Skeletal Muscle Tissue Engineering System to Mimic In Vivo Development

A Major Qualifying Project Submitted to the Faculty of WPI in partial fulfillment of the requirements for the Degree of Bachelor of Science

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Abstract

Skeletal muscle atrophy can occur for a number of reasons including degenerative diseases and age-related sarcopenia. Current pre-clinical studies for regeneration therapies are solely reliant on animal models, which do not accurately mimic human tissue and chemistry. The purpose of developing this device was to provide a reproducible manner of creating an in vitro skeletal muscle model that will aid in preclinical therapy testing. The device was designed to maintain a sterile environment for tissue culture, which provides anchorage, periodic strain, and generates an electric field to stimulate contraction. The intended output of the device is the controlled culture of a minimal functional unit of skeletal muscle that surpasses current standards of in vitro and in vivo pre-clinical models.

Executive Summary

Introduction

Skeletal muscle tissue is one of the most prevalent tissues in the human body and is responsible for all voluntary movement. This tissue is primarily composed of cells known as myocytes that fuse during embryonic growth to form mature muscle fibers (Grefte et al. 2007). There also exists a population of satellite progenitor cells in the basement membrane of the tissue. These satellite cells may be activated to repair damaged tissue by re-entering the cell cycle and differentiating into mature muscle cells thus functioning in muscle tissue formation. However, the regenerative capacity of mature muscle tissue is somewhat limited, especially in instances of intense damage such as that caused by severe trauma or extensive muscular atrophy (Grefte et al. 2007). In instances of intense muscle tissue damage the tissue is replaced by a combination of newly formed muscle fibers and collagenous scar tissue, which is weaker than the previously healthy tissue. Muscle regeneration may also be seen to decrease concurrent with aging as the satellite cell population decreases and the cells become less proficient at remodeling tissue.

A consistent means of evaluating clinical therapies is necessary to test and study various treatments aimed at enhancing muscle regeneration. Tissue engineering is a sought-after mechanism of accomplishing this goal as it would provide for simple, reproducible testing and would remove the need to move straight to *in vivo* testing in animal or human subjects. The development of a tissue engineered skeletal muscle construct would ideally be the minimum functional unit representative of a mature muscle and would serve as a platform on which to efficiently assess the effectiveness of therapeutic treatments for muscle regeneration.

Skeletal muscle tissue is constantly undergoing stimulation during and after its development. Muscle tissue is anchored to bone with tendons to assure its security within the body. All voluntary motion is then directed by voltages discharged by motor neurons adjoined to the tissue. Muscle movements involve contractions and relaxations, which may be characterized by muscular stretching that aligns the fibers that make up the tissue **(**Kjær et al. 2006). In order for an *in vitro* tissue engineering skeletal muscle system to work, it must be able to similarly provide

these electrical and mechanical stimuli to the generated tissue constructs, as well as an anchoring point, so that the maturation process mimics that in the body.

Design

The design for a system that would facilitate the development of functional tissue engineered muscle constructs should promote tissue self-assembly without the use of a scaffold to direct myocyte fusion. Myocytes are cued to fuse together with the proper growth factors to incite terminal differentiation. In the body, skeletal muscle tissue is anchored to bone via tendon attachment; this system allows tension to be provided to muscle so that the muscle tissue may strengthen while it matures. The *in vitro* tissue constructs should have points of anchorage as well to provide for this same tension. Finally, the aforementioned electrical and mechanical stimuli are very important considerations in this tissue engineering system to accurately form functional tissues.

Several different designs were brainstormed that would permit tissue self-assembly surrounding points of anchoring and that would also provide for mechanical and electrical stimulation in a controlled manner during development. After comparing the various options to determine the best and most feasible approach within our constraints, the team elected to use a design that actuated with a lever arm protruding through a rubber septum that was inserted into the culture dish lid. The use of a rubber septum allowed for the lever arm to attach to the external motor and also pass through into the dish to actuate the tissue construct while maintaining sterility throughout the interface. The design was further supplemented by two platinum tip electrodes for electrical stimulation.

The complete device design is based upon the use of a 35mm cell culture dish. Within this dish is an actuation system that utilizes two interlocking plates, one of which is held stationary while the other attaches to the lever arm and may move unilaterally. These posts protrude from each of the plates, and serve as points of anchorage for the tissue construct, permitting a linear region of tissue to exist in the midst of the device. The posts were created from polycarbonate to allow for deflection

To direct the formation of the tissue construct, and to mitigate stress concentrations, a mold was designed to promote a consistent cross sectional area throughout tissue construct, while simultaneously allowing the tissue to be anchored about the aforementioned posts. A reusable

PDMS mold was used for the negative mold, which could easily be autoclaved before the creation of a sterile agarose mold. Agarose was chosen for cell culture due to its noted low surface affinity for cell-adhesion, allowing for self-assembly of the differentiating muscle fibers. The mold fits around the posts and enables the motion of the actuating plate to be directly translated into strains within the tissue.

In order to produce controllable, periodic strains, a linear actuating stepper motor was attached to the lever arm. An Arduino microcontroller and driver were placed on a protoboard to allow for facile programming and control of the motor. This set up allowed for easy selection of strain magnitude, as well as rate of application.

Testing and Desired Results

Several tests were developed to test the functionality of the device. The design had to repeatedly and accurately strain tissue to a 10% greater length (+0.5 mm) comparative to its original length (5.0 mm). This was able to be verified through photos and basic measurement tools.

Programming validation for the motor was also a simple procedure. The desired outcome was for the motor to stretch the muscle over 100ms in time, and at a frequency of 2 seconds.

Proof of concept testing was designed to ensure the device would have the intended results. The device was designed to facilitate fiber alignment through processes similar to what occurs naturally within the body. Since the device used two different methods, electric current and mechanical actuation, to align the fibers, facilitated by anchoring posts, a controlled experiment was designed to observe the effects of each method individually. The first procedure called for C2C12 myoblasts to be seeded into the mold without any post anchoring at a concentration of 1 million cells throughout the mold. These cells were proliferated in DMEM with 10% FCS solution, and differentiation would be initiated two days before seeding by using DMEM with 2% horse serum. They were then allowed to develop for two days before seeding into the mold. No stimulation would be given to these control cells, and alignment would be observed under a microscope.

