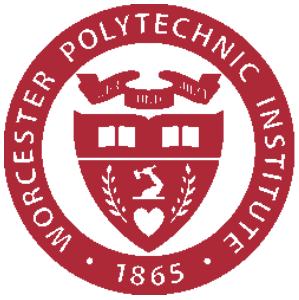
PROJECT NUMBER RLP-1401



WPI

Mechanical and Electrical Stimulation Device for the Creation of a Functional Unit of Human Skeletal Muscle *in Vitro*

A Major Qualifying Project Submitted to the Faculty of
Worcester Polytechnic Institute
in partial fulfillment of the requirements for the
Degree of Bachelor of Science in Biomedical Engineering By

_____
Keegan Cole

_____
Natalia Henao

_____
Tori Miller

_____
Kellie Pawelski

April 2015
Advisor: Raymond L. Page, PhD

Table of Contents

Table of Figures

Table of Tables

Authorship

Cell culture and tissue seeding were mainly handled by Natalia, Tori, and Kellie. Solidworks CAD designs for the final device were created and modified by Tori, and all submissions to the Rapid Prototyping Lab were completed by Keegan. Construction of the device and testing were completed by all team members.

All group members contributed equally to the writing and editing of the report.

Acknowledgements

The team would like to first and foremost acknowledge their advisor Raymond Page, PhD for his constant support and guidance throughout the project. Without his help, the team could not have performed to their full potential. Additionally, the team would like to thank the following people for countless hours of support, aid, and advice:

Jason Forte

Lisa Wall, Lab Manager

Tom Partington, Machine Shop/Lab Manager, Unit Operations Lab

Sakthikumar Ambady, PhD

Alyssa Fidanza

Denis Kole, PhD

Abstract

Duchenne (DMD) and Facioscapulohumeral (FSHD) Muscular Dystrophies affect one in 3,500 males and one in 15,000 people in the United States, respectively (Nowak 2004, Rahimov 2013). Currently, developing therapeutics for these diseases is hindered by the inability to test on human tissue in beginning trials and the genetic limitations of animal models. The purpose of the project was to design a device that would allow for the formation of a functional unit of human skeletal muscle tissue completely *in vitro*. Development and maturation of muscle tissue in the body occurs by mechanical and electrical cues, which researchers have yet to provide to engineered skeletal muscle in combination. The device design features a hydraulic system for mechanical actuation of tissue, capable of use within an incubator, and an electrical stimulation system provided via a separate lid. The device design provides positive and negative cyclic strain at 5%, 9%, and 15%, along with 3V/cm of electrical stimulation to mature self-assembled mouse skeletal muscle tissue. In the future, this device can be used to mature human skeletal muscle tissue for the purposes of developing and testing therapeutics for muscular dystrophies.

Executive Summary

Introduction

Muscular Dystrophy is a group of genetic diseases that weaken muscle and decrease mobility leading to death in many cases. Current therapeutics only treat the symptoms of the diseases, but there are still no cures or treatments that stop or reverse the progression. However, a promising route toward treatment is being researched in the field of skeletal muscle tissue engineering through the development of models that mimic the function of natural human muscle. Both *in vivo* and *in vitro* models of skeletal muscle are used as tools to improve the precision of therapeutic strategies such as drug screening and efficacy testing of pharmaceuticals. Thus, a model that mimics skeletal muscle tissue, and allows for the testing or development of therapeutics for Muscular Dystrophy, would significantly impact research for understanding how to treat muscular dystrophies.

Both *in vivo* and *in vitro* models of skeletal muscle are limited in how effectively they are capable of mimicking functional muscle tissue. Animal models, such as rodents, are used for *in vivo* testing of therapeutics. Yet, there is a significant genetic difference between rodents and humans in terms of muscular diseases. One specific example is FSHD, where the D4Z4 repeat sequence, unique to primates, mutates and leads to the expression of a cytotoxic protein. Thus, when a pharmaceutical treatment for muscular dystrophy demonstrates efficacy in animal models, it ultimately fails in human clinical trials due to the dissimilarities in DNA (Rahimov 2013). Another limitation of animal models is that they are expensive and time consuming. On the other hand, current *in vitro* models are unable to successfully provide the necessary environment or stimuli to produce biomimetic engineered skeletal muscle tissue. These *in vitro* models lack the ability to effectively provide both electrical and mechanical stimulation in the same system, which are important factors in the maturation of skeletal muscle (Grossi 2007). With these drawbacks in

mind, there is a need for a device that produces accurate models of skeletal muscle tissue in order to study how muscular diseases can be treated.

Overall, the goal for this project was to develop a device that applies both mechanical and electrical stimulation to self-assembled skeletal muscle tissue. For the purpose of the project, tissue was differentiated from the myogenic C2C12 cell line. Stimulation provided by the device mimics the dynamic properties found *in vivo*. This device improves upon past models by incorporating both mechanical and electrical stimulation and decreasing the number of cells used in each construct, allowing for nutrient diffusion and prevention of tissue necrosis.

Device Design

The purpose of the device is to mechanically actuate and electrically stimulate skeletal muscle tissue constructs *in vitro*. It also needs to support self-assembly of skeletal muscle tissue. Based on the unique constraints of the project, designs were considered that were biocompatible, fit within an incubator, and maintained a sterile environment.

The device itself consists of a polysulfone box, featuring one stationary platform and one moveable platform, connected to a syringe that penetrates the device's wall. On the platforms are stainless steel posts, upon which an agarose mold for tissue formation is placed. This mold is formed in a two-step process. It begins with a base mold of MED610 material, altered from a prior design, with which a PDMS negative is formed, cured, and sterilized. From the PDMS, agarose is poured to form the final tissue construct mold. Each mold can form three tissues by self-assembly, utilizing posts and notches to anchor the tissue. The structure of the mold is shown in Figure 1.

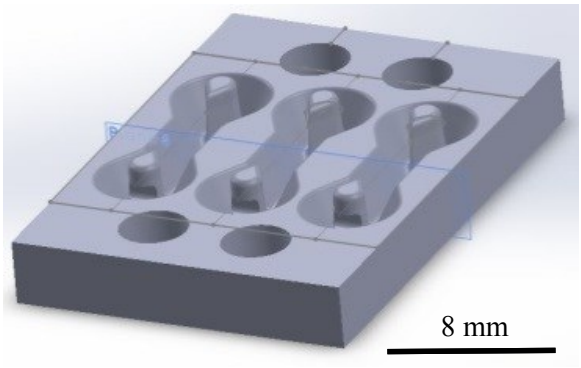


FIGURE 1. CAD DESIGN OF TISSUE MOLD

Two lids of polycarbonate are also provided, one that features stainless steel rods that contact media within the device to electrically stimulate the tissue. The device system utilizes a syringe pump capable of infusion and withdrawal for mechanical actuation and a BIOPAC system for electrical stimulation. The entire device can be seen in Figure 2.

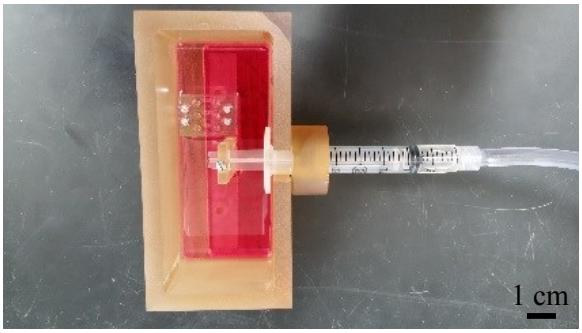


FIGURE 2. DEVICE WITH SYRINGE PUMP TUBING AND TISSUE MOLD IN DIFFERENTIATION MEDIA

Testing and Validation

To determine if the device could impart negative and positive strain to the tissue constructs, testing was performed using a syringe pump and LAS EZ microscope software. Preliminary tests were performed to establish the correlation between the syringe volume

displacement and the movement of the device platform. Testing was then done for the 5%, 10%, and 15% strain that would be applied to the tissue. Multiple trials were completed to determine which volume displacement of the syringe corresponded to the device platform displacement needed for the required strain. Since the tissue constructs are one centimeter in length, each millimeter movement ideally equals 10% strain. Five trials were completed for each strain, with averages seen in Table 1.

TABLE 1. DISPLACEMENT AVERAGES OF THE SYRINGE VOLUME AND DEVICE PLATFORM

Syringe Volume Displacement	**Device Platform Displacement**	**Strain**
0.03 mL	0.50 mm +/- 0.05	5%
0.06 mL	0.90 mm +/- 0.1	9%
0.1 mL	1.51 mm +/- 0.06	15%

To validate that the correct electrical stimulation was delivered at the tissue location, electrodes from a BIOPAC system were placed in contact with the media in the device and reading electrodes were placed at the site of the constructs. The system was programmed to deliver 3V for a 4ms duration with a one second rest period. This was done continuously for 30 seconds. Three different trials were performed using this set-up. The average peak voltage for the entire 30 seconds of each trial was then calculated and can be seen in Table 2.

TABLE 2. AVERAGE PEAK VOLTAGE FOR EACH ELECTRICAL STIMULATION VALIDATION TRIAL

Trial #	**Average Peak Voltage (V)**
1	3.04
2	3.06
3	2.98

Conclusion

The device design was created with the main purpose of allowing the formation of a functional unit of skeletal muscle tissue completely *in vitro*. The design was composed of three essential parts: mechanical actuation, electrical stimulation, and formation of self-assembled skeletal muscle tissue. Mechanical actuation was provided by a syringe system that was successful at providing both positive and negative strain. The stainless steel rods, along with the BIOPAC system, were effective at producing the necessary electric field to stimulate the tissues. Lastly, the agarose molds allowed for skeletal muscle tissue self-assembly. Additionally, the device is scalable and was developed to maintain an aseptic environment and remain functional within incubator conditions. Due to complications with the mold, the skeletal muscle tissues were not able to be stimulated with the device. This design offers promising potential for future studies aiming to create a functional unit of human skeletal muscle tissue for the testing and development of new therapeutic strategies.

Chapter 1: Introduction

Muscular dystrophies are a group of genetic diseases that weaken muscle and decrease mobility. One in 3,500 males in the United States is born with Duchenne Muscular Dystrophy, which is the most common pediatric form of the disease (Nowak, 2004). Currently, there is no specific treatment that can stop or reverse the progression of this disease, as available therapies only treat symptoms. However, a promising route toward treatment is being researched in the field of skeletal muscle tissue engineering through development of skeletal muscle that mimics natural human muscle to provide a model for diseases such as Duchenne Muscular Dystrophy, for therapeutic development. Both *in vivo* and *in vitro* models of skeletal muscle are used as a tool to improve the precision of therapeutic strategies such as drug screening and testing the efficacy of pharmaceuticals. Thus, a model that mimics skeletal muscle tissue and allows for the testing of therapeutics for muscular dystrophies would significantly impact research for understanding the processes of the disease and how to treat it.

Both *in vivo* and *in vitro* models of skeletal muscle present limitations on how effectively they are capable of mimicking functional muscle tissue. For example, *in vivo* study of engineered skeletal muscle occurs within animal models (mostly rodents). Yet, there are significant differences between rodents and humans when it comes to muscular diseases. One specific example of a disease in which an animal model is not available is Facioscapulohumeral Muscular Dystrophy (FSHD). FSHD occurs when the D4Z4 repeat array is mutated, which causes the expression of a cytotoxic protein; this repeat array is unique to primates, and so there is not a natural rodent model of the disease. Thus, when a pharmaceutical treatment for muscular dystrophy is proven to be effective on rats, it ultimately fails in human clinical trials due to dissimilarities in DNA (Rahimov 2013). Another limitation for animal models is that they are expensive and time consuming. *In vitro* models, on the other hand, fall short of successfully

providing the necessary environment or stimuli to produce biomimetic engineered skeletal muscle tissue. Current *in vitro* models lack the ability to effectively provide both electrical and mechanical stimulation in the same system, which are important factors in the maturation of skeletal muscle (Grossi 2007). Furthermore, although *in vivo* and *in vitro* animal models are presently used to study skeletal muscle and muscular dystrophy, they are inefficient at mimicking human skeletal muscle, due to molecular and maturation limitations. With these drawbacks in mind, there is a need for a device that models skeletal muscle tissue more accurately in order to study how muscular diseases can be treated. Therefore, the team's device will aim to develop a functional unit of skeletal muscle that can be used for development of therapeutic strategies.

Overall, the goal for this project is to, completely *in vitro*, mimic the dynamic properties that lead to *in vivo* levels of maturation and function of skeletal muscle tissue. This will be achieved by creating a device that applies both mechanical actuation and electrical stimulation to engineered 3D skeletal muscle tissue. First, seeding cells into a non-adhesive hydrogel and allowing these to self-assemble will create the 3D tissue constructs. The mold itself will mimic myotendonous junctions with two posts to provide anchorage points when the tissue is stimulated. For the mechanical actuation, the device will apply positive and negative strain to the individual tissue constructs after 3D tissue formation. Additionally, electrical stimulation will be provided to the tissue via electrodes. The requirements for a successful minimum functional unit of skeletal muscle will be based on whether or not the mechanical actuation and electrical stimulation increase the myofiber diameter and density throughout the entire construct. By increasing these myofiber features, the constructs will better mimic components of skeletal muscle tissue. The success of this project relies on the ability of the device to provide a suitable environment and physiologically, clinically relevant conditioning regimen that aids in maturation and production of a minimal functional unit of skeletal muscle tissue. This device should improve upon past models in terms of incorporating both mechanical and electrical stimulation, decreasing

the number of cells used in each construct, and increasing the biomimetic properties of the *in vitro* tissue to resemble and function like human skeletal muscle tissue.

Chapter 2: Literature Review

The following chapter details background on skeletal muscle tissue, muscular diseases and the resulting clinical need for a tissue model, as well as current models for skeletal tissue engineering. Skeletal muscle tissue is formed from fused myofibrils that produce a striated, multi-nucleated structure. In the case of small injuries, this muscle tissue repairs itself by signaling the migration of satellite cells to the location of injury. In cases of gross skeletal muscle injury or disease, such as Duchenne Muscular Dystrophy, the muscle cannot repair itself adequately, resulting in non-function, leading to a complete loss of dystrophin or expression of nonfunctional dystrophin in myofibers. Combined with respiratory insufficiency and cardiac failure, this disease can lead to premature death by the mid-20s (Rahimov 2013). Thus, the treatment of skeletal muscle diseases and injury requires the development of new therapeutics that must be tested in a model similar to that of the human body. Currently, animal models such as mice cannot serve as a platform for diagnostic screening and testing due to genetic differences in DNA. The development of a skeletal muscle tissue construct that mimics native muscle in the body can provide a model to test potential new therapeutics for muscle disorders.

2.1 Muscle Physiology

In order to understand the need for a tissue engineered model of human skeletal muscle tissue, the basic physical and functional properties of muscle tissue need to be understood. The human body contains three major muscle systems: skeletal muscle, smooth muscle, and cardiac muscle (Muscle Physiology 2007). Skeletal muscle connects to the skeleton via tendons and is tailored to produce force and direct movement. Smooth muscle is found within the walls of all blood and lymphatic vessels and controls their functions, mainly contraction. The heart is

comprised of cardiac muscle which plays a role in both the contraction and relaxation pumping mechanisms of the heart. Striations are visible in both cardiac and skeletal muscle due to the presence of contractile sarcomeres. However, only skeletal muscle can be controlled voluntarily (Muscle Physiology 2007). Both cardiac and smooth muscle rely on nerve signaling from the brain to perform required functions without conscious input.

2.1.1 Formation and Development of Skeletal Muscle

The development of skeletal muscle begins in the embryo with the signaling of skeletal myogenesis coming from molecules in nearby tissues. Muscle progenitor cells originate in the somite, which is a segmented portion of paraxial mesoderm located on either side of the neural tube and notochord in the embryo (Buckingham 2001). When the signaling of skeletal myogenesis occurs, muscle progenitor cells migrate from the somite to certain locations to form muscle masses in the body. For this to occur, muscle progenitor cells receive dorsal signals to enter into the myogenic program. The signals initiate expression of myogenic differentiation genes Myf5 and MyoD. When Myf5 is activated, it subsequently activates MyoD (Buckingham 2001). However, when Myf5 is absent, which is crucial in myoblast proliferation and myogenesis, a third gene Pax3 is responsible for regulating early muscle formation. Pax3 then activates MyoD and promotes the survival of the cells (Buckingham 2001). Thus, all three genes are important for muscle cell differentiation and development in the early embryonic stage. Differentiation results in myoblast cells from the myogenic progenitor cells, which will proliferate and align to form myotubes. From these structures, muscle fibers arise to form complete skeletal muscle tissue (Buckingham 2001).

2.1.2 Skeletal Muscle Structure and Function

The function of skeletal muscle tissue is closely related to its structure, which is hierarchical. Terminally differentiated muscle cells are known as myocytes, which arise from myoblasts during growth in the embryonic stage and later in life as well. These myocytes fuse to form a muscle fiber, or myofiber. The structure of myofibers is cylindrical, and each one is approximately 10-100 μm in diameter, depending on location and function (Jones 1990). The formation of muscle fibers occurs when myocytes fuse together to form multinucleated myotubes. To aid in function, the fibers contain contractile proteins, energy stores, and signaling molecules including AMPK, CaMKs, and p38 mitogen activated protein kinase p38 MAPK (Jones 1990). The myofiber overall is mostly composed of smaller myofibrils, which make up approximately 80% of the muscle fiber volume while the remaining space is occupied by extracellular matrix (Marieb 2012). As shown in Figure 4, the A-band, composed of myosin filaments, overlaps with actin filaments along the entire length of the muscle fiber, which gives it an alternating appearance, indicating striations. Each myofibril is enclosed by the sarcoplasmic reticulum, which allows for uptake and release of calcium ions to the proteins within myofibrils that are necessary for contraction. The structure of a singular myofiber is shown in Figure 5.

For the purposes of contraction, myofibrils are composed of repeating sections of sarcomeres, which are the basic functional units of muscle that allow for contraction stimulated by motor neurons (Jones 1990). The contractile proteins myosin and actin are found within these sarcomeres. These important proteins are responsible for sliding the myofilaments found within the sarcomeres. The placement of actin with respect to myosin in the sarcomere is visualized in Figure 4. The C-zone contains the myosin filaments, and the Z-band is the position where the actin filaments are attached from end to end of the sarcomere. Actin polymerizes into a double helical structure by splitting ATP into ADP + P_i (Jones 1990). As such, 90% of ADP in muscle is found bound to actin. Actin also contains tropomyosin, which blocks binding sites on the myosin

filaments. Calcium is required in order to release the tropomyosin from the binding sites, exposing the binding sites and allowing for myosin heads to bind to actin during contraction. Myosin can bind to both ATP and actin, but only to the latter when it is exposed to calcium ions. Thus, if no calcium is present, contraction will not occur (Jones 1990).

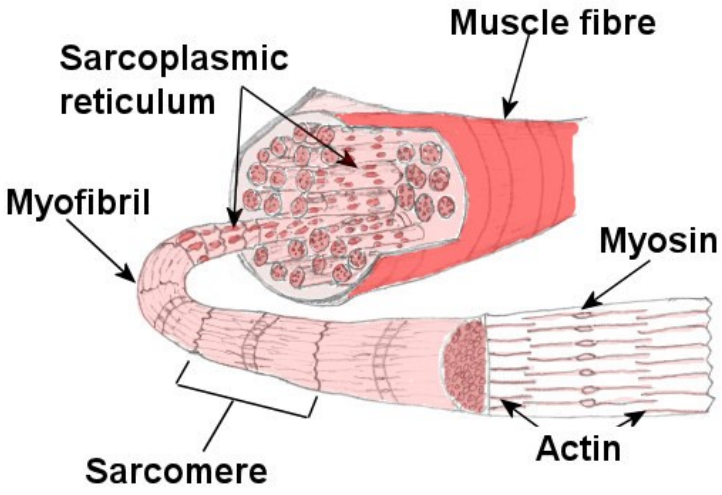


FIGURE 3. ANATOMY AND COMPOSITION OF A MUSCLE FIBER (JONES 1990)

Contraction of muscle fibers occurs upon stimulation of muscle cells. During this stimulation, motor neurons generate an action potential allowing for the release of acetylcholine from axon terminals. Acetylcholine will bind to receptors on the plasma membrane and generate an electrochemical signal. This signal will travel to the transverse tubule and cause calcium ions to be released from the sarcoplasmic reticulum. The calcium ions attach to tropomyosin, which undergoes a conformational change, exposing the myosin binding sites (Jones 1990). When myosin binds to actin, the sarcomeres shorten in length through the hydrolysis of ATP to ADP + P_i, causing the muscle tissue to contract.

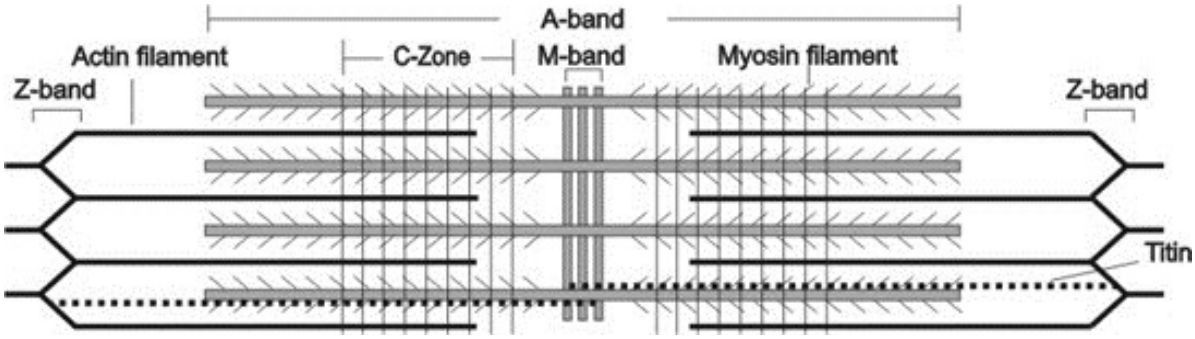


FIGURE 4. DIAGRAM OF A SARCOMERE (LUTHER 2014)

Although skeletal muscle tissue is mainly composed of myocytes, it also consists of an extracellular matrix (ECM), which aids in the force transmission of muscle fibers along with both the maintenance and repair of muscle tissue (Gillies 2011). The ECM bears the majority of the muscle's passive load, suggesting that the range of motion and stiffness of muscle is influenced by the properties and specific composition of the ECM. The structure of muscle ECM is comprised of three main sections and several important proteins. From the innermost layer to the outermost layer around the entire construct, the layers are shown in Figure 5. The endomesium wraps around individual muscle fibers, the perimesium wraps around groups of muscle fibers, and the epimesium wraps around the entire muscle construct.

The endomesium is the load bearing network which transmits the force generated by the muscle. The perimesium contains transverse collagen fibers that interconnect muscle fibers at certain points. These points also contain focal adhesions and intracellular domains, suggesting that the perimysium may be involved in cell signaling (Gillies 2011). The epimesium is primarily composed of very large collagen bundles and contains linkages to connective tissue for force transmission between muscles (Gillies 2011). The grouping of muscle fibers is known as a fascicle as shown in Figure 5. Throughout the skeletal muscle ECM, the major structural protein is collagen, which accounts for 1-10% of muscle mass (Gillies 2011). The endomesium, perimesium, and epimesium contain type I and type III collagen. These collagens have high

tensile strengths which are important for muscle contractions because they aid at the absorbtion the forces during contraction.

