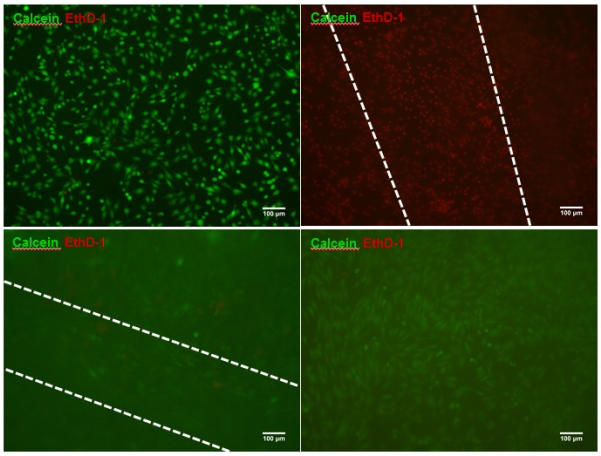

Figure 105: Results from cell viability experiment with cells on top of perfused gel with a single channel. All are merged images of LIVE/DEAD stain. Top row: Positive control of cells cultured in media (left) and cells on top of microengineered vascular network perfused with media both directly over a channel (left) and at the end of the hydrogel (right). White lines depict location of channel walls below cells.

Once cells were shown to be maintained on the surface of fibrin gel containing a microengineered vascular network, the cell viability in an adjacent fibrin layer was assessed.

5.5.3 Adjacent Layer Cell Viability Assay

The application of the work in this project in the Pins Lab is the creation of stacked, alternating myocardial and microvascular fibrin hydrogels. A cell viability assay of a fibrin hydrogel adjacent to the vascular network would indicate the effectiveness of this system. Once

it was shown that the perfused microengineered vascular network and bioreactor system maintained viability of cells seeded directly on top of the fibrin microvascular network, the viability of cells in adjacent layers was assessed using the protocol found in Appendix D: Protocols: Cell Viability in an Adjacent Layer on Top of Perfused Single-Channel Gels.

5.5.3.1 Trial 1

In this trial of cell viability of an adjacent layer to the perfused microengineered vascular network, the adjacent layer was added to the microfluidic chamber and then perfusion was initiated at 0.002 mL/min. This flow rate was maintained for twenty-four hours.

As seen in Figure 106, all cells were dead. The green seen in the image is background, base-level fluorescence.

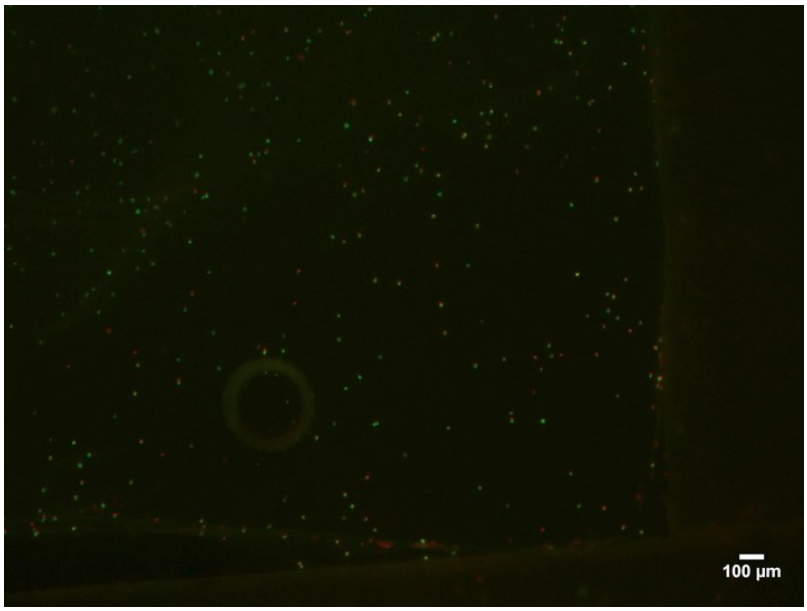

Figure 106: LIVE/DEAD merged image of cell-seeded fibrin hydrogel adjacent to perfused microengineered vascular network.

5.5.3.2 Trial 2

Trial 1 cell viability assay of an adjacent layer was unsuccessful as adjacent gels contained only dead cells. It was hypothesized this was because it took too long for media to reach the cells in the adjacent layer before they died.

In this trial of cell viability of an adjacent layer to the perfused microengineered vascular network, the adjacent layer was added to the microfluidic chamber and then perfusion was initiated at 0.01 mL/min for thirty-five minutes before lowering the flow rate to 0.002 mL/min for the rest of the twenty-four hours.

As seen in Figure 107, all cells in the adjacent layer were not viable.

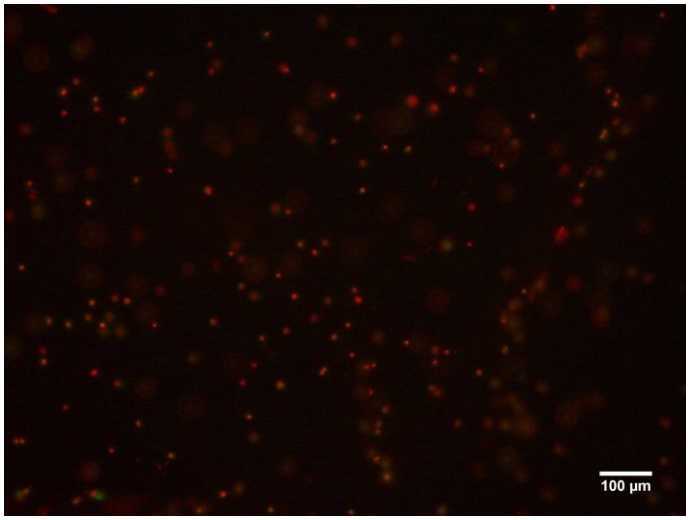

Figure 107: LIVE/DEAD merged image of cell-seeded fibrin hydrogel adjacent to perfused microengineered vascular network.

5.5.3.3 Trial 3

Trials 1 and 2 were unsuccessful and this was hypothesized to be because it took too long for media to reach the adjacent layer before cells died.

In this trial of cell viability of an adjacent layer to the perfused microengineered vascular network, the microengineered vascular network hydrogel was pre-perfused with media at 0.01 mL/min for one hour in an attempt to have media close to the top of the hydrogel when the adjacent layer was added. The adjacent layer was then added to the microfluidic chamber and then perfusion was lowered to 0.003 mL/min. This flow rate was maintained for the remained of the twenty-four hours.

As seen in Figure 108, all cells in the adjacent layer were dead.

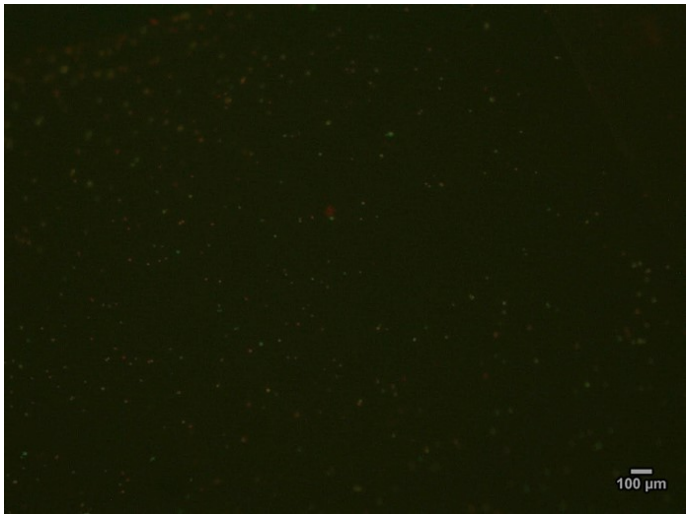

Figure 108: LIVE/DEAD merged image of cell-seeded fibrin hydrogel adjacent to perfused microengineered vascular network.

5.5.3.4 Trial 4

Trials 1, 2, and 3 failed and it was hypothesized that the low cell viability was not due to lack of media, but lack of oxygen.

In this trial of cell viability of an adjacent layer to the perfused microengineered vascular network, there were a few changes made. Cells were seeded not only in the adjacent layer, but throughout the microvascular hydrogel. The gelatin channel was flushed with media instead of PBS to "pre-load" the fibrin with some media. Gelatin flushing always is completed with a flow rate of 0.01 mL/min. Finally, one microfluidic chamber did not have the top coverslip viewing window in place while the other was sealed as usual. The adjacent layer was added to the microfluidic chamber and then perfusion was initiated at 0.002 mL/min. This flow rate was maintained for twenty-four hours.

As seen in Figure 109, cells remained viable after twenty-four hours in the positive control in which the cell-seeded gel was immersed in a well with media, and in a cell-seeded gel adjacent to a microengineered vascular network in an open microfluidic chamber. Cells did not remain viable in the negative control in which the cell-seeded gel was immersed in a well with

PBS and in a cell-seeded gel adjacent to a vascular network in a fully sealed microfluidic chamber.

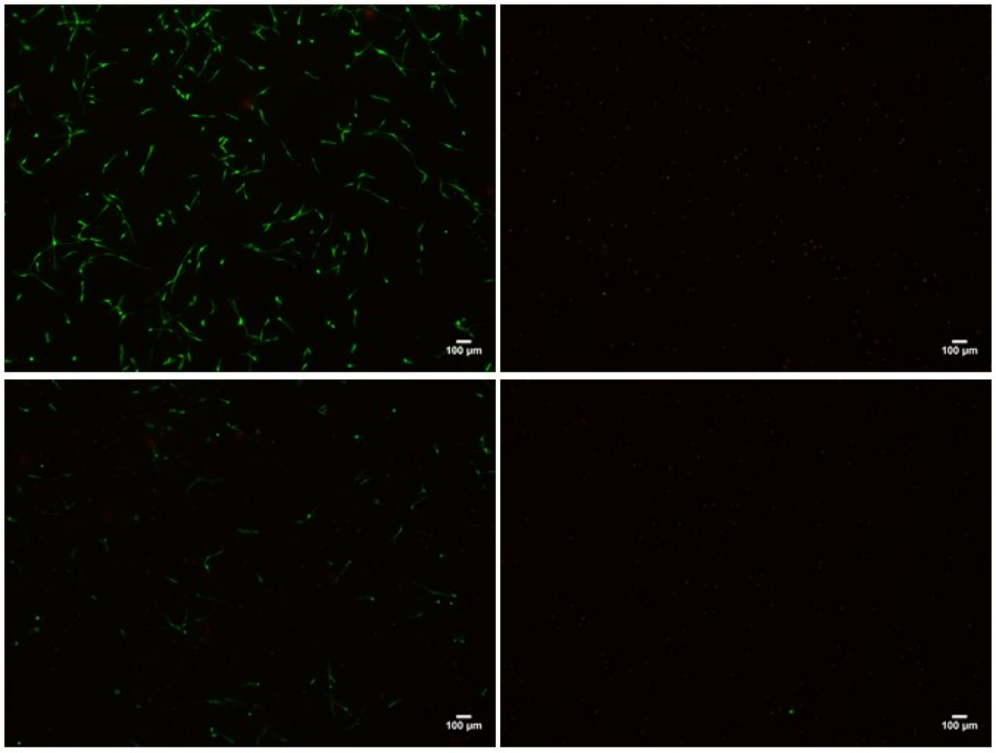

Figure 109: LIVE/DEAD merged images. Top row: Positive control of cells in hydrogel in well with media (left) and negative control of cells in hydrogel with PBS (right). Bottom row: Cells in adjacent layer in open microfluidic chamber (left) and cells in adjacent layer in sealed microfluidic chamber (right).

As seen in one representative image in Figure 110, cells throughout the full thickness vascular network hydrogel did remain viable in the open microfluidic chamber while cells in the vascular hydrogel in the fully sealed bioreactor had little to no viability.

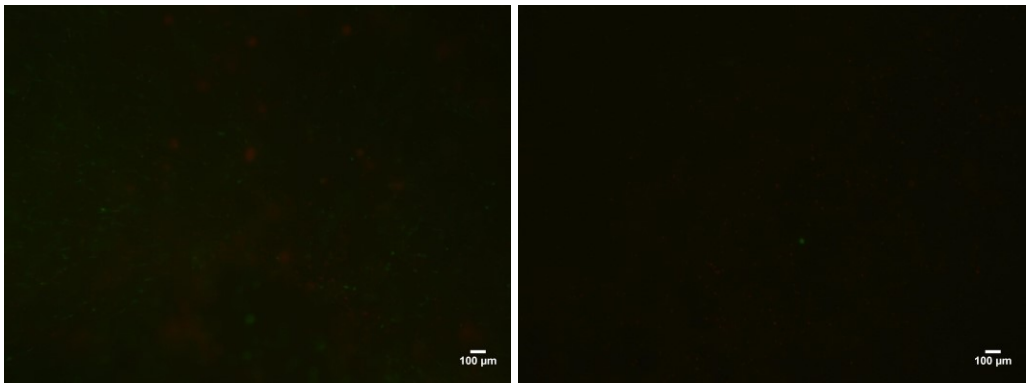

Figure 110: LIVE/DEAD merged images. Cells seeded within vascular network hydrogel in open microfluidic chamber (left) and fully sealed microfluidic chamber (right).

Chapter 6: Final Design and Validation

The purpose of this project was to design, develop, and characterize a bioreactor that provides continuous flow through a thin hydrogel scaffold containing a microengineered vascular network. Through the verification experiments performed in Chapter 5: Design Verification, the final designs of the microvascular network and bioreactor were selected, and the fabrication process was finalized. The team then validated the final design through a new set of experiments. These tests were set to evaluate the device along the objectives and functions defined in Chapter 3: Project Strategy.

In terms of the microvascular network, the qualitative assessment was based on the network's ability to sustain physiologically relevant flow and the fabrication method's ability to form channels of physiologically relevant diameters. Along with that, the assessment evaluated the network's ability to be stacked and the reproducibility of the microvascular network. It also assessed the network's endothelialization and the cost effectiveness of network fabrication.

The following sections discuss the qualitative assessment of the bioreactor design's ability to limit gas permeability, to allow for reproducibility, the use of multiple samples, ease of use and manufacturing, material cost, and cost effectiveness. Along with the objectives, the constraints and functions were taken into account. The team was able to successfully meet all of the defined constraints. The team was able to fabricate a microvascular network which maintained its patency and was able to sustain a physiologically relevant flow rate. The engineered vasculature also showed potential capacity to supporting an adjacent cell seeded layer. The team additionally developed a bioreactor system that can maintain viability of a small tissue construct through the use of a microfluidic chamber component. The chamber assists in the microvascular network fabrication and holds the tissue construct in a sterile environment

over an extended period of time. The team was able to complete the project in twenty-eight weeks and remain within the given budget of $780. In this chapter, the team described the final designs and how the project's objectives were achieved. Along with that, the limitations of the tests performed are discussed in addition to the relationship between the project and topics including economics, environmental impact, societal influence, political ramifications, ethical concern, health and safety issues, manufacturability, and sustainability.

6.1 Final Design

The gelatin sacrificial method was selected as the final fabrication method for the microvascular network. Based on the studies described in Chapter 5: Design Verification, this final design achieved most of the objectives and all functions described in Chapter 3: Project Strategy, while following the set constraints and specifications. The detailed protocol for microvascular network fabrication using the gelatin sacrificial material approach can be found in Appendix D: Protocols.

The Pressure Square Microfluidic Chamber, as described in Chapter 4: Design Process, was machined by Thomas Partington in Goddard Hall's machining shop of Worcester Polytechnic Institute. The base of the microfluidic chamber and the simple mold components were made out of PTFE. The top of the microfluidic chamber was made of POM and used a silicone gasket to improve the sealing between the microfluidic chamber parts. In addition the stainless steel plates allowed for distributed pressure improving the sealing and stability. The protocol for machining the microfluidic chamber can be found in detail in Appendix D: Protocols: Microfluidic chamber fabrication process.