To observe the effects of the anchoring on the muscle tissue development, the cells would again be seeded into the mold using the same culture procedures. This time however, two of the polycarbonate posts would be placed into the mold to allow for the cells to anchor themselves.

Again, no stimulation would be given to the cells. Alignment would be observed under a microscope.

In attempting to test the system as a complete functional unit, a number of design flaws were discovered that ultimately precluded device from working as intended. Of these, the greatest issue found was that the tissue molding system could not easily be removed from the culture dish without damaging or losing the tissue construct. Moderate tissue formation was seen in the dish but this was not able to be successfully stimulated in such a manner to accurately replicate *in vivo development.*

Conclusion

After completion of the project, we were able to determine that the separate mechanisms designed for our system were functional, but further design modifications are necessary to combine them into one working unit. The most prominent issue faced was preserving the safety and integrity of the tissue while extracting the agarose gel mold from the petri dish, an obstacle that could not be overcome within the time limits. However, the tissue molding system, mechanical stimulation mechanism, and electrical stimulation mechanism were all confirmed on individual bases to function as desired. In applying certain key design modifications during future project iterations this design concept should prove to be fully functional and successfully promote biomimetic skeletal muscle tissue formation.

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Chapter 1: Introduction

One of the most predominant tissues in the human body is skeletal muscle tissue. Skeletal muscle accounts for up to 40% of the body's mass and is responsible for all voluntary locomotion (Delbono et al, 2007). Because everyday body function is so heavily dependent on skeletal muscle, loss of this tissue can be detrimental to quality of life.

One of the most astounding features of skeletal muscle is its capacity for regeneration. Minor damage or injuries that occur to the tissue can be repaired by myogenic precursor cells, commonly referred to as satellite cells. These cells, which normally lie dormant in the basement membrane of the muscle, are activated by the release of Hepatocyte Growth Factor upon damage to the muscle fiber (Grefte et al. 2007). Once the satellite cells are recruited to the damaged area, they attach to the site of injury, and differentiate into mature and functional myofibers. The new muscle tissue that forms from this process may be vascularized and innervated to integrate with the surrounding tissue and bring it back to full functionality. Unfortunately, it is in cases of serious damage or trauma, atrophy, or disease, that skeletal muscle is not completely recovered. Fibrous tissue is recruited to the site instead, which greatly limits the functional capacity of the muscle tissue. The end result will be scarring and a partial loss of tissue function.

Efforts to develop therapeutic treatment for muscle loss have led to the field of skeletal muscle tissue engineering. Research in this field attempts to surmount the shortcomings and develop viable means of regenerating muscle tissue. This is accomplished by developing muscle tissue constructs *in vitro* that mimic natural tissue structure and function and may be used in therapeutic testing. However, the field of skeletal muscle tissue engineering is not without flaw. Problems in the field include the difficult procedures that are required to form and subsequently reproduce tissue constructs, and that the tissue does not possess nearly the same strength as does *in vivo* muscle tissue. The complexity of the *in vivo* environment also proves quite difficult to mimic in an *in vitro* laboratory setting, adding additional challenges to this goal.

Many tissue constructs that have been developed *in vitro* are done so on synthetic scaffolds that provide structure and support for the cells. While this technique produces viable tissue, it is not ideal as it does not mimic natural tissue formation, and does not achieve densities

found in human tissue. A more preferable alternative involves directing muscle cells to self-assemble into a myofiber and generate their own extracellular matrix for support.

After conducting a number of preliminary engineering design assessments, a design proposal was developed to address these needs. This design attempts to allow for self-assembly of muscle cells into a fully formed muscle fiber that will then be mechanically and electrically stimulated to direct fiber maturation and test the functionality of the tissue construct. Further research and analysis directed the development of the tissue engineering system design. In this system, a gel mold was used to direct tissue self-assembly in conjunction with the proper and necessary growth factors. The tissue construct was anchored to two posts to provide support and tension during formation. Mechanical and electrical stimulation aided in alignment and maturation.

Chapter 2: Literature Review

Skeletal muscle is a complex tissue system that is vital to everyday human movement. Unfortunately, there are many cases in which this tissue may be afflicted with a number of adverse conditions that damage and weaken it. Due to its limited regenerative capacity, skeletal muscle is not able to fully recover from more intense injury and alternative means of rehabilitating this tissue are required. The overarching aim of this project is to develop a means of accurately recapitulating fully formed skeletal muscle tissue *in vitro*. This chapter examines the background information pertaining to skeletal muscle tissue and clinical efforts to develop muscle tissue constructs for therapeutic testing.

2.1 Muscle Physiology

The human body is composed of three major muscle systems: skeletal muscle: smooth muscle, and cardiac muscle. Controlled by the autonomic nervous system, smooth muscle and cardiac muscle are both involuntary tissues. Cardiac muscle is responsible for contractions of the heart and smooth muscle controls all other involuntary mechanisms of the body, including food digestion and waste excretion. Skeletal muscle, directed by the somatic nervous system, is responsible for all voluntary motion in the body; it governs locomotion and requires cognitive control to function (Buckingham et al. 2003).

2.1.1 Skeletal Muscle Formation and Maturation

Along the neural tube of developing vertebrate embryos are somites, which are divisions of mesoderm that will become the myotome, dermatome, and sclerotome as the embryo develops. Of these, the myotome is the segment that will differentiate into muscle tissue, with the other two forming the dermis and vertebrae, respectively. The epaxial region of the myotome gives rise to the back muscles of the body, and the remaining muscles stem from the hypaxial region. During this time, muscle progenitor cells are seen to delaminate from the hypaxial myotome and migrate to the locations that are to become the dorsal and ventral muscle masses (Buckingham et al, 2003).

The earliest stages of myotome development occur with the delaminating progenitor cells. Delamination, as well as migration, of these cells is dependent on the presence of c-met, a

tyrosine kinase receptor that interacts with hepatocyte growth factor (HGF) to outline the path of migration (Buckingham et al., 2003). The delaminating progenitor cells begin to down-regulate the transcription factor Pax3 and activate myogenic regulatory factors (MRFs), subsequently becoming myoblast cells. The myoblasts then begin expressing a number of different factors that lead to differentiation into myocytes that fuse together to form a continuous layer of muscle fibers that is the myotome (Grefte et al., 2007).

During the formation of the myotome, another group of cells also delaminates and migrates to this same developing region of the body. These cells express Pax3 as well as Pax7 but do not express any of the same myogenic factors present into the cells that are to become myocytes. These cells instead differentiate into the satellite cells of the muscle tissue (Grefte et al., 2007).