Another important component found in all skeletal muscle ECM is proteoglycans. Proteoglycans are structures that contain a core protein attached to several polysaccharide glycosaminoglycan (GAG) chains. The most prevalent proteoglycans in skeletal muscle ECM contain chondroitin sulfate and dermatan sulfate GAG chains (Gillies 2011). Negatively charged GAG chains on proteoglycans can store and release growth factors to promote growth of the skeletal muscle, which is especially important in the endomesium to promote growth of muscle fibers (Gillies 2011). Proteoglycans can also bind to collagen and influence the structure of the ECM though growth factors. These proteoglycans aid in the linkage of the basement membrane to the endomesium. This connection is involved in the transmission of force from the myofiber to the tendon (Gillies 2011). A common family of small leucine-rich proteoglycans (SLRPs) is often found in skeletal muscle ECM (Gillies 2011). SLRPs have been shown to actively bind to and alter the organization and assembly of collagen fibers in the ECM (Dellett 2012). In addition to proteoglycans, integrins, transmembrane receptors that link the internal cell to its external ECM, contribute to cell signaling and aid in the migration of myoblasts (Gillies 2011).

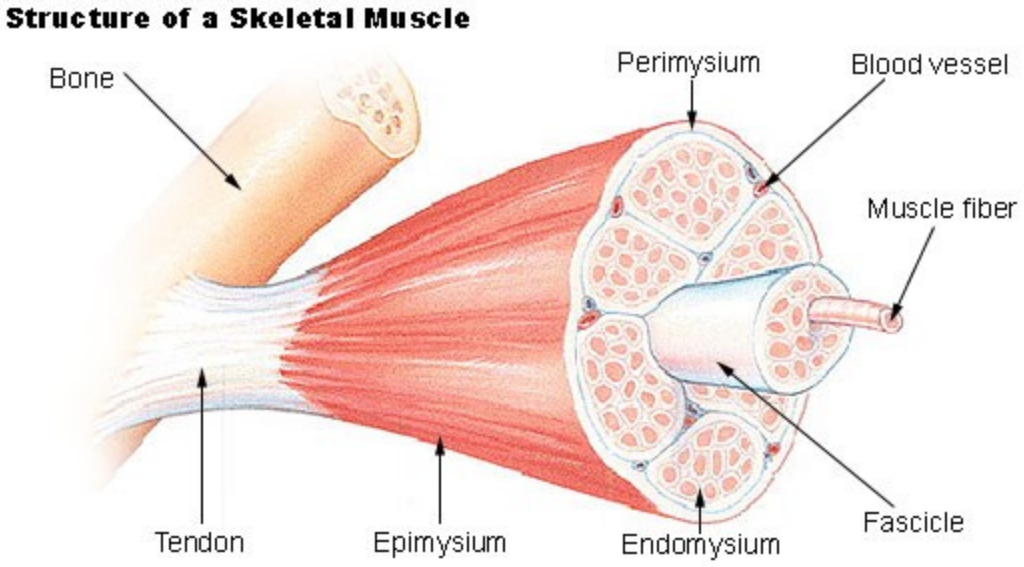


FIGURE 5. STRUCTURE OF SKELETAL MUSCLE (GILLIES 2011)

2.1.3 Repair of Skeletal Muscle Tissue

Skeletal muscle lacks the ability to repair itself without the aid of satellite cells. Thus, when muscle tissue is damaged, progenitor satellite cells will be activated and directed to migrate to the wound site and repair the damage. Small injuries are able to be repaired endogenously while large injuries result in scar tissue formation and the loss of functional muscle tissue. Satellite cells are derived from embryonic myoblasts that reside dormant between the plasma and basement membranes of muscle fibers and are activated following damage to the muscle fiber tissue (Turner 2012). The resulting repair follows a specific sequence, shown in Figure 6 and consists of three phases. The initial destruction and inflammatory phase is characterized by the necrosis of myofibers and inflammatory response from the cells. The tearing of myofibers exposes their contents, leading to an influx of calcium to the extracellular environment, which activates nearby calcium-dependent proteases to disintegrate the fibers. Complete destruction of the fibers is prevented by cytoskeletal proteins. People with muscular dystrophy lack these cytoskeletal proteins, such as dystrophin, which inhibits proper linkage between the cytoskeletal and the extracellular matrix results in skeletal muscle weakness and death of muscle tissue.

The repair phase is characterized by the phagocytosis of necrotic muscle fibers, the addition of new muscle fibers, and scar tissue formation. Macrophages migrate to the wound site and digest the necrotic muscle fibers. Through signaling from fibroblast growth factor (FGF) and hepatocyte growth factor (HGF), the satellite cells activate and migrate to the injury site (Turner 2012). Satellite cells will differentiate into myoblasts and fuse with each other or existing muscle fibers to form new muscle tissue. The satellite cell pool is a heterogeneous population and these cells are already predetermined despite their stem-cell-like qualities (Turner 2012). Additionally, muscle-derived stem cells (MDSC) have been shown to contribute to this satellite pool (Turner 2012). Fibroblasts secrete collagen to form scar tissue that bridges the gap between the remaining functional muscle fibers and the newly formed tissue. Finally, the remodeling phase is

characterized by the reorganization of muscle fibers, the remodeling of scar tissue, and the restoration of function to the muscle tissue (Turner 2012). However, if the damage is too severe, fibroblasts will form scar tissue that prevents muscle fiber formation and regaining of function.

Figure 6 shows the repair process of a large muscle defect (a) with volumetric muscle loss. When the muscle is injured (b), the cellular debris is removed and satellite cells begin to invade the area. The satellite cells begin to differentiate (c) into myoblasts and fibroblasts cause the formation scar tissue. Existing myoblasts fuse with myotubes that the satellite cells have formed (d) resulting in new muscle tissue formation. In volumetric muscle loss, large accumulation of scar tissue (e) prevents complete re-formation of myofibers. Therefore, the distal tissue (f) does not have any neuromuscular junctions and becomes dennervated (Turner 2012).

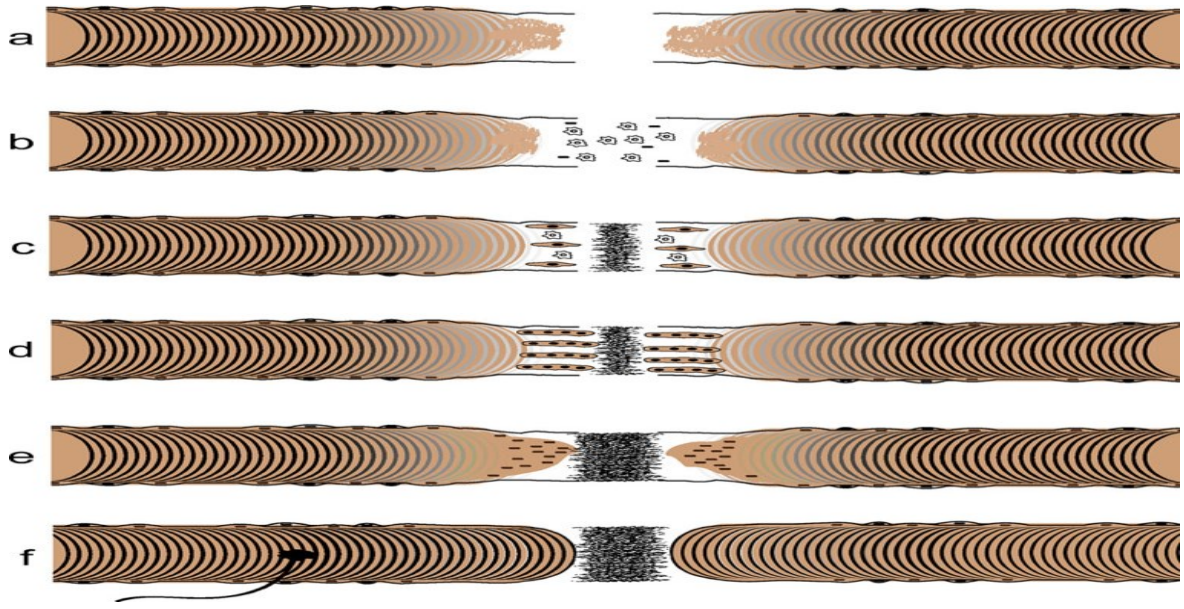


FIGURE 6. STAGES OF SKELETAL MUSCLE REPAIR FOLLOWING VOLUMETRIC LOSS (TURNER 2012)

2.2 Clinical Need and Significance of Skeletal Muscle Tissue Engineering

Skeletal muscle diseases and disorders cause complications in patients and severely impact quality of life. Additionally, volumetric muscle loss caused by accidents and extremity wounds can produce the same symptoms as skeletal muscle disorders (Grogan 2011). The body has the potential to repair minor damage to skeletal muscle, such as tears during routine exercise and overexertion. Following repair, the muscle generally regains its original function without surgical intervention. However, if muscle becomes diseased or is traumatically injured, it cannot fully regain function due to the deposition of collagenous scar tissue at the injury sites. For muscular diseases such as Duchenne Muscular Dystrophy, the development of pharmaceuticals can potentially aid in the treatment of these diseases. However, drug developers must test therapeutics pre-clinically, in models that can replicate or act similarly to the human body.

Traditionally, animal models are utilized to evaluate treatments prior to pre-clinical or human testing. However, the genetic variations from animal models differ dramatically from that of humans, partially due to the molecular composition of the extracellular matrix and the differences in genomic makeup. Additionally, pinpointing the specific gene responsible for producing muscular diseases can be difficult in some cases, as many are fairly large and complex (Chamberlain 2002). Although some animal models offer very close estimations, such as the pig for cardiac procedures, for the purposes of skeletal muscle tissues, animal models cannot fully replicate the human body before clinical testing, which can result in clinical failure (Mestas 2004). Skeletal muscle tissue engineering can help to study and treat muscular diseases and damage, as it provides a benchtop tool for testing human skeletal muscle tissue to test therapeutics and drug efficacy treatments for these diseases, so that proper muscular function can be restored to patients.

2.2.1 Muscular Diseases, Injuries, and Their Treatments

There are multiple diseases and injuries that can lead to diminished function and structure of skeletal muscle. Particular patient characteristics and activities can increase the likelihood of dramatic muscle injury and loss. Treatment for muscle injury can differ depending on the cause and extent of the damage. Treatment for muscular diseases depends on the type and extent of the disease. Treatment can take the form of pharmaceuticals, clinical intervention, or physical therapy. However, many treatments can only relieve the symptoms of muscular diseases.

2.2.1.1 Muscular Dystrophy

Muscular dystrophy includes genetic disorders in which muscle strength and volume gradually decline (Quercia 2011). One common disorder is Duchenne Muscular Dystrophy, which affects one in every 3,500 male births in the United States (Quercia 2011). As muscular dystrophies stem from a variety of causes, there is no one specific treatment or cure. The current treatment is patient specific and involves improving muscle and joint function and slowing deterioration through physical and occupational therapy, steroids, and surgery.

Treatment for symptoms of muscular dystrophy depends on what location in the body is afflicted with the disease, the extent to which the disease has progressed, and often differs for each patient. For example, muscular dystrophy around the airway might be treated by manually opening and respirating the airway, although this does not treat the condition itself (Respiratory 2004). Another general option lies in the administration of oral corticosteroids, which have been shown to increase muscle mass and slow deterioration. However, the continued use of corticosteroids produces undesirable side effects and is not generally recommended (Respiratory 2004). Despite this, research looks to treat the muscle tissue itself rather than the disease or its causes. New therapeutics are being developed that can potentially aid in muscle diseases, but FDA requirements for testing these therapeutics are difficult to achieve. Animal models cannot

accurately replicate the conditions of many muscular disorders in the human body. Thus, a benchtop model for testing potential new therapeutics to treat skeletal muscle tissue is required to improve the lives of those suffering from muscular disease.

2.2.1.2 Sarcopenia

Sarcopenia is a loss of muscle mass and strength that comes with aging, affecting approximately 30 to 50% of persons over age sixty (Paddon-Jones 2009). Sarcopenia is characterized by a decline in muscle efficiency and muscle protein synthesis (Morley 2011). On average, beginning at age 30, sarcopenia causes 3-8% lean muscle mass reduction for every ten years following (Paddon-Jones 2009). Thus, by age 80, a patient with aggressive sarcopenia can lose around half their muscle volume. This dramatically decreases the contractile function of skeletal muscle and quality of life. It contributes greatly to frailty and instances of falls and fractures in older adults.

The standard treatment for sarcopenia involves medication and physical therapy. Medicinal supplements can include testosterone and growth hormones. Testosterone has shown to improve the strength of sarcopenia patients, but has many disadvantages such as aggression and an increased risk of thrombosis, which overall prolong a poor quality of life (Jones 2009). Growth hormones have shown to increase muscle mass but has no appreciable effect on functional strength (Jones 2009). Beyond medicine, exercise can be suggested to help relieve the effects of sarcopenia but cannot treat the disease itself. Therapeutics designed to target the disease itself and help to restore muscle tissue could greatly improve patients' lives if suitable models exist to test the drugs' potential.

2.2.1.3 Muscle Injury, Rhabdomyolysis, and Volumetric Muscle Loss

Non-disease related muscle injury arises due to damage of the muscle tissue. Rhabdomyolysis is the breakdown of skeletal muscle tissue that occurs after laceration and traumatic injury to the muscle (Bagley 2006). It affects approximately 26,000 patients yearly in the United States alone (Sauret 2002). From the years of 2008-2012, the frequency of rhabdomyolysis occurrence in U.S. service members increased by 30%, attributed to extreme exertion (MSMR 2013). Beyond dramatic muscle damage, rhabdomyolysis can also lead to further life-threatening complications. When muscle is damaged, its cells release calcium, myoglobin, and other molecules into the extracellular environment. Up to a certain extent, released myoglobin is bound in the blood plasma. However, past approximately 100 g of skeletal muscle damage, myoglobin circulates to the kidney, blocking function and causing acute renal failure (Sauret 2002). Thus, rhabdomyolysis affects both the structural integrity of skeletal muscle and the proper functioning of other organs in the body. Due to these complications, treatment of rhabdomyolysis concerns maintaining proper renal function (Sauret 2002). However, if a skeletal tissue model were developed to replicate this exertion, the efficacy of a drug developed to combat this release of myoglobin in times of extreme muscle exertion could be tested and potentially given to service members or other high-activity groups.

Volumetric muscle loss can also occur due to high-energy civilian trauma or combat-related extremity wounds (Grogan 2011). High-energy trauma refers to injury by force that transfers high amounts of kinetic energy to the body, resulting in tissue damage (Jovanovic 2002). High-energy trauma results in death in 35-63% of cases, depending on the area's income and medicinal care (Jovanovic 2002). Approximately 5.8 million annual deaths occur worldwide due to high-energy accidents, wartime combat, and violent acts (Hettrich 2012). Extremity wounds in combat account for 64% of soldiers determined unfit for duty (Li 2014). Thus, engineering

methods to study and aid in treatment of volumetric muscle loss can positively impact the outlook of patients of muscle disorders and traumatic injuries.

The current gold standard treatment for volumetric muscle loss is the use of autogenic muscle flaps (Li 2014). These flaps cover defects in the muscle and return partial function to the muscle (Neill 2011). However, the flaps do not improve the structural restoration of the muscle and often result in functional deficiencies at the site of flap transplant (Li 2014). Often, patients request amputation of the entire limb due to poor treatment of the muscle loss. Other surgical options include autologous tissue transfer from one site of the body to the injury site and free muscle transplantation. Free muscle transplantation can successfully restore function and joint movement (Grogan 2011). However, the procedure is very complicated, very expensive, and time consuming, making it a nonviable option for all patients. For example, if the patient suffers from muscular disorder throughout the body, there will be no viable site present from which to retrieve muscle to transplant. There is also a chance for donor site morbidity. One non-surgical option is bracing, which allows for gradual physical therapy and is easily removed; however, it is expensive and patient specific, only treating the symptoms and not the cause (Grogan 2011). As such, these complications lead to the need for therapeutics that promote the regeneration and functionalization of skeletal muscle tissue. Testing of therapeutics requires reliable, cost-effective, and efficient benchtop models of human skeletal muscle tissue.

2.2.2 Need for a Human Skeletal Muscle Tissue Model to Evaluate Potential Therapeutic Options

Many studies that test the potential of therapeutics or any upcoming biomedical application use animal models to test *in vivo.* There are certain muscular diseases, such as Facioscapulohumeral Muscular Dystrophy (FSHD), that are present in humans but not in murine or other animal models. Currently, FSHD is one of the most prevalent types of muscular dystrophy; however, the specific mutation causing FSHD affects a DNA repeat sequence that is

not found in mice and rats, preventing the use of a murine model to test any potential therapeutic treatment (About FSHD 2014). Although the DNA of humans and mice is relatively similar, there are key differences that allow such diseases to occur in humans alone.

There are also molecular differences, particularly in the immunological system, that cause different reactions to therapies. For example, hundreds of treatments for HIV and neuroprotection have shown effective in animal models but have failed in humans (Greek 2013). Also, approximately 90% of drugs proven successful in animal models fail in initial clinical trials (Drake 2013). This failure results from the inherent immunological differences between humans and mice; for example, CD4 glycoprotein is expressed on human macrophages, but is absent from mice macrophages (Mestas 2004). MHC II expression is also present on human T-cells and absent in mice (Mestas 2004). These immunological differences between mice and humans can result in undesirable results during clinical trials and potential failure overall.

The use of animal models can be time consuming and expensive. In order to enable understanding of native skeletal muscle and produce effective treatments for the aforementioned muscular diseases, a biomimetic model of human skeletal muscle tissue is required, to serve as a tool for testing therapeutics for skeletal muscle diseases. This model would ideally serve as an accurate representation of *in vitro* skeletal muscle similar to *in vivo* muscle. This model would allow for the study of drug delivery systems, drug efficacy, and therapeutic methods for certain muscular diseases, directly applied to human tissue without the added complication of animal models that might not produce clinically relevant results. Additionally, a benchtop biomimetic skeletal tissue model will provide more predictive results for the therapeutics in the human body, and the entire process will be cheaper and quicker than using animal models. This will save both money and time in preclinical testing and potentially avoid the use of animal models altogether.

2.3 Current Skeletal Muscle Tissue Engineering Methods

There are several current methods to engineer models of skeletal muscle tissue, including 2D culture and 3D tissue constructs using tissue scaffolds. There are also *in vitro* techniques to better mature *in vitro* muscle to function like native muscle, such as mechanical and electrical methods, which aid in the stimulation of skeletal muscle tissue. However, there lacks a model that combines all of these aspects into one mimetic design of *in vivo* skeletal muscle formation and function. Current methods do not fully generate *in vitro* what is found *in vivo*. They are inefficient at providing a combination of cell attachment, migration, proliferation, and differentiation provided by the extracellular matrix (ECM). They also fail to mimic the electrical and mechanical stimulation provided by the central nervous system and the stress loading that muscles undergo during stretch or exercise (Powell 2002). The lack of these different components does not allow for a fully functional skeletal muscle unit that represents *in vivo* skeletal muscle, showing small diameter muscle fibers, low myofiber density, and excessive exogenous ECM (Powell 2002).

2.3.1 Tissue Scaffolds

The goal of tissue scaffolds is to mimic the features of body tissue in terms of initial mechanical stability, immune response, architecture, and porosity. Many scaffolds are composed of synthetic materials, usually polymers. Recent approaches to forming tissue scaffolds include harvesting from naturally occurring sources, such as the decellularized ECM of skeletal muscle of animal models. These ECM-based tissue scaffolds can be recellularized to create potential functional constructs for tissue repopulation. Depending on the source, application, and other factors, scaffolds are developed from various types of gels and components, including collagen, hyaluronic acid, laminin, fibrin, chitosan, silk fibroin, alginate, and agarose (Wolf 2014).

Collagen is the most commonly purified and naturally derived ECM component used in scaffolds (Herbage D, 2000). Often times, collagen type I is used, because it is the most abundant

ECM protein in many adult tissues. Studies have shown that collagen promotes cellular growth, and collagen substrates can modify the morphology, migration, adhesion, and differentiation of cells (Herbage D, 2000). Hydrogel scaffolds can also be made from hyaluronic acid (HA), a glycosaminoglycan found in the ECM. Hydrogels based on HA have been shown to promote myogenesis *in vitro*. Previous studies have shown that a HA-cell hybrid has the potential to support constructive and functional remodeling in a volumetric muscle loss injury model, but one of the drawbacks to these scaffolds was that neuromuscular junction function was not shown (Baiguera S, 2014). Beyond collagen and HA, hyaluronic chitosan, silk fibroin, alginate, and agarose can be used for tissue scaffolds (Wolf 2014). All substances are well tolerated *in vivo* and can take the form of both gels and solutions, depending on temperature and other factors.

Tissue scaffolds offer a number of benefits. The materials are suitable to construct 3D structures that are biocompatible, biodegradable, and porous. Scaffolds can feature an optimized architecture that creates sufficient surface area, allowing for cell attachment, growth, and proliferation. Also, scaffolds can be adjusted to have favorable mechanical properties and suitable degradation rates (Judith 2012). Overall, tissue scaffolds ideally offer a base structure for cell anchorage, proliferation, and differentiation (Webber 2014). However, real tissue scaffolds are not ideal and feature several drawbacks that limit their use in tissue engineering. Due to the increased densities of the tissue constructs using scaffolds, culture media and nutrients cannot diffuse through the scaffold to the center of the tissue, resulting in tissue necrosis.

2.3.1.1 Limitations of Scaffolds

A major drawback of tissue scaffolds is that they do not fully recapitulate the complex ultrastructure and bioactivity of ECM (Wolf 2014). With such a large number of components, molecules, and signals in the ECM, it is difficult to recapitulate the native ECM environment *in vitro*. There are also mass transport limitations when using tissue scaffolds. Vascularization of scaffolds also poses a problem, as the diffusivity of oxygen and nutrients of the scaffold is not as

effective as natural ECM and this leads to necrotic tissue in many of the current models. The distance at which cellular viability can be maintained in terms of diffusivity through a tissue must be less than 100 micrometers (Bland et al, 2012).

Tissue engineering is moving away from rigid, 2D culture plates, but 3D gel scaffolds might still constrict and alter the formation of tissue due to poor mechanical properties. Scaffolds made from polymers provide undesirable mechanical properties, such as a high elastic modulus, leading to stress shielding of the tissue. Stress shielding prevents the tissue from taking on full loads, leading to weakening of tissue and eventual cell death (Liu 2004). Gel scaffolds also have varying degradation rates and could present the possibility of toxicity and alteration of pH of the local surroundings causing adverse side effects. Although the use of tissue scaffolds helps to improve growth and proliferation of cells making it possible to go from 2D to 3D, it does not address all the factors of creating an *in vivo* mimetic environment for tissue growth *in vitro* (O'Brien, 2011).

2.3.3 Skeletal Muscle Models

Standard cell culture on a 2D culture dish fails to mimic the *in vivo* environment in which cells naturally grow. Models of skeletal muscle tissue, particularly in the form of 3D tissue constructs, provide a possible solution to some of the limitations of 2D cellular culture. Native tissue is not 2D, so the 3D formation of the tissue constructs allows the cells to behave more like they would *in vivo*. This provides a more accurate representation of how the tissue will react to stimulation, therapeutics, drugs, and other stimuli, when in the body (Sawkins 2013).

2.3.3.1 3D Tissue Constructs

Tissue constructs of human skeletal muscle tissue can be utilized for pharmaceutical testing for new drugs to treat muscular diseases and disorders. Several attempts at forming a biomimetic skeletal muscle tissue construct have been documented, but many contain drawbacks

such as requiring large amounts of cells or resulting in necrosis of the tissue due to poor diffusion of nutrients.