When using the machined Pressure Square Bioreactor, all pieces needed be sterilized prior to use. When casting fibrin within the bioreactor mold, the following steps should be taken.

First, an 18 mm x 18 mm glass coverslip should be placed in the pocket of the base. As described in Section 5.3 Continuous Perfusion, the gelatin mold, either single or bifurcated, should be placed within the two pieces of the mold. The plugged needles are next inserted into the inlet and outlet holes, and the fibrin is cast. Once the fibrin has polymerized, another 18 mm x 18 mm coverslip is placed on top of the mold sealing the fibrin gel system. The top half of the bioreactor is placed and the screws are screwed in to ensure a tight fit so that leakage cannot occur. The final CAD drawings can be seen in Appendix E: Final Design. Figure 115 shows the exploded view of the microfluidic chamber. Figure 116 - Figure 120 show the 2D drawings of the machined pieces. In addition the parts list for the chamber is located in Appendix E: Final Design. The plugged needles are replaced with new needles, and the system is moved into the incubator in order to melt the gelatin hydrogel. Using warm PBS, the gelatin is flushed out, and a patent channel is formed.

6.2 Objectives Achieved

The following section describes how the final designs achieved the objectives determined in Chapter 3: Project Strategy. The section includes details on how the microvascular network and bioreactor follow the set specifications and remained within the design space limited by the constraints.

6.2.1 Microvascular Network

6.2.1.1 Physiologically Relevant Dimensions and Flow

The defined objective was that the fabricated microvascular network needed to have a diameter ranging between 11 and 1000 μm and be able to sustain a flow rate ranging between 0.0015 and 0.21 mL/min. Using the gelatin sacrificial method and the available gauge needles, the team was able to fabricate channels which mimic the sizes of arterioles. The system was also

able to sustain its patency, when perfused at flow rates between 0.001 and 0.005 mL/min for an extended period of time.

6.2.1.2 Stackable

One objective of this project was to create a system that would allow for the stacking of fibrin hydrogels. In its simplest form, one gel containing a microengineered vascular network and another containing cells would be able to be stacked. This objective was accomplished as seen in Section 5.5.3 Adjacent Layer Cell Viability Assay. The ultimate goal of stacking cell-seeded layers adjacent to the microvascular network was to maintain cell viability. This was shown, at least preliminarily, in Trial 4 of the Adjacent Layer Cell Viability Assay in which cell seeded in the adjacent layer remained viable. Though the top of the microfluidic chamber remained open since no glass coverslip was added, because cells remained viable after twenty-four hours, the system perfused media through the microengineered vascular network hydrogel into the cell-seeded adjacent hydrogel. In conclusion, this project developed a system that allowed for the stacking of two fibrin hydrogels and produced preliminary data that cell viability in the adjacent layer was able to be maintained.

6.2.1.3 Reproducible

From the tunability study and reproducibility analysis, described in Chapter 5: Design Verification, the team demonstrated that the fabrication method created microvascular networks of similar diameters. For example, the average diameters of gelatin sacrificial molds after 25 minutes of dehydration for 20G and 23G needles were 453 μm ± 8% and 270 μm ± 8% respectively. Additionally, the team showed that the final diameter of the channel is dependent on the initial size of the sacrificial mold. This would allow the user to adjust the final resulting

microvascular network dimensions based on the extrusion needle used to create the sacrificial mold.

6.2.1.4 Endothelializible

After the client meetings and discussions, the endothelialization objective of the microvascular network was ranked second to last. As such, this want was addressed last in the project strategy. Due to the time constraint on the team, endothelialization of the microvascular network was not achieved. This could be potentially addressed in future work on the project.

6.2.1.5 Cost Efficient

The average cost of the materials necessary to fabricate a single microvascular network is $ 0.0672, calculated based on the components in Table 27. These values were calculated based on the cost of the raw stock materials from providers and the amount used in the fabrication of the designed microvascular network. The finalized microvascular network fabrication protocol, as written in Appendix D: Protocols, minimizes any possible waste of materials. The protocol allows for any excess materials to be frozen for later use, which significantly reduces the amount of waste.

Table 27: Cost of materials necessary for the fabrication of a single microvascular network

Material	Cost per one microvascular network fabrication ($)
Fibrinogen	$64.4*10^{-3}$
Thrombin	$2.02*10^{-3}$
PBS	$0.106*10^{-3}$
$CaCl_2$	$38.3*10^{-6}$
Gelatin	$0.255*10^{-3}$
HEPES Buffered Solution	$0.404*10^{-3}$
NaCl	$0.426*10^{-6}$
Total	0.0672

6.2.2 Bioreactor

6.2.2.1 Controlled Gas Permeability

Through the sealing test described in Chapter 5: Design Verification, the results suggested that a sealed microfluidic chamber is gas impermeable. By analyzing the cell viability of an open microfluidic system and a completely sealed microfluidic system, the team hypothesized that by sealing the chamber, the only oxygen that could be delivered to the system came from the media. Cells died much faster when in the sealed chamber, compared to being in an open chamber with no coverslip. When a closed chamber was perfused through, the cells remained alive, proving that the cells were receiving enough oxygen and nutrients from media.

6.2.2.2 Maintain Sterility

The microfluidic chamber maintained sterility over a five day period. The silicone gasket, stainless steel plates, and screws successfully seal the chamber. Using all three provided a tight seal of the chamber; it proved to be more effective than using one or two methods alone. The

chamber was sterilized in 70% ethanol for ninety minutes, rinsed with distilled water three times, and then filled with media. This setup remained in the incubator for five days and resulted in no microbial growth, proving the method of sterilization to be successful.

6.2.2.3 Reproducible

The dimensions of the microfluidic chamber are intricate and the small scale of the features can be difficult to reproduce. However, with the correct tools and experience, the dimensions could be reproduced easily. The flow through the chamber is subject to no change between experiments because there is no change in the dimensions of the chamber between uses. The only modulation in the flow would come from any size differences in the microvascular channel during the channel formation process.

6.2.2.4 Multiple Samples

Currently the microfluidic chamber is self-contained for a single fibrin gel with a single cell scaffold. This means cross contamination is avoided by isolation of each sample to its own chamber. Future versions may be designed to accommodate multiple samples. This design would simply need to extend the length of the current chamber and create multiple slots for the mold to sit into, as opposed to only one. There would be some features that must be determined before designing a microfluidic chamber that can accommodate multiple samples such as whether or not each sample needs its own perfusion system or if all samples would be perfused at the same time with one pump. Another consideration would be whether each slot would be growing tissue with the same properties or not.

6.2.2.5 Ease of Use

The microfluidic chamber is a very small device making it simple for the user to set up, transport, and perform experimentation. The average set up time for the chamber is about

twenty-two seconds when not using vacuum grease. However, when vacuum grease is being used in between all layers, the average time increased to one minute and forty-three seconds. The chamber must be transported from the lab bench where set up occurs, to the incubator where it would be attached to the whole bioreactor system. The microfluidic chamber is small enough to transport it with one hand, easily. Currently, the most time consuming step is cleanup. The vacuum grease can be messy and difficult to remove off the chamber. Cleanup takes about four minutes to ensure all the grease has been washed off. The chamber is washed with warm water and soap.

6.2.2.6 Cost Effective

The microfluidic chamber cost $0 to produce. This was because only little material was required to manufacture the part, so scrap from the machine shop in Goddard Hall was used. Glass coverslips cost close to $0.03 per piece and two are used in the chamber totaling $0.06. Also, two 20G needles are used which costs $0.10 per needle, totaling $0.20 if the chamber were to be manufactured. The materials used, POM and PTFE, could be purchased in bulk and the same could be done for the slides and needles. Producing this microfluidic chamber at large quantities would cost very little for the material and associated parts. Also, the chamber is reusable, minimizing costs to the user.

6.3 Impact of Device

The overall purpose of the following sections is to discuss the impacts the device has in areas including economics, environment, health and safety, society, politics, ethics, manufacturing, and sustainability.

6.3.1 Economics

By designing a microvascularization method, the team helped advance the development of the cardiac patch in the Pins Lab towards reaching clinically relevant sizes. A cardiac patch could offer a regenerative treatment for myocardial infarcts, which is readily available and regenerates the patient's myocardium, restoring function to the damaged site. By creating a bioreactor system that could assist in the fabrication of and sustain the multilayered cardiac scaffold, the team created a non-expensive method to create the replacement cardiac patches. If FDA approved, it is possible that this treatment could become the industry gold standard, as it overcomes many limitations of current treatments. Another possible advantage is that this approach would make the treatment more available, which would increase the number of treated patients while increasing the standard of living. If implemented, this treatment could increase the cost of fibrinogen, since there would be larger demand for tissue constructs. This in turn would promote the creation of companies that focus on providing this treatment and its required materials. Major challenges that need to be overcome to reach that stage are the development and finalization of the cardiac patch design, scaling up the design, FDA regulatory approval with animal testing and clinical trials, and finally the marketing and distribution of the treatment.

6.3.2 Environmental Impact

The designed bioreactor system and the engineered microfluidic network could have an impact on the environment. The microvascular network is made using gelatin, natural protein, fibrin, and natural polymers, all of which would be disposed of as a biohazard waste. Once biohazard waste is brought to the appropriate facility, it is incinerated and dumped into landfills. Incinerators release mercury and dioxins into the environment. Biohazard waste incinerators are one of the country's leading emissions sources of toxic and bioaccumulative pollutants. Also, the

diesel trucks which travel far distances to deliver the medical waste to incinerators also emit toxic pollutants. The pollutants that are released into the atmosphere threaten the public's health (Wormer et al., 2013). Since the final design involves cell culture, the supplies needed to maintain these cultures would follow safe biohazard disposal protocols, which would ensure that the materials would be discarded safely into the environment.

Currently, the chamber top is made from polyoxymethylene (POM). In regards to polyoxymethylene, there are no environmental impacts that exist. The material is not dangerous or of any harm to nature. However, the melting point of polyoxymethylene is 167°C and if this temperature is reached or surpassed, the generation of formaldehyde gas could occur. Since the steam autoclave temperature reaches 121°C, the polyoxymethylene should not reach its melting point (Council on Dental Materials, 1991). Because the difference between the melting of polyoxymethylene and the temperature of the steam autoclave is only 46°C, it may be a safer option to sterilize with ethanol.

The base of the bioreactor is made up of polytetrafluoroethylene (PTFE). PTFE has been shown to release toxic chemicals that could potentially be carcinogenic (Ellis DA, 2001). PTFE will endure temperatures up to 230°C for about 2.3 years before degradation occurs and toxic chemicals begin to be released (Simon, 1998). Regardless, for this project, the designed microfluidic chamber does not expect to reach temperatures that high. If it is decided that components of the bioreactor need to be disposed of, the user must follow appropriate solid biohazard disposal procedures. Since PTFE is a stable material, once cleared of biologicals, it can be disposed to a landfill or recycled. Most of the components of the whole bioreactor system are recyclable and reusable, which reduces the risk of waste accumulation.

6.3.3 Societal Influence

As discussed previously, the project aims to assist in the research and development of a cardiac patch, which is still in its early stages. If the overarching research project is successful in creating a cardiac patch as a treatment for heart failure, the need for heart donors would decrease and the cost for treatment could be reduced, making it more affordable for more people. One drawback of this is that there is may be a chance that the number of organ donors would actually decrease. This could be due to the public perception that tissue scaffold treatments have nullified the need for organ donors. However, by offering a more affordable treatment, which has lower associated risks than whole heart transplant, the standard of living of patients could improve. Based on the AHA statistics, the average cost of a heart transplant is approximately 1.2 million dollars. The team expects the cardiac patch treatment to cost a fraction of a heart transplant because the fabrication cost would be lower than the cost of finding and transporting an available live heart.

6.3.4 Political Ramifications

This project has insignificant political ramifications, as there would no direct effects on the industry or commercial market. If commercialized, it is possible that well developed countries would implement these treatments first, since these countries would have access to the research, materials, and funding to provide them. The increase in standard of living would likely make these countries more favorable, which could potentially lead to migration of people from less developed countries. The bioreactor system design and microvascular network design are in the initial stages of research and development, which means significant work, needs to be done before the engineered cardiac patch becomes marketable and accessible to the public.

If the device were to include embryonic stem cells, there would be perceived political issues. In 2001, President George W. Bush banned federal funding for embryonic stem cell lines that were recently created. This left only twenty-one cell lines fit for research using federal funding. In 2009, President Barack Obama introduced an Executive order that lifted the ban Bush executed and allowed the National Institutes of Health (NIH) to introduce new criteria for stem cell research. The new criteria allowed for hundreds of embryonic stem cell lines to be studied through federal funding. However, difficulties with stem cell studies still exist as the Dickey-Wicker amendment is still in place. This prevents federally funded scientists from creating new embryonic stem cell lines as they are not allowed to create or cause harm to embryos (Murugan, 2009). This would prove to be an issue with this project if scientists choose to use embryonic stem cells for the creation of the cardiac patch, since private funding would be required.

6.3.5 Ethical Concerns

For the scope of this project, there are few ethical concerns. One ethical concern is the isolation of proteins from animals used in the design. Fibrinogen, thrombin, and gelatin are proteins acquired from animal sources, two bovine and porcine sources respectively. If demand for tissue engineered cardiac patches increased, the need for these animals could increase as well.

The designs from the project are going to be contained within the lab space, where ethical norms are overseen by the Institutional Animal Care and Use Committee (IACUC). Ethical concerns could arise when the bioreactor system and microvascular network are used to fabricate a cardiac patch for an actual patient. There would need to be oversight that would control the patient's biopsied cells from being used for any other purpose than agreed on by the patient.

Tissue engineering includes the ethical concern of potential abuse of the regenerative approach. By regenerating tissue, researchers try to improve the function and structure of damaged tissue. This could, however, be used to create enhanced humans, which is similar to the ethical concern with embryonic gene therapy. The only way to regulate this and prevent this extreme from becoming reality is to strictly regulate who has access to the tools and materials necessary. These concerns, however, relate to the finalized and implemented treatment.

6.3.6 Health and Safety Issues

In terms of the bioreactor, it is safe for the user to use. None of the components of the bioreactor system produce harmful chemical fumes because they are all made of stable materials that are not reactive at room temperature, 22°C, and incubator temperature, 37°C. The device does not produce any harmful chemical byproducts. The main two cautions that must be taken is using glass coverslips because they are easy to break if not handled carefully and handling the input and output needles. Mishandling of these could potentially cause injury by punctures and cuts. The user must follow proper procedures for disposal of sharps, as well as follow standard procedures for working with blood-borne pathogens. In terms of the vascular network, because it is still within the initial phases of design, more tests will need to be completed to understand its safety and reliability. None of the materials used to fabricate the microvascular network are toxic, so the user is at low risk, as long as he or she follows proper lab safety procedures and protocols.

In order for the researched cardiac patch to be FDA approved, the designed system needs to go through thorough inspections and regulatory tests, as this treatment approach would most likely be treated as a 510k device. There is a chance that the fibrin sourcing will need to change, since currently for research bovine fibrinogen is used, which may not be the best option for

human implantation. Since fibrinogen and thrombin are blood derived, they must be screened for viral prions prior to use.