2.1.2 Structure and Function of Skeletal Muscle Tissue

The standard muscle cell is known as the myocyte. Myocytes develop from myoblasts during embryonic stages of growth and fuse together to form functional muscle fibers. As a result of this fusion process, myocytes are multinucleated. These cells are composed of many myofibrils, rod-shaped chains segmented into sarcomeres. Sarcomeres themselves are the basic contractile functional units of muscle tissue, and they are made up of contractile actin and myosin myofilaments. The myofibrils are aligned in parallel to form the muscle fibers, with thousands of myofibrils making up each fiber. Muscle fiber structure may be seen in Figure 1.

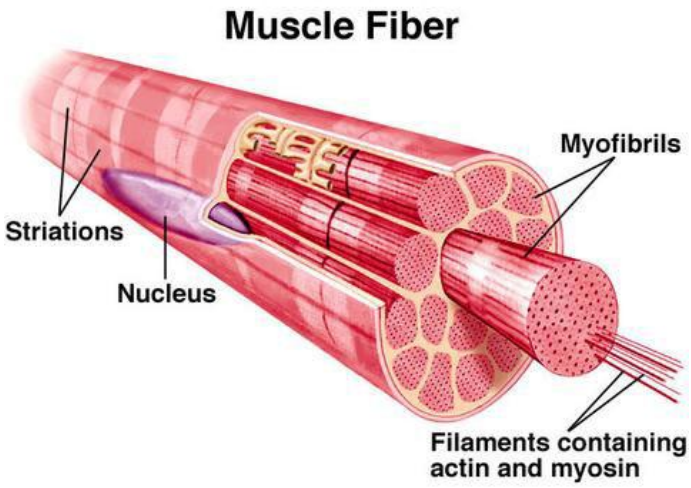

Figure 1: Muscle Fiber Structure (Jeon, JS. Muscle Fiber. 2012.)

Muscular contraction is controlled by motor neurons. At the neuromuscular junction of each muscle fiber, a motor neuron axon attaches to the motor end plate. The neuron generates an action potential that is passed through the axon terminal to the motor end plate. Once sensed, this causes calcium channels to open, allowing for Ca^{2+} ions to pass into the cell. The neurotransmitter acetylcholine is then released by exocytosis and binds to receptors at the motor end plate, opening channels that allow sodium ions to move inward and potassium ions to exit the cell. Different concentrations of sodium and potassium pass through the channels, creating local depolarization that diffuses across the membrane surface and instigates muscle contraction. During this contraction period, the thick and thin filaments pull on one another as regions known as the I-bands and H-zone of the sarcomere shorten, causing the Z-lines to move closer together, as seen in Figure 2.

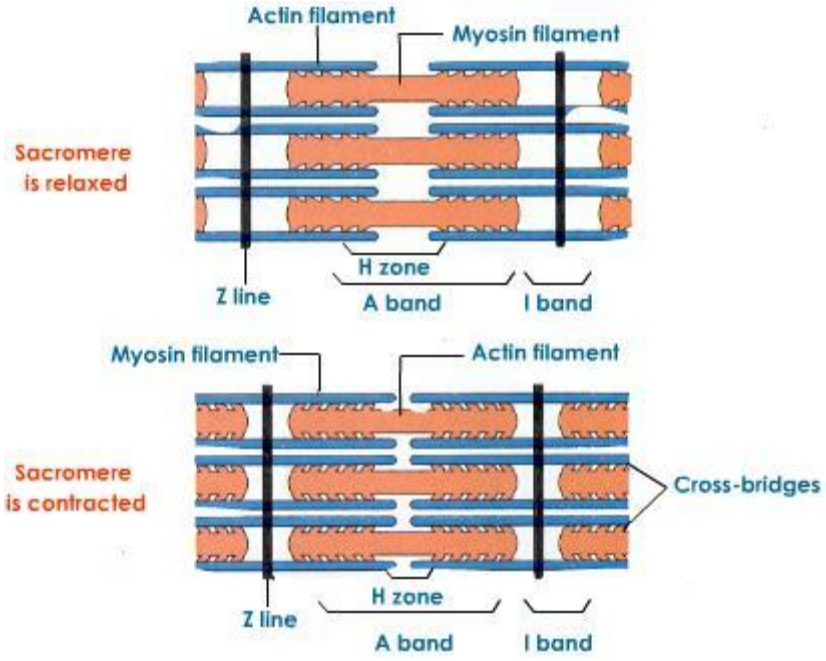

Figure 2: Regions of a Sarcomere during Relaxation and Contraction (Shreastha, 2010.)

While myocytes and satellite cells are considered the primary components of skeletal muscle tissue, there are a number of additional constituents present. Along with the aforementioned motor neurons that stimulate contraction, blood vessels run throughout and supply necessary nutrients to the cells, and there is an extensive extracellular matrix (ECM) that provides a number of functions for the tissue. Specifically, the ECM provides for cell attachment, migration, proliferation, and differentiation. It is also used in bearing loads applied

to muscle tissue and has been implicated to function in response to muscular disease and injury. Three layers of skeletal muscle ECM exist, the first of which is the endomysium. This layer of ECM surrounds individual myofibers and serves as the load-bearing network on these fibers. The next layer is the perimysium; this layer is largely undefined but it is known to surround bundles of myofibers, known as fascicles. Finally, the epimysium encases the entirety of the muscle (Gillies et al., 2011). The coupled structure of skeletal muscle tissue and extracellular matrix is seen in Figure 3.

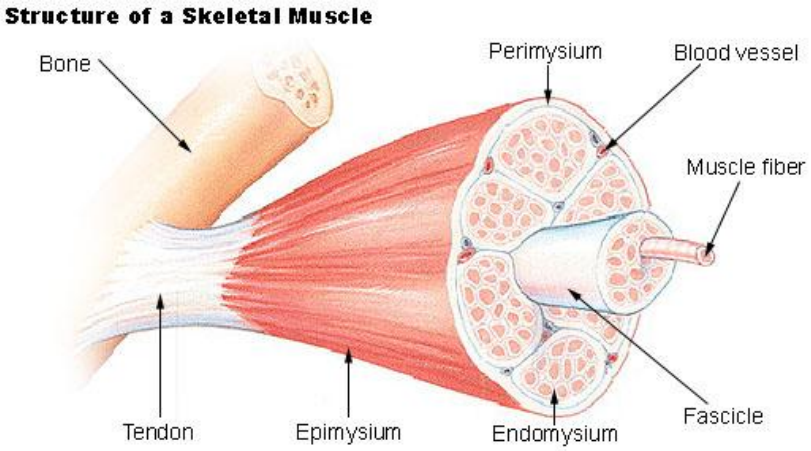

Figure 3: Skeletal Muscle Tissue and ECM Structure (Structure of a Skeletal Muscle, 2012.)