Many previous MQPs have developed skeletal muscle tissue constructs in a dog-bone shaped mold, using two posts around which the tissue forms. In 2011, a team utilized a dog-bone shaped mold with a V-shaped channel that resulted in myofibers of approximately 160 μm in diameter. The resultant tissue construct is seen in Figure 7. The purpose of the V-shaped channel was to obtain a small myofiber diameter; yet, the construct itself still required 3 million C2C12 cells (Aschettino 2011). As seen in Figure 7, one major problem of this design was the length of the channel in the center, which required use of a high number of cells.

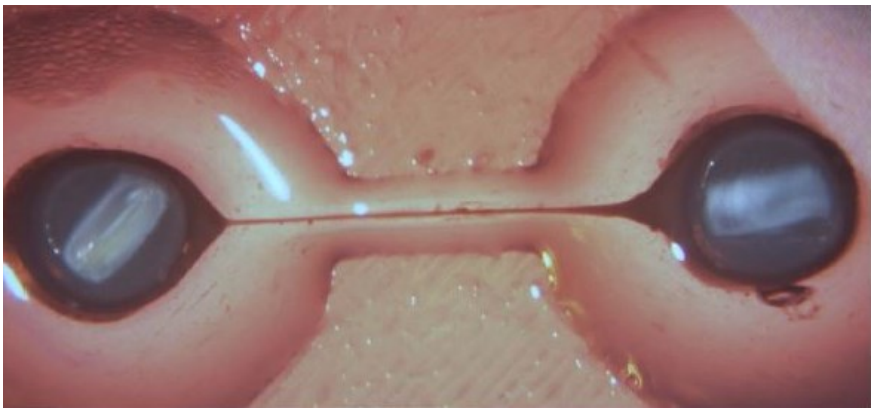


FIGURE 7. SKELETAL TISSUE CONSTRUCT AT 5 DAYS MATURATION, NO SCALE PROVIDED (ASCHETTINO 2011)

A later MQP project formed tissue constructs with a seed cell count ranging from 4.5 million to 7 million (Almeida 2013). For the purposes of skeletal tissue modeling, a high cell count for seeding tissue constructs is impractical and costly. Additionally, large tissue constructs increase the possibility of tissue necrosis. In one study, a skeletal muscle tissue construct ring was formed using the combination of magnetite cationic liposomes and magnetically labeled C2C12 cells (Fujita 2011). Aligned, multinucleated myotubes were observed in the muscle tissue, and myogenin and myosin heavy chain were detected successfully. However, this construct required the use of 700,000 cells per ring (Fujita 2011). This is an improvement in number from previous

studies, yet still fairly large. Thus, the ideal tissue construct will utilize a smaller cell count to optimize materials, while still providing an accurate model of skeletal muscle tissue.

2.3.3.2 Mechanical Actuation of Skeletal Muscle Models

Scaffolds and 3D tissue constructs build towards the eventual goal to mimic *in vivo* skeletal muscle development. However, these systems lack two main functions: measurable and customizable mechanical and electrical stimulation. Mechanical stimulation is crucial to the proper growth and modeling of skeletal muscle tissue in order to replicate native muscle. Muscle fibers require force when growing to develop strength along the appropriate axis. Mechanical stimulation can affect myofiber diameter, cell number, and myofiber composition (Liao 2009). It also helps to regulate the proliferation and differentiation of myoblasts (Grossi 2007). Mechanical stimulation has also been shown to improve myofiber alignment and myoblast differentiation (Powell 2002). Additionally, mechanical stimulation increases the ultimate tensile strength and tensile modulus of engineered tissue (Gauvin, 2011). This results in overall improvement in strength and elasticity of the muscle that cannot be achieved without the application of mechanical stimuli, although these values have yet to become close to native tissue. For example, using mechanical actuation has improved myofiber diameter to around 10 micrometers, but native tissue diameters average 50 micrometers. Additionally, mechanical actuation has increased myofiber density to approximately 10%, but this fails to compare to native density at around 80%.

Mechanical stimulation applied to skeletal muscle tissue is either static or dynamic. Static stimulation is the application of a fixed strain without any cyclic movement, while dynamic stimulation results in cyclic strain which replicates *in vivo* muscle movement. Skeletal muscle is matured by mechanical actuation, because the application of strain onto muscular cells from the ECM or other forces generates a reactive strain from the cytoskeleton of the cells to the tissue's adhesion point (Grossi 2007). A device that successfully provides mechanical stimulation has

been shown to produce 5, 10 and 15 % strain on the tissue. This has been shown to increase myofiber diameter, density, and area (Powell 2002).

2.3.3.2.1 Powell Mechanical Cell Stimulator (Powell 2002)

In a study using human bio-artificial muscles (hBAM), mechanical cyclic strain was applied using a Mechanical Cell Stimulator, seen in Figure 8. The hBAM were stretched, initially at strains of 5% for each 2 day period, which eventually led to 15% strain after 4 days. Repetitive stretching and relaxing of the tissue for eight days increased the hBAM mean myofiber diameter and area (Powell 2002). Providing this continuous workload on the muscles allowed them to mature better than static controls, resulting in increased numbers of muscle fibers and myofiber diameters. Additionally, unidirectional stretching of these muscle fibers for 36 – 40 hours along with cyclical stretching for 2 – 3 weeks had shown to both orient and organize the myofibers into parallel arrays along a principal line of strain, compared to static cultures (Powell 2002).

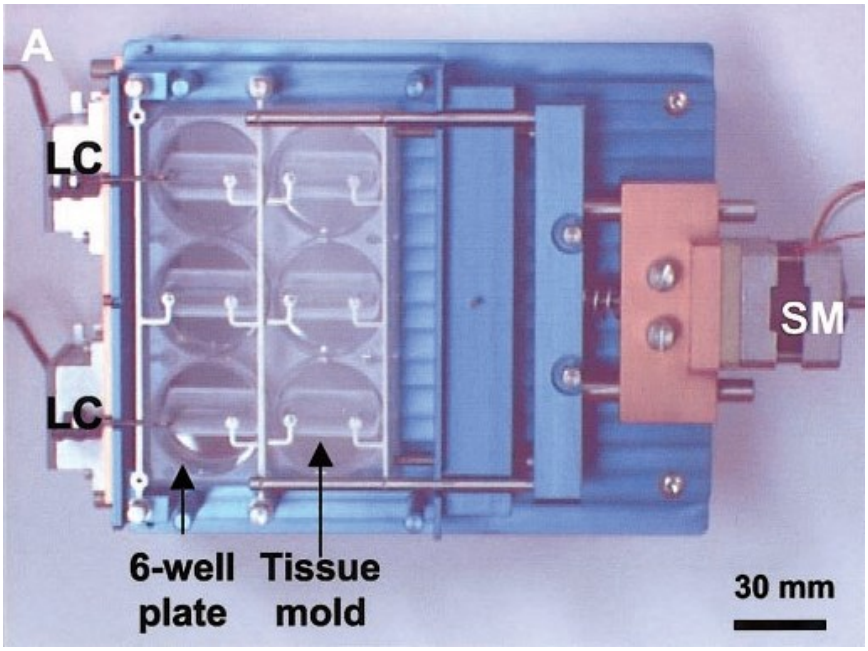


FIGURE 8. MECHANICAL CELL STIMULATOR WITH STEPPER MOTOR (SM) AND LOAD CELLS (LC) THAT MEASURE MUSCLE TENSION (POWELL 2002)

However, this study observed that their myofiber distribution did not resemble *in vivo* human skeletal muscle. Approximately 80% of *in vivo* muscle is composed of muscle fibers, while their tissue engineered hBAM were only comprised of about 2-15 % of muscle fibers with the rest being exogenous ECM, due to poor nutrient diffusion (Powell 2002). In addition, *in vitro* hypertrophy observed increased the myofiber diameters, but not to values of *in vivo* skeletal muscle. After 16 days of mechanical stimulation, the hBAM myofiber diameters only reached approximately 8 μm, compared to *in vivo* myofibers, which range from 10 – 100 μm (Powell 2002). They attributed this result to the lack of innervation and other differences to native tissue.

2.3.3.2.2 Boonen Ramp vs Cyclic Loading Study (Boonen 2010)

It is important to determine whether the tissue will be actuated with ramp or cyclic mechanical loading. The effect of ramp stretch followed by dynamic stretch on skeletal muscle tissue was investigated in a study by (Boonen 2010). In this study, uniaxial ramp stretch of 0-2% was applied to tissue over a period of 2 days, followed by uniaxial intermittent dynamic stretch of 2-6%, at a repeat of 3 hours stretched and 3 hours relaxed (Boonen 2010). Stretch was kept below 10% at all times. The stretch was applied to C2C12 and murine muscle progenitor cells (MPC) in both a 2D and 3D environment. There were no remarkable results in 2D cultures, because both stimulated and non-stimulated tissue formed similar myotube structures and underwent similar differentiation and maturation. In 3D cultures, mechanical stimulation had no effect on C2C12 sarcomere formation and furthermore prevented cross striation formation in the MPCs. Dynamic stretch resulted in a reduction in maturation and MyoD expression, and no proliferation increase was observed. Overall, the combined ramp and dynamic stretch protocol decreased the maturation of C2C12 and MPCs in both 2D and 3D culture environments. As such, it is not a recommended protocol for engineering skeletal muscle tissue (Boonen 2010).

2.3.3.2.3 Vandenburgh Mechanical Cell Stimulator

A tissue culture system was developed that can mechanically stimulate cells growing on an elastic plastic substratum in a 24 well chamber. The collagen-coated substratum, where the cells were attached, was stretched by a Mechanical Cell Stimulator. The muscle tissue could be stretched and relaxed by a stepper motor with linear accuracy of 30 μm. A computer, which was interfaced with the cell chamber, was used to control the activity of the mechanical stimulation. Primary avian skeletal myoblasts were used and they proliferated and fused into multinucleated myotubes. Results suggested that applying uni-directional mechanical stress on skeletal muscle cells causes the developing myotubes to orient parallel to the direction of movement while applying continuous cyclic stress (stretch-relaxation) orients the myotubes perpendicular to the direction of movement. Application of non-continuous cyclic stress did not result in myotube orientation, because it did not allow for strengthening of the tissue. The study's limitations were that the cells were grown in a culture dish (2D) and the myotube density only increased slightly (Vandenburgh, 1988).

2.3.3.3 Electrical Stimulation

The ability to apply electrical stimulation to developing engineered skeletal muscle *in vitro* is also important to the proper growth of the tissue. Since skeletal muscle *in vivo* is stimulated by neuron activity, electrical stimulation augments the development and maturation of the tissue. Lack of electrical stimulation has been shown to lead to muscle weakness and necrosis (Ito 2014). Thus, the inclusion of electrical stimulation is integral to the fabrication of functional skeletal muscle tissue (Ito 2014).

2.3.3.3.1 Vandenburgh Electrical Stimulation with Flexible Posts (Vandenburgh 2008)

In a study using flexible posts of 150-350 μm in radius, engineered muscle tissue was attached to the flexible posts and applied with electrical stimulation. With this protocol, the tissue will contract a certain distance relative to the radii of the posts, illustrated in Figure 9 (Vandenburgh 2008). Upon stimulation, the muscle tissue produced tetanic conractile (active) force to deflect the posts (Vandenburgh 2008). In this study, caps at the top of the posts prevented the muscle tissue from sliding up the posts upon stimulation, to produce accurate results. An image motion detecting system was used to capture the post deflections and calculate the active force generation reported. It was determined that, when muscle tissue generated increasing levels of active force by electrical stimulation, the myoblasts differentiated into aligned post-miotic muscle fibers that also expressed sarcomeric proteins, as is seen in Figure 9 (Vandenburgh 2008). Electrical stimulation of the tissue directly affected the fiber structure. Thus, the inclusion of electrical stimulation in an *in vivo* mimetic device for skeletal muscle tissue growth is equally as important as mechanical stimulation but for different biological reasons.

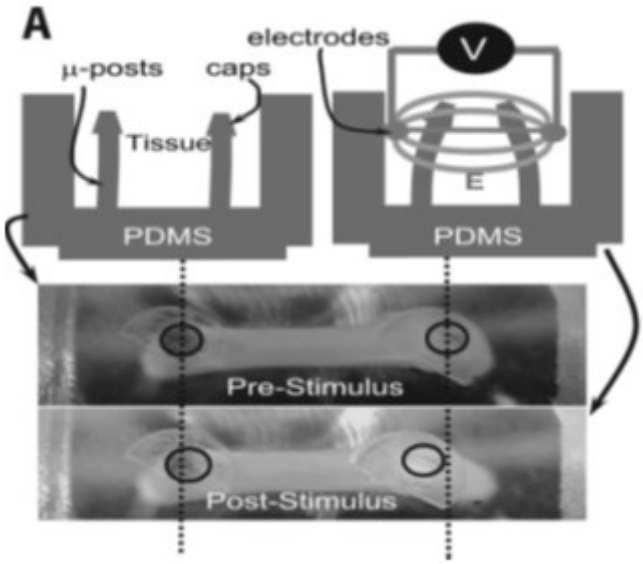


FIGURE 9. MBAM ACTIVE FORCE GENERATION, DETAILING THE LOCATION OF ELECTRODES OVER THE POSTS AND THE TISSUE PRE- AND POST- STIMULUS, SCALE NOT PROVIDED (VANDENBURGH 2008)

2.3.3.3.1 Donnelly Electrical Bioreactor (Donnelly 2010)

A bioreactor that controlled different parameters of electrical impulse such as pulse amplitude, width, frequency and worked to test ratio could improve physiology and function of muscles *in vitro.* Current tissue engineering approaches aid in the development of skeletal muscle tissue, they allow for the formation of primary myotubes. Despite the advantages of these techniques there has not been a system that successfully provides electrical stimulation that mimics the impulses received *in vivo* by the central nervous system. Such electrical stimulation allows for the formation of secondary and adult muscle fibers. It was the goal of this study to develop a bioreactor system that could be used to stimulate muscle in either two or three dimensions *in vitro.* They further demonstrate how the physiological stimulation parameters can directly affect response to stimulation in 2D cell culture and the function of 3D engineered tissues.

Cellular responses were assessed for the 2D electrical stimulation at different frequencies and force production due to electrical stimulation was studied for the 3D muscle samples. The stimulator circuit was designed to provide programed electrical impulses at amplitudes up to 50V and pulse widths down to 50us. Pulse circuit changes the circuits to different desire stimulation voltages and then discharged through muscle tissue, cell monolayers or 3D muscle constructs via parallel metal electrodes using a growth medium as a conductive path. A six well plate is used as the base to hold the six tissue samples for 3D application and fits within an incubator. The lid of the base plate has six sets of parallel electrodes, Figure 8 shows the rapid prototype of this study.

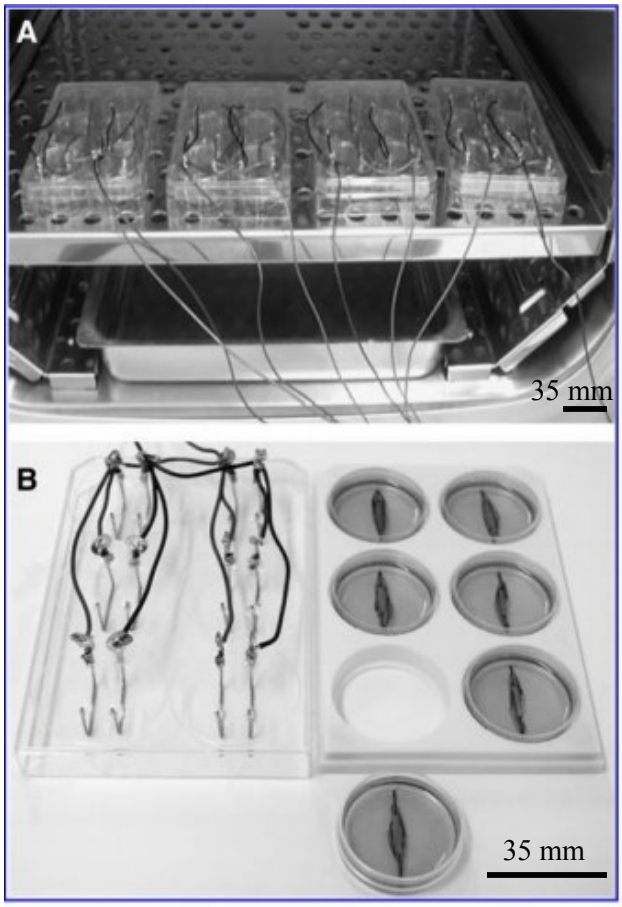


FIGURE 10. BIOREACTOR PROTOTYPE (A) 2D STIMULATING BIOREACTOR, (B) 3D STIMULATING BIOREACTOR (DONNELLY 2010)

U-shaped stainless steel electrodes were embedded into the lid, 10 mm and 20 mm apart, for 3D and 2D applications respectively. Crip connectors were attached to the end of the electrodes to then be connected to a stimulator circuit. For 2D cell culture, C2C12 were cultured, and experiments took place after 5 days of differentiation at which point the myotubes were fully formed. After stimulation, cells were collected and the supernatant was removed for protein determination. For 3D cell culture, 100,000 cells were added to the 6 mm silk structures and fresh proliferation media was given every other day until the cells were 90% confluent. These cells were then switched to differentiation media for 2 days. Following, the 2D cells were stimulated at 10 Hz (4x0.3 ms pulses delivered in 400 ms trains with a 3.6 ms recovery) or 100 Hz (40x0.3 ms pulses delivered in 400 ms trains with a 3.6 ms recovery). The 10 Hz protocol lasted three hours,

and the 100 Hz lasted 30 minutes. In 3D culture, the constructs were stimulated with 4x0.1 ms pulses delivered in 400 ms with a 3.6 ms recovery. This stimulation was provided for 7 days.

Low frequency electrical stimulation resulted in no effect on the rate of muscle protein synthesis, but the higher frequency showed a statistically significant increase in protein synthesis. In 3D constructs, application of 1.25 V/mm tended to increase force production. This study observed an increase in force production, but also demonstrated that high *in vitro* voltage stimulation negatively affected 3D engineered muscle performance and caused electrochemical damage. Fused tetanus was also observed with stimulation frequencies in excess of 3 Hz. Therefore, a different protocol and lower frequencies were suggested to provide electrical stimulation to skeletal muscle tissues in order to prevent tissue damage (Donnelly et al, 2010).

2.3.3.3.2 Park Electrical Stimulation in Collagen Scaffolds (Park 2008)

In a study conducted by Park et al., the authors seeded C2C12 in collagen scaffolds and tested the effects of various electrical frequencies and voltages (Park 2008). After standard co-culture for three days, the cell and collagen constructs were placed in dishes that contained carbon rods connected to platinum wires. These wires were attached to a custom made electrical circuit that enabled the authors to control the frequency and voltage of the electrical stimulation. They tested voltages of 2, 5, or 7 V and frequencies of either 1 or 2 Hz. The constructs were then continuously stimulated for five days at each variable. The authors found that the cells were more uniformly distributed with the 1Hz/5V, 1Hz/7V, 2Hz/5V, and 2Hz/7V stimulated groups. Furthermore, the groups stimulated with 2Hz showed elongated and developed cells layers containing muscle cells. They also found that constructs stimulated at 5V with 2Hz formed better developed myotubes and constructs stimulated with 5V had improved development of sarcomeres.

With this study, there were distinct limitations. One of these limitations included a low uniform muscle cell density throughout the entire construct. Although the groups stimulated with

2Hz showed less collagen and more muscle cells throughout the entire construct, apoptosis was still present. Additionally, the size and density of myofibers were not quantified in this study.

2.3.3.3.3 Ito Continuous Electrical Pulse Stimulation (Ito 2014)

In a study testing the application of continuous electrical pulse stimulation (EPS), C2C12 muscle tissue constructs were electrically stimulated with parameters ranging from 0.1-0.5 V/mm, with 2-10 ms widths, at 0.5-2 Hz frequencies (Ito 2014). The parameters of typical native, neural EPS stimulation are 0.5 V/mm, 2ms, and 1 Hz, as determined in a previous study (Radisic 2004). It has been shown that stimulation over 2.5 V/mm and frequencies over 3 Hz cause damage to the tissue (Ito 2014). C2C12 cells stimulated with electrical pulses at 0.3 V/mm with 4 ms width and 1 Hz frequency showed a 4.5 increase in force production, from a "load" of 50-60% peak twitch force, measured after 14 days (Ito 2014).

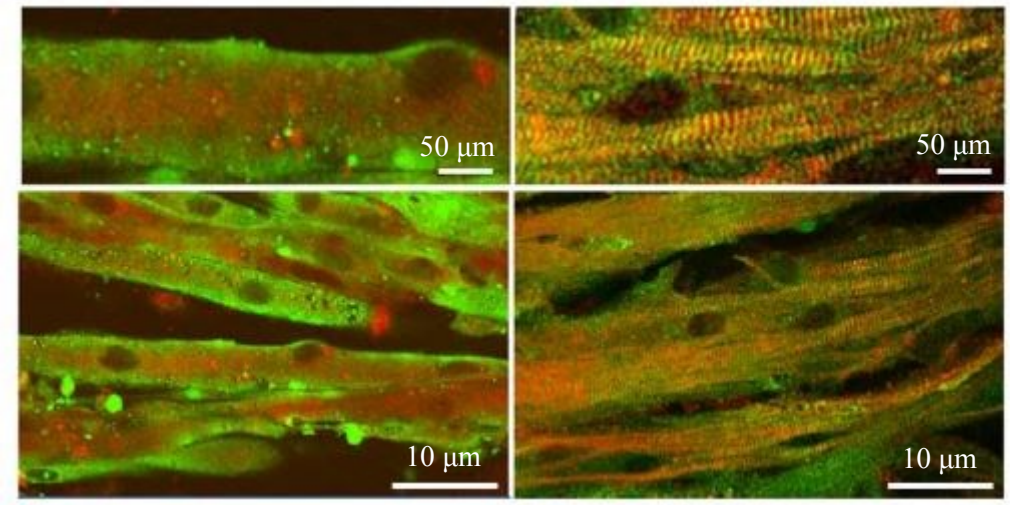


FIGURE 11. TISSUE CONSTRUCTS AT DAY 14, STAINED FOR ALPHA-ACTIN (GREEN) AND F-ACTIN (RED). TISSUE ON THE LEFT RECEIVED NO ELECTRICAL STIMULATION, WHEREAS TISSUE ON THE RIGHT DID. (ITO 2014)

However, despite the favorable force production results, the increased pulse width (4ms) provided in this study has shown to cause direct calcium release from the sarcoplasmic reticulum, which differs from normal behavior *in vivo* (Ito 2014). Due to this result, the tissue did not contract in a

way similar to contraction found in native tissue. As such, further modifications are required to mimic *in vivo* stimulation of the muscle tissue.

Chapter 3: Project Strategy

3.1 Initial Client Statement

A client statement presents certain details of the project in which, through interaction with the client, the goal of the project is clarified. The following client statement was given by the client, Dr. Raymond Page:

> "A tissue engineered skeletal muscle system would enable the study of skeletal muscle tissue formation, maturation and the potentiality of cells entirely in vitro that could be used to approximate the utility of their use for the replacement of lost or damaged skeletal muscle tissue. The goal of this project is to design and produce a system that recapitulates skeletal muscle fiber structure, into which myogenic cells can be seeded such that skeletal muscle tissue is formed. The system must be either produced aseptically or must be sterilizable and fit into an incubator in order to permit study of live cultures over time. The engineered system should further be amenable to the study of effect of mechanical strain and electrical stimulation on muscle fiber maturation and contractile function."

After reading the initial client statement, the team met with the client to clarify the goals of the project and a revised client statement was written.