6.3.7 Manufacturability

The casing that is considered the “microfluidic chamber” can be manufactured simply with appropriate tools. The chamber needs to be manufactured with precision so that the system ensures proper sealing of the chamber. The screw holes must be drilled with a small enough tolerance to ensure the inlet and outlets fit tightly to avoid leakage. Manufacturing the inside corners of the bottom piece can be very challenging, since the machining device does not allow for sharp corner precision. A possible approach to this would be using a milling device to make the well initially, and then using a sawing device to cut the corners, making precise sharp corner which match to the mold edge. Besides these few components that require more precaution and attention, the microfluidic chamber is simple to manufacture and many could be reproduced quickly. The current prototype has been manufactured by hand at WPI. With appropriate CAD drawings, the part could be outsourced to professional medical device manufacturing companies, which have access to the materials necessary for the fabrication and machines with micrometer precision milling and cutting,

6.3.8 Sustainability

In order to achieve the fibrin microvascular network, significant amounts of unrecoverable energy is necessary. The network is fabricated using natural polymers and proteins which need to be isolated from animal sources. In order to purify and separate the desired materials, large amount of energy is necessary. Large amounts of energy is used to create the purified solutions necessary for cell media. During the fabrication process, energy is

expended to keep the biosafety cabinet and incubator functional, which are necessary for the sterile fabrication of the microvascular network.

Significant energy is needed to manufacture the bioreactor components. The syringe pump system required energy to fabricate, since energy would be expended to build the device from raw materials, and then to fabricate the pieces to the right size and dimensions. After that, addition energy is needed to assemble the system precisely. For the microfluidic chamber, significant energy would be expended to make the initial raw materials, and then to machine them to into the proper dimension to fit together.

For sustaining a tissue construct, energy would be expended to autoclave the microfluidic chamber, and to keep the syringe pump functional, as it runs on electricity. Additional energy would be expended by the incubator, which sustains conditions favorable for cell growth. The current bioreactor design allows for repeated use of the system, which means the energy necessary to fabricate the components is only once over extended periods of time. This is also true for the isolation and purification processes of the materials needed for microvascular network fabrication. The largest amount of energy would be consumed by the repeated sterilization of the bioreactor components, and the continuous function of the incubator to keep favorable conditions and of the biosafety cabinet to maintain a sterile environment for cell work.

6.4 ISO Standards

ISO International Standards ensure that products are safe, reliable and of good quality. The most important standard for the team's application is ISO 10993: Assessing the biocompatibility of medical devices and materials. This was examined by testing the biocompatibility of the material used through multiple experiments (ISO 10993, 2009). When cells were placed into the bioreactor system with constant perfusion, no death occurred, proving

the material to be biocompatible. Next is ISO 11737: Sterilization of Medical Devices. This standard is in place to determine what population of microorganisms might be on the product, test the sterility, and validate/maintain the sterilization process (ISO 11737, 2006). Tests were completed to analyze the sterility of the microfluidic chamber using 70% ethanol. The tests yielded no microbial growth, proving the sterility of the system. ISO 13022: Medical products containing viable human cells are the next standard that team must adhere to. (ISO 13022, 2012). This standard was not implemented during this project, but if the design were to be manufactured for clinical use, this standard must be implemented. The final standard that applies is ISO 16792: Technical product documentation using CAD. Computer-Aided-Design was used to design the microfluidic chamber and this standard was followed to ensure proper dimensioning, labeling, and documentation (ISO 16792, 2015).

Chapter 7: Discussion

Heart disease is the leading cause of death in the United States and the most common form is a myocardial infarction. A myocardial infarction causes the death of myocardium which is then replaced by scar tissue reducing the functionality of the heart because no healing occurs. Current treatments either are not readily available or are only used to prevent further damage. Most importantly, no treatments regenerate the patient's myocardium. There is a clear need for an off-the-shelf solution that regenerates functional tissue (Emmert, Hitchcock, Hoerstrup, 2013). The team was able to design and develop a bioreactor system as well as microvasculature that sustained the viability of C2C12 cells. This consisted of two main components: the microfluidic chamber and the microengineered vascular network in a fibrin hydrogel.

7.1 Discussion of Results

The purpose of this project was to design, develop, and characterize a microengineered vascular network in a fibrin hydrogel and a bioreactor that provides continuous perfusion. Once continuous perfusion was established, the diffusion of nutrients through the hydrogel and the viability of cells were to be assessed. As described in earlier portions of this report, through interviews and meetings with the client and users, the design team developed a list of objectives and constraints. Some of the high-level objectives for the project were being reproducible, of physiologically relevant dimensions, stackable, easy to use, and cost effective. From the list of objectives, the team developed functions which included providing continuous perfusion, maintaining sterility, allowing for visualization, providing diffusion of nutrients, and maintaining cell viability. Over the course of the project, each of these functions were achieved.

7.1.1 Microengineered Vascular Network

A microengineered vascular network was designed and developed using 10% gelatin in PBS as a sacrificial mold.

7.1.1.1 Gelatin Sacrificial Mold Method Development

A method to produce both a single cylinder and a sacrificial mold with one or two bifurcations, transfer to a microfluidic chamber, maintain structure in fibrin, and form a perfusable channel was designed and developed. To develop this method, much iteration was attempted to solve problems as they arose.

The method of loading liquid gelatin into a syringe, cooling to form a gel, extrusion through a needle onto a PDMS surface enabled rapid production of single sacrificial mold cylinders. To create bifurcated channels, a method was developed in which the solid gelatin was heated to its gel transition temperature, between 25℃ and 30℃ for a concentration of 10%, and printed. This method allowed for the production of single- and double-bifurcations in a user-friendly, non-time-consuming way.

7.1.1.2 Transfer and Placement in Microfluidic Chamber

Unless the sacrificial mold was to be produced in the microfluidic chamber, it would have to be transferred to the chamber. Immediately after extrusion, it was not possible to handle the mold. The dehydration process on the benchtop for thirty minutes enabled the mold to be lifted, cut, and maneuvered into the microfluidic chamber.

To overcome the low density of gelatin relative to water, it was critical to hold the mold in place to prevent it from rising to the top of the fibrin solution. The developed method includes inserting the mold into the inlet and outlet needles of the microfluidic chamber. This kept the sacrificial mold in the center of the fibrin throughout the polymerization process. The final

design in this project has a relatively thick microengineered vascular network hydrogel of around 3000 μm. The ultimate goal is to create thin microvascularized hydrogel. This method of keeping the sacrificial mold straight and in the plane of the inlet and outlet needles would be critical as the microvascular network hydrogel is scaled down in thickness to ensure the mold does not bend and reach the surface of the fibrin gel,

7.1.1.3 Channel Formation

The ultimate goal of forming sacrificial mold in microvascular engineering is to remove it and form a channel. The team developed a method of forming a channel by warming the entire sample in an incubator for ten to fifteen minutes to fully melt the gelatin and then perfusing with warm PBS for more than ten minutes at a flow rate of 0.1 mL/min to ensure full gelatin removal. This same process was able to be used for the fabrication of single and bifurcated channels.

7.1.1.4 Tunability of Channel Diameter

It was first determined that the initial sacrificial mold had an outer diameter not significantly different than the inner diameter of the corresponding extrusion needle. It was then shown that there was a significant difference between this initial mold diameter and final diameter of the formed channel. It was determine that the final channel diameter could be predicted through a linear relationship with the initial mold diameter immediately after fabrication. The implication of this is that one could tune the desired channel diameter by changing the needle through which the sacrificial mold is extruded. Additionally, using needles of gauges smaller than 19G would result in channels with physiologically relevant diameters on the scale of arterioles.

7.1.1.5 Reproducibility of Sacrificial Mold Production

It was shown that the fabrication of cylindrical sacrificial molds in this project was reproducible with respect to the diameter of the molds. The average diameter and percent standard deviation of gelatin cylinders were measured and calculated at three time points, zero minutes (immediately after extrusion), twenty-five minutes, and seventy-five hours for two different gauge needles, 20G and 23G. As seen previously in Chapter 5: Design Verification, the average diameter immediately after extrusion is approximately equal to the inner needle diameter. At all-time points for both needle sizes tested, the percent standard deviation was 10% or less. The team concluded this showed sufficient reproducibility. Also important to note is that there is little difference between the diameters at twenty-five minutes and seventy-five hours. From this it can be concluded that the gelatin sacrificial molds decreasing of size is mostly complete by twenty-five minutes after extrusion and that this is a sufficient amount of time for dehydration.

The ability to form reproducible, tunable, perfusable channels in fibrin within a biomaterial has implications to tissue engineering. Through the implementation of the microengineered vascular network process designed in this project, it was hypothesized the passive diffusion limit of oxygen and nutrients could be overcome. By overcoming this limitation, scaffold development can continue to produce tissues constructs of clinically relevant sizes, for example a one centimeter thick cardiac patch (Kawel et al., 2013).

7.1.2 Bioreactor System

The team designed and developed a microfluidic chamber in a perfusion bioreactor system that successfully provided continuous perfusion of a physiological rate for twenty-four hours. The bioreactor system includes the microfluidic chamber, a syringe pump, and collection

flask. In addition, the team designed and developed a microvasculature in a fibrin gel that sustained C2C12 cells 1200 μm above the network. In the following sections the results and limitations of the design testing is discussed.

7.1.2.1 Perfusion Testing

One of the functions of the bioreactor was to allow for continuous perfusion. During the perfusion test it was shown that the bioreactor system was able to continuously perfuse one milliliter of liquid through the microfluidic chamber successfully at flow rates of 0.01 mL/min, 0.1 mL/min, and 1 mL/min. The system allowed for the perfusion of water in and out without disruption. This means the system can sustain perfusion through the microfluidic chamber to provide the sample with the necessary nutrients for growth. The longest the team perfused through the microfluidic chamber was twenty-four hours. A limitation of this test is that the duration was only one day and therefore does not prove the ability to perfuse over the original desired length of three weeks.

7.1.2.2 Leakage Testing

The leakage test resulted in no leaked fluid. This means the system is sealed and can sustain perfusion over the course of experimentation. The bioreactor system can both perfuse liquid through and not leak, showing the system is properly sealed. This proves that the microfluidic chamber can contain fibrin before and after polymerization without compromising the structure of the gel. Because of this, the chamber can be used without losing volume in the experimentation area or contaminating the sample for up to twenty-four hours. The limitation of this test is that it was only performed for twenty-four hours. Future experiments should perform leakage tests for longer periods of times to understand the microfluidic chambers ability to remain properly sealed.

7.1.2.3 Setup Time

An objective of the bioreactor was ease of use. If the setup time for the microfluidic chamber is minimal, it contributes to the ease of use of the entire system. As seen in Section 5.2.3 Setup Time of Microfluidic Chamber, the average of the three trials took 98.43 seconds to complete. The duration of the setup decreased with each trial. This shows that after becoming familiar with the system and the process becomes more fluid, the time it takes to complete is reduced. Overall, a setup time of approximately 100 seconds requires a minimal time contribution to the setup of the system which lightens the burden on the user and demonstrates its ease of use. A limitation of this experiment includes that it did not include the time it takes to create the gelatin sacrificial mold, place the sacrificial mold, allow for diluted thrombin and fibrinogen aliquots from the freezer to thaw, create the fibrin scaffold, etc. because these steps can greatly vary in set-up time depending on different conditions. The setup time calculation strictly applies to the setup of the microfluidic chamber itself and does not include any of the microengineered vascular network components or the other components of the bioreactor system as a whole.

7.1.2.4 Dimension Validation

By validating the dimensions of the microfluidic chamber, the accuracy of the machining process can be tested. Based on the results, the highest percent error that occurred for any measurement was with the silicone gasket, which had a percent error of -6.000%. This is because the gasket is stretched when it sits in the grove of the POM top piece of the chamber which causes stretching and for the width of the gasket to decrease in size. The next highest percent error occurred with the PTFE chamber top viewing window length, which had a percent error of 3.47%. Overall, since the percent error for all fabricated components is less than 4%, the

machining process proved to be effective. A limitation of this experiment includes that it was only performed on one fabricated microfluidic chamber. Although two were made, the dimension validation test was only performed on one. Another limitation is that this dimension validation was performed after the microfluidic chamber was used a few times. Because of the malleable material used, there could be slight variation in dimensions after each use, especially screw holes.

7.1.2.5 Sterility

A function of the bioreactor was to maintain a sterile environment. The sterility test ran on the microfluidic chamber resulted in no microbial growth as seen in Figure 93 and Figure 94 in Section 5.2.5 Sterility Testing and suggested the completion of this objective. Over the five day incubation period, no bacteria was able to enter the chamber and grow. This suggests the sterilization technique of 70% ethanol was successful. This also shows the chamber successfully remains sealed and can maintain an aseptic environment during experimentation. This was successfully completed with the use of a silicone gasket and screws that compressed the microfluidic chamber. A limitation of this test is that the duration was only five days and therefore does not show sterility over the original desired length of three weeks. In order to prove the completion of the objective, a bioburden assay should be performed according to the FDA and ISO 11737 standards (ISO 11737, 2006).

7.1.3 Continuous Perfusion

Previous work in the Pins Lab focused on developing a microengineered vascular network using the bonding of a pre-vascularized hydrogel to a single flat hydrogel (Bornstein, Gagnon, Moutinho, & Reyer, 2015). Continuous perfusion was not able to be established due to improper sealing of the two hydrogels as well as at the input and output of the network. This

design of the microengineered vascular network presents solutions to these problems and as a result, continuous perfusion was shown to be possible. The vascularized hydrogel in the current method is formed in one piece which eliminates the need to bond hydrogels together.

These two strategies subsequently allowed for continuous perfusion after removal of the sacrificial mold. Perfusion was accomplished at physiologically relevant flow rates within the ranges of arterioles *in vivo*.

7.1.4 Diffusion Assay

Based on the gathered images, the team was able to measure the diffusion distance from the channel wall. From the data gathered, the relationship between the diffusion distance and times was modeled. A linear curve fit was used, since the team expected that the diffusion through the gel system would be at a steady state.

The team found out that in the initial ten seconds of the diffusion, the model was logarithmic. This can be explained by the concentration gradient between the solution inside and outside of the channel. After the initial ten seconds, the slope stabilized and remained linear until the final data point reached when the assay was completed after forty minutes. This time limit was selected, because at thirty minutes, the diffusion boundary had reached the wall of the mold. Because of the manner that the FITC spread out of the channel, this lead the team to believe that rather than simple diffusion, the FITC molecule was actually perfusing through the system, being carried with the solution away out from the channel. Because of that, the FITC diffusion boundary was able to reach 1600 μm. This lead the team to conclude that the fabricated microvascular network could potentially sustain the cell viability of an adjacent cell-seeded gel.

7.1.5 Cell Viability Assays

7.1.5.1 Preliminary C2C12 Survivability

From the gathered data from the experiment, a few conclusions were drawn. The first is that PBS is effective and sufficient as a negative control in cell viability experiments as determined by loss of cell viability after four and eight hours at all areas outside of the edge of the wells. It was hypothesized that residual media remained at the edge of the wells in which cells retained viability. By comparing Figure 100 and Figure 101, it can be seen that the air and PBS have similar effects on cell culture, as a significant portion of the cell culture died in both conditions. The second conclusion that was drawn is about the time length of the cell viability experiments. By comparing Figure 101 and Figure 102, there is little to no change in the air and PBS conditions. The cell culture in the cell media seems to have increased between the eight hour time point and twenty-four hour time point. This means that either amount of hours is sufficient time to determine C2C12 cell viability relative to PBS or air negative controls.

It is important to note a drawback to our analysis. The wells in the plate used for the experiment have a slight curvature upward toward the center. This creates a small groove at the edge of the wells. Because of this depression, it was difficult to completely remove the cell media from the systems. It is for this reason it is believed living cells were observed in the air wells at later time points. Media was not completely aspirated and this residual media allowed some cells to survive. In order to avoid this, we recommend using flat wells, thoroughly rinsing the non-media systems with PBS, and / or carefully aspirating all fluid along the edge of the wells.

7.1.5.2 Cells on Top of Perfused Single Channel Viability Assay

The cell viability assay on top of a perfused microengineered vascular network was a critical validation of the bioreactor and microengineered vascular network designs.