The main structural element of skeletal muscle ECM is collagen, with collagen type I and III being the predominant isoforms in fully developed tissue. A small amount of elastin is also present to provide for elastic behavior in the tissue **(**Kjær, 2003). Several proteoglycans, highly glycosylated protein molecules, have additionally been demonstrated as very important to the development of skeletal muscle tissue and ECM. The proteoglycan decorin, consisting of a glycosaminoglycan chain of chondroitin sulfate and dermatan sulfate, has been proven to interact with collagen and direct the formation of the collagen fibrils. Heparan sulfate proteoglycans such as syndecan and glypican are verified as important in tissue development, with syndecan serving as a differentiation inhibitor that is highly expressed early in cell development and down-regulated later on, and glypican displaying the opposite pattern and helping stimulate differentiation. Additional proteins that have been shown to be important in the development of the ECM are integrins; it has been shown that integrins are largely responsible for directing myoblast migration and adhesion to the extracellular matrix (Velleman, 1999).

2.1.3 Regeneration of Skeletal Muscle Tissue

Whereas many other tissues in the body are fully capable of regenerating themselves over time, skeletal muscle tissue does not possess this same capacity. In the event that the injury to muscle tissue is relatively low and there is minimal volumetric muscle loss, the muscle satellite cells will be able to fully remodel and repair the tissue; if the extent of the damage is too great however, full regeneration will not occur. Regardless, muscle tissue restoration is a multi-step process consisting of degenerative and regenerative phases.

Necrosis of muscle fibers initiates degeneration of muscle tissue. This tends to occur after trauma to the muscle, during which the sarcolemma, the cell membrane for muscle cells, is disrupted. Myofibers then become increasingly permeable and the intracellular concentration of proteins such as creatine kinase rises. It is believed that calcium concentrations in the cell also rise at this point, triggering proteolysis that furthers degeneration. Also occurring during the degenerative phase, inflammatory cells are activated and release signaling molecules that direct neutrophils and macrophages to the site of the injury. These cells are responsible for phagocytosing cellular debris, which include necrotic cells (Chargé and Rudnicki, 2004). Figure 4 outlines the process of muscle regeneration following injury.

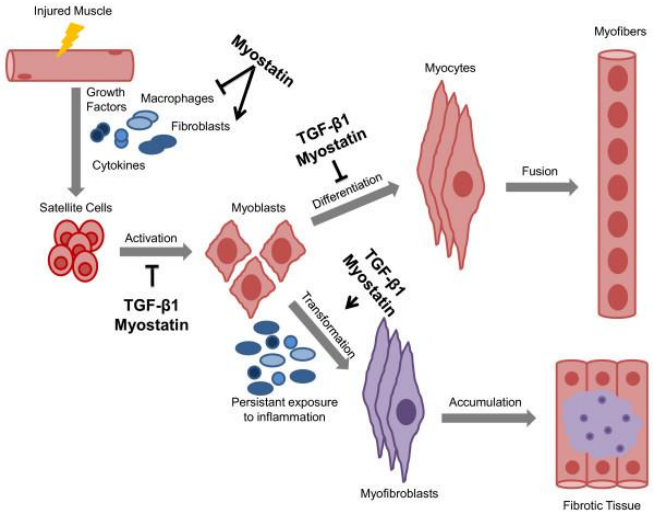

Figure 4: Regeneration of Damaged Muscle Tissue (Burks et al. 2011)

While working to clear the damaged tissue of cellular debris, macrophages also activate myogenic cells in the area. These myoblasts migrate to the site of injury and proliferate extensively. Eventually, Pax3 and Pax7 are down-regulated while myogenin and Mrf4 are up-regulated; some of the myoblasts enter terminal differentiation and the remaining cells return to a

quiescent state. The differentiated myoblasts then fuse to each other in the process of forming new myofibers or fuse to the damaged myofibers in an effort to repair them. Concurrent with this series of events is scar tissue formation, begins with the formation of a blood clot at the initial injury site. Fibroblasts travel to the site of the blood clot and break it down, replacing it with fibrous tissue that is predominantly collagen. This scar tissue provides mechanical strength to the new muscle tissue and provides a point of anchoring for the fibroblasts; however, as fibroblast proliferation gets more excessive, the scar tissue that is formed becomes increasingly dense (Baoge et al, 2012). Muscle fibers formed through this period are smaller and possess centrally located nuclei, which move toward the fringes as the fibers grow and expand. Regenerative capacity in aging muscle is typically less efficient as the number of satellite cells present in the tissue declines and the cells become less adept at remodeling damaged tissue (Grefte et al, 2007).

2.2 Clinical Need, Significance of Tissue Engineering and Skeletal Muscle Applications

In order for this design project to be feasible, a need must be established. Prior scientific research and clinical work has shown that this project is justified in its undertaking and that successful completion will do much to advance contemporary biomedical engineering work.

2.2.1 Tissue Engineering Background

Tissue Engineering is the application of engineering principles to biological cell culture. Tissue engineering requires the use, regulation, and manipulation of various chemical, physical, and other cellular factors for the intended purpose of improving, replacing, or mimicking of biological functions (Bach et al. 2004). In order to research and provide solutions to problems in the medical industry, tissue engineering uses living cells as a tool for the development of new techniques and innovations.

Tissue engineering is a field that covers a broad range of applications. In many industries, tissue engineering is used to create biomimetic tissues that allow for the accurate study of various drug delivery methods, the efficacy testing of pharmaceuticals, or the production of cellular products (MacArthur, 2005). The development of pharmaceuticals for human use requires a substantial amount of testing, including preclinical testing and six separate phases of

clinical testing (Guidance, 2006). Currently, preclinical testing is conducted *in vivo* on animal subjects to begin determining the drug's efficacy, toxicity, and pharmacokinetic information. Due to the ethical concerns with animal testing, as well as the significant differences in biology between humans and other animals, alternatives for animal testing need to be pursued. Tissue engineering proves to be a promising model to accurately test pharmaceuticals *in vitro*. Although refinement of these processes is still necessary, the tissues created are being constantly improved to better represent the human *in vivo* environment.