3.2 Design Objectives

The initial client statement was revised and the requirements for the project were clarified. This was done through research about the limitations of previous attempts at this project and through interviews with the client. A list of objectives and attributes are important to determining the success of the design and thus, the device will ideally contain the following:

1. Provide anchorage points
2. Allow for tissue formation, self-assembly, and initial maturation before stimulation
3. Low cell count for minimal functional unit for tissue constructs
4. Allow alignment of tissue
5. Create entirely viable tissue

6. Constructs should contain high myofiber densities
7. Constructs should mimic the in-vivo muscle fiber size or be close to it
8. Impart mechanical deflection: uniaxial strain
9. Simulate twitch and tetanic contractions of tissue through electrical stimulation
10. Results need to be reproducible and precise
11. Measure displacement of tissue from contraction
12. Device supports incubator conditions while providing an aseptic environment and remaining biocompatible

The most important objectives were to create an entirely viable tissue construct and achieve *in vivo* skeletal muscle tissue standards through the use of mechanical and electrical stimulation. Within this minimal functional unit it is necessary to have a low cell count to establish a model system that can cut down on both expense and timing. Also, a small construct is ideal to prevent necrosis of the cells in the tissue. If the construct is too large, the media, which is the nutrient source, cannot actively diffuse to cells located further than 50-150 microns from the perimeter (Bland et al, 2012). High myofiber density, along with muscle fiber diameter, is another requirement that will determine the contractile properties of the muscle tissue. Therefore, having a higher density of myofibers, and greater force production per unit area, with larger diameters will result in increased functionality that will successfully mimic human skeletal muscle tissue (Powell, 2002).

Moreover, anchorage points are essential in order to replicate the role of tendons and bones so that the muscle tissue can properly contract in a controlled and aligned manner (Marieb, 2012). It is also necessary to provide an aseptic environment for the tissue culture in order to maintain cell viability. If an aseptic environment is not established, contamination of the cells will compromise the results. Passive strain is applied throughout the maturation and differentiation of the tissue. Small amounts of strain must be applied to the tissue in order for it to mature. Without

providing this passive strain, the muscle tissue will eventually atrophy (Powell 2002). If too much strain is provided, the muscle tissue will rupture. Additionally, action potentials from the central nervous system allow for electrical field-mediated tetanic contraction of the muscle tissue. Electrical stimulation will direct tetanic contraction and result in an increase in contractile strength. Further, all experiments must be reproducible to obtain consistent results for success. This would allow for the design to move forward into possible high-content clinical stages involving human skeletal muscle tissue and for the team to fulfill the requirements set by the client.

3.3 Design Constraints

The team developed design constraints after reviewing the client statement and strategy for the project:

- The device must fit in the incubator
- The team has a budget of $624
- Aseptic environment must be provided
- Project needs to be completed within 28 weeks

Allowing the cells, located within the device, to mature and grow is a necessity. Thus, the device has to meet the size requirements of an incubator so that the cells can be maintained at incubator conditions of 37 °C, 5% $C0_2$, and 95% humidity (Janice L West, 2011). In addition, to maintain sterility within the cells, the device has to allow for an aseptic environment that can be sustained. This would prevent any contamination from reaching the cells and skewing the data. Lastly, the team has 28 weeks to finish the project with a budget of $624 to spend.

3.4 Revised Client Statement

The revised client statement was written after meeting with the client, Dr. Raymond Page. The final statement represents the overall goal of the project in a concise and direct form.

"Design a system that allows for 3-D skeletal muscle tissue formation in vitro. This device should improve upon previous attempts and enable the application and study of mechanical and electrical stimuli to engineered skeletal muscle in order for the in vitro tissue to mimic mature human tissue. This may prove useful to future applications that study how muscular diseases occur and how they can be treated through the application of new therapeutic strategies. The device has to provide an aseptic environment and fit inside an incubator."

Chapter 4: Alternative Designs

4.1 Introduction

The team identified important attributes that were needed for the project success. Within this chapter, the team will explore the different needs that are relevant for the project. This chapter will also introduce the team's alternative designs along with their advantages and disadvantages. Furthermore, the chapter will explain the preliminary experiments that will be performed in order to test the feasibility of the chosen design concept.

4.2 Needs Analysis

The team decided the needs and wants of the device design, as detailed below. Needs are absolute requirements for the features of the device. Wants would enhance the overall design, but are not necessary for the ultimate success of the design.

4.2.1 Needs

- Impart mechanical deflection: uniaxial strain
- Provides anchorage points
- Simulate tetanic contraction of tissue through electrical stimulation
- Minimal functional unit

4.2.2 Wants

- Measure contractile force
- Measure mechanical actuation
- Measure electrical stimulation
- Perform histological staining on matured tissue constructs
- Provide means for easily adjusting mechanical and electrical stimulation

4.2.3 Analysis of Device Design

Imparting mechanical deflection is an important part of creating a functional unit of skeletal muscle tissue that is biomimetic to native tissue. After reviewing literature on how this can be accomplished, it was determined that the device needs to impart a strain of five percent for two days, ten percent for another two days, and fifteen percent for four days (Powell, 2002). This will be done after the tissue has matured for two weeks. In addition to this, tetanic contraction of the tissue needs to be applied while the tissue is stimulated mechanically. One previous study utilized an electrical field of 3 V/cm, a frequency of 1Hz, and a timing of 4ms (Ito, 2014). This study was determined to replicate what the team wanted to accomplish, thus the designed device needs to implement these parameters. Another important aspect that the device needs to include in order to replicate fully functioning skeletal muscle tissue is providing anchorage points for the tissue construct. Furthermore, the device needs to incorporate a smaller tissue construct, which has a lower cell count than what is used in the client's laboratory. Currently, the tissue construct is formed using 400,000 cells. An aspect of the device that the team would like to include, but does not necessarily need to include is the measurement of the contractile force of the tissue. Similarly, measuring the amount of mechanical and electrical stimulation that the tissue is receiving is not an essential component of the device. These parameters will be included in the experimental portion of the design to ensure that the proper application of stimuli is actually reaching the tissue. However, this does not need to be included in the final design of the device.

4.3 Design Metrics for Mechanical and Electrical Functions

The team will measure the success of the design based on its ability to correctly deliver mechanical and electrical stimulation. The team has determined the numerical values that the device must produce, as detailed below.

4.3.1 Mechanical Stimulation

Many previous studies have shown that providing mechanical stimulation to skeletal muscle tissue can aid in both the maturation and development of the tissue. Applying cyclic strain to the muscle tissue helps organize muscle fibers into parallel arrangements which more appropriately resemble a functional unit of skeletal muscle. These strains must be applied at low amounts at first to properly condition the muscle and prevent rupture of the tissue. Different protocols of stretching these muscle tissues are detailed below.

1. (Powell et al. 2002): Oriented the muscle tissue and help muscle development including increased elasticity, satellite cell activation and myofiber area. The HBAMs were stretched at 5% strain (1 mm) for 2 days (days8–10), 10% strain (2 mm) for 2 days (days 10–12), and 15% strain (3 mm) for 4 days (days 12–16). HBAMs that were not repetitively strained were used as controls. HBAMs were stretched to 5, 10, and 15% strain and held at this strain for 10 min before returning to the culture length (0% strain). After 10 min at the culture length, the strain routine was repeated for a total of four step stretches.

2. (Grossi et al. 2007): Used mechanical stimulation every day for an allotted time. One study did 2 seconds of strain and 2 seconds of rest for an hour every day.

3. (Moon et al. 2008): Strained the tissue for 5 minutes every hour for 5 days.

4. (Boonen et al. 2010): Protocol consisted of a 2-day uniaxial ramp stretch of 0–2%, followed by a uniaxial intermittent stretch regime of 2–6% dynamic stretch (3 h on, 3 h off). The ramp stretch did not affect maturation of C2C12 and MPC constructs significantly. However, orientation of myotubes in the direction of attachment between the anchoring points was present in both stretched and control constructs, but most prominent in C2C12 constructs. In conclusion, this research showed that a combined mechanical stimulation protocol in the physiological range is restricting maturation into functional muscle fibers both in 2D monolayers of cells cultured on

coated flexible membranes, and in fibrin constructs of C2C12 and MPCs. Finally they concluded that this mechanical stimulation protocol is not recommended for tissue engineering

4.3.2 Electrical Stimulation

Many studies have investigated the application of electrical stimulation to skeletal muscle tissue constructs. Electrical stimulation is characterized by its electrical field, frequency, and pulse duration. To date, not one combination of these attributes has been discovered to completely mimic the electrical impulses that are found *in vivo*. A number of previously conducted studies are summarized below. Their results can be seen in Chapter 2.

TABLE 3. SUMMARY OF PREVIOUS ELECTRICAL STIMULATION STUDIES ON SKELETAL MUSCLE TISSUE.

Study	**Electrical Field (V/cm)**	**Frequency (Hz)**	**Timing (ms)**	**Duration**
Thelen, et al 1997	3	2	6	2 days
Radisic, et al 2004	5	1	2	5 days
Pedrotty, et al 2005	6	0.5 - 10	0.5 - 250	1 - 14 days
Fujita, et al 2007	6.7	0.1 - 10	24	1 - 9 hr
Yamasaki, et al 2009	8.3	0.5 - 10	10	80 s
Ito, et al 2014	3	1	4	10 days

Electrical damage has been shown to occur in electrical fields over 2.5 V/mm and 3 Hz (Ito 2014). Based on successful results in previous studies, and for feasibility of this project, the team has chosen the following values for electrical stimulation. The team will stimulate the tissue using an electric field of 5 V/cm, at a frequency of 1Hz. The pulses of stimulation will occur for 4 ms at a time. The total duration of electrical stimulation will occur for 7 days. These values must be provided through electrical stimulation by the device.

4.4 Functions Means Table

Based on the objectives and needs analysis, the team has developed a table of means to achieve each required function of the device and have an organized way of exploring different methods to achieve these functions.

TABLE 4. FUNCTIONS-MEANS TABLE FOR ACCOMPLISHING OBJECTIVES OF DEVICE DESIGN.

Functions	Means					
Anchorage points	Metal	Plastic	PDMS	Curved hook	Agarose	MED 610
Mechanical Stimulation	Motor	Magnet	Pulley system	Hydraulics/ Syringe System	Gears/belt	Track/ wheel system
Electrical Stimulation	Stainless Steel Rods with BIOPAC System	Cobalt Electrodes	Magnetic field	Piezoelectric polymer	Platinum Iridium Electrodes	Copper electrodes
Strain Measurement	Video/ Images	Strain-gauge loading	Spring with constant k known			
Electrical output Measurement	BIOPAC ECG100C Amplifier	Oscilloscope	Voltage Meter			
Aseptic Environment	Package in sterile environment	Autoclave	Alcohol	Hydrogen Peroxide	Ethylene Oxide Sterilization	

This chart can be used to explore the various ways to accomplish the different aspects that the device needs to achieve. It can be used to more accurately determine what means would fulfill the client's needs for the project. The anchorage points need to be flexible enough to allow for strain when the tissue is mechanically deflected. Most importantly, the points need to allow for the negative strain of the tissue before it is mechanically stimulated and during the stimulation. They also need to allow for reliable anchoring of the tissue and not allow the tissue to slip off during mechanical deflection or tissue contraction (Powell, 2002). Furthermore, the material of the anchorage points cannot have any sharp points or have a geometric shape that can cause damage to the tissue. In addition to this, an aseptic environment must be provided for the

tissue to ensure its survival and prevent contamination or cell death. The means of accomplishing this can vary, but it is important that all aspects of the device are sterile.

In order to mechanically stimulate the tissue, the device needs to impart a certain amount of strain on the device at different times throughout the stimulation duration period. The minimum amount of strain that the device needs to apply on the tissue is five percent and the maximum is fifteen percent (Powell, 2002). This was decided after researching other studies that exhibited results in increasing myofiber diameter in tissue constructs along with the area percentage of myofibers throughout. For the electrical stimulation, it is important to ensure that the method used does not damage the tissue. Also, the method used needs to apply the chosen amount of frequency and electrical field for a certain amount of time. The chosen parameters were, 3V/cm at a frequency of 1 Hz and width of 4ms (Ito, 2014). These parameters follow under a range of values that closely replicate the neural stimulation of skeletal muscle tissue *in vivo*.

During the experimental process of testing the device, the ability to measure the strain and electrical output that reaches the tissue is important. These measurements will aid at the understanding of how the tissue construct reacts to the different parameters of mechanical and electrical stimulation. Choosing a method to see these changes in the tissue is essential to the success of the project. Another function needed to be incorporated into the experimental process is measuring the cell density within the tissue constructs. The method used should also allow for the measuring of the myofiber diameter and the area percentage of myofibers, which will help the team determine if the methods that are being utilized are more successful than other devices and results are closer to human skeletal muscle tissue. By comparing all the means of achieving the functions the team was able to come up with detailed conceptual designs, which are shown in the next section.

4.5 Alternative Concepts

Within this section the team introduces and explains four alternative designs based on previous metrics and the functions and means table. From these four alternative designs, the chosen design will undergo further modification to become the final device design.

4.5.1 Alternative Design 1: Moving Lid

This design is intended to use a six or twelve well plate. The idea of being able to stimulate several samples at the same time immediately converts the design into a solid candidate to provide both the mechanical and electrical stimulation to the tissues, making the overall process more efficient. Additionally, the amount of media will be decreased which will reduce the cost of experiments. Within each well, a steady pole would be present that will remain static. On the contrary, the lid of each well will contain a moving pole. The tissue is cultivated and matured in a separate petri dish and then transported to the mechanical and electrical stimulation system. When the lid is closed and the tissue is inside of the six or twelve well plate, the moving pole gets incorporated inside the well, providing anchorage points to the tissue and making it possible for the strain to be applied. The moving poles in the lid are attached to a bar passing through the center of the plate which is then attached to a stepper motor. The stepper motor will provide the cyclic strain needed to align the tissues simulating the in vivo stimuli. Additionally, this system will have a base that will hold the six or twelve well plate as seen below.

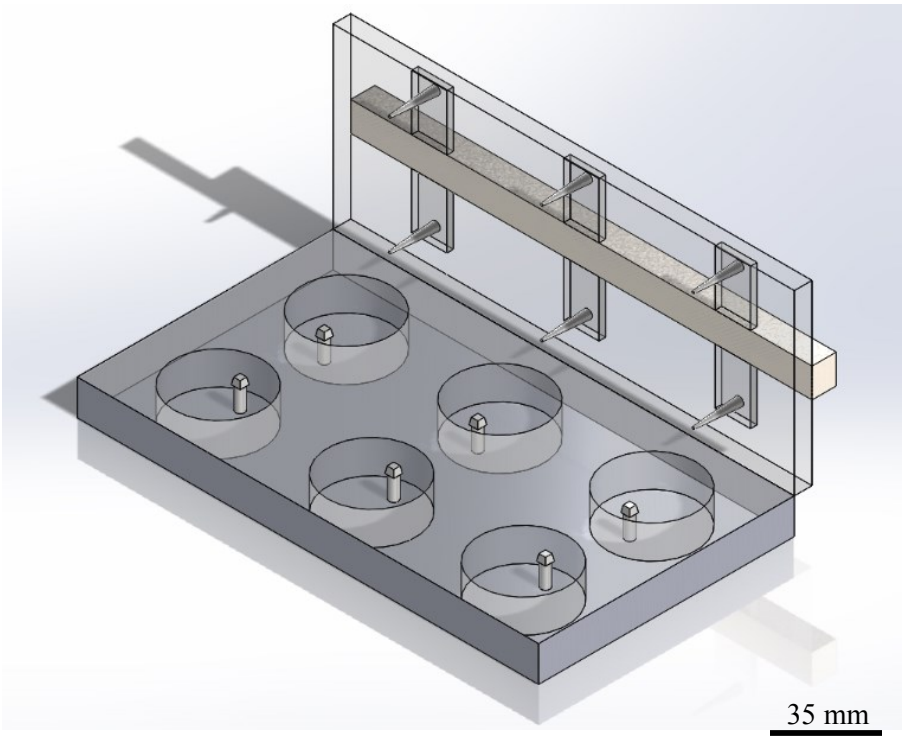


FIGURE 12. CAD MODEL OF MOVING LID ALTERNATIVE DESIGN

Electrical stimulation will be transmitted through electrodes. Perpendicular to the posts, two holes will be drilled in the lid. Through those holes, platinum wires will enter the media and conduct an electrical impulse. Although this system is a promising candidate to reduce the cost and increase the efficiency of the experiments, it will not be precise at holding the tissues due to smaller changes in tissue sizes. Tissues will need to be transported from the petri dish into the system. When transporting and anchoring multiple tissues within the system, as well as removing the lid, damage to the tissues can occur.

4.5.2 Alternative Design 2: Electromagnet System

This design utilizes an electromagnet to mechanically stimulate the muscle tissue. A single well would be used containing two posts that act as anchoring points for the tissue construct. One of the poles would be moveable with a magnet component embedded into it. The

other pole would be static and remain stationary during stimulation. Each post would have caps on the ends to prevent the muscle tissue from sliding off. An external magnetized bar would be used to modulate the movement of the magnetic post. A channel would be made in front of the magnetic pole so that it could be moved linearly back and forth. An electromagnet would be placed on a bar underneath the magnetic post that is connected to a stepper motor. A cylindrical pin would connect the magnetic bar and the stepper motor to constrain the linear movement. This would allow the magnetic post to move in both the positive and negative direction so that the muscle tissue can be strained. Two electrodes would be placed through the top of the lid to provide electrical stimulation to the muscle tissue through the media.

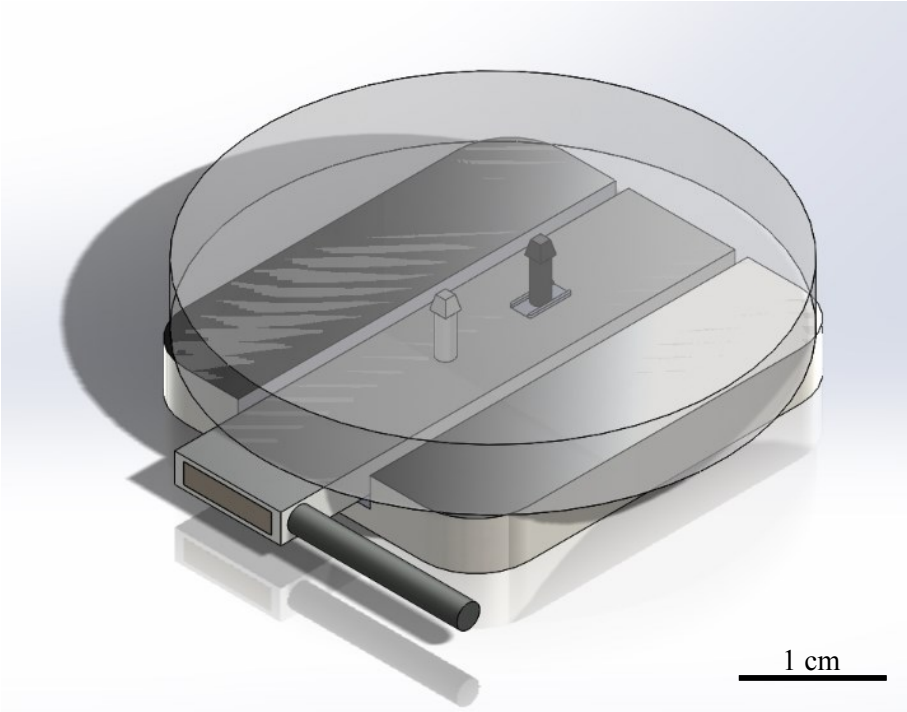


FIGURE 13. CAD MODEL OF ELECTROMAGNET SYSTEM

The disadvantage of using magnets is that they can sometimes not be precise due to the delay in interaction between the embedded magnet and the external magnet. The magnets would have to be strong enough to overcome the friction that is working against the displacement of the magnetic post. Otherwise, this delay could make it hard to maintain reproducibility and precision

during the experiments. Additionally, magnets are restricted to certain metals which means the magnetic post must be biocompatible and not interfere with the development of the skeletal muscle tissue.

4.5.3 Alternative Design 3: Wire System

This proposed design features two posts, one stationary and one placed into a track system on the bottom of the petri dish. The moveable post contains two notches, one for the tissue and one for a suture wire. The suture wire is inserted into a hook-like notch in the moveable post above the medium and tissue construct. The wire exits the dish through a rubber septum and connects to pulley motor that winds and releases the wire to pull the post and mechanically stimulate the tissue. For the purposes of electrical stimulation, an electrode will be incorporated into the design through the rubber septum as well. The electrode wire will be inserted through the septum and allowed to contact the medium to emit electrical stimulation. The electrode will be attached to one side of the inside of the petri dish.

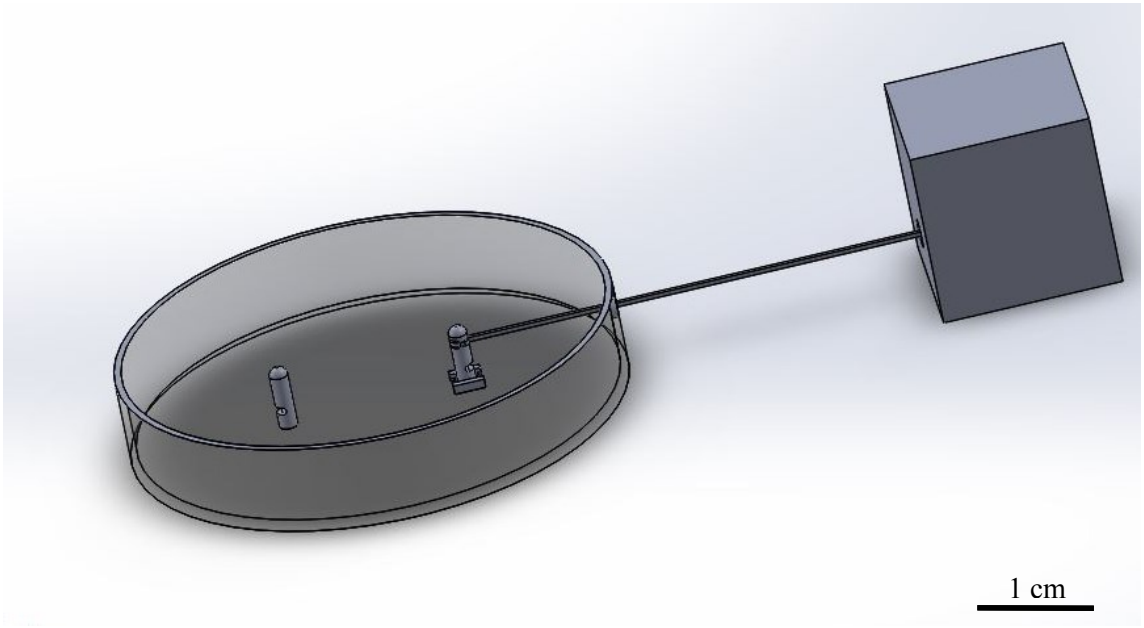


FIGURE 14. CAD MODEL OF WIRE SYSTEM

This wire design provides a system to mechanically and electrically stimulate the tissue construct. However, it has several complications that should be addressed before it can be produced. First, following transfer of the tissue to the posts, the user is required to slip the suture onto the removable post. Additionally, there is a chance for complications in the rubber septum. The suture wire and electrode wire might interfere with each other, causing friction or contamination by pulling bacteria into the system. The electrode system will also require protection from corrosion. Finally, the track system in the petri dish might cause complications in manufacturing due to small size.