Various flow rates were used in this assay for different reasons. A flow rate of 0.01 mL/min is always used to clear the liquid gelatin. Once the gelatin was cleared and to mimic physiological flow rates for arterioles, a flow rate of 0.001 mL/min was selected. Due to an error by a team member, neither the media perfused or PBS perfused samples were perfused overnight. Because cells remained viable, the team concluded that continuous perfusion of the system was not actually a requirement to maintain cell viability. Even though the flow rates varied throughout the experiment, though not a continuous perfusion experiment, this is still a controlled experiment because all perfused samples received the same variable flow rates.

This assay showed that media perfused microengineered vascular networks maintained cell viability of C2C12 cells on top of the hydrogel 1200 μm from the top of the perfused channel, a distance six times the 200 μm static diffusion limit of oxygen. Additionally, cells at the edge of the hydrogel, at a distance of around 4700 μm remained viable. The implications of this are that a perfusable microengineered vascular network has the capability of maintaining cell viability at distances greater than the passive diffusion limit of oxygen.

The next step, which is more directly relevant toward the goal of creating a stackable alternating microvascular, myocardial layered cardiac patch, would be to assess cell viability of a layer adjacent to the vascular network.

7.1.5.3 Adjacent Layer Cell Viability Assays

Through multiple trials of the cell viability assay of a cell-seeded fibrin hydrogel adjacent to a perfused vascular network, it was hypothesized that the cells were not remaining viable, not

due to lack of nutrients media, but due to lack of oxygen. It seemed that the microfluidic chamber was so well sealed that the amount of oxygen entering the system in the media was not sufficient.

Trial 4 tested to see whether this hypothesis was correct. The results suggested that in the microfluidic chamber without the top coverslip, allowing oxygen to access the top of the gel, cells in both the adjacent layer and throughout the 3400 μm thick vascular network hydrogel remained alive. These results raise some interesting points. It seems in a sealed microfluidic chamber, there is not sufficient oxygen to keep any cells alive. In a microfluidic chamber that is open on the top, cells remain viable throughout the entire thickness of the microvascular network and adjacent layers so there is sufficient oxygen for all cells to survive. The cell viability in the adjacent layer did seem higher than within the vascular network hydrogel. Based on the literature, oxygen should only passively diffuse 200 μm into the hydrogel so oxygen delivered to the rest of the hydrogel should come from the perfused media.

Future tests of this system would include the use of a gas permeable membrane, such as a thin silicone sheet, on top of the microfluidic chamber that would allow for gas exchange in addition maintenance of sterility with just the microfluidic chamber.

7.2 Comparison to Other Studies

7.2.1 Microengineered Vascular Network

The design of the microengineered vascular network presented in this project report is created using a novel, tunable method of extruded sacrificial molds made of 10% gelatin. As described in detail in Chapter 2: Literature Review and Chapter 4: Design Process there are multiple methods for developing *in vitro* vascularization including angiogenesis and vasculogenesis, the formation of new blood vessels by endothelial cells from existing vessels or

de novo respectively, bonding of pre-vascularized hydrogels, and cylindrical rod extraction (Hasan et al., 2014; Lovett, Lee, Edwards, & Kaplan, 2009). Each of these methods, however, has distinct limitations. Vascularization by angiogenesis or vasculogenesis is a time-consuming process and it can be days or weeks before vasculature is formed (Laschke et al., 2006). In this time, the development of thick scaffolds is difficult because perfusion is not yet possible. In addition, it is difficult to control the formation of vascularization (Laschke et al., 2006). The method of bonding pre-vascularized hydrogels is limited by inadequate sealing of the two gels as well as difficulty sealing the inlet and outlet (Bornstein, Gagnon, Moutinho, Reyer, 2015). The cylindrical rod extraction method is limited by the possibility of damage to the hydrogel due to shear upon rod removal and lack of geometry options since it is only possible to create a single, non-bifurcated channel based on feasibility studies in this report. The sacrificial mold method developed in this project overcomes each of these limitations. Compared to the days or weeks with the angiogenesis method, the time required from formation of a gelatin sacrificial mold to channel fabrication is vastly decreased to around an hour. There is also much greater control over the formation of the microvasculature since the resultant channels are of the approximate size and geometry of the mold.

Compared to the method of bonding pre-vascularized hydrogels, the method developed in this project avoids the problems of bonding two hydrogels together and improper sealing at the input and output of the network. The sacrificial mold is placed in the microfluidic chamber before adding the fibrin components and the gel is created in one piece. This eliminates the need to even consider bonding hydrogels together. The method in this project includes placing the sacrificial mold into the inlet and outlet needles of the microfluidic chamber which integrates the formed channel into the microfluidic chamber. The sacrificial mold method also has distinct

advantages over cylindrical rod extraction. In this project there was no evidence of damage to the fibrin hydrogel due to shear upon removal of the sacrificial mold as opposed to common damage with the rod extraction. Additionally bifurcations are possible with the sacrificial method as opposed to only being able to form single channels with rod extraction.

7.2.2 Bioreactor

The team's final bioreactor system included a syringe pump, microfluidic chamber, and collection flask. A similar bioreactor system was created by Warren Grayson et al. (2008). Their bioreactor system focused on using tissue engineering of bone to allow 6 tissue constructs to be maintained at the same time, while allowing for continuous perfusion and imaging with a microscope. This system is similar to the team's bioreactor in that both included a pump and microfluidic chamber; however the bioreactor system of Grayson et al. did not have a collection flask. Instead, the media continued to flow through a loop. Another difference is that the team's bioreactor made use of a syringe pump while that of Grayson used a peristaltic pump.

An additional difference between the two designs is that the microfluidic chamber of Grayson used the same media for six tissue samples. With the team's design, a different chamber would be used for each sample, thus making use of a different input media syringe for each chamber. The chamber of Grayson was entirely transparent, as it was made with polycarbonate plastic, while the only part of the team's chamber that was transparent was the glass coverslip viewing window; the rest of the team's chamber was made of materials including PTFE and POM. Both designs, however, made use of silicone gaskets. The team's bioreactor used a silicone gasket between the top lid of the chamber and the top glass coverslip. The chamber of Grayson used silicone gaskets to separate the parts of their microfluidic chamber. Additionally, both could be imaged with a microscope as they both had a large enough transparent portion to

allow for viewing of the sample and also both adhered to a height profile appropriate for imaging. The team used stainless steel plates and 4 - 40 screws to secure the system and maintain sterility, while the other chamber makes use of a Petri dish lid, which can easily be removed. This could also be seen as a benefit, however, to allow for easy access to the samples when desired.

Another similar microfluidic chamber was created by Mirjam Fröhlich and focused on the perfusion of 3D tissue scaffolds (Fröhlich et al., 2010). Their system used a peristaltic pump, microfluidic chamber and a media reservoir. The system flows from the reservoir, through the pump, into the microfluidic chamber, then back to the reservoir. The team's system flows one way, starting in the syringe pump and ending in the collecting flask. Their microfluidic chamber used a silicone gasket as well; however, it was a sheet gasket rather than a gasket. They also used a layer that contained pre-made channels for the media to be perfused through. This layer restricts the number of tissue samples the device can sustain at one time. The team's microfluidic chamber has space for a hydrogel, whereas the chamber created by Mirjam Fröhlich only contained a glass slide with a tissue scaffold on top. The other microfluidic chamber utilized threaded nozzles to allow for the addition of tubing to be secured. Both the team's and Fröhlich's chamber take advantage of the rigidity stainless steel to provide structure and pressure to maintain the sterility of the chamber. Both microfluidic chambers kept the height profiles low to allow for imaging under a microscope.

Chapter 8: Conclusions and Recommendations

8.1 Conclusions

The challenge presented to the team was to design a bioreactor system and a novel microvascular network for the growth and vascularization of a cardiac patch. The current methods of cardiac repair are non-regenerative methods such as coronary artery bypass, percutaneous coronary intervention, and heart transplant. An engineered cardiac patch would fulfill this need with an off-the-shelf regenerative treatment. The major challenge is creating vasculature which would efficiently deliver nutrients and gases necessary to sustain cell viability throughout the scaffold.

The team designed a microvascular network using a sacrificial material protocol and a bioreactor system with a custom designed microfluidic chamber to hold the tissue construct. The team developed a fabrication procedure for the microvascular network, which allows for the formation of channels with diameters similar to arterioles. The fabrication protocol also allows the user to tune the diameter of the final fabricated channel in a reproducible manner. Using this method, the vascularization formed maintained its integrity and patency.

The team also designed, developed, and tested a bioreactor system that would sustain the microengineered vascular network and an adjacent cell-seeded layer. The components of the bioreactor are a syringe pump that allows for precise control over a physiologically relevant flow rate, a custom microfluidic chamber, which is compatible with the microvascular network fabrication process, and a collection flask. The designed system was able to sustain cell viability of fibrin construct seeded with cells over twenty-four hours.

Once the bioreactor system and microvascular network were designed, the team studied the perfusion through the system, characterized the diffusion through the microvascular network,

and performed a cell viability assay to assess the effectiveness of the designs. The team determined that the bioreactor can provide continuous perfusion at a constant flow rate. After that the team studied the microvascular network's ability to sustain the provided perfusion. Both of these experiments showed positive results, as the bioreactor pump allowed the user to precisely control the flow rate and that the engineered microfluidic chamber was able to withstand the flow without losing its integrity or patency. Once continuous perfusion was successfully established through the microvascular network, it was necessary to characterize the diffusion through the system. Using a fluorescent dye, the team hypothesized that the microvascular network would be able to deliver nutrients to and sustain an adjacent layer. Perfusion of the microengineered vascular network successfully maintained cell viability of C2C12 cells on top of the perfused hydrogel. The cell viability of an adjacent layer showed that bioreactor system and the microvascular system are able to sufficiently deliver nutrients to the cells of the adjacent layer, but the bioreactor system as design may be unable to provide sufficient oxygen to sustain the cell viability.

In summary, the team designed a functional microvascular system with channels mimicking the size of arterioles and a bioreactor system that can sustain continuous perfusion and hold the construct in an aseptic environment. A number of recommendations are made to the designs to improve their functionality and efficiency.

8.2 Recommendations

8.2.1 Microvascular Network Design

8.2.1.1 Using a Permeable Cover Cell Viability

The first recommendation the team has for future work on the microvascular network is to test cell viability of an adjacent layer on top of the microvascular network in a microfluidic

chamber with a gas permeable cover. The team tested the cell viability of an adjacent layer in an open microfluidic system, and the assay showed promising results with high percentage of cells remaining alive both in the adjacent layer and throughout the entire 3400 μm thick hydrogel. This, however, made it more difficult to maintain an aseptic environment. The team recommends testing the use of a clear, gas permeable materials such silicone in place of the glass coverslips, improving the cell viability of the adjacent layer, while retaining an aseptic environment.

8.2.1.2 Scaling Down the Microvascular Network Channel Diameter

In this project, the team aimed to achieve a microengineered vascular network channels which have diameters within the range of arteriole diameters. In order to mimic *in vivo* vasculature at the lowest level, the next step would be to scale down the microvasculature to capillary diameter ranges. This would allow for more complex microvascular geometries to be created as well as increase the network's efficiency in delivering nutrients and gases throughout the finalized fibrin construct.

8.2.1.3 Developing and Designing More Complex Network Geometries

In addition to scaling down the microengineered vascular network's size, it would also be important to scale up the complexity of the network. This project developed channels that were either a single lumen or had one bifurcation. The team recommends increasing the complexity to better mimic a capillary bed and to bring the vascularization closer to cells within the hydrogel.

The gelatin sacrificial material was shown to be molded in PDMS as single cylinders, single-bifurcations, and multi-bifurcations as seen in the Sacrificial Mold - Photolithography Feasibility Study. Forming a mold, introducing liquid gelatin, allowing to cool, and removing gelatin remains a very promising solution to forming more complex and smaller network geometries.

8.2.1.4 Endothelialization of Microvascular Network

Endothelialization of the microvascular network, the lowest ranked objective, was the only objective the team was unable to achieve due to the time constraint. By endothelialization the microvascular network, two major aspects would be improved. The *in vitro* system would mimic *in vivo* even closer since the channels would be structurally closer to actual blood vessels. The second aspect is that by successfully endothelializing the microvasculature, the system has the potential to sustain blood perfusion.

8.2.1.5 Perfusing and Sustaining a Multilayered Construct

The next step in the development of a multilayered cardiac patch would be to perfuse and sustain multiple microvascular and myocardial layers respectively. This would require some adjustment to the bioreactor to be able to hold the multilayered system, as well as some possible changes to the microvascular layer fabrication method so that the gel can retain its patency even with the addition layer weight.

8.2.2 Bioreactor System Design

8.2.2.1 Microfluidic Chamber

The current model of the microfluidic chamber is able to achieve each of the objectives and functions of the system. However, there is always room for improvement. Below, different aspects of the current design are further discussed and recommendations are made on how they can be improved.

8.2.2.1.1 Chamber Shape

The current shape of the chamber creates the need for the stainless steel plates to distribute the pressure across the surface area of the device. Having a hexagonal design with more screws evenly placed around the perimeter would eliminate the need for the plate because

the pressure would be distributed better. Also, the reduction in pieces would make the device easier to use during setup.

8.2.2.1.2 Viewing Window

The current viewing window on the top most portion of the microfluidic chamber is 10 mm x 10 mm. However, these dimensions could be wider to allow the user to see more of the sample during testing in addition to allowing more of the sample to be imaged with a microscope. Glass coverslips come in a variety of dimensions, making the integration of a larger coverslip into a modified microfluidic chamber simple.

8.2.2.1.3 Screw System

Currently the system uses four 4 - 40 stainless steel screws to keep all components of the chamber in place. However, this system requires the use of an Allen wrench and can seem tedious when setting up more than one microfluidic chamber. An alternate approach could be the use of quarter turn fasteners that require one turn each before they are secured in place and ensure the system is tightened.

8.2.2.1.4 Simple Mold Thickness

The simple mold is three millimeters thick. However, this demands a thick fibrin layer which requires the media to diffuse further to reach the cells on top of it. By reducing the thickness of the mold, a thinner fibrin layer could be made which would reduce the distance the media needs to perfuse to reach the cells on the system and provide them nutrients.

8.2.2.1.5 Input & Output Hole Stops

Currently, it is difficult for the user to determine when to stop pushing the 20G needles into the input and output holes of the chamber. This can lead to damaging the fibrin layer that has been created if the needles are inserted too far. Even if they are inserted carefully, they could

potentially move when transporting the system as well. To prevent this altogether, small stops could be integrated into the input and output holes that would allow the needle to reach its intended place and would prevent it from moving further into the system.

8.2.2.2 Media Oxygenation

One alteration to the bioreactor system would be the addition of an oxygenator. This component would increase the oxygen concentration in the perfused media, which would mimic the blood. Using a the completely sealed microfluidic chamber, the user would be able to evaluate the microvascular network's effectiveness in delivering oxygen and nutrients to cells in an adjacent fibrin layer, which would closely mimic the conditions *in vivo.*

8.2.2.3 Chamber Testing

A few recommendations can be made to further validate the microfluidic chamber, discussed in Chapter 7: Discussion. When validating the dimensions of the chamber, the measurements should be made immediately following fabrication before any testing is done. This would eliminate any changes to the dimensions that could be caused by sterilization or experimentation. The sterility testing performed on the chamber only suggested the completion of the objective. In order to prove this, a bioburden assay should be performed according to the FDA and ISO 11737 standards (ISO 11737, 2006). Additionally, long-term sealing tests should be run on the microfluidic chamber to ensure the device remains sealed and sterile.