Engineered tissues are also being developed for the purposes of regenerative medicine. Regenerative medicine is the process of restoring or establishing normal functionality of biological systems through the replacement of cells, tissues, or organs (NIH Fact Sheet, 2006). Each year, approximately seven thousand deaths are reported to the U.S. Department of Health & Human Services for patients awaiting the availability of a viable organ for transplant (Waiting List, 2008). These are examples of lost lives that could have been avoided with ample resources and well-developed engineered organs. Today there are several examples of commercially available engineered tissue products. One such example is Dermagraft, a cultured dermis layer which is used to treat and begin the healing process for foot ulcers in diabetics, a symptom which affects approximately 600,000 patients each year (Gentzkow et al., 1996).

2.2.2 Clinical Significance of Skeletal Muscle Tissue Engineering

Several diseases and conditions have proven the significance of tissue engineering as a field. As there is no cure for many of these diseases, tissue engineering offers potential hope to many of these hopeless ailments.

Muscular dystrophy, commonly referred to as MD, is a group of debilitating disorders that leads to muscle weakness and loss (Muscular Dystrophy, 2012). MD is inherited and involves an aberration in the genetic code relating to certain proteins involved in muscle function and structure (Bushby, 2000). Muscular dystrophy affects approximately one in every 3,500 individuals and has symptoms which include drooling, frequent falls, trouble walking, drooping eyelids, and loss of muscle volume. Currently, there is no cure for muscular dystrophy, and physical therapy serves as the only the treatment for its symptoms.

Malignant tumors in skeletal muscle are called Rhabdomyosarcomas (Rhabdomyosarcoma, 2012). Typically diagnosed in children and teens, about two-thirds of

those affected are under the age of ten. Tumors can be found in the extremities, neck, trunk, and head (such as in nasal cavities, in the throat, or muscles around the cervical spine). Treatment of this soft tissue tumor, if malignant, is often a combination of radiation and chemotherapy; surgical removal is often necessary if initial treatments fail. When surgical removal of the tumor is required, surrounding tissue is often extracted during the open surgery, this results in significant trauma and muscle tissue damage.

Sarcopenia is a third cause of muscle loss which occurs during the natural process of aging. Aging, an inactive lifestyle, kidney disease, heart disease, diabetes, or changes in hormones can increase risk of muscle atrophy. Sarcopenia leads to weakness and frailty, which may incur an increase in falls and traumatic injury (Northwest Primetime, 2012). Currently, sarcopenia due to aging is not curable, but can be prevented or reduced through healthy diet, as well as consistent exercise.

Despite muscle tissue's ability to heal minor wounds, traumatic injuries can damage the structure of skeletal muscle beyond natural and biological repair. Muscle tissue injuries of this degree include lacerations, which are common, as well as crushing, which can lead to rhabdomyolysis, the rapid breakdown of muscle tissue (Rhabdomyolysis, 2012). Traumatic injury affecting muscle is a common injury among military personnel. An autograft, tissue transfer from another viable source of the same patient, is commonly used to help aid in the repair of traumatic injuries. Unfortunately, additional damage to the patient is done in order to harvest the tissue graft, which leads to donor site morbidity and additional muscle loss (Extremity Trauma, 2006).

2.2.3 Need for in vitro Skeletal Muscle Model

Within the medical field, there is a serious need for a model that accurately mimics the *in vivo* tissue properties of skeletal muscle. A 3D skeletal muscle tissue model which accurately represents the *in vivo* environment would give researchers the opportunity to perform tests and initiate preclinical trials on new treatments for debilitating muscle diseases. Currently, research surrounding the development of 3D models for *in vitro* skeletal muscle tissue is being performed, but an optimized gold-standard predicate has not yet been created.

Preclinical trials for pharmaceuticals and other treatment options are currently performed on animal models which gives rise to a couple of major concerns. A first concern is the

anatomical and physiological differences between humans and other animals, making them an imperfect model for these tests. When preclinical tests are performed on an animal, the focus on the study is to gain knowledge about a drug's nonhuman efficacy, pharmacokinetics, and toxicity information. Unfortunately, animal models cannot be used to accurately predict human biochemical reactions. Another concern with these preclinical studies involves the ethics surrounding animal research. The large amounts of animals sacrificed for medial testing procedures gives rise to concern amongst animal activists and scientists alike. Therefore, a biomimetic *in vitro* model would be one solution which would accurately represent the conditions within the body and allow for more accurate data collection without raising any ethical concerns. Such a model would allow for accurate evaluation of insipient pharmaceuticals, but the development of this model also comes with many challenges and limitations which must first be overcome or designed around.

2.3 Potential Methods and Materials

There are many possible ways to develop a device; however there are distinct benefits and setbacks associated with each method. By gathering as much data as possible, we were able to choose the technique that worked best for the group and the constraints presented by the project.

2.3.1 Tissue Scaffolds

Since the inception of tissue engineering as an independent research field, tissues have mainly been developed amid artificial scaffolds. These scaffolds are made from the same materials that constitute natural extracellular matrix and attempt to mimic the structure and function of the natural ECM. Necessary for consideration in development of an artificial scaffold are material and mechanical properties, porosity, and architecture amongst other concerns (Lim, 2012). Though these scaffolds have been used for many years in all ranges of tissue culture and are able to effectively imitate natural ECM, the use of these structures has yet to be perfected and they still suffer from numerous drawbacks, including that artificial scaffold use does not allow for accurate replication of the natural tissue formation seen *in vivo*.

This project aims to develop a naturally forming skeletal muscle tissue construct, and implicit within this criterion is that no artificial scaffold should be used. Current scaffolding techniques were researched to provide a foundation of knowledge on the strengths and

drawbacks of this technique as well as to develop alternative means of providing structural support and developmental cues for our maturing tissue.