4.5.4 Alternative Design 4: Hook Design

The team's design utilizes a stationary post and hook anchoring system that provides mechanical stimulation through a stepper motor setup. Overall, the design is intended for a six well plate, but can be first tested on a single petri dish system that allows for scalability. The tissue construct is anchored using a stationary pole on one end and a hook that moves on the other. Both the pole and the hook will have caps in order to prevent the tissue from sliding off the anchoring system. Furthermore, the hook is attached to a wire that is wound around a spindle. The spindle moves, allowing the wire to unwind or wind around it, through its connection with a stepper motor. When the stepper motor is activated, the wire will move the tissue uniaxially to provide tension on and off the tissue. To provide the necessary electrical stimulation, electrodes will be placed in the media of the well. The general conceptual design of the mechanical component can be seen in Figure 15.

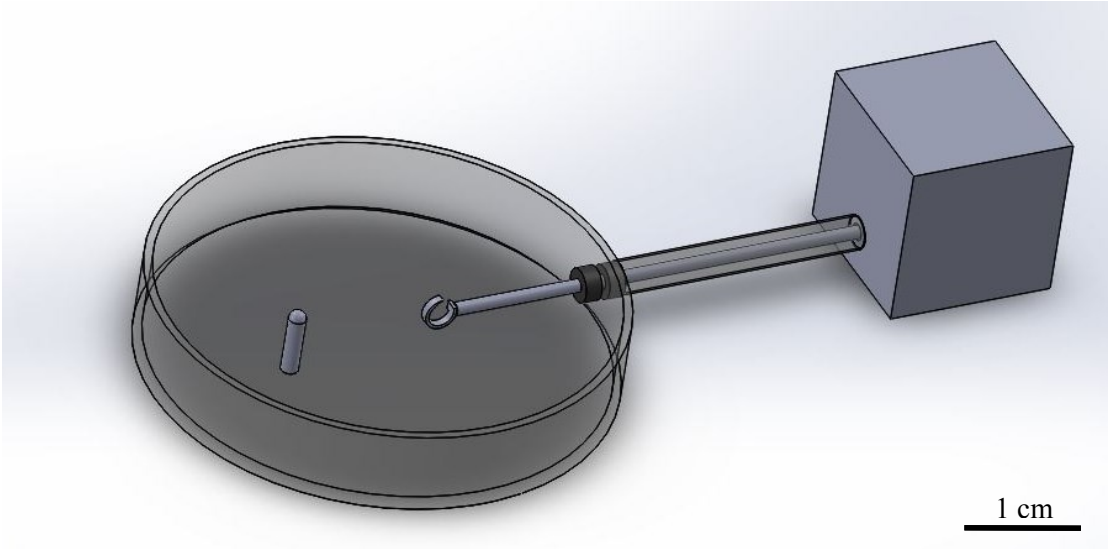


FIGURE 15. CAD MODEL OF HOOK DESIGN

One advantage to this setup is that the motor will be located outside the incubator, which will prevent corrosion of the electrical components. A disadvantage is that the accuracy of the string and motor method is currently unknown and needs to be tested through experimentation. In addition, the process of getting the tissue constructs onto the post and hook system will be difficult without breaking the construct and keeping everything sterile. Lastly, there is the possibility of the wire breaking if there is too much tension put on the individual wire or if the wire itself has low fatigue tolerance.

4.6 Design Comparison Chart

In order to assess what design concept to move forward with, the team decided to complete a design comparison chart as shown below in Table 5.

TABLE 5. DESIGN COMPARISON CHART RANKING THE FOUR ALTERNATIVE DESIGNS AGAINST EACH OBJECTIVE OF THE DEVICE DESIGN.

Objective	**Electromagnet**	**Moveable Lid**	**Wire Pull**	**Hook**
Anchorage points	5	2	3	4.5
Mechanical stimulation	3	4	4	5
Tetanic contraction by electrical stimulation	4	4	4	4
Aseptic culture environment	3	3	3	5
Incubator compatible	2	4	2	4
Manufacture below budget	2	4	3	5
Biocompatibility	4	5	5	4
TOTAL	**23**	**26**	**24**	**31.5**

Each design concept was ranked in a scale from 1 to 5, with 1 being the least likely to achieve the objective and 5 being the most appropriate at meeting each objective. The hook design was chosen based on these criteria due to its highest scores in each objective category.

4.7 Prototypes and Proof of Concept

This section contains an overview of the prototype development of the final chosen alternative design. The team initially chose the hook design shown above after completing the design comparison chart. Here the team will assess advantages and disadvantages of each prototype and how that led to further modifications for the next prototype..

4.7.1 Prototype 1: Hook Design

This prototype was developed in order to determine if the assembly would work as a whole and provide the mechanical actuation to the tissue constructs. For the purpose of the prototype, elastic bands were used to model the skeletal muscle tissue. This design consists of the following materials shown in Table 6 representing the proposed components of the final design.

TABLE 6. CHART OF MATERIALS FOR PROTOTYPE. (LEFT) FINAL PROPOSED MATERIALS, (RIGHT) COUNTERPART MATERIALS USED FOR PROTOTYPE

Component	**Prototype Material**
Wire/Bar	Wooden dowel
Post (polycarbonate)	Hairbrush bristle
Tissue	Dental elastic
Culture dish (35 mm)	Culture disk (100 mm)
Motor	Manual power
Hook	Hook (brass)
Adhesive	Crazy glue
Machining	Power drill
Culture media	Water

Initially the team modified a 100 mm culture dish to insert a bar and hook through the wall of the dish. The alternative designed proposed a wire instead of a bar, which was found to be insufficient at holding the weight of the hook. Within the dish, a hair brush post was glued to the base of the dish to model the stationary post of the design. Two different petri dishes were used, one with the post directly glued to the bottom and the other with a hole drilled into the bottom for insertion of a post through the base to visualize two different options. This was done to explore how the stationary posts could remain fixed without detaching. To determine whether or not the design could strain at 5, 10 and 15%, elastics were placed on the stationary post and the hook and manually actuated. The prototype is shown in Figures 16 and 17.

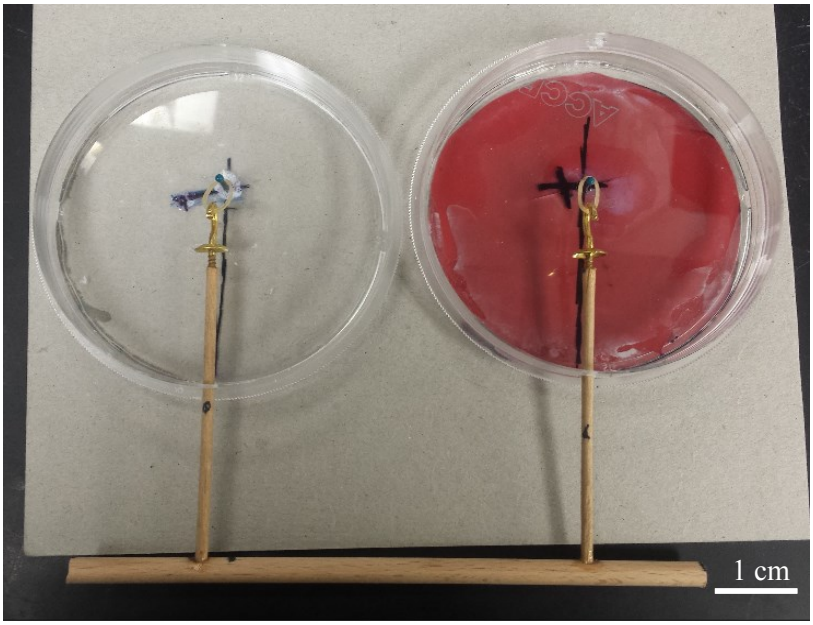


FIGURE 16. AERIAL VIEW OF PROTOTYPE 1

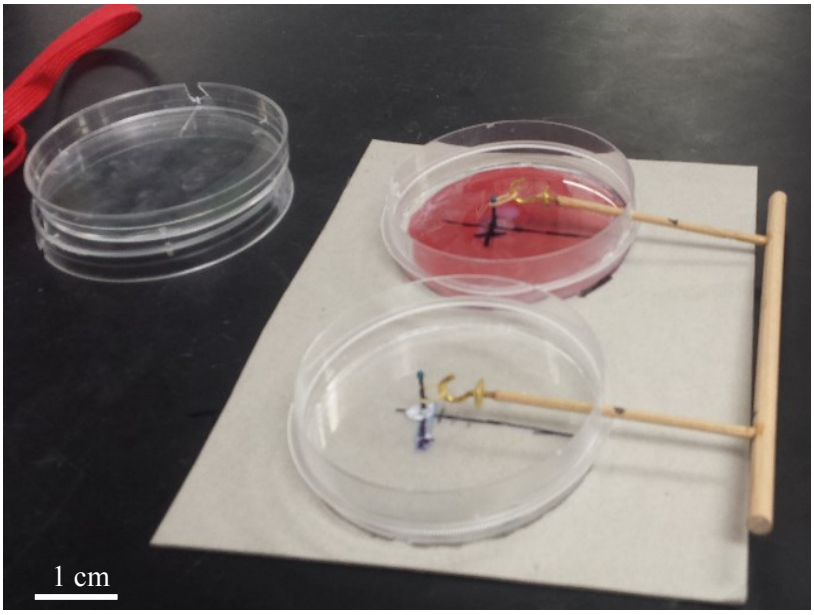


FIGURE 17. ANGLED VIEW OF PROTOTYPE 1

From this prototype the team was able to determine that the assembly itself worked as a whole and it was able to provide positive mechanical strain at 5, 10 and 15 % (Powell, 2002). However this design proved to be ineffective at providing negative strain. Additionally, the

prototype could only stimulate one tissue construct at a time, which would increase both time and cost. It was also found that the hole in the petri dish was too low and was unable to hold a sufficient amount of liquid to cover the entire tissue construct. The process of moving the bar in and out of the plate could introduce bacteria or contamination to the tissue. Based on these limitations the team decided to move forward with a different approach.

4.7.1 Prototype 2: Platform/Hydraulic System

The second prototype was developed in order to solve previous limitations observed during the first prototype. This design consists of the following materials shown in Table 5 representing the proposed components of the final design.

TABLE 7. CHART OF MATERIALS FOR PROTOTYPE. (LEFT) FINAL PROPOSED MATERIALS, (RIGHT) COUNTERPART MATERIALS USED FOR PROTOTYPE

Component	**Prototype Material**
Moving platform	Aluminum
Post (polycarbonate)	Hairbrush bristle
Tissue	Dental elastic
Culture dish	Lid of a pipet tip box (3.5 x 5 in)
Syringe pump	Hydraulic syringe pump
Adhesive (medical glue)	Crazy glue
Machining	Power drill
Culture media	Water

For the second prototype, the team used a hydraulic syringe system to provide the mechanical actuation in order to solve the problem of contamination to the system. As the plunger of the syringe was the only part of the hydraulic system to contact the internal compartment of the dish, the syringe's septum provides protection against contamination. The dish was modified from a circle to a rectangle (3.5 x 5 inches) in order to house multiple tissue constructs. The hook system was changed to a moving platform with multiple posts that lined up with the stationary posts as shown below in Figures 18 and 19.

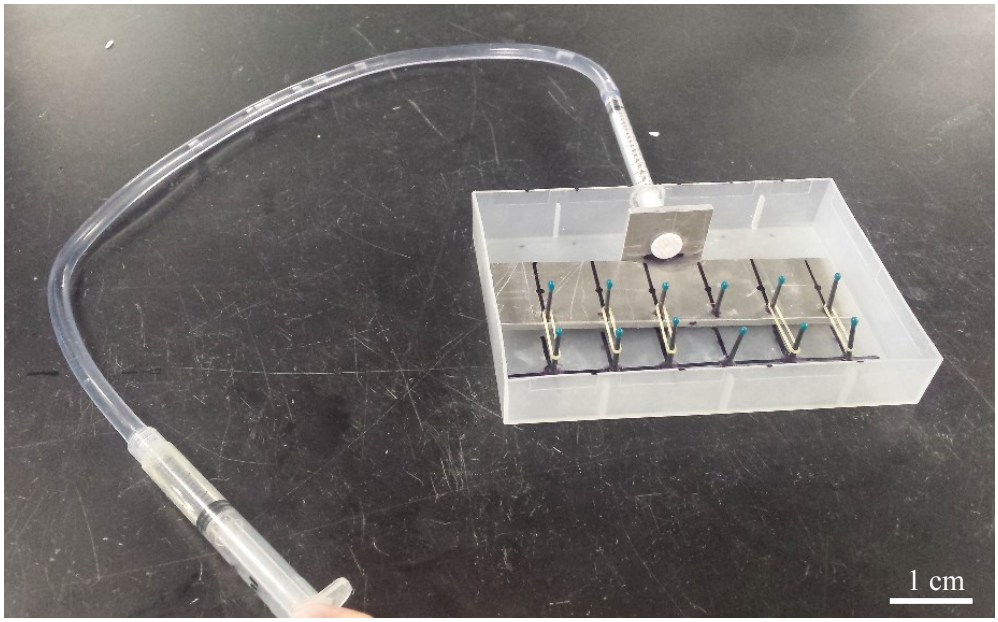


FIGURE 18. PERSPECTIVE VIEW OF PROTOTYPE 2, WITH ELASTICS STRETCHED

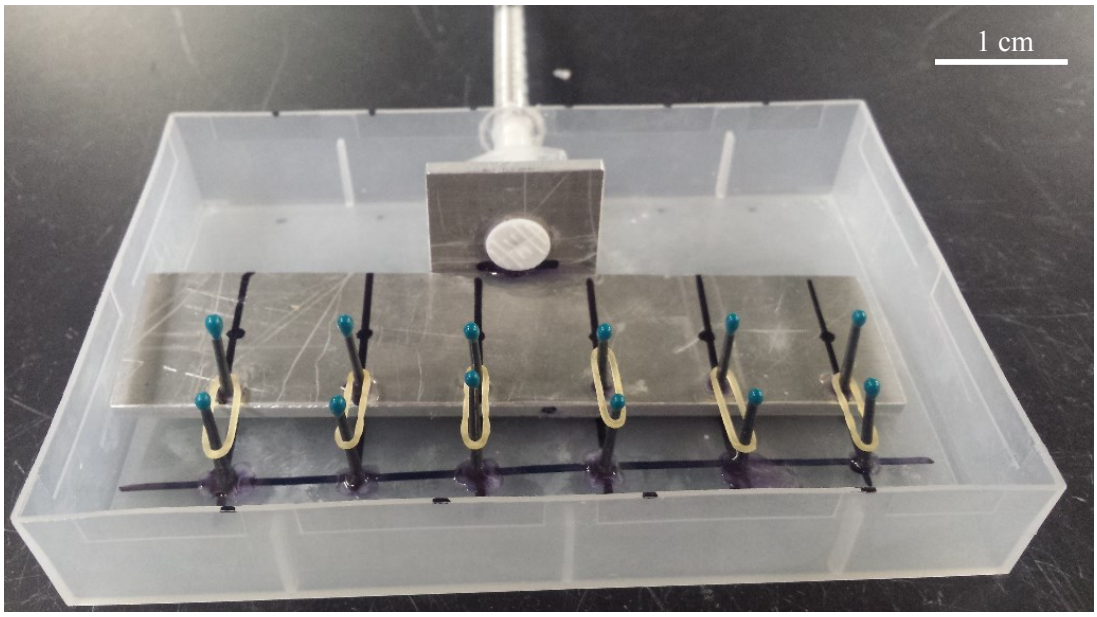


FIGURE 19. CLOSE-UP VIEW OF PROTOTYPE 2 INNER PLATFORM AND POST SYSTEM, WITH ELASTICS RELAXED

Several issues were encountered with this prototype. The main problem was the introduction of air bubbles into the hydraulic system that prevented and delayed the response of the plunger that was attached to the moving platform. Additionally, due to the "L" shape of the platform, the petri dish wall height was not sufficient to allow the use of a lid. It was also observed that the stationary posts were lower in height compared to the posts on the moving platform. Attaching the moving platform to the plunger is an obstacle for the future assembly of the device, since it would be difficult to perform in an aseptic environment. In order to address these concerns, the team proposed an increase in wall height of the petri dish, the use of degassed liquid, a modified technique for attaching the platform to the syringe plunger, and a second platform to raise the stationary posts to the height of the moving posts. These modifications will be incorporated into the final device design.

Chapter 5: Design Verification

5.1 Formation of Tissue Constructs

Originally, the tissue constructs were formed on an agarose mold, of a dog-bone shape, in which the mold design was provided to the team by a graduate student who was working on a similar project. The mold design was 3D printed using a rapid prototyping machine (Objet260) that was available to the team on the Worcester Polytechnic Institute (WPI) campus. These molds, printed out of acrylonitrile butadiene styrene (ABS) plastic, were then cast with Polydimethylsiloxane (PDMS) to create the negative mold. Prior to casting the agarose, the PDMS was autoclaved. Under sterile conditions, agarose was cast into the negative PDMS mold to make the final mold as seen in Figure 20. The myogenic C2C12 cells were then seeded, with differentiation media, into each of the three wells in the laboratory hood as seen in Figure 21. After seeding the tissues, the molds were placed inside the incubator and stored in differentiation media.

After 24 hours, the molds were taken out of the incubator and imaged. The tissue formation of the original mold can be seen in Figure 21 below.

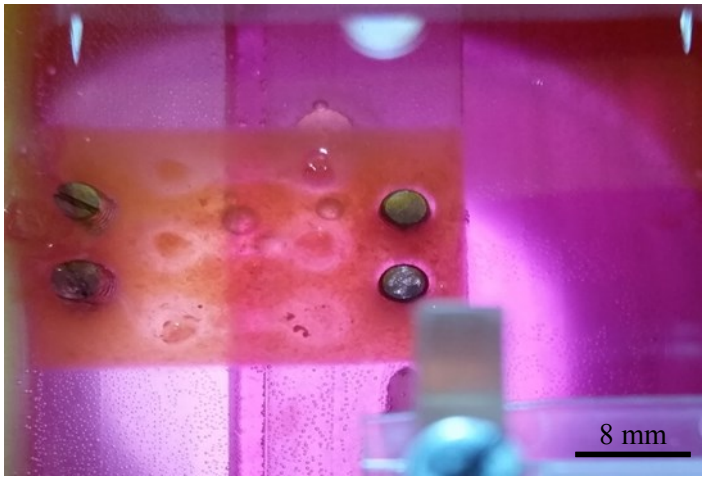


FIGURE 20. AGAROSE MOLD PLACED IN FINAL DEVICE

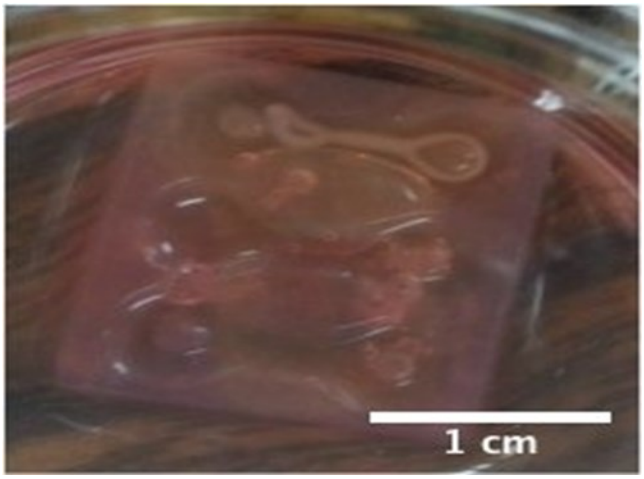


FIGURE 21: ORIGINAL AGAROSE MOLD WITH SEEDED TISSUES IN EACH WELL. SOME WELLS EXHIBITED BETTER TISSUE FORMATION THAN OTHERS.

After making modifications to the final device, a new mold was designed in SolidWorks which incorporated notches to the posts to better anchor the tissue. Additionally, two holes on each side were added for the placement of screws to hold the mold in place during mechanical actuation. The image of the SolidWorks design can be seen in Figure 22 below. This modified mold was 3D printed out of ABS plastic using the Objet260 rapid prototyping machine. The team decided there would be no need to cast an agarose mold, considering the tissues would be seeded into the modified mold, placed in the device, and be interchangeably removed. At first, the team planned to cut the plastic mold in the center to allow for mechanical actuation upon placement in the device. However, the tissues seeded into the modified mold exhibited poor formation because ABS plastic was not biocompatible according to the rapid prototyping guidelines. Additionally, the team encountered problems with preventing leakage of cells and media from the plastic mold during tissue formation. A solution using NIPAM was attempted, but ultimately failed due to temperature differences and lack of a method to hold the mold together during solidification. Thus, MED-160, a biocompatible material, was 3D printed instead of the ABS plastic, and the casting procedure, detailed above, was performed again to make a final agarose mold.

Once the agarose mold was made, the myogenic C2C12 cells were seeded, after incubating for 24 hours in differentiation media, into each of the three wells. For both molds, the original and modified, the tissues were formed by self-assembly. Figure 23 depicts the agarose mold inside a petri dish with the tissues in each well.

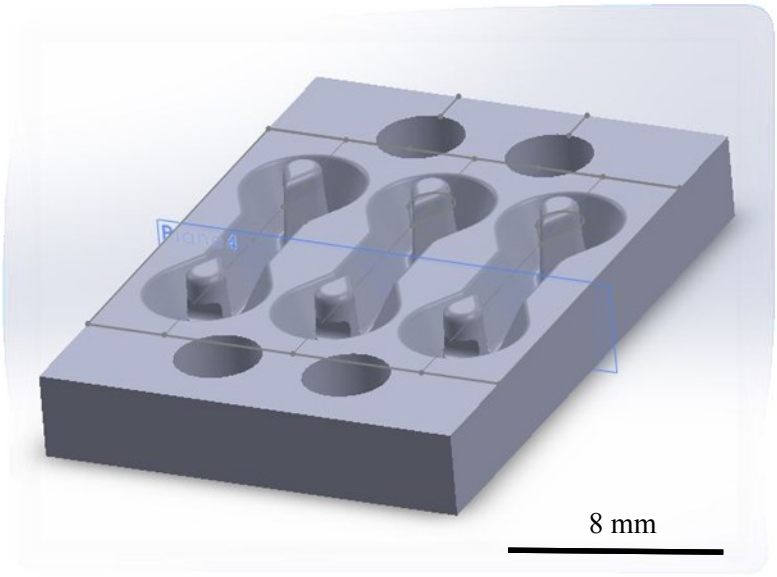


FIGURE 22. SOLIDWORKS DESIGN OF THE MODIFIED MOLD WITH AN EXTENDED LENGTH AND THE ADDITION OF HOLES AND NOTCHES

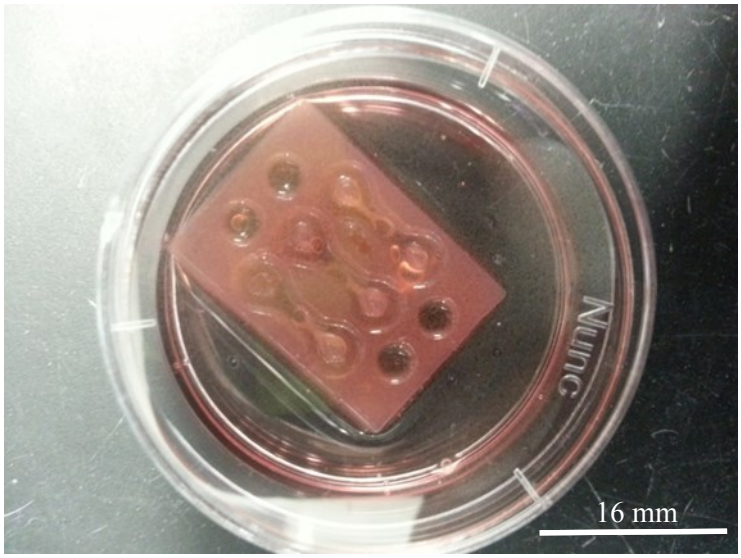


FIGURE 23. MODIFIED AGAROSE MOLD WITH SEEDED TISSUE IN EACH WELL. THE TISSUES SHOWN RUPTURED AT THE POSTS

5.2 Mechanical Actuation Testing

Before the tissues were mechanically actuated, a conditioning regimen was created and used in order to strengthen and develop the muscle tissue. Table 8 below describes the specific parameters that will be followed when the tissues undergo mechanical actuation. For the first three days, the tissues will be mechanically strained at 5% for a total of 3 sets of 5 stretches and relaxations. These tissues will be strained at a rate of 0.635 mm/s using a syringe pump for a total time of two seconds. After three days, the mechanical strain of the tissue will be increased. The cyclic strain applied to the tissues will increase to 10% for three additional days. The tissues will be mechanically stretched and relaxed 5 times and allowed to rest for 30 seconds before repeating the same procedure. The strain rate will remain the same and the total time of stimulation will be four seconds. Lastly, for the remaining two days, the cyclic strain applied will increase to 15% and the number of sets will remain the same. The tissues will be stretched and relaxed five times, allowed to rest for 30 seconds, and repeated twice more. The strain rate, again, will remain the same and the tissues will be stimulated for six seconds.