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Appendix A: Timelines

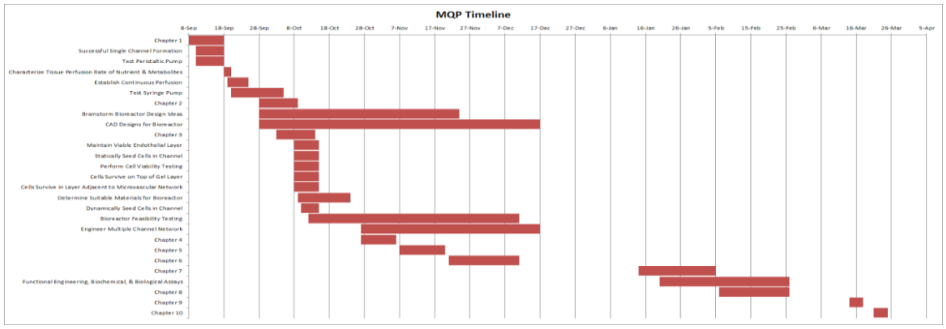

Figure 111: Year-long MQP Gantt chart

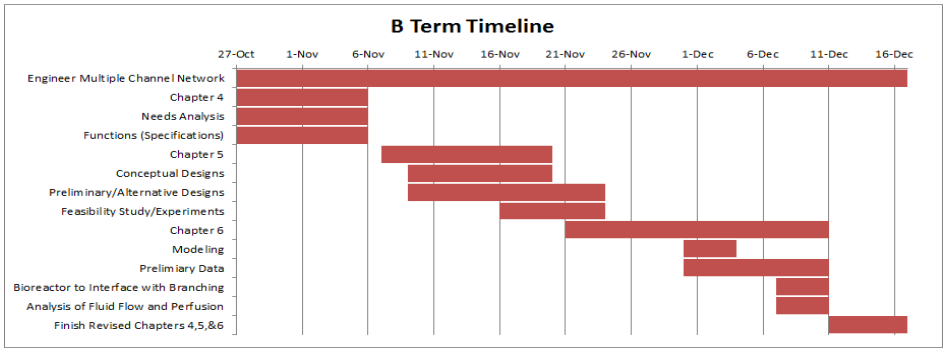

Figure 112: B Term MQP Gantt chart

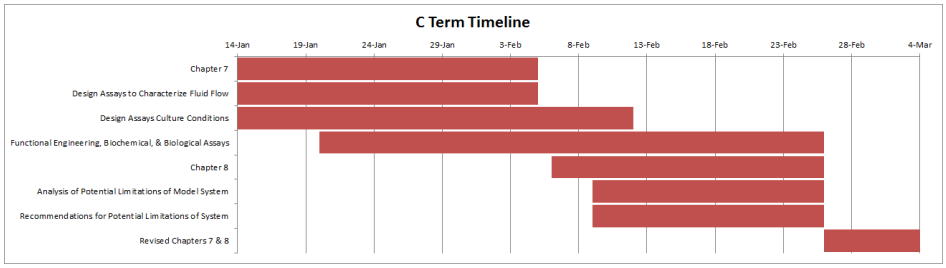

Figure 113: C Term MQP Gantt chart

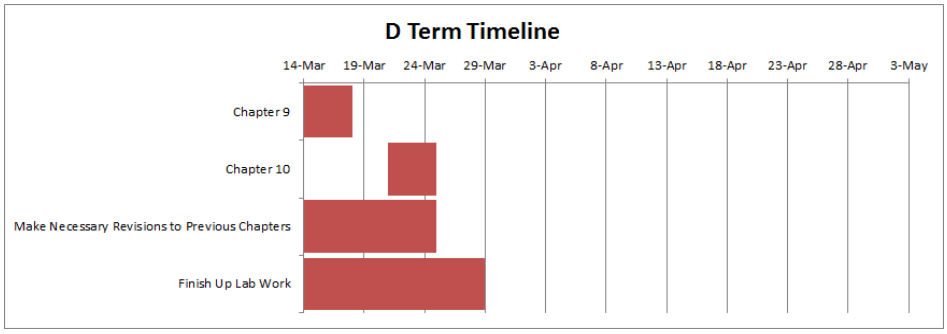

Figure 114: D Term MQP Gantt chart

Appendix B: Decision Matrices

Table 28: Team PCC for bioreactor

Objectives: Bioreactor (Team)						
Goal	**Ease of Use**	**Cost Effective**	**Controlled Gas Permeability**	**Reproducible**	**Multiple Samples**	**Total**
Ease of Use		1	0	0	0	1
Cost Effective	0		0	0	0	0
Controlled Gas Permeability	1	1		1	1	4
Reproducible	1	1	0		1	3
Multiple Samples	1	1	0	0		2

Table 29: Team PCC for microvascular network

Objectives: Vascular Network (Team)							
Goal	**Reproducible**	**Cost Effective**	**Physiologically Relevant Dimensions**	**Endothelializable**	**Physiologically Relevant Flow**	**Stackable**	**Total**
Reproducible		1	1	1	0	0	3
Cost Effective	0		0	0	0	0	0
Physiologically Relevant Dimensions	0	1		1	0	0	2
Endothelializable	0	1	0		0	0	1
Physiologically Relevant Flow	1	1	1	1		.5	4.5
Stackable	1	1	1	1	.5		4.5

Table 30: Client PCC for bioreactor

Objectives: Bioreactor (Professor Pins)						
Goal	**Ease of Use**	**Cost Effective**	**Controlled Gas Permeability**	**Reproducible**	**Multiple Samples**	**Total**
Ease of Use		1	0	0	0	1
Cost Effective	0		0	0	0	0
Controlled Gas Permeability	1	1		1	1	4
Reproducible	1	1	0		1	3
Multiple Samples	1	1	0	0		2

Table 31: Client PCC for microvascular network

Objectives: Vascular Network (Professor Pins)							
Goal	Reproducible	Cost Effective	Physiologically Relevant Dimensions	Endothelializable	Physiologically Relevant Flow	Stackable	Total
Reproducible		1	1	0.5	0.5	0.5	3.5
Cost Effective	0		0	0	0	0	0
Physiologically Relevant Dimensions	0	1		1	0.5	0	2.5
Endothelializable	0.5	1	0		0.5	0	2.0
Physiologically Relevant Flow	0.5	1	0.5	0.5		0.5	3
Stackable	0.5	1	1	1	0.5		4.0

Table 32: User 1 PCC for bioreactor

Objectives: Bioreactor (Obrien)						
Goal	Ease of Use	Cost Effective	Controlled Gas Permeability	Reproducible	Multiple Samples	Total
Ease of Use		1	0	0	0	1
Cost Effective	0		0	0	0	0
Controlled Gas Permeability	1	1		1	1	4
Reproducible	1	1	0		1	3
Multiple Samples	1	1	0	0		2

Table 33: User 1 PCC for mirovascular network

Objectives: Vascular Network (Obrien)							
Goal	Reproducible	Cost Effective	Physiologically Relevant Dimensions	Endothelializable	Physiologically Relevant Flow	Stackable	Total
Reproducible		1	1	1	0	1	4
Cost Effective	0		0	0	0	0	0
Physiologically Relevant Dimensions	0	1		0	0	0	1
Endothelializable	0	1	1		0	0	2
Physiologically Relevant Flow	1	1	1	1		1	5
Stackable	0	1	1	1	0		3

Table 34: User 2 PCC for bioreactor

Objectives: Bioreactor (Carnes)						
Goal	Ease of Use	Cost Effective	Controlled Gas Permeability	Reproducible	Multiple Samples	Total
Ease of Use		1	0	0	0	1
Cost Effective	0		0	0	0	0
Controlled Gas Permeability	1	1		0	1	3
Reproducible	1	1	1		1	4
Multiple Samples	1	1	0	0		2

Table 35: User 2 PCC for microvascular network

Objectives: Vascular Network (Carnes)							
Goal	Reproducible	Cost Effective	Physiologically Relevant Dimensions	Endothelializable	Physiologically Relevant Flow	Stackable	Total
Reproducible		1	1	1	1	1	5
Cost Effective	0		0	0	0	0	0
Physiologically Relevant Dimensions	0	1		1	0	0	2
Endothelializable	0	1	0		0	0	1
Physiologically Relevant Flow	0	1	1	1		0	3
Stackable	0	1	1	1	1		4

Table 36: Pairwise comparison chart of sub-objectives of "Ease of Use"

Ease of Use	A	B	C	Total
A. Ease of transport		1	1	2
B. Ease of set-up	0		0	0
C. Easy fluid control	0	1		1

Table 37: Pairwise comparison chart of sub-objectives of "Reproducible"

Reproducible	A	B	C	Total
A. Dimensions		0	1	1
B. Flow	1		1	2
C. Stacking of multiple layers	0	0		0

Appendix C: Client Meetings

Client Meeting - 9/2/15

We met with our clients, Meg & Megan. The following is the information we gathered.

- The dimensions of the fibrin scaffold should at largest be:
    - Length: 2cm
    - Width: 1 cm
    - Height usually 300 µm
- The longest duration the scaffold would need to survive in storage is 3 weeks, most often is 2 weeks.
    - 3 weeks is the longest cells need to be able to survive
- Media needs to be changed every other day
- The only financial budget/limitation is the team budget
- Other needs:
    - Ease of use
    - Short setup time
    - Maintenance, sterilization
        - Autoclave
        - Fits in incubator
        - Pulsatile flow
        - Secure layers - reproducible stacking method for multiple layers
        - Closed and breathable
    - Incubator dimensions:
    - 18 in tall, 18.5 in wide, 18.5 inch deep
    - Hood: 15 in deep
- Reviewed Gantt chart

Client Meeting - 11/6/15

- After grant rewrite, endothelialization has become a low priority
- Stay away from zinc, use stainless steel
- Non-uniform diameters of tubes in fibrin/thrombin does not matter as long as they are straight
- Use a syringe pump, not a peristaltic pump
- For vascular network instead of doing small branches try 2 bigger ones or 4 media ones
- Agaros might be good for the sacrificial mold
- Try casting fibrin around the tube
- Does the fibrin gel go straight up against the Pluronic or does the Pluronic have some area that it cannot let material get close to
- Call tech support and ask if Pluronic sticks to the material we are using
- Pins Lab has biocompatible screws
- Make sure material does not corrode in incubator

- Concerned 3D printing will not allow for a tihgt seal
- PTFE tape or binder clips
- Proved can form fibrin gel in one piece with complex geometries
- Cons: more difficulty aligning channel with the inlet and outlets
- Pro: no attachment or adhesion problems

Client meeting - 11/13/15

During meeting, we presented the alternative bioreactor designs and received feedback

Tray/dish concept

- Maybe instead of building the interior tubes and inlets, make the designs with just the holes so an inlet/tube could be inserted throughout the hole when needed
- There might be "cross-talk" currently so design to have walls that can separate each sample
- Look into ways that ensure an airtight seal. Maybe gasket tape?
- For transparent material, look into using silicon, which is also gas permeable

Petri Dish Concept

- Same idea; make it so you can insert tube/inlet separately
- Probably not easy to machine with holes
- Maybe use a drill...not best option
- Not airtight. DIfficult to make it

Pressure Square

- Only issue was 60 degree turn of vascular and graft and Meg's layer. But Meg proposed that all layers be oriented in the same way and she can orient her threads onto the layer in a 60 degree fashion instead

Mold

- Instead of having a bottom, can we make the side walls a little thicker to maintain integrity and stability
- Might be hard to machine or print such a small sized piece
- Make sure all parts and materials of this mold are biocompatible

Problish with lab testing: cylindrical rod extraction method tears the gel and when being perfused causes buildup of liquid

- Look into other methods of cutting the wire or polishing the end to make it smooth

- Contact machine shop to see what might be available
    - Laser cutting
    - Polishing
    - Filing down ends

Client Meeting - 12/4/15

After a conversation about the meanings of each objective for both the bioreactor and the vascular network, there was a lot of confusion about the definitions of each objective.

Bioreactor PCC

Ease of Use	1
Cost Effective	0
Controlled Gas Permeability	4
Reproducible	3
Multiple Samples	2

These new scores are based off unanimous agreement between the advisor, clients, and teammates

Vascular Network PCC

Reproducible	3
Cost Effective	0
Physiologically Relevant Flow	4.5
Endothelializable	1
Stackable	4.5

Review on molds: Clients favorite mold is the insert mold. This mold is most user friendly when it comes to the use of a sacrificial material. Also, because of its alignment ability, it is the most secure mold.

We then spoke about next tests we should do with molds and bioreactor.

Needle infusion

Perfusion

Leakage tests

Client Meeting - 2/8/16

- Clients were updated on progress
- Manufacturing of bioreactor is in progress with Tom Partington in Goddard Hall
- Channels continue to be successfully formed

Appendix D: Protocols

Solution Preparation Protocols

$CaCl_2$ Solution Preparation (40 mM)

Objective: To make a $CaCl_2$ solution, which is used to activate the thrombin

Materials:

$CaCl_2$ (MW = 110.98)

diH_2O

Conical tube

Beaker

Pipetter

Procedure:

1. Add 0.1776 g of $CaCl_2$ to 40 mL of diH_2O to a small beaker
2. Mix well and transfer to conical tube
3. Store at 4℃

HEPES Buffered Saline (HBS) Solution Preparation

Objective: To make HBS solution, which is used in fibrinogen and thrombin aliquot preparation

NOTE: HBS has 20 mM HEPES and 0.9% (w/v) NaCl

Materials:

diH_2O

NaCl (MW = 58.44)

HEPES (MW = 238.34)

Beaker

Capped bottle

Graduated cylinder

Procedure:

1. Add the following reagents to 200 mL of diH_2O in a beaker
    a. 2.25 g of NaCl
    b. 1.1915 g of HEPES
2. pH the solution to 7.4 using NaOH and HCl
3. Bring the volume of solution to 250 mL with diH_2O
4. Transfer the solution to the bottle and store at room temperature

1X Phostpage-buffered saline (PBS) Solution Preparation

Objective: To make 1X PBS solution, which is used in fibrin gel formation and as an experimental negative control.

Materials:

diH_2O

10X PBS solution

Graduated cylinder

Capped bottle

Stir bar

Procedure:

1. Measure 400 mLof diH_2O
2. Add 50 mLof 10X PBS to the solution
3. Add 50 mLof diH_2O and bring the final volume to 500 mL
4. Mix well and pH the solution to 7.4
5. Store at room temperature

C2C12 Complete Media Preparation Protocol

Objective: Make C2C12 complete proliferation media, which is used to sustain the cells, while in culture

Materials:

Ham's F12

DMEM

FBS

Pen/Strep

(Amphotericin B)

Beaker

Capped Bottle

Pipetter

Procedure:

NOTE: Since this is cell culture media, the whole process needs to be done in a clean biosafety cabinet with sterile tools and containers, otherwise you risk contamination

1. (day prior) Sterilize a capped bottle to contain the complete media
2. Bring all materials into the biosafety cabinet
3. Mix the following solutions in the prepared sterile bottle
    a. 220 mLof Ham/s F12
    b. 220 mLof DMEM
    c. 50 mLof FBS
    d. 5 mLof Pen/Strep
    e. (5 mLof Amphotericin B)

NOTE: We add amphotericin B at gateway, because we have had fungal problems in the past

4. Mix the solution well, label, and store in the 4°C fridge

0.05% w/v Fluorescein isothiocyanate (FITC) Staining Solution Preparation

Objective: To make FITC, which is an auto-fluorescent dye. In our particular project, this was used to image diffusion through the microvascular network.