2.3.1.1 Gels

Gels have been used in tissue engineering for quite some time. Materials such as PDMS, agarose, and other hydrogels all have very similar likeness to body tissue in terms of architecture, mechanical properties, and potential porosity. Hydrogels in general are water-based, and absorb this water due to their cross-linked network of polymers and their ability to encapsulate water between molecules. (Ahmed, 2008). They can be used as scaffolds to develop 3D tissue constructs as well as vessels for growing cells. A specific use of PDMS as a scaffold would be to create a porous architecture to populate with bone cells in order to create a bone scaffold. This material would be biocompatible and allow cells to proliferate and differentiate properly inside of the scaffold in order to better integrate with the body (Hayakawa, 2003). Unfortunately, PDMS is time consuming to create and can be more labor intensive than needed.

Agarose can be used in a similar manner by creating a porous construct of the material and shaping it as needed. In one study of linear axonal regeneration, agarose gel was used as a scaffold to promote linear growth and arrangement (Breckon, 2006). This material is easy to use and also easy to sterilize. Media, growth factors, and other culture elements can be added to the agarose construct, making it an attractive choice as a scaffold.

2.3.1.2 Collagen

Collagen is another potential scaffolding material for tissue engineering. This material, along with fibrin, is a natural material that integrates well with tissue. In the extracellular matrix of many cells, collagen is a predominant factor that contributes toward mechanical stability of cell attachment. It is widely recommended for use in tissue engineering because of its molecular structure that is extremely compatible with cell engineering (Efrimescu, 2011). Collagen itself can be used as a scaffold, or it can be combined with gels in order to make a sturdier imitation matrix.

2.3.1.3 Fibrin

Fibrin is made of base units called fibrinogen that have a tendency to self assemble in a fashion that is relatively stable. Fibrin hydrogels have been widely used as a scaffold for adipose,

cardiovascular, ocular, liver, skin, and other types of tissue engineering. These gels have the ability to enhance angiogenesis as well as neural extension (Ahmed, 2008).

2.3.1.4 Disadvantages of Scaffolds

Though scaffolds promote tissue growth and formation, they also hinder the potential in tissue engineering. Unfortunately, having an externally synthesized material that is foreign to the body will never work as successfully as natural body tissue composition. Therefore, the body will never fully integrate many of the scaffolds into its system without some potentially adverse side effects.

Hydrogels have three main disadvantages. Shrinkage of hydrogel will cause the tissue around the scaffold to deform as well. While this might not affect the tissue growth immensely, it has potential to develop tissue in an unnatural fashion, which may cause side effects once implanted in the body. Gels also have low mechanical stiffness, which can be disadvantageous when used in load bearing applications. Finally, gels have a tendency to degrade prematurely before tissue growth and development have completed. All these problems can be treated, but not fully resolved (Ahmed, 2008).

Many polymeric scaffolds also do not offer the proper mechanical properties for their intended use. Therefore, if this is a load bearing structure, and the elastic modulus is higher than the surrounding tissue, this may cause stress shielding, killing the cells around the scaffold. Ultimately, this may do more damage than good if too many cells are killed. Degradative side effects of certain scaffolds may also be of concern (Ma, 2004). Some materials release acidic or basic byproducts as they degrade and alter the pH of the surrounding environment. Others may release toxic ions. These are all things that must be considered when using scaffolds.

In essence, while in many scaffolds can be useful, a more natural method of tissue engineering is in need. This is the force that has fueled the development of 3D tissue engineering.

2.3.2 Creating 3D Tissue Constructs

One specific instance with similar goals to this project was carried through by co culturing nerve cells with fibroblasts to create a 3D construct. The fibroblast cells were seeded onto a plate coated with laminin, and then neural cells were then seeded atop this layer. When nerve cells migrated and created a network that covered about 70-80% of the plate they were

seeded in, the media was changed and TGF- β was added. The layers were pinned down, and within a few days, a 3D construct was created (Adams 2010).

2.3.3 Mechanical Stimulation

To produce an accurate engineered tissue construct, it is important to mimic *in vivo* conditions as closely as possible. Skeletal muscle tissue in the body constantly undergoes cyclic strain during both the development period and in everyday use as mature tissue. Therefore, cyclic mechanical stimulation in engineered muscle tissue is important to the proper development of the tissue. Studies have shown increased cellular proliferation, myofiber organization and extracellular matrix (ECM) synthesis through the use of mechanical stimulation. Mechanical stimulation of monolayer skeletal muscle cultures have also been known to affect gene regulation, endogenous protein expression, protein accumulation, protein localization, and metabolic activity in a manner consistent with changes that occur in vivo (Powell et al. 2002).

The regimen of mechanical stimulation has been proven to make a difference in the degree of proliferation and maturation of the muscle tissue. The percent strain as well as the duration of cyclic strain is each affects the development of the muscle tissue in different ways. Many studies have been conducted with strain percentage varying from 5% to 25%. All these studies oriented the muscle tissue and help muscle development including increased elasticity, satellite cell activation and myofiber area (Powell et al. 2002). However, only the most recent studies with 10% strain induced both proliferation and differentiation (Grossi et al. 2007). These studies called for 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 (Grossi et al. 2007). Another study strained the tissue for 5 minutes every hour for 5 days (Moon et al. 2008). Mechanical stimulation is very important to the development of in vivo skeletal muscle tissue and must be accomplished in order to mimic *in vivo* muscle tissue. The results of a study by Moon et al. are displayed below:

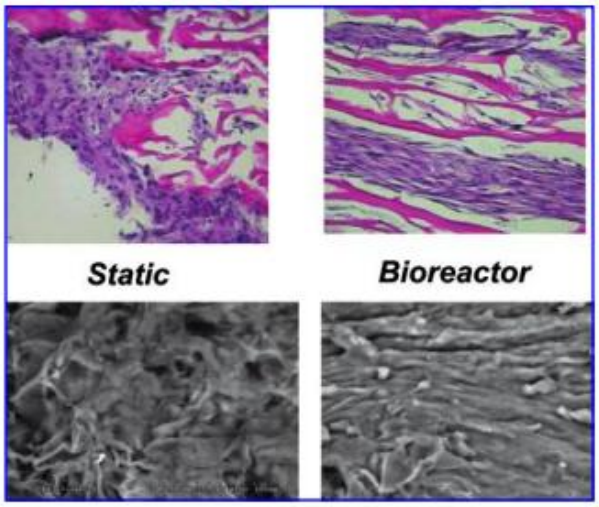

Figure 5: In Vitro Myofiber Development. Static development is displayed on the left side, and mechanically stimulated muscle is shown on the right. SEM imaging was used for the bottom images and H&E stain used for the top images. (Moon et al. 2008).