TABLE 8. CONDITIONING REGIMEN THAT WILL BE FOLLOWED TO MECHANICALLY ACTUATE THE SKELETAL MUSCLE TISSUE

Days	**Strain (%)**	**Times/day**	**Reps of stretch/Relaxation**	**Rest period between sets**	**Strain rate (mm/s)**	**Time of stimulation (s)**
1-3	5	3	5	30 seconds	0.635	2
4-5	10	3	5	30 seconds	0.635	4
6-7	15	3	5	30 seconds	0.635	6

Determination of these parameters was decided by evaluating a previous mechanical stimulation experiment on skeletal muscle tissue (Powell 2002). In their experiment, they increased the amount of cyclic strain provided to each tissue every two to four days (Powell

2002). Thus, the team followed a regimen that is very similar to the procedures they followed which would allow the team to compare testing results to theirs.

5.3 Electrical Stimulation Testing

Prior to applying electrical stimulation to the muscle tissues, parameters for the testing were established. The electrical stimulation parameters are detailed in Table 9 below. The tissues will be applied a voltage of 3 V/cm at a frequency of 1 Hz with a width of 4ms. This procedure will occur for four hours a day for a total of seven days. The electrical stimulation will be provided to the tissues through a Biopac system. The voltage will be transmitted through conducting copper wire that is twisted around six 3-16 stainless steel needles located at different locations on the lid of the device. These needles, contained by rubber septa, will extend below the lid and into the media that is present within the device.

While many studies have investigated applying electrical stimulation to skeletal muscle tissue, not one combination of voltage, frequency, width, and duration has been discovered that will work the best for the electrical stimulation of skeletal muscle tissue. After reviewing the study by Ito et al, and based on their success, these values for the electrical stimulation testing were chosen (Ito 2014).

TABLE 9. ELECTRICAL STIMULATION PARAMETERS THAT WILL BE FOLLOWED TO ELECTRICALLY STIMULATE THE SKELETAL MUSCLE TISSUE

Voltage	Frequency	Width	Duration	Overall
3 V/cm	1 Hz	4 ms	80 s/day	1 week

In order to test the electrical stimulation concept, a prototype lid was created with the components detailed above. Figure 24 below illustrates the prototype and its components. Copper

wire was used as the conducting material and the lid was composed of the top section of a 12 well plate. To test this, the BIOPAC system was setup and connected to the copper wire while salt water was placed inside the plate to simulate the media. The BIOPAC system was turned on and 10 V were applied, measured by a voltage meter. Shortly after, the salt water started to effervesce, indicating that the electrical stimulation was being provided. For the prototype, excessive voltage was applied in order to test the concept. In the final device, using the BIOPAC input lines and placing them within the media, the delivered electrical field could be determined at each of the points where the tissue would be located in the device. This ensured that each point in the device received the correct electrical stimulation, such that all tissues would be evenly stimulated throughout the device. After verifying the concept of the prototype, the final lid was milled out from polycarbonate and assembled into the device, as seen in Figure 35.

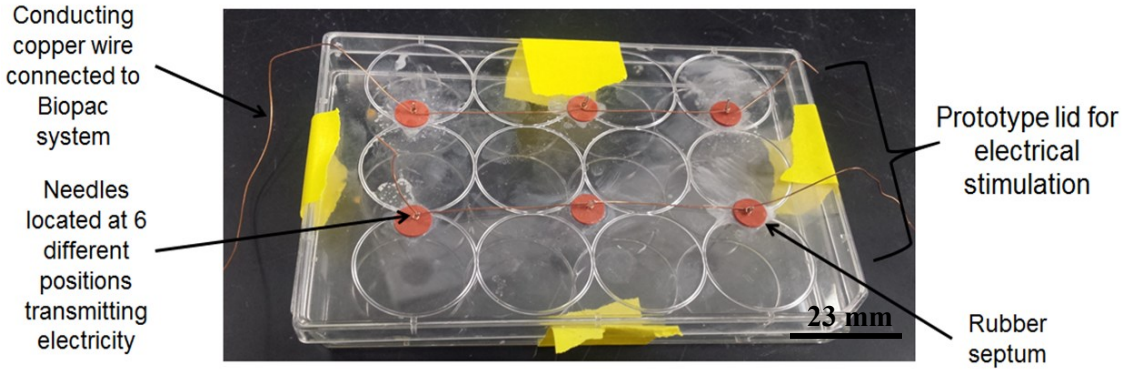


FIGURE 24. PROTOTYPE FOR THE LID OF THE FINAL DEVICE THAT WILL APPLY THE ELECTRICAL STIMULATION

Chapter 6: Final Design and Validation

After considerations made during the testing of the prototypes, a final design was created and manufactured. This chapter explains the design of the device and why aspects of the design where chosen.

6.1 SolidWorks Device Design

After reviewing the shortcomings of the prototypes, the final design drawing was proposed. In order to accommodate the syringe system without allowing media to leak through the opening in the side of the device, the team increased the wall height of the container. Furthermore, the team desired the ability to mechanically and electrically stimulate multiple tissue constructs at the same time. Thus, the idea of having enough space for twelve tissue constructs was implemented by creating a device with a length of ten centimeters long, five centimeters wide, and five centimeters tall. Another limitation from the previous prototype that was solved with the final design was the unequal height of the posts on the moving platform and the floor of the container. To fix this, a 6mm step was designed for one side of the wall. The exact dimensions of the device can be seen in Figure 25, while the dimensions of the accompanying lid can be seen in Figure 26. The final assembly of the device allows for the moving platform to line up with the step, which keeps the posts at the same level. Further, the placement of the syringe was considered. After computing the maximum height and the level of media needed to cover the tissue constructs, the center of the syringe was placed through the wall of the device at a height of 35mm from the bottom. This height ensured that the media would not come into contact with the syringe and any possible leakage from the hole the syringe was placed in would be prevented. A side assembly drawing of the device and syringe system can be seen in Figure 27. Lastly, to hold

the tissue molds within the device, four posts per mold were placed in the position of the associated hole in the tissue mold.

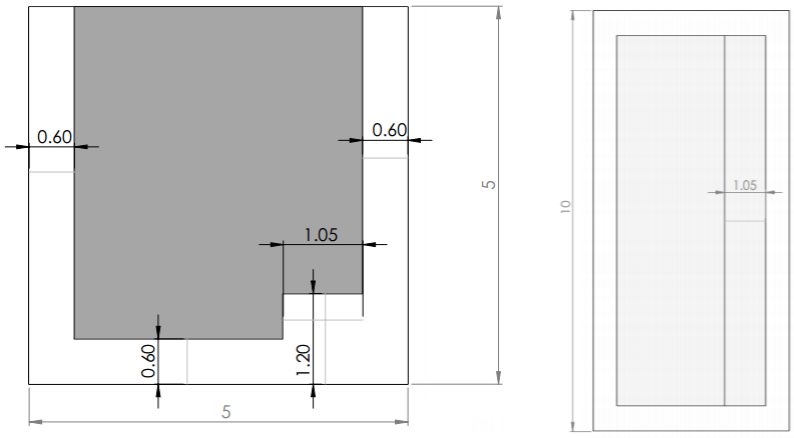


FIGURE 25. DIMENSIONS OF THE FINAL DEVICE (IN CENTIMETERS). THE PICTURE ON THE LEFT SHOWS A SIDE VIEW, WHILE THE PICTURE ON THE RIGHT SHOW A TOP VIEW

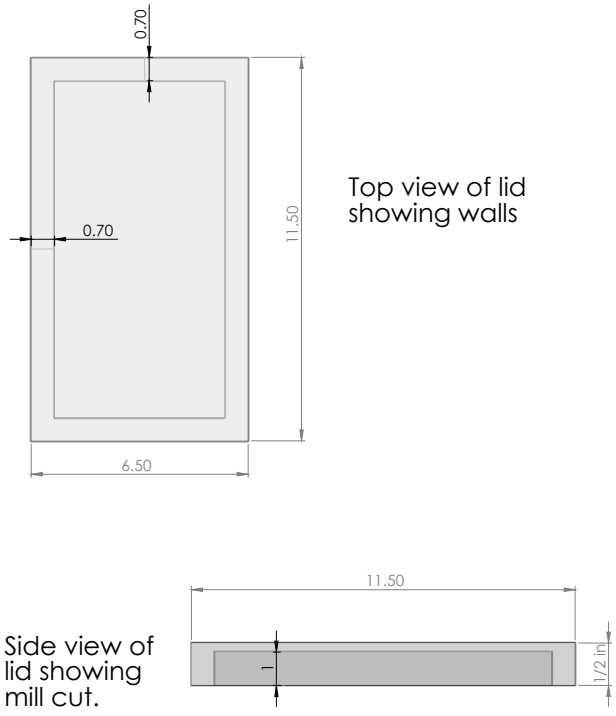


FIGURE 26. DIMENSIONS OF THE FINAL DESIGN OF THE LID OF DEVICE (ALL DIMENSIONS GIVEN IN CENTIMETERS, UNLESS NOTED)

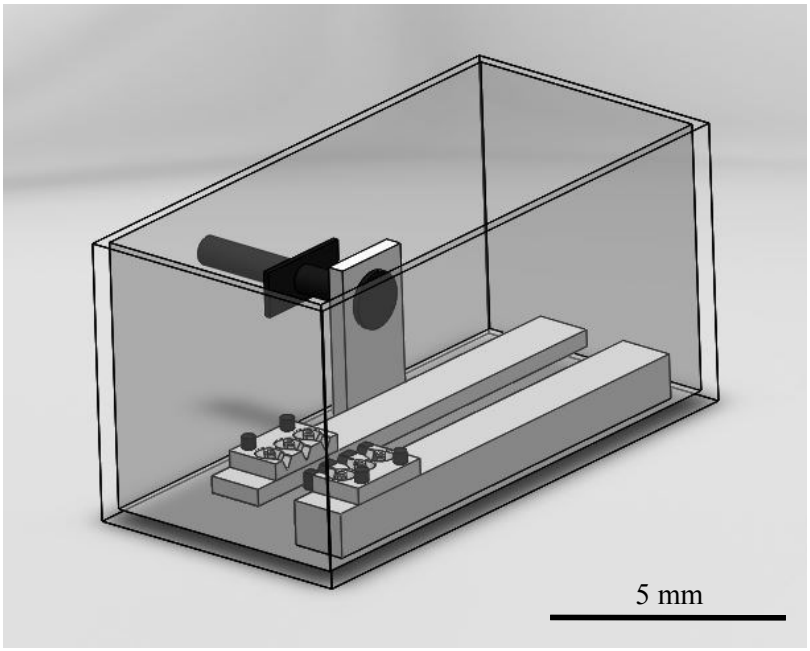


FIGURE 27. DEVICE ASSEMBLY WITH SYRINGE AND TISSUE MOLD PLACEMENT (EXACT DIMENSIONS NOT SHOWN)

6.2 Tissue Mold Design

The final design of the tissue mold included a few modifications from the current mold that the team was working with. To prevent the tissue from slipping off the posts while maturing, the posts within the tissue construct design were heightened. Further, a notch was included at the top to ensure that the tissues did not come off if they contracted upward. To solve the problem of anchoring the tissue molds to the moving platform and step, two holes were placed on either end of the mold. This allowed permanents posts in the device to be placed in the holes to hold the mold in place. A picture of the final tissue mold can be seen in Figure 20.

6.3 Mold Placement and Tissue Formation

When ready for use, the agarose molds were placed into the device using sterile forceps, by aligning the holes of the mold over the stainless steel guiding posts in the device.

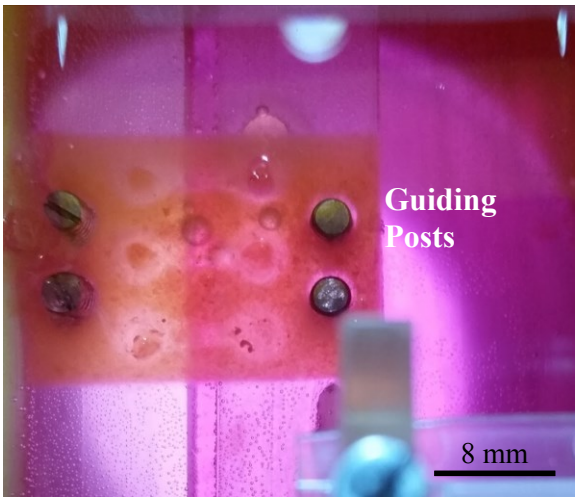


FIGURE 28. INCORPORATION OF MOLD INTO DEVICE, WITH GUIDING POSTS

To seed the tissue into the molds, C2C12 cells were cultured, maintaining less than 50% confluence in order to prevent cell-to-cell contact, which would initiate terminal differentiation. Terminal differentiation is undesirable because it prevents the cells from differentiating into myocytes that fuse to form myofibers. Once the desired number of cells was cultured, cells were re-suspended at a density of 400,000 cells per 35 μL of differentiation media. This cell solution was then pipetted into each well of the mold. After 24 hours, the tissue was formed, and at this point, sterile forceps were used to cut the agarose mold along the line seen in Figure 29 to allow for mechanical actuation of the device and tissue. Unfortunately, the tissues could not be incorporated into the device because the notch modifications did not survive the transfer process to the agarose molds, which resulted in the tissue slipping off the posts. As such, no further testing was performed on the tissue.

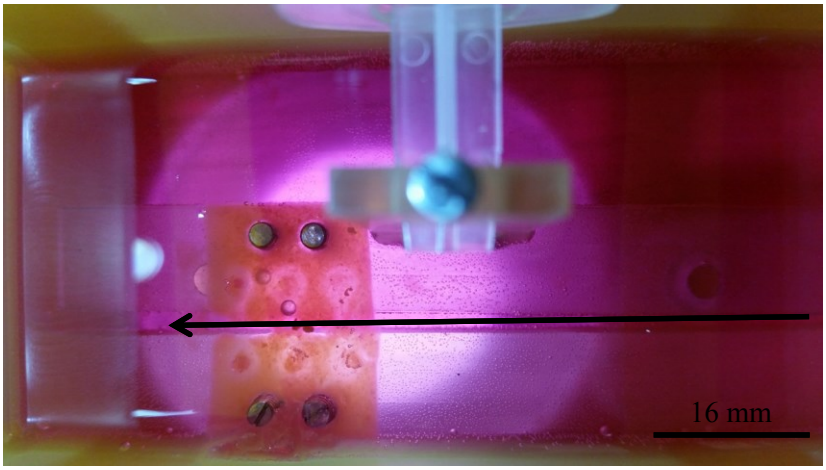


FIGURE 29. INCORPORATION OF MOLD INTO DEVICE, SHOWING LINE FOR CUTTING MOLD FOR MECHANICAL ACTUATION

6.4 Device Materials and Manufacturing

When considering the materials that would work for the design, the team wanted to choose materials that were biocompatible with cells and autoclavable. The chosen material was then tested through a direct contact cytotoxicity test. After, the final device was manufactured and assembled.

6.4.1 Material Biocompatibility Testing

The final material chosen for the device itself was polysulfone. Originally, the team wanted to utilize polycarbonate for the container, but polycarbonate sheets at the thickness the team required were not easily obtained. A block of polysulfone was chosen because of its ability to be autoclaved and its availability to the team in the dimensions that were required for the device. To validate that the material was biocompatible with cells, the team performed a direct contact cytotoxicity test using a six-well plate. First, a small piece of polysulfone was placed into one well of the plate. Another well was designated as the control group. 100,000 C2C12 cells were then pipetted into each of the wells, and three milliliters of proliferation media was added to each. As documentation, pictures of the control group and the cells in contact with the

polysulfone were taken at 10x magnification. Figure 30 shows the pictures of both groups on the day the cells were added to the wells. The cells were visualized after two days, shown in Figure 31, and the cells in contact with the material were compared to the control group. Since the cells were similar in each well and alive, polysulfone was validated as biocompatible.

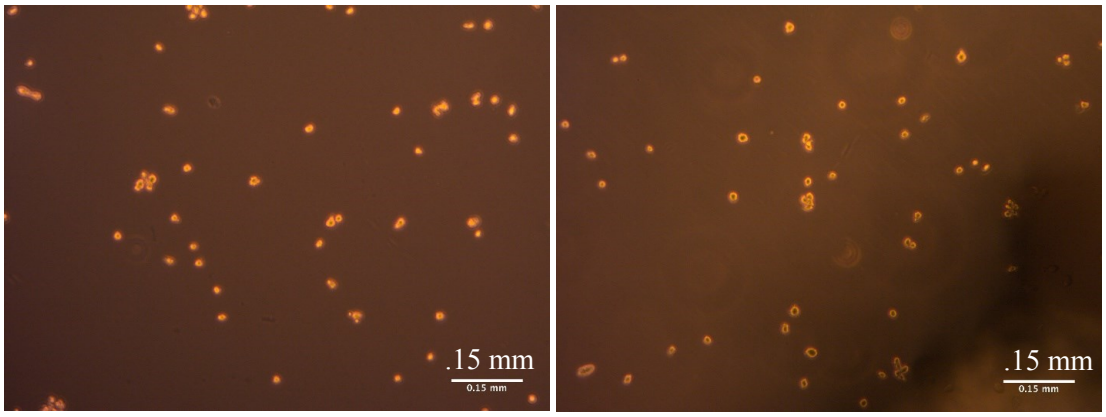


FIGURE 30. DIRECT CONTACT CYTOTOXICITY TEST DAY 1. THE CONTROL GROUP (LEFT) IS IN CONTACT WITH ONLY THE POLYSTYRENE OF THE CULTURE DISH. THE PICTURE ON THE RIGHT SHOWS THE CELLS ALSO IN CONTACT WITH POLYSULFONE (PLACED IN LOWER RIGHT OF PICTURE). SCALE BARS ARE IN MILLIMETERS.

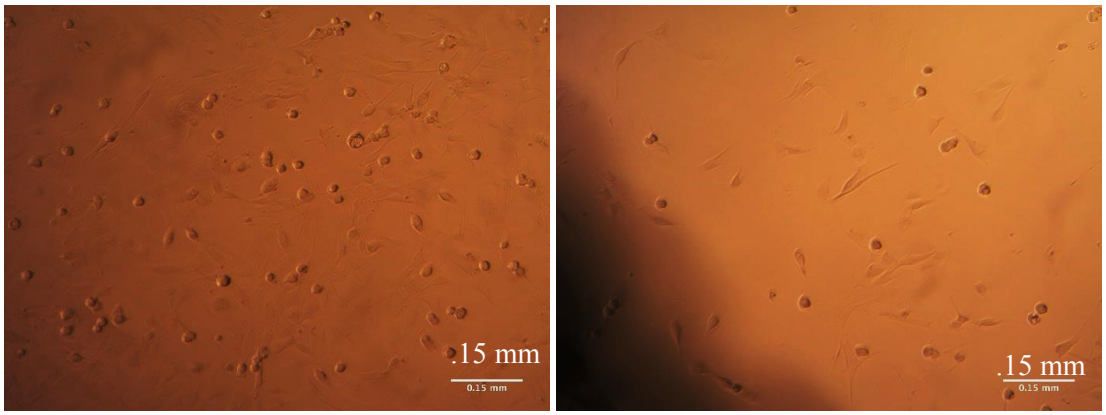


FIGURE 31. DIRECT CONTACT CYTOTOXICITY TEST DAY 3. THE CONTROL GROUP (LEFT) IS IN CONTACT WITH ONLY THE POLYSTYRENE OF THE CULTURE DISH. THE PICTURE ON THE RIGHT SHOWS THE CELLS ALSO IN CONTACT WITH POLYSULFONE (PLACED IN LOWER LEFT OF PICTURE). THE SCALE BARS ARE IN MILLIMETERS.

6.4.2. Final Device Assembly

The device box was milled to the specifications shown in Figure 25 using the polysulfone material. Leftover material was utilized to make the movable platform that was attached to the syringe in the box. Stainless Steel 304 screws were then used to assemble the platform. Additionally, the leftover polysulfone material was also used to create a collar on the outside of the device to hold the syringe in place. The material used for the posts holding the tissue molds consists of standard 5-40 stainless steel screws that were smoothed down to accommodate the standard one-eighth holes positioned in the tissue molds. For the lid of the device, polycarbonate was chosen since it is clear and would enable the team to see the tissue constructs within the device. Figure 32 and 33 show the final assembly of the device using the materials mentioned.

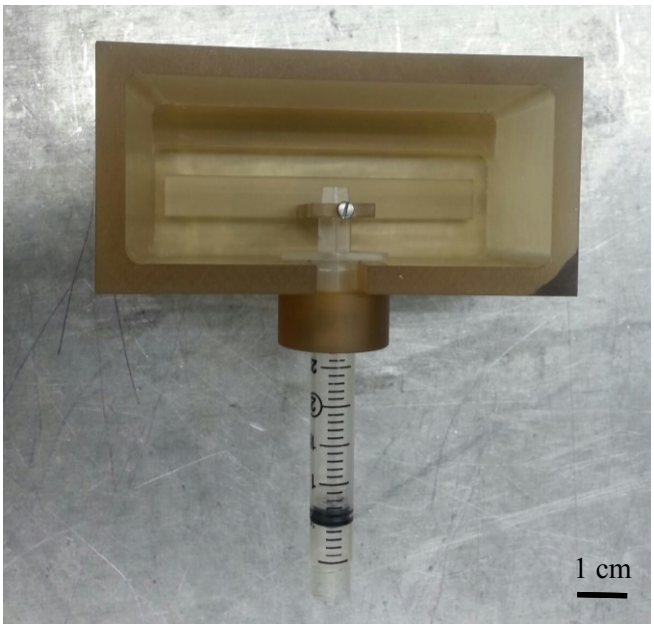


FIGURE 32. TOP VIEW OF FINAL ASSEMBLED DEVICE WITH SYRINGE AND MOVABLE PLATFORM

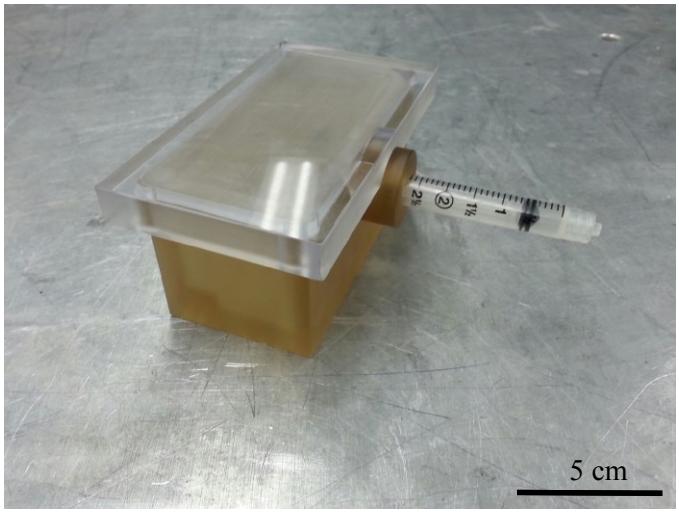


FIGURE 33. SIDE VIEW OF ASSEMBLED DEVICE WITH SYRINGE AND LID

6.5 Final Device Protocol Validation

To test that the chosen mechanical and electrical protocols could be performed with the final device, both a syringe pump and BIOPAC system were utilized.