Materials:

FITC(solid)

Sterile diH_2O

50 mLconical tube

Pipetter

Procedure:

NOTE: the recommended solution concentration is 1 mg/mL, but this can be adjusted to match the experiment's need

1. Mix 0.05 g of FITC(s) into 45 mLof diH_2O into the conical tube

   ·Make sure to protect the solution from light, so that the dye does not photo bleach

2. Add another 5 mLof diH_2O, washing the weighting boat
3. Mix well
4. Store in the 4°C fridge
5. Dilute if necessary

Fibrinogen and Thrombin Aliquot Preparation

Objective: Preparing fibrinogen and thrombin stock aliquots, which are used for dilution and fibrin thread creation.

Materials:

Fibrinogen (82022, MP)

Thrombin (T4648, Sigma)

$CaCl_2$ (MW = 110.99)

NaCl (MW = 58.44)

HPS

Micropipette

Conical tubes

Eppendorf tubes

Procedure:

NOTE: Not all fibrinogen in product is clottable. This needs to be taken into account. Previous optimization of results was done with product from Sigma Aldrich (SA)

NOTE: Current product is from MP Biomedicals

NOTE: To get the % protein and % clottable, review the certificate of analysis, which comes in with the product. It might be different for each batch

Originally, the aliquots are made to concentration of 70 mg/mL, based on SA's procedure. Using that, determine the concentration of the new product needed to have an equivalent concentration of effective protein (EP) as SA's aliquots.

To dissolve 1000 mg:

Fibrinogen Aliquot (70mg/mL)

1. Weigh 1.00 g of fibrinogen and pour in conical tube.
2. Measure X (specifically calculated for each batch) of Hepes buffered saline (HBS) and add to fibrinogen in conical tube
3. Place conical tube on rocker plate, adjusting position every 30 - 40 min until fibrinogen is no longer visible on the inside of the tube

NOTE: There may be clumps visible in the solution, which is OK

4. Measure 1 mLaliquots in eppendorf tubes and store at -20℃

Thrombin Aliquot (40U/mL)

1. Add 25 mLHBS to bottle of 1 KU thrombin
2. Mix well
3. Aliquot 200 µl into eppendorf tubes and store in -20℃

NOTE: Final concentration should be 8 U/ 200 µl

Fibrinogen and Thrombin Diluted Aliquot Preparation

Objective: Preparing diluted fibrinogen and diluted thrombin aliquots for fibrin gel scaffolds

Materials:

Fibrinogen (82022, MP) Stock Solution

Thrombin (T4648, Sigma) Stock Solution

HBS

40 mM $CaCl_2$

PBS (can be substituted with cell media if using cells)

PDMS - curing agent & elastomer base

Eppendorf tubes

Centrifuge tubes

Procedure:

Diluted fibrinogen solution (2X solution) (dFb solution)

1. Pipette 137.8 µL of fibrinogen stock solution into a centrifuge tube
2. Add 862.2 µL of HBS into centrifuge tube
3. Mix carefully
4. Split in 200 µL aliquots of dilute Fb
5. Final concentration = 11 mg/mL

NOTE: Mix gently to avoid polymerization

Diluted thrombin solution (2.35 U/mL) (dTh solution)

1. Pipette 58.75µL of Thrombin stock solution into centrifuge tube
2. Add 941.25µL HBS to the tub
3. Mix (triturate)
4. Split into aliquots of 50 µL of dilute Th

Pluronic F127 Sacrificial Material

Adapted from (Müller, Becher, Schnabelrauch, & Zenobi-Wong, 2013)

If available, perform the preparation of the poloxamer solution in a cold room (4 °C). If not available, place a glass bottle in a beaker filled with ice-cold water. At higher temperatures the poloxamer will be above the gel point and will not dissolve properly.

Procedure:

1. Add 15 mL of ice cold PBS solution into a glass bottle and stir vigorously using a magnetic stirrer. (Note: for Pluronic F127 in DI water, replace all PBS with DI water).
2. Weigh 6.125 grams of poloxamer and add it in small amounts to the cold PBS. Wait until the poloxamer has partially dissolved before adding more.
3. Stir the solution until all poloxamer has dissolved.
4. Add cold PBS until a final volume of 25 mL is reached. The final concentration will be 24.5% w/v.
5. Stop stirring the solution and let it rest at 4 °C until bubbles and foam in the solution have disappeared. Bubbles that are trapped within the gel will be transferred to the printer cartridge and will lead to defects in the printed sacrificial molds.
6. Keep the solution at 4 °C until 30 min before the experiment.

Fibrin Gel Preparation Protocol

Objective: Fabricate a fibrin hydrogel.

Materials:

Diluted Fibrinogen (dFb)

Phosphate Buffered Saline (PBS)

or cell media

or cell suspension

Diluted Thrombin (dTh)

40 mM $CaCl_2$

Procedure:

1. Thaw frozen dFb and dTh in hot water bath (5 - 10 minutes) to ensure complete melting.
2. To make 1 mL of fibrin gel
    a. Add 150 µL of PBS, cell media, or cell suspension to 335 µL of dFb. Mix thoroughly (10 - 15 times with micropipette) Handle dFb with care to avoid polymerization before desired.
    b. Add 80 µL CaC1 to 100 µL dTh. Mix thoroughly.
    c. Add two solutions together, mix thoroughly, and add to desired location.
3. Let polymerize for 30 minutes.

Vascularization Protocols

Cylindrical Rod Extrusion

Objective: Fabricate a single straight channel in fibrin gel using the cylindrical rod extraction method (for more information look in Chapter 5: Design Verification)

Materials:

40 mM $CaCl_2$

Diluted Thrombin (dTh)

Non - sterile PBS

Diluted Fibrinogen (dFb)

0.02% Pluronic F127 solution

Micropipette

Eppendorf tubes

Fibrin well/mold

Steel wires

Perfusion polyethylene tube

Procedure:

Wire Fabrication

1. Measure needed length of the wires
2. Cut the wires to length using wire cutters or saw
   Note: Cutting with these techniques results in defects on the tip of the wires
3. Using a fine file, smoothen out the edges on the wire tips to remove any defects and sharp edges.

Fibrin Channel Fabrication

1. Coat the wire tips with Pluronic F127.
    1. Put the wire tips, which are going to be used, in a small beaker
    2. Pour 0.02% Pluronic solution into the beaker, until all tips are submerged

3. Leave the tips in the solution for 10 - 15 min
4. Aspirate Pluronic, remove the wire tips, and leave to air dry before usage

2. Prepare solutions for fibrin gel (for 500µL of final gel)
    1. Once the fibrinogen and thrombin are taken out of the freezer, thaw for 5 - 12 min
        1. Put the fibrinogen aliquot to thaw in the hot water bath (the water bath will ensure that all of the fibrinogen is completely thawed)
        2. Leave the thrombin aliquot to thaw on the lab bench
    2. Mix 335µL fibrinogen with 75µL cell media using micropipette in an Eppendorf tube (handle the fibrinogen with care to prevent polymerization)
    3. Mix 50µLof the thrombin with 40µL $CaCl_2$ in another Eppendorf tube

3. Making the fibrin gel
    1. Place the wire tip through the perfusion well
    2. Using a micropipette, transfer and mix the thrombin+$CaCl_2$ solution to the fibrinogen+cell media tube
    3. Using the same micropipette, quickly transfer the new solution to the prepared well (speed is essential to prevent the fibrinogen from polymerizing)
    4. Leave the fibrinogen mixture to polymerize in the well for at least 30 min
4. Fabricating the channel
    1. Once the fibrin is fully polymerized, slowly remove the wire
    2. Clean the wires to remove any residual protein

Formation of Channels from Gelatin Sacrificial Materials

Objective: To fabricate a microvascular channel using gelatin as a sacrificial material

Materials:

Microfluidic system

Dilute fibrinogen

Dilute thrombin

CaCl2 (40 mM)

1X PBS

Syringe pump

Syringe

Tubing

Gelatin Sacrificial Mold Fabrication

Solution preparation initially adapted from (Golden & Tien, 2007).

Formation of gelatin cylinders (for a single channel)

1. Prepare a 10% w/v (g/100 mL) solution of porcine gelatin in (1X) PBS.
2. Heat gelatin to 37°C in hot water bath to liquefy.
3. Pull appropriate volume of liquid gelatin into 1 mL syringe.
4. Let reform gel (10 - 15 minutes).
5. Attach needle with desired diameter (ex: 20G needle = 603 μm inner diameter ~= initial gelatin cylinder outer diameter)
6. Extrude cylinders to desired length (~1.5 cm) onto PDMS (found to be a non-stick surface).
7. Dehydrate cylinders for 30 minutes. They can then be handled and moved into the mold.

Formation of gelatin bifurcations (for a bifurcated channel)

1. Heat the syringe to 25 - 30°C, which is the range of the gelatin gel transition temperature. The gelatin will exist in a part liquid-like, part gel-like state. This allows the user to print a bifurcation that maintains its shape (gel-like property) yet forms connections (liquid-like property).
2. Heating can be done with the hand. Heat until gelatin leaving needle begins to flow without being extruded.
3. Draw bifurcation while slowly extruding.

Channel Formation via Sacrificial Mold

Formation of channels from gelatin sacrificial materials.

1. Place sacrificial mold into in microfluidic chamber mold.
2. Insert “plug” needles into inlet and outlet of bioreactor so that gelatin inserts into the needles. (This will hold the cylinder in place and prevent it from floating to the top of the fibrin.)
3. Add top of the inner mold, binder clips to hold pieces tightly together, and add appropriate volume of fibrin components. Add 340 μL of fibrin solution slowly or even dropwise to avoid “pushing” the gelatin mold in one direction. If fibrin is added only to one side, the gelatin mold will end up curving away from that side and the channel will have a high curvature.
4. Allow fibrin to polymerize for 30 minutes.

5. Construct remainder of microfluidic chamber. Remove binder clips,
6. Heat sample to 37°C to melt gelatin for 10 - 15 minutes. Warning: Perfusion to flush gelatin before complete melting can damage the gel and channel.
7. Flush liquid gelatin with 1X PBS sufficiently (>10 minutes) at a flow rate of 0.1 mL/min.

Fibrin Gel Diffusion Assay

Objective: To characterize the diffusion rate, the diffusion limit, and the diffusion coefficient through a single-channel gel.

Set up: 4 samples prepared

Materials:

1x PBS

0.25% Pluronic

Diluted fibrinogen aliquot (11 mg/mL)

Diluted thrombin aliquot (2.35 U/mL)

FITC stain (0.625 mg/mL)

Channel fabrication mold on glass slide

Steel wires

1cc and 3cc Syringe

Tubing and 20G needles

Procedure:

Preparation

1. Prepare the FITC stain
2. Mix the 0.2% Pluronic stock solution
3. Take out a small amount with a 1cc syringe
4. Put the steel needles into the syringe to coat (15 min)
5. Take out dTh and dFb to thaw (5 min)
6. Prepare the molds

- Clear the inlet and outlet tube
- Check for leaks

Casting the Gel

1. Fill the tubing with 1x PBS
2. Place the steel wire into the molds
3. Mix the solutions (650 µl total = 4 sample)
    - dFb – 435.5 µl
    - PBS – 97.5 µl
    - dTh - 65 µl
    - $CaCl_2$- 52 µl
4. Leave the mixture to polymerize (30 min)
5. Extract the needle
    - Needs to be done rapidly so there is no tearing in the gels
6. Perfuse a small amount of PBS to test the channel's integrity

Diffusion Testing

1. (30 min prior) Turn on the fluorescent mercury lamp
2. Load a 3cc syringe with FITC solution
    - Keep in the dark to prevent photo bleaching
3. Setup the syringe pump to the microscope
4. Replace the PBS syringe with the FITC syringe and place onto the syringe pump
5. Start perfusion initially at a high rate, until the dye reaches the inlet, then drop it off to the desired perfusion rate (eg. 0.005 mL/min)
6. Image and collect data
    - The initial 10 seconds, if possible collect a video
    - After that image every 30 - 60 seconds
7. Clean up everything

8. Analyze the images based in ImageJ

C2C12 Survival Assay

Objective: To study the C2C12 cell viability in unfavorable conditions for cell proliferation

Materials:

12 well plates

0.25% Trypsin

Complete c2c12 cell media

1X PBS

Pipette

Centrifuge tube

Procedure:

1. Check the C2C12 culture for contamination
2. Bring the flask into the hood and aspirate the media
3. Trypsinize the cells for 5 mins
4. Add 5 mL of media to deactivate the trypsin
5. Move the cell suspension to a centrifuge tube
6. Using a hemocytometer, determine the cell count
7. Centrifuge the cell suspension
8. Aspirate the solution, leaving behind the cell pallet
9. Add media amount to desired cell density
10. Add desired cell seeding to each well
    a. 3 wells for media condition
    b. 3 wells for PBS condition
    c. 3 wells for air condition

11. Leave the cells to attach to the well bottom (2 hours)
12. Aspirate media from all wells
13. Add corresponding solution to each well
14. Leave in the incubator
15. Using a live/dead stain, collect fluorescent imaging
    a. At 4 hours
    b. At 8 hours
    c. At 24 hours
16. Analyze cell viability by calculating percent cell survival

Cell Viability on Top of Perfused Single-Channel Gels

Purpose: To determine cell viability on top of perfused single-channel gels.

Set Up:

· 6 samples prepared

- Experimental: Media Perfusion (n = 3)
- Control: PBS Perfusion (n = 3)

Materials:

C2C12 full media

· 219 mL F12 (1X)

· 219 mL DMEM (1X)

· 50 mL Fetal Bovine Serum

· 5 mL Penicillin Streptomycin (Pen Strep)

· 5 mL Amphotericin B

· 2 mL Aprotinin

70% Ethanol

Diluted fibrinogen aliquot

· 137.8 µL fibrinogen stock solution

- 862.2 µL HEPES Buffered Saline (HBS)

Diluted thrombin aliquot

- 58.75 µL thrombin stock solution
- 941.25 µL HBS
- Calcium chloride (CaCl2) (40 mM)

Cell culture devices on glass slides

Glass coverslips

~2.5 in. steel wire (279 µm diameter)

C2C12 cells – Mus musculus myoblasts

5 mL Trypsin (0.25%)

LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells

- Calcein AM (Live-Green)
    - Live cells: ubiquitous intracellular esterase activity converts calcein AM to calcein.
- Ethidium homodimer-1 (EthD-1) (Dead-Red)
    - Enters through damaged membranes, binds to nucleic acids

Sterilization:

NOTE: Before starting the ethanol submersion, check for clots and blockages in the tubing and needles using a syringe

- Sterilize all components via submersion 70% ethanol for 90 minutes. Components include:
    - Cell culture devices on glass slides
    - Tubing
    - Needles
    - Steel wire
    - Adaptors
    - Coverslips
- Rinse tubing with DI water to ensure all ethanol is removed.
- Aspirate ethanol and allow to air dry overnight or rinse in DI water three time for 5 minutes.
- “Sterilize” or clean as best as possible the K210 Syringe Pump with 70% ethanol prior to use using a spray and wipe method.
- Sterile filter all fibrin gel reagents using a 0.2 µm filter.

Perfusion Preparation:

- Remove top shelf from the incubator. If the syringe pump is placed on the top shelf, it is too close to the temperature sensor and the incubator will alarm.
- Replace the plug from the bottom incubator with the one from the top, unused incubator (and place the bottom plug into the top incubator).
- Stay and watch the outputs on the incubator to ensure no CO_2 and heat are escaping.
- Place "sterilized" syringe pump on left side of incubator so it is as far away from the thermometer as possible.
- Coat portion of steel wires that will be pulled through the gel with Pluronic F127 for 10 - 15 minutes
- With tubing connected on both sides, put steel wire through tubing and across cell culture device.