2.3.4 Electrical Stimulation

In vivo muscle tissue matures not only because of mechanical stimulation, but due to neuron activity in the tissue as well. Likewise, *in vitro* muscle tissue needs some form of neuron-like stimulation to properly mature and align the fibers. An electrical voltage can simulate this neuron activity to a certain degree. Without electrical stimulation the muscle tissue can become weak and necrotic due to its lack of alignment (Thelen et al. 1997). On the contrary, muscle tissue enhanced with electrical stimulation during development have shown greatly accelerated maturation (Langelann et al. 2010). An experiment preformed by Fujita et al. proved that alignment was also greatly improved through electrical stimulation. The results of this experiment are shown below:

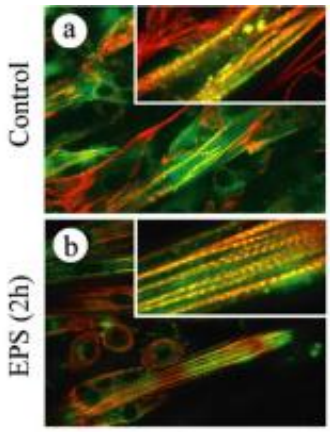

Figure 6: Myofibers enhanced with an electrical stimulation regimen. The top figure displays myofibers developed without stimulation, and the bottom figure displays fibers that have aligned better as a result of the stimulation (Fujita et al., 2007).

Much like mechanical stimulation of *in vitro* muscle tissue there are many different factors to consider in developing an electrical stimulation regimen. These factors include duration, voltage, amperage, and timing. The roles of these factors, however, are not completely understood and a template for electrical stimulation for maturing muscle tissue does not exist. Studies electrical fields have ranged from 0.6 V/cm to 8.3 V/cm. The duration is equally different ranging from 14 days to 80 seconds. A summary of the studies is below in Table 1.

Table 1: Summary of Electrical Stimulation Studies

Reference	Summary
Thelen et al. 1997	Used C2C12 2-D structure in an electric field of 3 V/cm^3 with a pulse frequency of 2 Hz and duration of 6 milliseconds. Stimulation was continued for 13 days and increased expression was shown.
Pedrotty et al. 2005	Used Rabbit Myoblasts in 3-d PGA structure with an electric field of 0.6 V/cm. A pulse frequency of 0.5-10 Hz and duration of 0.5 -250 ms was used for 14 days. This showed increased proliferation but had no effect on differentiation.
Fujita et al. 2007	Used a 2-D C2C12 structure in an electric field of 6.7 V/cm. A pulse frequency of 0.1-10 Hz and duration of 24 ms was used for 1-9 hours 8 days after differentiation. This accelerated sarcomere assembly.
Yamasaki et al. 2009	C2C12 grown with collagen for 2D and 3D in an electric field of 8.3 V/cm. A pulse frequency of 0.5-10 Hz and duration of 10 ms was used for 80 seconds of stimulation 6 and 12 days after differentiation. The contractile performance of the tissue was similar for both the 2D and 3D tissues.

Electrical stimulation aids in the progression of skeletal muscle tissue as seen from the above table. However, more research must be conducted to create a protocol to help tissue mature and mimic *in vivo* tissue.

Chapter 3: Project Strategy

The goal of this project was to design and model an *in vitro* skeletal muscle tissue mold and stimulation device that mimics *in vivo* muscle tissue. This chapter outlines the steps taken to define, prioritize, and address the goals of the client. In order to revise the initial client statement the team identified and prioritized objectives, functions, and constraints that must be fulfilled for the project to be successful. Using the defined objectives, functions and constraints the initial client statement was then revised. Finally, the project approach was defined by the group.

3.1 Initial Client Statement

An initial client statement is often broad, unfocused, and includes an implied solution (Dym, 47). The project team's first step is to clarify the client's needs by asking questions. Dr. Raymond Page prepared client statement for the team read:

> "Currently, the laboratory uses extruded fibrin microthreads with human skeletal muscle derived cells seeded onto the surface and transplanted into SCID mouse skeletal muscle injury models to study the effect of various cell derivation and culture methods on functional tissue regeneration. The use of animals is time consuming and costly which severely limits the number of parameters that can be evaluated. Currently, the microthreads are produced first and then cells with myogenic potential are seeded onto the microthreads using a rotational cell seeding system. The limitations of this system include the ability to only achieve a cell density limited to the surface area of the microthreads and the system is not compatible with long term culture to evaluate the differentiation potential of the cells *in vitro*. For cylindrical tissue such as skeletal muscle fibers to form, the cells must degrade the microthread material and proliferate and migrate into the core. The proliferation phase of the cell cycle is not compatible with the quiescent phase required for cell fusion and matrix synthesis needed for skeletal muscle tissue formation. This could to lead to premature breakdown of the tissue structure before the seeded cells can synthesize new matrix. An optimal situation would involve a system where cells could be seeded at the density required for cell fusion and tissue formation. However, the current microthread production process involves a stretching and drying step to produce axially aligned fibers, which is not compatible with seeding the cells within the microthreads at the time of formation.
>
> 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 through the initial client statement the team met with the client and asked questions to clarify the problem and the goal of the project.

3.3 Design Objectives

After revising the client statement and refining the desired outcomes of the project, a process in which our design would meet these outcomes was formulated. First, the desired outcomes were defined as objectives, functions, or constraints. According to Dim & Little's "Engineering Design", design objectives are defined as characteristics desired by the client, and are used to create goals and ensure the quality of the design (Dym, 6). Objectives for this project were organized into main and sub objectives.