6.5.1. Mechanical Protocol Validation

To determine if the device could impart negative and positive strain to the tissue constructs, testing was performed using a syringe pump and LAS EZ microscope software. Preliminary tests were performed to establish the correlation between the syringe volume displacement and the movement of the device platform. Testing was then done for the 5%, 10%, and 15% strain that would be applied to the tissue. Multiple trials were completed to determine which volume displacement of the syringe corresponded to the device platform displacement needed for the required strain. Since the tissue constructs are one centimeter in length, each millimeter movement would ideally equal 10% strain. Five trials were completed for each strain, with averages, standard deviation, and target strain seen in Table 10. Figure 34 also shows how the syringe volume displacement corresponds to the platform displacement. As can be seen in the

table, strains of 5%, 9%, and 15% were accomplished when utilizing the syringe pump, which was close to the target strains.

TABLE 10. DISPLACEMENT AVERAGES OF THE SYRINGE VOLUME AND DEVICE PLATFORM, ACHIEVED STRAIN, AND TARGET STRAIN

Averages			Target
Syringe Volume Displacement	**Device Platform Displacement**	**Strain**	**Target Strain**
0.03 mL	0.50 mm +/- 0.05	5% +/- 0.5	5%
0.06 mL	0.90 mm +/- 0.1	9% +/- 1.0	10%
0.1 mL	1.51 mm +/- 0.06	15% +/- 0.6	15%

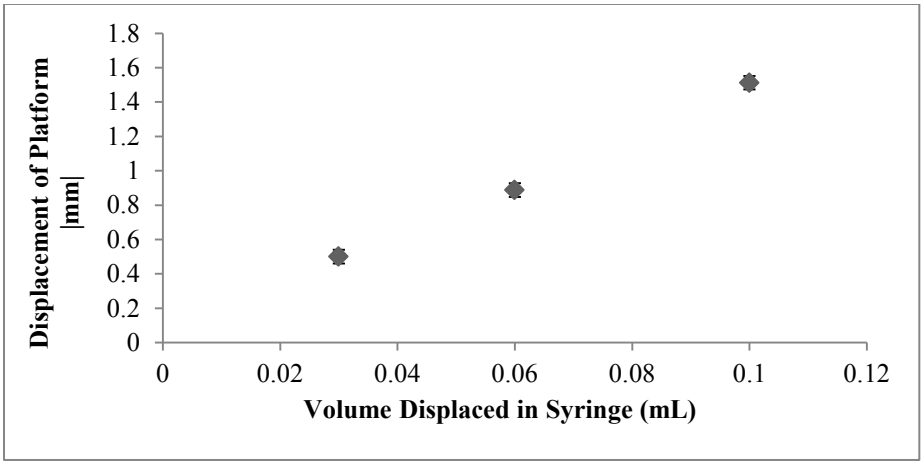


FIGURE 34. RELATIONSHIP BETWEEN THE SYRINGE VOLUME DISPLACEMENT AND THE MOVING PLATFORM DISPLACEMENT

6.5.2. Electrical Protocol Validation

To validate that the correct electrical stimulation was delivered at the tissue location, electrodes from a BIOPAC system were placed in contact with the media in the device and reading electrodes were placed at the site of the constructs. The set-up with the device and electrodes can be seen in Figure 35. The system was programmed to deliver 3V for a 4ms duration with a one second rest period. This was done continuously for 30 seconds. Three

different trials were performed using this set-up. The average peak voltage for the entire 30 seconds of each trial was then calculated and can be seen in Table 11 and Figure 36. As can be seen in both the table and the figure, the impulse voltages for each test were close to the target impulse voltage of 3V.

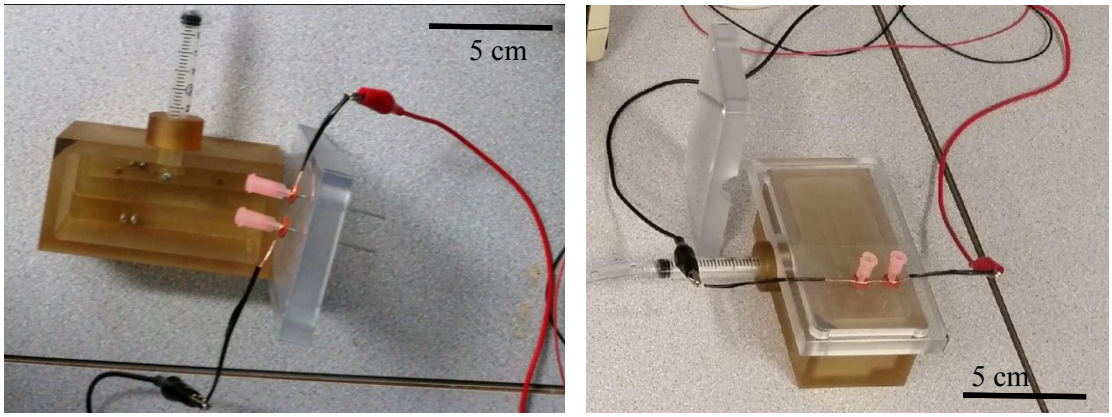


FIGURE 35. DEVICE AND BIOPAC ELECTRODE SET-UP FOR ELECTRICAL PROTOCOL VALIDATION

TABLE 11. AVERAGE PEAK VOLTAGE AND TARGET VOLTAGE FOR EACH VALIDATION TEST

Test #	Average Peak Voltage (V)	Target Voltage (V)
1	3.04 +/- .1	3
2	3.06+/- .09	3
3	2.98+/- .12	3

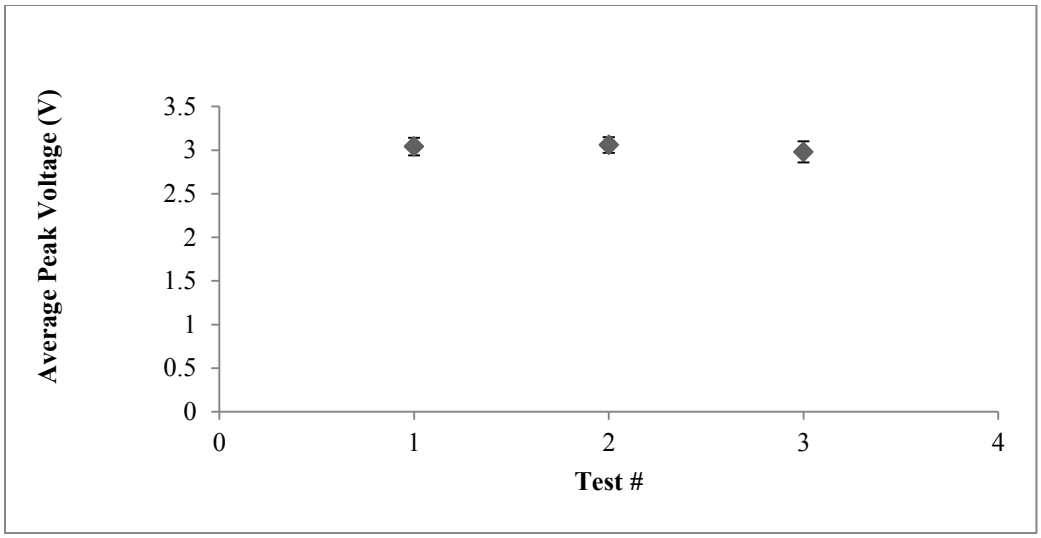


FIGURE 36. AVERAGE PEAK IMPULSE VOLTAGE FOR EACH VALIDATION TEST

Chapter 7: Discussion

This chapter reviews the objectives and goals of the project and how they have been met through the device design and subsequent testing. It also details any limitations of the project's data and outcomes. Finally, this chapter includes discussion on the various influences, ramifications, and considerations of the project, in regards to its effect on economic, environmental, societal, political, ethical, health and safety, manufacturability, and sustainability concerns.

7.1 Tissue Culture

For the purposes of tissue culture, the project design built upon a mold previously developed in the 2013 MQP "Skeletal Muscle Tissue Engineering System to Mimic *In vivo* Development" and later modified by Alyssa Fidanza. Many of the project's stated objectives were met through the use of this mold. The mold provided anchorage points, allowed for tissue self-assembly at a low cell seeding count, and formed aligned tissue within the diffusion limit for media and nutrients. Past MQP projects used a seeding cell density from 1 million – 7 million cells per construct. This modified tissue mold allows for a cell seeding density of 400,000. However, as the mold was originally designed to be a pattern for an eventual agarose mold, the mold itself offered no clear solution for actuating the tissue. Thus, it would fail to meet the objectives to promote high myofiber density or match *in vivo* myofiber diameter, because these objectives relied on the ability to provide stimulation to the tissue. In the past, the agarose mold proved difficult to work with, because of its fragility and ease of damaging tissue when compromised. So, the team chose to seed tissue into the original plastic mold for stability. Additionally, the team encountered issues when seeding the cells into the mold itself, namely the tendency for the tissue to slip off of the posts. The mold also had no way to be incorporated into a

device. As such, the team modified the mold with higher posts with notches, which would act as more effective anchoring points for the tissue. The team also extended the mold to include holes that could slip over a second set of posts in the device system. Although the notches failed to survive transfer from the MED610 mold to the agarose, these modifications were able to incorporate the mold into the device. Future modifications can be added to ensure that the tissue is better anchored by the notch modification.

If the tissue were able to be stimulated within the device, several methods could be used to document its maturation and conditioning. Characterization and morphological studies on histological samples of tissue could determine if the objectives of high myofiber density, *in vivo*-like myofiber diameter, and completely viable tissue are actually reached by the mold and device designs. The team would use immunohistochemistry myosin staining and Masson's Trichrome Blue to visualize the alignment and size of myofibers and to ensure that the tissue does not have a necrotic core or large sections of collagen.

7.2 Mechanical Actuation and Electrical Stimulation

For the purposes of the device, the project objectives were to mechanically actuate and electrically stimulate the tissue within the device. Additionally, the device had to allow for measurement of strain in the tissue. Past project teams chose to actuate the device with a stepper motor or similar device. However, the humid conditions of the incubator did not allow for the motor to work within the incubator, and programming the motor proved tedious or difficult. Additionally, several projects encountered problems in the connection points between the motor and the device, leading to slipping and other noise in the actuation. To resolve these problems, the team chose to incorporate a hydraulic, closed system into the design. By using a long polyethylene tube between the two syringes of a syringe pump, the pump could remain outside of the incubator if tissue were to be actuated within the incubator. The syringe pump system also

reduced the risk of noise in actuation due to a better engineered solution. These modifications allowed the device to better achieve the objective of mechanical actuation. In terms of electrical stimulation, many past projects were unable to incorporate this component into their device. By manufacturing two separate lids, one for normal viewing and one for electrical stimulation with stainless steel rods, the project met the objective of providing electrical stimulation.

Mechanical actuation was validated by programming an NE-4000 syringe pump to infuse and withdraw specified volumes of mineral oil within the tubing. Strain measurement was validated by utilizing video and resolution-calibrated measurement with a Leica microscope and the LAS EZ microscope software. The tissue assembly mold produces tissues at 1.0 cm in length; thus, a measured displacement of 1.0 mm in the device would correspond to 10% strain in the tissue. This simple conversion was used to validate the strain provided by the device without the incorporation of tissue itself. Ultimately, the team was able to achieve strains of 5, 9, and 15%. Due to the small size and relative weakness of the tissue, it is not expected that incorporation of the tissue would alter the displacement of the device by the syringe pump. Delivery of an electrical stimulation was validated by the BIOPAC 100C ECG Amplifier and reading electrodes, placed at the site of the tissue if tissue were incorporated into the device. The BIOPAC software reported the correct electrical stimulation amounts. Upon incorporation of tissue into the device, it is expected that all validated functions would perform as they did without tissue. Thus, the device was successful in providing measurable mechanical actuation and electrical stimulation.

7.3 Limitations

One particular limitation of the data is that the team has not tested experimentally the most optimal regimen for mechanical actuation and electrical stimulation. The focus was to ensure that the device could indeed apply stimulation at specified values and that this stimulation resulted in some improvement in the maturation or development of the tissue. Therefore, further

work must be done with the device to develop the best regimen for stimulation of the tissue. However, because this was not the focus of the project, the team does not consider it a limitation of the project overall.

7.4 Project Impact

All engineering and design projects carry impact in several different areas, including the environment, ethics, health and safety, and economics. This section details the influences, ramifications, and considerations that the device design and project have on society in a broader context.

7.4.1 Economic Considerations

Several economic considerations must be taken into account concerning the device design. The manufacturing of the device itself is a large, but fixed, cost of approximately $120. The team was able to avoid incurring any charge for labor required for the device manufacturing, but in future development of the device, this labor cost must be factored into the overall cost.

Beyond manufacturing is the cost of supplies and reagents. Purchase of the C2C12 myogenic cell line, or any line desired by the research, can potentially be a one-time cost and can be expanded exponentially. However, the cost of storage and passaging materials for the cells factors into the overall cost of the design. Proliferation media used by the project team cost approximately $130 per 500 mL. Additionally, media usage in the design is higher than average, at approximately 30 mL per 12 tissues. Changing the differentiation media would cost approximately $3 each time. However, it is believed that media changes will be far less frequent, if occurring at all, throughout the duration of tissue maturation and testing, whereas devices that incorporate smaller well designs for tissue assembly would require much more frequent media changes due to the metabolism of the tissue. Depending on how long the cells are maintained in

proliferation media before tissue formation, the cost of developing one tissue should be around $10-12, without utilities and other related costs.

In regards to the economy of everyday life, the device has the potential to decrease the cost of therapeutic drug testing. Currently, animal models are required for testing new muscular dystrophy therapeutics, which can become costly. Keeping of animal models requires materials, staff, supplies, and other requirements which can total hundreds of thousands of dollars for the research facility. The project device does not require additional supplies or staff beyond what is typically required in a cell-culture research lab. Thus, the team's device has the potential to eliminate the need for animal models in testing, which can impact the economics of medical research and drug discovery.

7.4.2 Environmental Impact

The team's device is made of mainly polysulfone and 304 stainless steel, which is 8-12% nickel. Although preferable, 316L medical grade stainless steel was not available during the project; the team would plan to switch to 316L in future modifications. Currently, polysulfone is not biodegradable, but it is recyclable. Stainless steel is also not biodegradable but is reusable. Thus, the environmental impact would be limited if it were ensured that the device were recycled at the end of its product life cycle. Fortunately, the device is intended to be used continuously, and the only consumable materials specific to the device are the media and cells required to form tissues. The product life of the device is predicted to last for several years or more, because the time required to develop and test therapeutics is typically on this scale. Thus, the amount of waste produced by disposing the product, if it were to happen, should be infrequent and minimal overall. The device does utilize media which requires serum; however, the team believes that the environmental impact from this usage is minimal because serum is already a standardized item in the field of research.

7.4.3 Societal Influence

The team's device aims to provide a way to form biomimetic skeletal muscle tissue for the eventual purpose of developing and testing therapeutics for muscular dystrophy. Thus, the production of the device will further warrant medical research into muscular dystrophy. This will have a great impact on the lives of those living with muscular dystrophy, as well as their families, friends, and communities, because it will bring research one step closer to developing treatments, and possibly cures, for these diseases. The device might open research into the development of therapeutics for other diseases, based on systems that form biomimetic models of cardiac tissue, liver tissue, and more. Thus, the social influence of the team's device could potentially extend beyond muscular dystrophy into further branches of medical research. This research has the potential to benefit the lives of many people who suffer from various diseases. Additionally, because the device can create a model of human skeletal muscle tissue, decreasing the need for animal models in drug testing, as was discussed in the Literature Review, the expected results for clinical trials could be much more promising. Thus, clinical trials might potentially have a greater chance of success, and their participants might have a lesser chance of risk, due to previous testing of the drug onto human tissue.

7.4.4 Political Ramifications

Some cultures worldwide will be adverse to the use of this device, due to religious or other reasons, as it will eventually use a human cell line. However, the use of animal models poses an equal political concern. Thus, the decision to use any therapeutics developed as a result of this device will remain in the hands of each individual culture. In terms of the global market, the device could increase the global economy, slightly, because its use will create more opportunities for the development of therapeutics in a more streamlined testing manner. More research will require more grants, time, and materials, which will stimulate global economy. Additionally, this device would enter the global market as an aid for researching therapeutics and

could potentially be used beyond researching muscular dystrophy, and so it could provide competition to other pre-existing methods. This competition would both enhance the global market and the research done across the world.

7.4.5 Ethical Concern

Eventual use of the team's device will result in a human, biomimetic model of skeletal muscle tissue for therapeutic development and testing. With this tissue model available, animal models will not be required for medical testing. This alternative to animal models could be seen as favorable, because it decreases the amount of animals used in medical research. In terms of addressing a good and satisfying life, the device will provide a way to test and develop therapeutics for muscular dystrophy, which will greatly improve and possible save the lives of those who suffer from it. Currently, muscular dystrophies like Duchenne Muscular Dystrophy have no cure and no survivors, and so it is ethically imperative to develop therapeutics for the diseases; otherwise, with a lack of advancement in research for these diseases, they will continue to impact and claim many lives. Thus, the device offers ethical concern in that it is providing a way to continue research that is ethically required.

7.4.6 Health and Safety Issues

The project device has the potential to have a large impact on the health of those suffering from muscular dystrophy. Successful usage of the device should create a platform for testing drugs in terms of type, concentration and dosing, and other factors. There are many reasons for variation in animal models, including gender differences, littermates unevenly split throughout the trials, changes in care and handling, and loss of disease phenotype in disease models (Perrin 2014). As all tissues formed in the device will be stimulated in the same manner, there should be less variation in testing results than is seen in animal model testing. Additionally, the device provides a way to test therapeutics for muscular dystrophy with human tissue, rather

than animal models, which should expedite testing and produce more favorable predictions for clinical trials. Although the model itself is not all-encompassing of factors in the human body, it should prove more alike to native human tissue than animal models. Thus, the health of those suffering from muscular dystrophy will benefit from this device. In terms of safety, the team does not see any posed risk of personal safety to the general public or, when used correctly, the users of the device. When used incorrectly, the device may pose an electrical hazard due to the incorporation of electrical stimulation.

7.4.7 Manufacturability

Manufacturability is a large concern in device production, taking into account how many materials are required, how they are processed and machined, how they are assembled, and how to streamline the process. The team's device was manufactured from bulk polysulfone and polycarbonate, as well as several stainless steel fasteners and screws. The tissue molds were produced using MED-160 plastic from the Higgins Labs Rapid Prototyping Lab. In the development of the device, bulk polysulfone was milled down into a box and cut into several additional pieces to form the other components of the device. This decision resulted in wasting about 140 cm^3 of polysulfone. In the future manufacturing of the device, both the box and internal components can be injection molded, as polysulfone is a thermoplastic, which can allow for easier mass production of the device, and minimal waste. Additionally, the team modified standard 5-40 stainless steel screws for attachment and fastening. In the case of manufacturing, these screws could potentially be made in bulk by an outside company or in-house. As for the syringe pump system, two standard BD 3.0 mL syringes were used with non-distendable polyethylene tubing. Practically, the syringes and tubing would be purchased from a supplier. Finally, the tissue mold could also be molded in-house. In terms of manufacturability and material obtainment, the tissue mold could be changed to be made of polysulfone as well.

Overall, the team utilized a machining shop for the purposes of manufacture. In the future, manufacturing would require a facility that housed an injection molding machine, assembly robotics or laborers, and an autoclaving and packaging system to ensure sterility of the device. This system can easily be modeled off of pre-existing production operations for cell culture dishes and other products. The implementation of these changes should not prove too difficult in order for mass production, and as such, the device could easily be reproduced for future use.

7.4.8 Sustainability

Production of the device will require a factory setting in which plastic and stainless steel can be processed and assembled. This factory setting will require the input of electricity, gas, and water, but given the current usage of natural resource and utilities, this additional usage should be negligible. However, both polysulfone and stainless steel, which comprise the bulk of the device, are recyclable materials. Manufacturing could make use of recycled polysulfone and stainless steel in order to further increase the sustainability of production. By utilizing injection molding techniques, rather than milling, far less waste will be produced in the manufacturing of the device, increasing the sustainability. The team's device is easily standardized, since no part of it contains any customized components; this standardization can reduce the amount of material, time, and energy required to produce the device, maintaining a fair level of sustainability.

Chapter 8: Conclusion and Recommendations

The team was successful at meeting most of the objectives pertaining to the individual parts of the project. Nevertheless, there are limitations that were encountered, for which the team has created future recommendations.

8.1 Device Recommendations

The height and width of the device proved to be one of the most noticeable limitations of the design. The height of the milled container is 5 cm, while the width is 10 cm. These dimensions make it difficult to maneuver the tissue molds inside the device while maintaining a sterile environment. The tissue molds were made of agarose, which are delicate and can easily tear while placing them inside the device. This not only compromises the molds, but also the viability of the tissues within them. Decreasing the height and increasing the width will provide more available working space, while increasing the length will allow more tissues to be stimulated. As of now, twelve tissues can be stimulated simultaneously, but if this number increases, the device will be more efficient in terms of time and cost. An additional limitation is that the lid of the device does not allow for visualization of actuation. This can be fixed by making a new lid out of polystyrene that does not require milling, providing the transparency that will allow for visualization.

8.2 Tissue Mold Recommendations

Tissue molds were made out of agarose. These molds proved to be effective in terms of maintaining cell viability and allowing the diffusion of media through the tissues during their formation. However, two main problems were found. The first problem was the transportation of the tissues into the device. Due to the fragility of agarose, it is necessary to be careful when maneuvering the molds to prevent tissue damage. The second problem was that the notch modification of the posts in the molds did not transfer to the agarose molds, which resulted in the tissues slipping off of the posts, as can be observed in Figure 21. Thus, the team chose to prototype the same molds out of MED-610, a biocompatible material. In practice, the center was cut out in order to allow for mechanical actuation, reducing the mold's fragility, while maintaining the notch modifications to prevent the slippage of the tissue. However, an additional problem was found at the moment of seeding the tissues. The middle cut-out region needed to stay connected to the rest of the mold while the tissue formed to prevent cells from leaking out of the mold. The team attempted to seal the mold with both agarose and temperature sensitive NIPAM. However, both methods failed, because there was no effective way to compress the mold while the material solidified. Thus, the team decided to instead incorporate the agarose mold into the final device. A future recommendation is to incorporate capped pins into the platforms of the device through the posts of the agarose mold. With this change, the tissue can be seeded into the agarose mold in the device, which will eliminate the need to transfer the mold and tissues; additionally, the capped pins will hold the tissues in place in the event of tissue slippage, allowing for complete tissue formation.