Cast Gels (All steps should be completed in biosafety cabinet):

- Prepare a new thrombin solution on the bench by mixing 200 µL of 40 mM CaCl2 with 250 µL of diluted thrombin aliquot. This is the approximate amount needed for 1 mL of fibrin gel because some volume is lost during sterile filtering.
- Use a 0.2 µm sterile filter to filter the new thrombin solution and diluted fibrinogen aliquot into sterile 2 mL centrifuge tubes.
- Make one set of gels for each condition
    - Add 75 µL of PBS to 335 µL of fibrinogen.
    - Mix 90 µL of thrombin - $CaCl_2$ and fibrinogen - PBS solutions and add 150 (or 120 µL) µL into each cell culture device.
- Let gels polymerize for 30 minutes.

Cell Culture (All steps should be completed in biosafety cabinet):

- Culture C2C12 mouse myoblasts in C2C12 full media.
- When sufficient cells are available add 5 mL of 0.25% Trypsin and incubate at 37°C and 5% CO_2 for 5 minutes or until cells have detached from the bottom of the flask.
- Neutralize trypsin with 5 mL of media and move cell suspension to a 15 mL conical tube.
- Remove a 10 µL sample and obtain a cell count.

- Centrifuge cell suspension at 1000 RPM for 5 minutes in IEC Centra CL2.
- Resuspend in appropriate volume to establish a concentration of 1 million cells / mL.

Cell Seeding:

- Add 100 µL of cell suspension to the top of the gel in each cell culture device
- Incubate for 4 hours at 37°C and 5% CO_2 to allow cells to adhere to fibrin gel

Perfusion:

- Attach needles and tubing to cell culture devices
- Prepare syringes with either media or PBS and attach to tubing.
- Place syringes in syringe pump and prime the tubing by running the pump until fluid fills all tubes and all air has been expelled.
- Aspirate media on top of fibrin gels.
- Cover each device with a glass coverslip to maintain sterility and prevent oxygen diffusion.
- Move all devices to the incubator, attach to tubing, and begin perfusion at a flow rate of 0.001 mL/min or 60 µL / hour.
- Perfuse for 20 hours at 37°C and 5% CO_2.

NOTE: Make sure to take the tubing's volume into account when preparing syringes for the perfusion

LIVE/DEAD Cell Staining:

- This staining protocol is from Jon Grasman's work with C2C12 cells and is similar to the example dilution protocol from Invitrogen.
- Thaw and centrifuge the reagents briefly.
- Add 4 µL of supplied 2 mM EthD-1 stock solution and 1 µL of supplied 4 mM calcein AM stock solution to 2 mL of sterile D-PBS (I used PBS since cells were not later being trypsinized). Vortex to ensure thorough mixing. Note: this represents 1/5 size batch of live/dead stain.
- Remove all media from the devices since media can react with Calcein AM causing high background fluorescence. Wash with PBS.
- Add 100 µL of PBS staining solution to each device.

- Incubate samples for 30 minutes.

Imaging – Leica inverted fluorescence microscope (Rolle scope):

- Insert the correct fluorescence cubes prior to imaging.
- Turn on the mercury lamp 15 - 30 minutes prior to imaging.
- Keep shutter closed whenever possible to avoid photobleaching of the samples.
- Appropriate images were obtained by taking six images total, two above the channel, two at the inlet and outlet, and two below the channel using the 10X objective.
- After imaging, merge images using ImageJ and perform analyses.

Cell Viability in an Adjacent Layer on Top of Perfused Single-Channel Gels

Purpose: To determine cell viability in an adjacent layer on top of perfused single-channel gels.

Set Up:

- 6 samples prepared
    - Experimental: Bioreactor samples perfused with media (n = 2)
    - Positive Control: Cell-seeded fibrin hydrogel in media (n = 2)
    - Negative Control: Cell-seeded fibrin hydrogel in PBS solution(n = 2)

Materials:

Bioreactor system

C2C12 full media

- 219 mL F12 (1X)
- 219 mL DMEM (1X)
- 50 mL Fetal Bovine Serum
- 5 mL Penicillin Streptomycin (Pen Strep)
- 5 mL Amphotericin

70% Ethanol

Diluted fibrinogen aliquot (11 mg/mL)

- 137.8 µL fibrinogen stock solution
- 862.2 µL HEPES Buffered Saline (HBS)

Diluted thrombin aliquot (2.35 U/mL)

- 58.75 µL thrombin stock solution
- 941.25 µL HBS

Calcium chloride (CaCl2) (40 mM)

Thrombin aliquot (40 U/mL)

Cell culture devices on glass slides

Glass coverslips

Gelatin hydrogel

C2C12 cells – Mus musculus myoblasts

5 mL Trypsin (0.25%)

LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells

Calcein AM (Live-Green)

- Live cells: ubiquitous intracellular esterase activity converts calcein AM to calcein.

Ethidium homodimer-1 (EthD-1) (Dead-Red)

- Enters through damaged membranes, binds to nucleic acids

Sterilization:

NOTE: Before starting the ethanol submersion, check for clots and blockages in the tubing and needles using a syringe

1. Sterilize all components via submersion 70% ethanol for 90 minutes. Components include:
    - Bioreactor Elements
    - Tubing
    - Needles
    - Adaptors
    - Coverslips
    - Beakers
2. Rinse tubing with DI water to ensure all ethanol is removed.
3. Aspirate ethanol and allow to air dry overnight or rinse in DI water three time for 5 minutes.
4. "Sterilize" or clean as best as possible the K210 Syringe Pump with 70% ethanol prior to use using a spray and wipe method.

5. Sterile filter all fibrin gel reagents using a 0.2 μm filter.

Formation of gelatin cylinders

1. See Gelatin Sacrificial Mold Fabrication protocol

Perfusion Preparation

1. Remove top shelf from the incubator. If the syringe pump is placed on the top shelf, it is too close to the temperature sensor and the incubator will alarm.
2. Replace the plug from the bottom incubator with the one from the top, unused incubator (and place the bottom plug into the top incubator).
3. Stay and watch the outputs on the incubator to ensure no CO_2 and heat are escaping.
4. Place "sterilized" syringe pump on left side of incubator so it is as far away from the thermometer as possible.

   NOTE: It's possible that the incubator will overheat due to the syringe pump running. Leave the door ajar with a note informing the other students why it's open. If the alarm sounds just silence it.

Formation of channels from gelatin sacrificial materials.

1. See Channel Formation Via Sacrificial Mold protocol.
2. Only proceed to the next step once the channel has been formed so that as soon as cells are ready to be added, they can be placed in the microfluidic chamber and perfused.

Cell Culture (All steps should be completed in biosafety cabinet)

1. Culture C2C12 mouse myoblasts in C2C12 full media.
2. When sufficient cells are available add 5 mL of 0.25% Trypsin and incubate at 37°C and 5% CO_2 for 5 minutes or until cells have detached from the bottom of the flask.
3. Neutralize trypsin with 5 mL of media and move cell suspension to a 15 mL conical tube.
4. Remove a 10 μL sample and obtain a cell count.
5. Centrifuge cell suspension at 1000 RPM for 5 minutes in IEC Centra CL2.

6. Resuspend in appropriate volume of PBS to establish a concentration of 1 million cells/mL. Use PBS if validating the microengineered vascular network – bioreactor system so that the only media the cells obtain is from the media perfused through the network.

Cell Seeding

· Create vellum paper frames with an inner square of 1 cm x 1 cm with side thickness of 0.15 cm.

· Add 105 µL of cell suspension (300,000 cell/mL) to each vellum frame.

Perfusion

1. Prepare syringes with media and attach to tubing.
2. Place syringes in syringe pump and prime the tubing by running the pump until fluid fills all tubes and all air has been expelled.
3. Move all microfluidic chambers to the incubator, attach to tubing, and begin perfusion at a flow rate of 0.003 mL/min.
4. Perfuse for 24 hours at 37°C and 5% CO_2.
5. Depending on the size of the syringe, it may be necessary to refill before the 24 hours are complete. When refilling, make sure outlet tubing is at the height of the microfluidic chamber so that when the inlet tubing is removed, fluid does not drain out of the channel and microfluidic chamber.

   NOTE: Make sure to take the tubing's volume into account when preparing syringes for the perfusion

LIVE/DEAD Cell Staining

This staining protocol is from Jon Grasman's work with C2C12 cells and is similar to the example dilution protocol from Invitrogen.

Thaw and centrifuge the reagents briefly.

Add 6 µL of supplied 2 mM EthD-1 stock solution and 1.5 µL of supplied 4 mM calcein AM stock solution to 3 mL of sterile D-PBS (I used PBS since cells were not later being trypsinized). Vortex to ensure thorough mixing.

NOTE: Adjust the final solution volume to the amount needed for each sample.

Remove samples from wells and the bioreactor devices. Wash with PBS to remove any residue media!

Add 0.5 mL of PBS staining solution to each sample.

Incubate samples for 30 minutes.

Imaging – Leica inverted fluorescence microscope (Rolle scope)

Insert the correct fluorescence cubes prior to imaging.

Turn on the mercury lamp 15-30 minutes prior to imaging.

Keep shutter closed whenever possible to avoid photobleaching of the samples.

After imaging, through ImageJ perform analyses.

Cell Viability in an Adjacent Layer on Top of Perfused Single-Channel Gels in an Open System and Seal System

Purpose: To determine cell viability in an adjacent layer on top of perfused single-channel gels in an open system, which has no top coverslip, and a sealed system, where the microfluidic chamber is completely closed.

* For this assay, the team used the protocol from Cell Viability in an Adjacent Layer on Top of Perfused Single-Channel Gels, with adjustments to improve the cell viability while preparing the samples.

The first adjustment was the gelatin was flushed out warm media over 40 minutes, in order to preload the microvascular network gel with media before the adjacent layer is placed on top.

The second adjustment was that the perfusion flow rate was 0.002 mL/min over 24 hours.

All other elements of the protocol were kept the same.

Microfluidic chamber fabrication process

Objective: To fabricate the pieces for the microfluidic chamber

Materials:

PTFE block

Stainless Steel block

POM

Silicon Gaskets

4 - 40 Screws

Procedure:

Microfluidic Chamber Bottom

1. Start with desired raw material.
2. Use band saw to cut the raw material to oversized dimensions of the desired piece.
3. Secure oversized piece into a vice on a Bridgeport 3 axis mill.
4. Cut the piece to the proper dimension on each side by using sets of parallel positioning bars.
5. To cut the center window, find the center of the piece using an edge finder. When the spring loaded edge finder kicks out slightly, it is on the edge. Set the digital display to zero. Repeat on perpendicular side.
6. Using a 3/8 in. mill bit, line up the center of the piece and begin to mill the window out. To determine the distance in one dimension needed use half the desired width and subtract the radius of the bit. Use the digital read out to determine depth of the cut.
7. To drill holes for the screws, based on drawing, again use the edge finder to find the proper spacing.
8. To drill inlet and outlet holes place the piece side up in the vice. Use the edge finder to determine the middle of the side and mill out the hole into the cutout window. Repeat on opposite side.

Microfluidic Chamber Top

1. Repeat steps 1 - 7 from bioreactor bottom. Use a square blank in the vice to secure piece due to the thickness of the desired piece.

Mold Piece

1. Start with raw material.
2. Use band saw to cut the raw material to oversized dimensions of the desired piece.
3. Secure oversized piece into a vice on a Bridgeport 3 axis mill. Use a square blank like when milling the bioreactor top.
4. Cut the piece to twice the thickness of the final desired piece. Leave extra material around piece for easier handling because the small dimension of the final mold.
5. Repeat steps 5, 6, and 8 from bioreactor bottom to cut out the window and inlet and outlet holes.
6. Next, mill away the top half of the whole piece to leave the desired height and semicircle inlet and outlet channels.

7. Mill the outer diameter to correct size.
8. Repeats steps 1 - 7 for second mold piece.

Stainless Steel Plate

1. Start with raw material.
2. Repeat steps 2 - 7 from Bioreactor Bottom. Use a square blank in the vice to secure piece due to the thickness of the desired piece.

20G Needles

1. 4 20G needles were cut and filed down to make the endings blunt instead of sharp.

Perfusion Test

Objective: To ensure different flow rates could perfuse through the chamber without the loss of liquid while using the appropriate amount of volume and expected time frame.

Materials*:*

Syringe pump

1 mL syringe

Water

Two pieces of 12 inch tubing

Two 20G needles

Microfluidic chamber

Parafilm

Collection flask

Pin

Procedure:

1. Plug in syringe pump and set the flow rate to 0.01 mL/min.
2. Fill syringe with 1 mL of water
3. Attach tubing to syringe
4. Attach other end of tubing to 20G needle

5. Assemble microfluidic chamber
6. Insert the needle with tubing attached to syringe into the inlet hole of the microfluidic chamber
7. Insert a second needle to outlet of microfluidic chamber
8. Attach tubing to that needle
9. Place parafilm over a collection flask and pierce a hole with a pin with the same diameter as the tubing
10. Place the end of the tubing into the hole in the parafilm so the water can perfuse into the collection flask once it has perfused through the system
11. Hit start on the syringe pump
12. Wait until the syringe is empty
13. Remove output tubing from collection flask and remove parafilm from top of collection flask
14. Measure the volume of water in the collection flask
15. Compare volume of water in collection flask to 1 mL
16. Empty collection flask
17. Fill a syringe with 1 mL of water
18. Attach to inlet tubing of system
19. Set flow rate of syringe pump to 0.1 mL/min
20. Repeat steps 12 - 19
21. Set flow rate of syringe pump to 1 mL/min
22. Repeat steps 12 - 16

Sealing Test

Objective: To ensure the microfluidic chamber creates a tight seal to prevent contamination.

Materials:

70% ethanol

Microfluidic chamber components

Syringe pump

$CaCl_2$ solution

2 mL syringe

C2C12 media

Fibrinogen

Thrombin

Gelatin

Two 20G needles

Collection flask

Parafilm

Two pieces of 12 inch tubing

Media

Procedure:

1. Sterilize components of microfluidic chamber through ethanol submersion. Submerge in 70% ethanol for 90 minutes
2. Remove components from ethanol after 90 minutes
3. Set up syringe pump and plug in
4. Prepare $CaCl_2$ solution following $CaCl_2$ Solution Preparation protocol
5. Fill a 3 mL syringe with complete C2C12 media
6. Prepare fibrinogen and thrombin following the Fibrinogen and Thrombin Diluted Aliquot Preparation
7. Create gelatin sacrificial mold fabrication following the Gelatin Sacrificial Mold Fabrication protocol
8. Create a channel within a fibrin scaffold following the Channel Formation Via Sacrificial Mold protocol
9. Set flow rate on syringe pump to 0.003 mL/min
10. Attach one end of tubing to 3 mL syringe containing media and other end to 20G needle of microfluidic chamber
11. Attach another 20G needle to outlet end of microfluidic chamber
12. Prepare collection flask by sealing with parafilm and piercing small hole
13. Place tubing into hole of collection flask
14. Hit start on syringe pump
15. When syringe is empty fill with another 3 mL of media
16. Set flow rate on syringe pump to 0.003 mL/min
17. Hit start
18. Run for 24 hours

Setup Time of Microfluidic Chamber

Objective: To ensure ease of use in order to not inhibit the users during experimentation

Materials:

Microfluidic chamber components

Timer

Procedure:

1. Separate all microfluidic chamber components
2. Hit start on timer
3. Place the PTFE base on the benchtop
4. Apply vacuum grease to an 18 mm x 18 mm glass coverslip
5. Place coverslip on PTFE mold bottom
6. Place PTFE mold bottom into base of PTFE chamber
7. Place PTFE mold top onto PTFE mold bottom
8. Place a glass coverslip on top of PTFE mold top
9. Place POM top of chamber onto assembly
10. Place stainless steel base plate beneath assembly
11. Place stainless steel top plate above assembly
12. Place screws in holes on four corners of the chamber
13. Use an Allen wrench to screw holes in place
14. Tighten system
15. Insert a 20G needle into the inlet hole of microfluidic chamber
16. Insert a 20G needle into the outlet hole of microfluidic chamber
17. Hit stop on timer
18. Disassemble entire system
19. Repeat steps 1 - 18 two more times
20. Take the average of three trials