8.3 Conclusion

Overall, the device design was created with the main purpose of allowing the formation of skeletal muscle tissue completely *in vitro*. The design was composed of three main parts: mechanical actuation, electrical stimulation, and skeletal muscle tissue self-assembly. The syringe pump system was successful at providing cyclic positive and negative strain of 5%, 9% and 15%. The BIOPAC system was successful at producing the necessary electric field to stimulate the tissues, with a voltage of 3 V/cm at a frequency of 1 Hz and 4 ms width. Agarose molds of 16 mm x 23 mm allowed for tissue self-assembly. The molds also provided anchorage points. Finally, a minimum functional unit size was achieved using less than 400,000 cells resulting in length of 1 cm and a diameter of approximately 150 μm at the center. The size of the tissues was small enough to allow for nutrient diffusion but large enough to withstand the positive and negative strain. The device was developed to maintain an aseptic environment and work within incubator conditions in order to maintain tissue viability. Due to time constraints, the tissues were not able to be stimulated with the device due to their constant slippage off the mold, as mentioned. The findings of the overall design indicate promising potential if recommendations are incorporated. This design can serve as a prototype for future studies aiming to create a functional unit of skeletal muscle tissue that can be used for the development or testing of new therapeutic strategies.

References

Armed Forces Health Surveillance Center. (2013). "Update: exertional rhabdomyolysis, active component, U.S. Armed Forces 2008-2012." Medical Surveillance Monthly Report, March.

Bagley, W.H., H. Yang, K.H. Shah. (2006). "Rhabdomyolysis." *Internal and Emergency Medicine. 2*(3), 210-218.

Baiguera S, Urbani L, Del Gaudio C.(2014). Tissue engineered scaffolds for an effective healing and regeneration: reviewing orthotopic studies. *Biomed Res Int.*

Bland E, Dréau D, Burg K. (2012). Overcoming hypoxia to improve tissue-engineering approaches to regenerative medicine. *J Tissue Eng Regen Med.*

Boonen K,J., Langelaan M,L., Polak R,B., van der Schaft D,W., Baaijens F,P., Post M,J. (2010). "Effects of a combined mechanical stimulation protocol: Value for skeletal muscle tissue engineering." Journal of Biomechanics 43(8):1514-21.

Buckingham, M. (2001). "Skeletal muscle formation in vertebrates." *Current Opinion in Genetics & Development*. *11*(4), 440-448.

Chamberlain, J.S. (2002). "Gene therapy of muscular dystrophy." *Human Molecular Genetics, 11*(20), 2355-2362.

Chevallay B, Herbage D.(2000). Collagen-based biomaterials as 3D scaffold for cell cultures: applications for tissue engineering and gene therapy. Med Biol Eng Comput,(2):211-8.

Dellett, M., Hu, W., Papadaki V., Ohnuma S. (2012). "Small leucine rich proteoglycan family regulates multiple signalling pathways in neural development and maintenance." *Dev Growth Differ 54*, 327-40.

Drake, A.C. (2013). "Of mice and men: what rodent models don't tell us." *Cell Mol Immunol, 10*, 284-285.

Donnelly K, Khodabukus A, Philp A, Deldicque L, Dennis RG, Baar K. (2010). A novel bioreactor for stimulating skeletal muscle *in vitro*. *Tissue Eng Part C Methods*, (4), 711-8.

Du Geon Moon, George Christ, Joel D. Stitzel, Anthony Atala, and James J. Yoo. *Tissue Engineering Part A*. April 2008, 14(4): 473-482. doi:10.1089/tea.2007.0104.

Enas M,Ahmed. (2013). "Hydrogel: Preparation, characterization, and applications" *Journal of Advance Research.*

Fidanza, Alyssa, Jonathan Fitzgibbon, Steven Greco, Todd Pfizenmaier. (2013). "Skeletal Muscle Tissue Engineering System to Mimic *In vivo* Development." *Worcester Polytechnic Institute.*

Fujita, H., et. al. (2007). “Accelerated de novo sarcomere assembly by electric pulse stimulation in C2C12 myotubes.” *Experimental Cell Research, 313*, 1853-1865.

Fujita, H., et. al. (2011). “Functional evaluation of artificial skeletal muscle tissue constructs fabricated by a magnetic force-based tissue engineering technique.” *Tissue Engineering, Part A: Tissue Engineering, 17,* 107.

Gauvin, Robert, et al. (2011). “Dynamic mechanical stimulations induce anisotropy and improve the tensile properties of engineered tissues produced without exogenous scaffolding.” *Acta Biomaterialia. 7*(9), 3294-3301.

Gillies, A. R., & Lieber, R. L. (2011). “Structure and function of the skeletal muscle extracellular matrix.” *Muscle & Nerve*. *44*(3), 318-331.

Greek, R., Manache, A. (2013). "Systematic reviews of animal models: methodology versus epistemology." *Int J Med Sci, 10*, 206-221.

Grefte, Sander, Anne Marie Kuijpers-Jagtman, Ruurd Torensma, Johannes W. Von Den Hoff. (2007). “Skeletal Muscle Development and Regeneration.” *Stem Cells and Development. 16*(5), 857-868.

Grogan, Brian F., and Joseph R. Hsu. (2011). “Volumetric muscle loss.” *Journal of the American Academy of Orthopedic Surgeons. 19*(3), 35-37.

Grossi, Alberto, Kavita Yadav, Moira A. Lawson. (2007). “Mechanical stimulation increases proliferation, differentiation and protein expression in culture: Stimulation effects are substrate dependent.” *Journal of Biomechanics. 40*(15), 3354-3362.

Harrison, B.C., Allen, D.L., Leinwand, L.A. (2011). “IIb or not IIb? Regulation of myosin heavy chain gene expression in mice and men.” *Skelet Muscle, 1,* 5.

Hettrich, C.M., Browner, B. (2012). “High-energy trauma.” *Best Practices & Research Clinical Rheumatology, 26*, 281-288.

Ito, A., Yasunori, S., Masanori, I., Kazushi, Y., Masahiro, F., et al. (2014). “Induction of functional tissue-engineered skeletal muscle constructs by defined electrical stimulation.” *Scientific Reports. 4*, 4781.

Janssen, I., Shepard DS, Katzmarzyk P.T., Roubenoff R., (2004). “The Healthcare Costs of Sarcopenia in the United States.” *Journal of the American Geriatric Society 52*, 80-85.

Janice L.W. (2011). Technical Bulletin: Equipment Maintenance-Incubators. *North Carolina State Laboratory of Public Health*, (4):2.

Jones, D. A., & Round, J. M. (1990). *Skeletal muscle in health and disease: a textbook of muscle physiology*. Manchester: Manchester University Press.

Jones, T.E., Stephenson, K.W., Kling, J.G., Knight, K.R., Marshall, T.L., Scott, W.B. (2009). "Sarcopenia--mechanisms and treatments." *J Geriatr Phys Ther, 32*, 83-90.

Jovanovic, M., Janjic, Z., Maric, D. (2002). “Principles of management of high energy injuries of the leg.” *Med Pregl, 55*, 437-442.

Judith R., Nithya M., Rose C, Mandal A.B. (2012). “Biopolymer gel matrix as a cellular scaffold for enhanced dermal tissue regeneration.” *Biologica.,* 40(4), 231-9.

Li, Mon Tzu A. Nick J. Willett, Brent A. Uhrig, Robert E. Guldberg, Gordon L. Warren. (2014). “Functional analysis of limb recovery following autograft treatment of volumetric muscle loss in the quadriceps femoris.” *Journal of Biomechanics 47*(9), 2013-2021.

Liao, Hua, and Guang-Qian Zhou. (2009). “Development and progress of engineering of skeletal muscle tissue.” *Tissue Engineering. Part B, Reviews 15*(3), 319-331.

Liu X., Ma P,X. (2004). “Polymeric scaffolds for bone tissue engineering.” *Annals of Biomedical Engineering,* 32(3), 477-86.

Luther, P. (2014, October 14). *Electron Tomography of Muscle Sarcomere*.

Marieb, E. (2012). Human Anatomy and Physiology (9th ed.). Pearson.

Mase, Vincent J, Jr, Joseph R. Hsu, Steven E. Wolf, Joseph C. Wenke, David G. Baer, Johnny Owens, Stephen F. Badylak, Thomas J. Walters. (2010). “Clinical Application of an Acellular Biologic Scaffold for Surgical Repair of a Large, Traumatic Quadriceps Femoris Muscle Defect.”*Orthopedics, 33*(7).

Mestas, J., Hughes, C.C. (2004). "Of mice and not men: differences between mouse and human immunology." *J Immunol, 172*, 2731-2738.

Morley, John E., Richard N. Baumgartner, Ronenn Roubenoff, Jean Mayer, K. Sreekumaran Nair. (2001). “Sarcopenia.” *Journal of Laboratory and Clinical Medicine. 137*(4), 231-243.

Muscle Physiology - Introduction to Muscle. (n.d.). *Muscle Physiology – Introduction to Muscle*. Retrieved September 26, 2014.

Neill, Turner J. and Stephen F. Badylak. (2011). “Regeneration of skeletal muscle.” *Cell and Tissue Research, 347*(3), 759-774.

Nowak, Kristen J., Kay E. Davies. (2004). Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. *EMBO 5*(9): 872-876.

O’Brien F. (2011). Biomaterials & scaffolds for tissue engineering. *Materialstoday*, (11), 88-95.

Paddon-Jones, D., Rasmuseen, B.B. (2009). "Dietary protein recommendations and the prevention of sarcopenia." *Curr Opin Clin Nutr Metab Care, 12*, 86-90.

Park, Hyoungshin, Rajat Bhalla, Rajiv Saigal, Milica Radisic, Nicki Watson, Robert Langer, Gordana Vunjak-Novakovic. (2008). Effects of electrical stimulation of C2C12 muscle constructs. *Journal of Tissue Engineering and Regenerative Medicine 2*(5): 279-287.

Perrin, S. (2014). Preclinical research: Make mouse studies work. *Nature, 507*(7493), 423-425.

Pedrotty, D.M. (2004). "Engineering skeletal myoblasts: roles of three-dimensional culture and electrical stimulation." *Am J Physiol Heart Circ Physiol, 288*(4), H1620-6.

Powell, Courtney A., Beth L. Smiley, John Mills, Herman H. Vandenburgh. (2002). "Mechanical stimulation improves tissue-engineered human skeletal muscle." *American Journal of Physiology-Cell Physiology. 283*(5), 1557-1565.

Quercia, Nada, and Monique Laberge. (2011). *The Gale Encyclopedia of Medicine: Muscular Dystrophy.* Detroit: Gale.

Radisic, M., et. al. (2004). "Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds." *Proc Natl Acad Sci USA, 101*(52), 18129-34.

Rahimov F., and Kunkel, L.M. (2013). Cellular and molecular mechanisms underlying muscular dystrophy. *JCB* (4): 499-510.

"Respiratory Care of the Patient with Duchenne Muscular Dystrophy." *American Journal of Respiratory and Critical Care Medicine, 170*, 456-465.

Sauret, J.M., Marinides, G., Wang, G.K. (2002). "Rhabdomyolysis." *Am Fam Physician, 65*, 907-912.

Sawkins, M. J., Shakesheff, K. M., Bonassar, L. J., & Kirkham, G. R. (2013). "3D Cell and Scaffold Patterning Strategies in Tissue Engineering." *Recent Patents on Biomedical Engineering*. *6*(1), 3-21.

Stern-Straeter, Jens, Frank Riedel, Gregor Bran, Karl Hormann, and Ulrich Reinhart Goessler. (2007). "Advances in Skeletal Muscle Tissue Engineering." *In Vivo (Athens, Greece), 21*(3), 435-444.

Thelen, M.H., Simonides, W.S., and van Hardeveld, C. (1997). "Electrical stimulation of C2C12 myotubes induces contractions and repressed thyroid-hormone dependent transcription of the fast-type sarcoplasmic-reticulum Ca2+-ATPase gene. *Biochem J, 321*(3), 845-848.

Turner, N. J., & Badylak, S. F. (2012). "Regeneration of skeletal muscle." *Cell and Tissue Research*. *347*(3), 759-774.

Vandenburgh, H., Shansky, J., Benesch-Lee, F., Barbata, V., Reid, J., Thorrez, L., et al. (2008). "Drug-screening platform based on the contractility of tissue-engineered muscle." *Muscle & Nerve*, *37*(4), 438-447.

Vandenburgh, H. H. (1988). A computerized mechanical cell stimulator for tissue culture: effects on skeletal muscle organogenesis. *In vitro* cellular & developmental biology, 24(7), 609-619.

Webber M,J., Khan O,F., Sydlik S,A, Tang B,C., Langer R.(2014). "A Perspective on the Clinical Translation of Scaffolds for Tissue Engineering." *Annals of Biomedical Engineering.*

Wolf, M., Dearth, C., Sonnenberg, S., Loboa, E., Dadylak, S. (2014). "Naturally derived and synthetic scaffolds for skeletal muscle reconstruction." *Advanced Drug Delivery Reviews*.

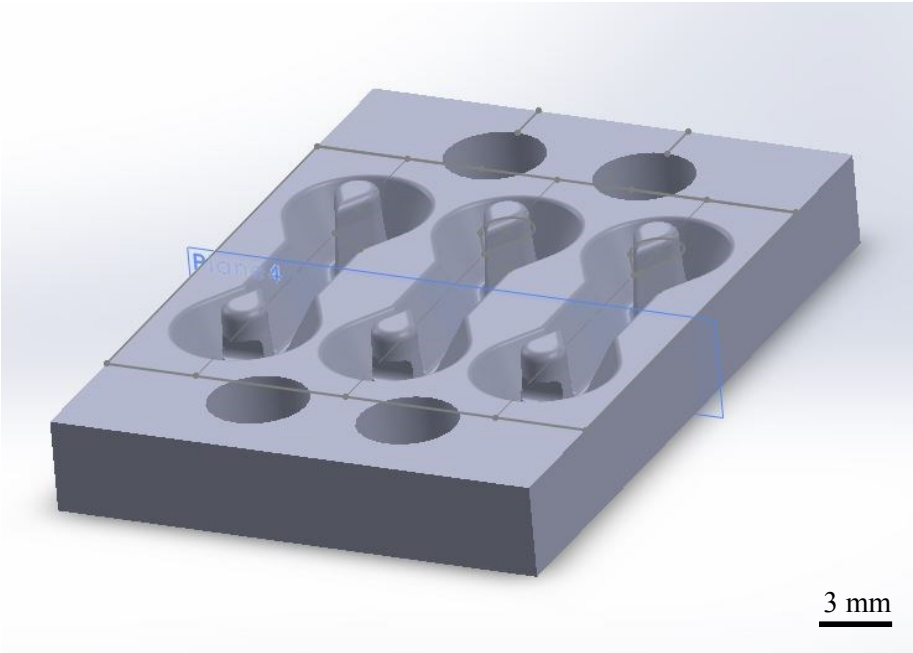
Yamasaki, K., et. al. (2009). "Control of myotube contraction using electrical pulse stimulation for bio-actuator." *J Artif Organs, 12*(2), 131-137.

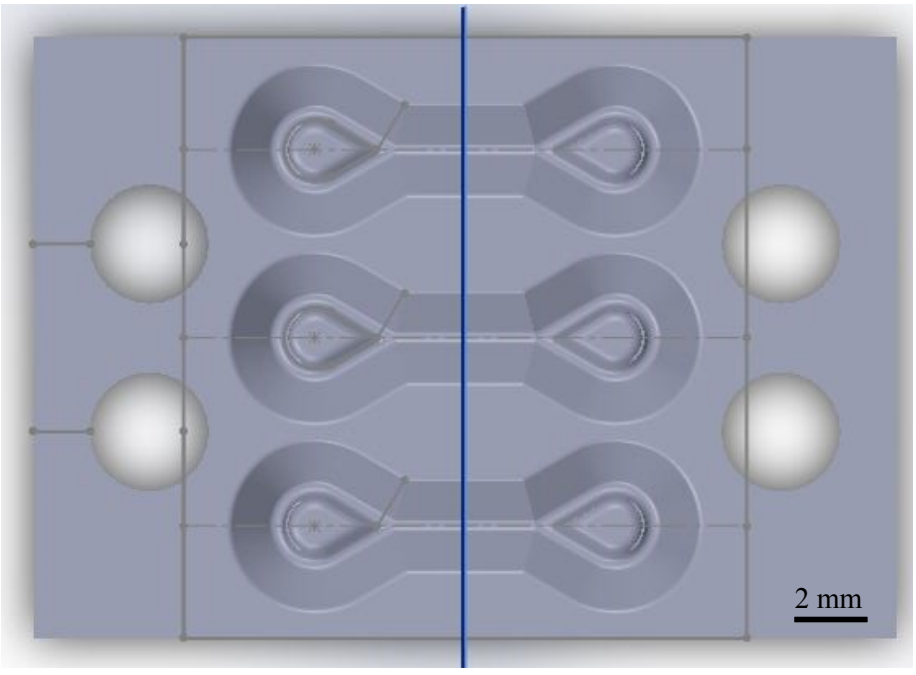
Appendix A: Budget Summary

The team began with a budget of $624. Many prototype materials did not incur costs, and ultimately most costs were encountered during the production of the final device. All encountered costs are documented in the following table:

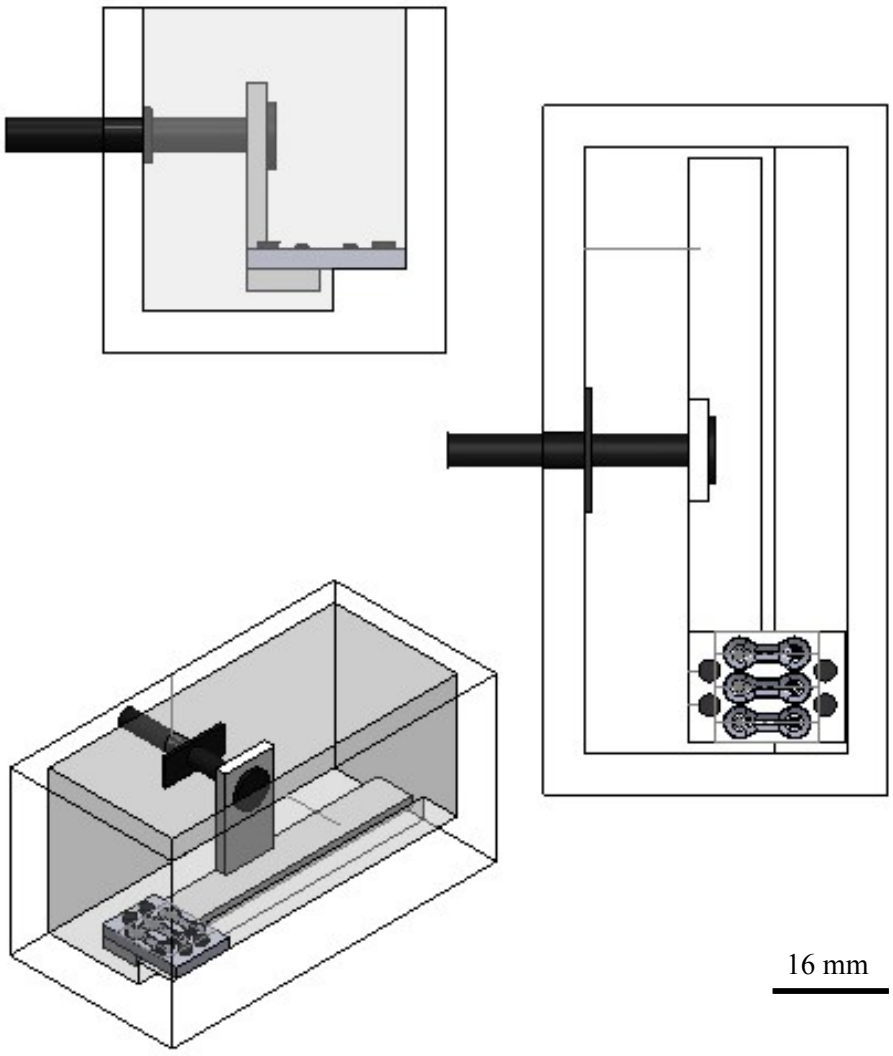
Item	**Cost**	**Budget Left**
Lab fee	$100	$524
Initial molds, ABS plastic	$5.15	$518.85
Bulk polysulfone and polycarbonate	$66.11	$452.74
Mineral oil	$11.06	$441.68
Extended molds, ABS plastic	$9.45	$432.23
Extended molds, MED-610	$4.98	$427.25
Standard 540 screws	$16.11	$411.14

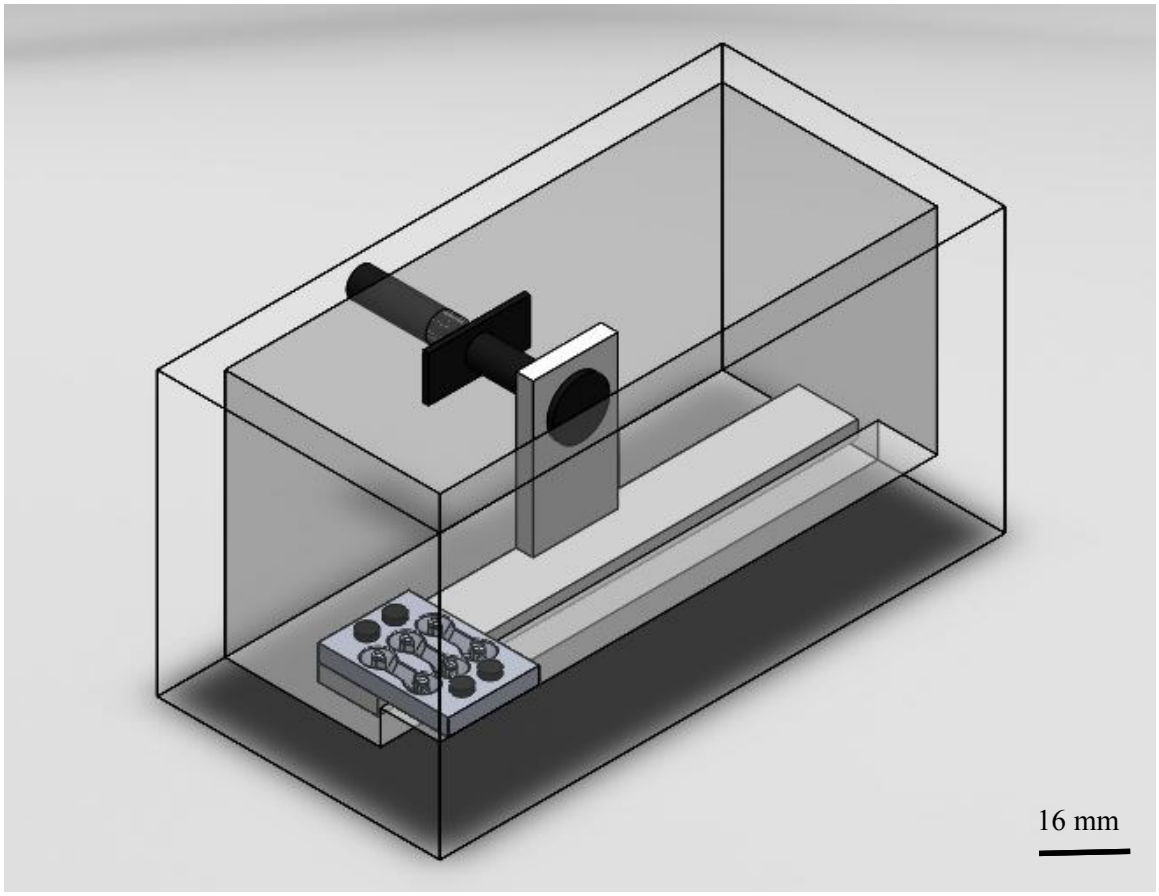
Appendix B: CAD Model, Modified Tissue Mold





Appendix C: CAD Model, Device




16 mm