Dimension Validation

Objective: To ensure the manufacturing process is accurate and reproducible

Materials:

Microfluidic chamber components

Microscope

Ruler

Procedure:

1. *Take images of various components of the microfluidic chamber including:
    a. Indentation in POM chamber top for silicone gasket
    b. Silicone gasket
    c. PTFE mold indentation
    d. Screw hole in POM
    e. Top view of bottom part of microfluidic chamber

f. Side view of PTFE bottom part of microfluidic chamber
g. Top view of POM top of microfluidic chamber
h. Side view of POM top of microfluidic chamber
i. Top view of Stainless Steel Top Plate
j. Side view of Stainless Steel Top Plate
k. Top view of Stainless Steel Bottom Plate
l. Side view of Stainless Steel Bottom Plate
m. Half of Simple Mold

*take photo from top view unless otherwise noted

1. For each measurement, make sure to measure it 3 times and to average the measurements
2. For a, measure the diameter of the indentation in 3 different places
3. For b, measure the diameter of the gasket in 3 different places
4. For c, measure the diameter of the indentation in 5 different places
5. For d, measure the diameter of the screw hole in 3 different places
6. For e, measure the full PTFE chamber base length on the right, top, left, and bottom
7. For e, also measure the PTFE chamber base pocket length on the right, top, left, and bottom
8. For e, also measure the PTFE chamber base viewing window length on the right, top, left, and bottom
9. For f, measure the PTFE chamber base height
10. For g, measure the PTFE chamber top length on the right, top, left, and bottom
11. For g, also measure the PTFE chamber top viewing window length on the right, top, left, and bottom
12. For h, measure the POM chamber top height
13. For i, measure the Stainless Steel top plate length on the right, top, left, and bottom
14. For j, measure the height of the Stainless Steel top plate
15. For k, measure the length of the Stainless Steel bottom plate
16. For l, measure the height of the Stainless Steel bottom plate
17. For m, measure the outer length of the PTFE mold on the right, top, left, and bottom
18. For n, measure the length of the viewing window of the PTFE mold on the right, top, left, and bottom
19. Ensure each set of similar measurements are averaged
20. Compare average to the length that was desired before machining
21. Calculate the error by subtracting the desired length from the average length
22. Convert those values to percentages
23. Calculate the standard deviation of the measurements

Sterility Testing

Objective: To ensure the microfluidic chamber can maintain a sterile environment of an extended time within incubator conditions

Materials:

70% ethanol

Microfluidic chamber

Media

Petri dish

Microscope

0.05% Methylene Blue

Distilled water

Procedure:

1. Sterilize components of microfluidic chamber through ethanol submersion. Submerge in 70% ethanol for 90 minutes
2. Remove components from ethanol after 90 minutes
3. Rinse with distilled water 3 times
4. Assemble the microfluidic chamber while remaining inside the biosafety cabinet with needles attached
5. Fill the chamber with media
6. Place chamber into a Petri dish and cover with lid
7. Transport Petri dish to incubator and let sit for 5 days
8. Once the 5 days have passed, remove the Petri dish, disassemble the microfluidic chamber and remove the bottom coverslip
9. Place coverslip under an upright bright - field microscope at 10x magnification
10. Image for bacteria
11. After imaging, place coverslip into a well and stained with 0.05% Methylene Blue
12. After 1 minute, wash the coverslip
13. Image washed coverslip under a microscope for bacteria

Appendix E: Final Design

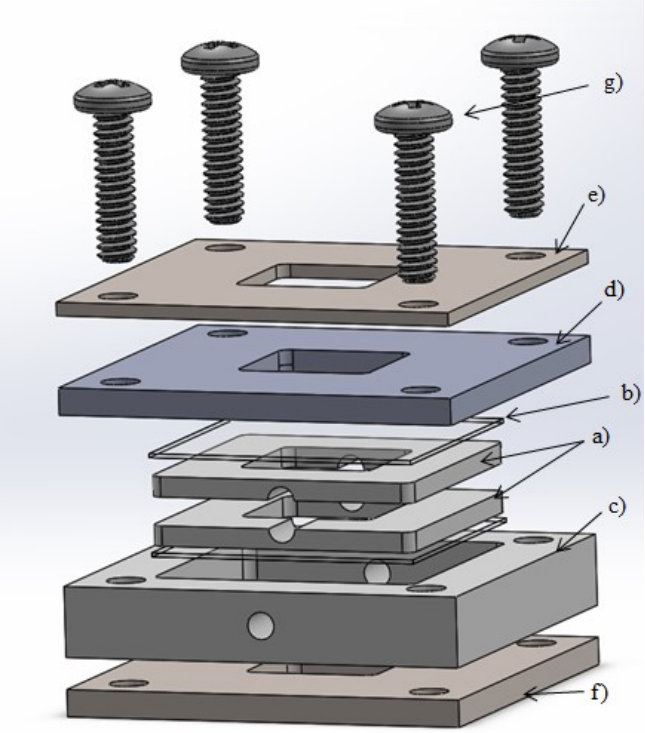

Figure 115: Exploded view of microfluidic chamber. a) Two Piece Mold, b) Glass coverslips, c) Chamber bottom, d) Chamber top, e) Stainless Steel top, f) Stainless Steel bottom, g) 4 - 40 screws

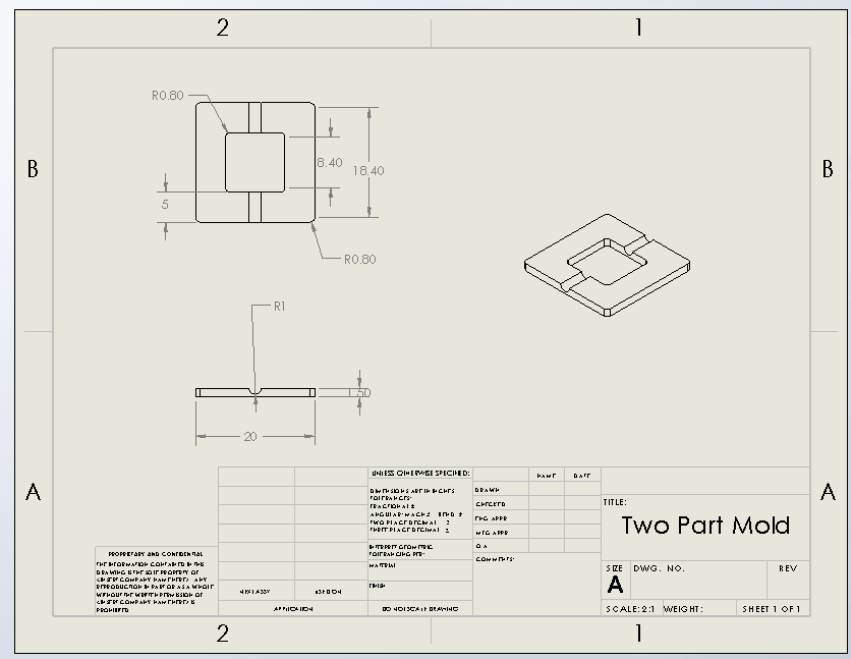

Figure 116: 2D drawing of one half of Two part Mold

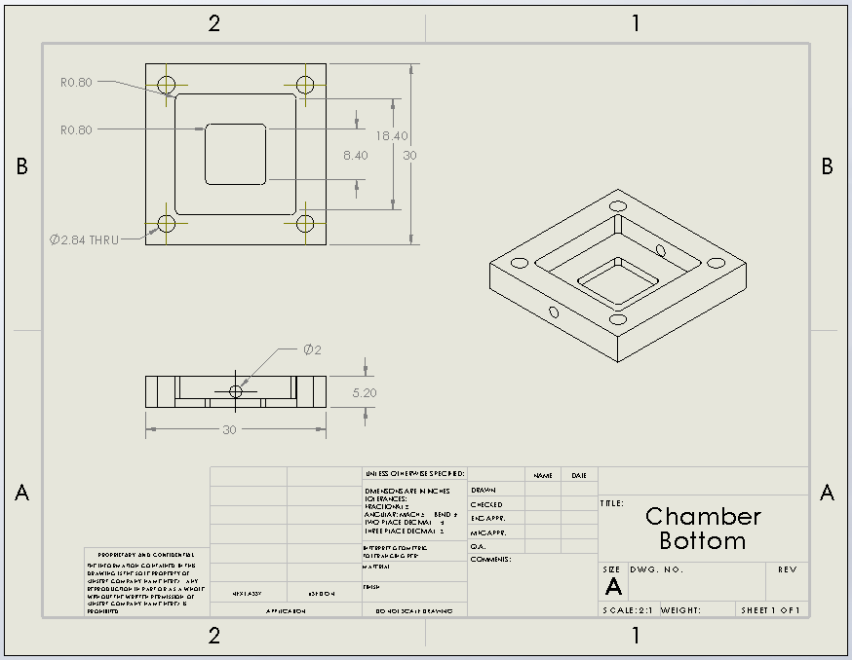

Figure 117: 2D drawing of chamber bottom

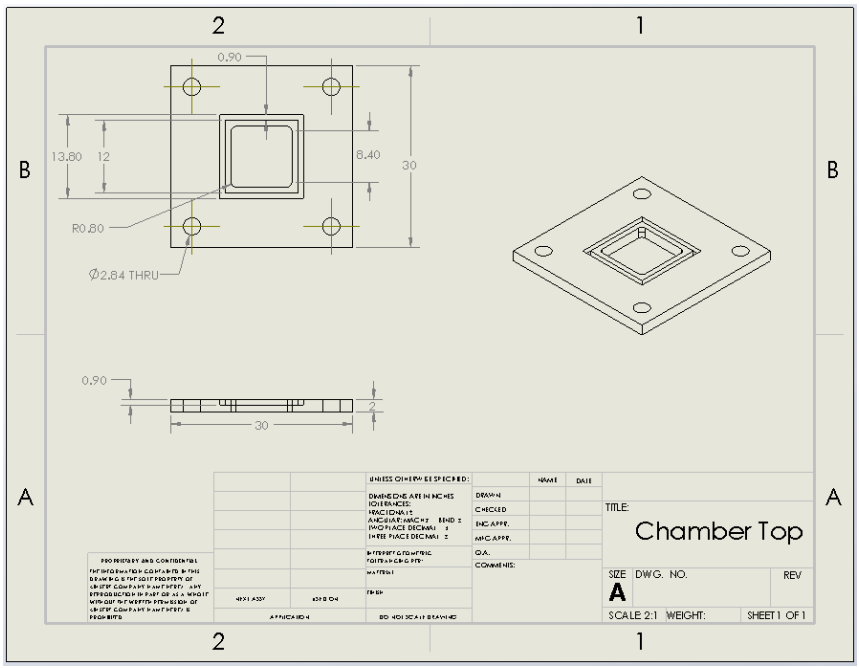

Figure 118: 2D drawing of chamber top

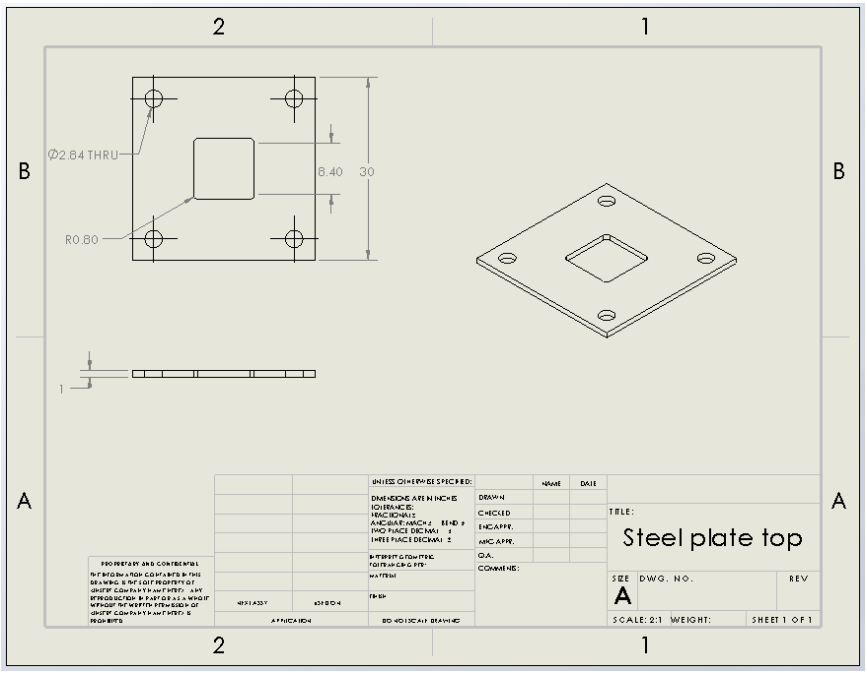

Figure 119: 2D drawing of Stainless Steel top plate

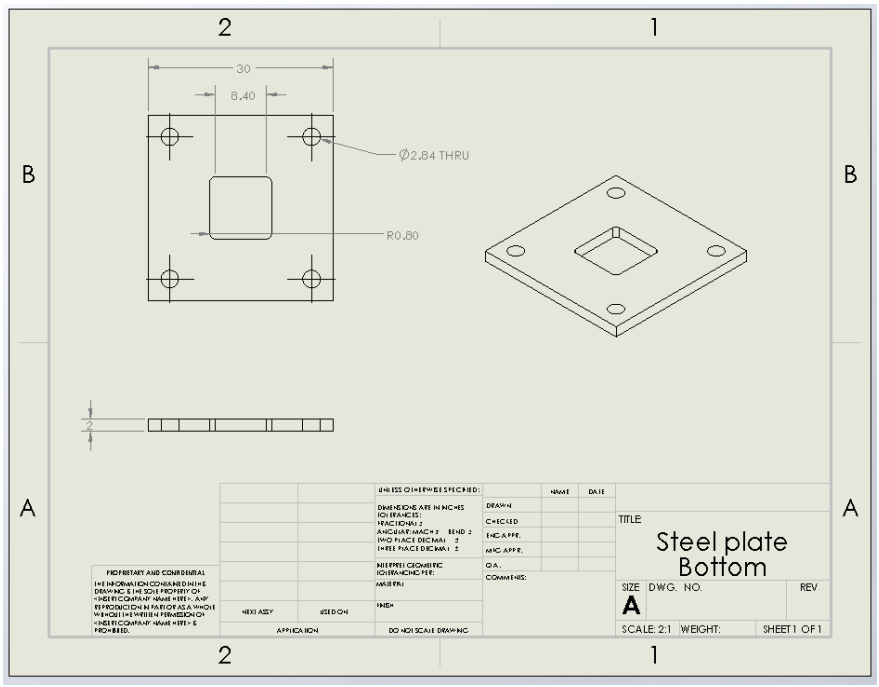

Figure 120: 2D Drawing of Stainless Steel bottom plate

Microfluidic Chamber Parts List

Table 38: Microfluidic chamber parts list

Material	Supplier	Product Number	Quantity	Description
PTFE	McMaster-Carr	8545K1	1 sheet	6’’ x 6’’, 1/4’’ thick
POM	McMaster-Carr	8738K49	1 sheet	0.093’’ Thick, 5ft
Stainless Steel	McMaster-Carr	9090K15	1 sheet	316 Stainless Steel, 2’’ x 12’’
Screws	McMaster-Carr	91735A105	1 pkg.	316 Stainless Steel, 7/16’’ Length, 4 - 40
Gasket	McMaster-Carr	5233T19	1 pkg.	1mm wide, 11 mm ID, Silicone
Glass Coverslips	Capitol Brand	M3453 - 1818	1 Box.	18 x 18 mm #1 Thickness