3.3.1 Tailored Objectives

The main and sub objectives were determined to be:

- Allow for the creation of scaffold-less 3D tissue construct
    - Support cell proliferation
    - Support cell specialization
    - Mimic biological tissue structure, directionality
    - Provide anchor points for muscle tissue attachment
- Mimics *in vivo* tissue
    - Mechanically stimulate tissue
    - Electrically stimulate tissue
- Results should be reproducible, consistent, and of high quality
    - Fiber size
    - Fiber strength
    - Fiber alignment
    - Culture conditions
    - Electrical stimulation magnitude, frequency, and duration
    - Mechanical stimulation magnitude, frequency and duration
- Able to be sterilized
    - Temperature resistant materials
    - Open design for steam to contact surfaces
- Able to be biocompatible
    - Desirable material surface properties
    - Prevent cell adhesion except where desired
    - Non-toxic
- Sufficiently user-friendly
    - Touch-points are clearly labeled
    - Automation of repetitive tasks
    - Clear instructions
- Low Cost
    - Manufacturing cost
    - Operating cost

In order to better understand the relative importance of each objective and establish a set of priorities, a pair-wise comparison chart was utilized. A pair-wise comparison chart generates a quantitative weight of importance for each object by comparing individual objectives against others. The pair-wise comparison chart, viewable in Appendix A, produced a prioritized list of our main objectives. From this chart, it was determined that the most important objective of our design would be the creation of scaffold-less, three-dimensional muscle tissue. Prioritizing the objectives allows for the design to better suit the needs of the client, and creates a foundation to generate solutions upon. An objectives tree was created to visually show the objectives and can be seen in Appendix B.

3.4 Constraints

The team devised the following list of constraints from the initial client statement and guidelines for the project:

- Must fit into an incubator
- Must be workable at physiological conditions
    - Mechanical components should retain function at incubator conditions (37 °C, 5% CO_2, 95% humidity)
- Must be able to be developed and machined with materials that are biocompatible and readily available
- Must be able to be sterilized or developed using pre-sterilized components
- Must remain within allotted MQP budget ($524)
- Must be completed within the MQP timeframe (25 weeks)

In order to sustain cell growth and development, the tissue and device must be kept in an incubator. Thus, it must fit the size constraints of the incubator and the materials must maintain their integrity at the incubation conditions of 37 °C, 5% CO_2, and 95% humidity. The materials used, specifically those that are coming into regular contact with the tissue construct, must be made from biocompatible materials that are easy to obtain and manufacture. In order to prevent contamination, the device must be constructed in a sterile manner or must otherwise be able to be sterilized, likely via autoclave. Finally, the project itself has a limited budget of $524 and timeframe of 25 weeks, so it must be completed within these parameters.

3.5 Revised Client Statement

After meeting with Dr. Page and creating and evaluating the objective, functions, and constraints the team created a revised client statement. The statement illustrated the desired outcome of the project in a short, concise statement and eliminated any bias from the client.

"Design an in vitro system to permit the development of a three-dimensional skeletal muscle tissue construct without the use of a scaffold. The system should be constructed to allow for mechanical and electrical stimulation during the tissue development process to aid in maturation."

3.6 Project approach

To provide direction for the completion of this project, the team developed steps toward building and testing a successful device. Although smaller accomplishments will be determined along the way, these steps serve as a basic outline to keep the team on track and successful.

3.6.1 Develop Cell Culture Techniques

Proper culturing techniques need to be developed to ensure cells are alive and functioning. Procedures that will allow for both proliferation and differentiation are required for successful cell culture. C2C12 cells are typically cultured by thawing the cells and then seeding them at about 1.5 million per flask, when using T75 flask. They are allowed to proliferate to about 50-60% confluency before being passaged (Quinn, 51).

Once cells have proven to proliferate, and are ready to be installed into the device, a culture technique that involves differentiation was needed. This allowed for maturation before seeding cells into the device so that they would not be completely shocked once placed in an environment that promoted differentiation. Ideally, a method to create a co-culture between fibroblasts and myoblasts will be used in order to create a natural ECM for the myoblasts to attach themselves to.

3.6.2 Develop Gel Mold

A gel mold is needed to provide structure for the development of the tissue construct. This mold will have to resist cell adhesion in order for a 3-D muscle construct to form. Ideally, this tissue will develop in a shape that would allow for anchoring to assist with mechanical stretching. This mold will also have to enable fibroblast and myoblast co-culture.

Mold development occurred in several steps. Well shape was considered to properly develop a shape that best enhanced fiber development. Agarose percentage was also addressed to ensure an appropriate consistency for the cells to develop upon. Finally, a method of mold production was developed to ensure a sterile mold can be created for cell culture.

3.6.3 Develop Mechanical Design/Procedures

The goal of this device is to both mechanically and electrically stimulate muscle cells, just as if they were in the body. Mechanical properties of muscle cells *in vivo* generally have shown that they experience an average of 10% strain during regular exercise (Moon, 2008). Regular exercise not only aligns and develops muscle fibers, but also helps to differentiate muscle cells that have yet to develop into mature myofibers. Using this knowledge, a method of stretching these muscle cells must be created in order to stimulate muscle growth. Although a 10% strain is definite, the frequency and length of each cycle needed to be determined through results found in research and experimentation.

3.6.4 Develop Electrical Design/Procedures

In order to simulate the process of a nerve giving a muscle cell an electrical signal, an electrical system was created that worked in conjunction with the mechanical system developed. This will work synchronously to send an electrical charge to the muscle during the mechanical stimulation. A regimen was developed through research and experimentation to determine frequency, voltage, and length of stimulation. Proper material to construct the electrodes was also found in order to prevent corrosion and therefore media contamination. Once this has all been determined, validation of the system was conducted to ensure its function.

3.6.5 Proof of Concept Testing without cells

After the main structure is developed and functioning independently, the device will be tested before seeding cells. This will ensure that the mechanical and electrical aspects of the project are working. Seeding the cells would cause additional unwanted variables in our testing so before they are added, individual proof of concept testing was necessary. This was accomplished by using a small rubber band to simulate stretching cells, surrounded by bubbling media due to the electrical voltage.

3.6.6 Total Proof of Concept Testing

After we proved that the mechanical and electrical aspects of this device were functioning properly, cells were seeded into the device. This final round of testing would demonstrate all features of the device and attempt to form fully functional muscle tissue. From here, adjustments were made to the device to make sure everything functioned as intended.

Chapter 4: Design Alternatives

4.1 Introduction

A revised client statement further clarifies the objectives of this project, but does not lead to a single desirable solution. There are many possible solutions that exist, each with tradeoffs of strengths and weaknesses. In order to ensure that the solution with the best means of operation is created, the project group generated multiple variations of different aspects of the design, and compared the methods in which they work. In order to generate a large number of designs, different brainstorming techniques were used.

4.2 Needs Analysis

In order to begin brainstorming solutions within the scope of this project, the proposed functions of the design were analyzed. A feasibility assessment was conducted on the list of functions to determine which functions were deemed necessary and which were thought to be less important.

The design 'needs' list consists of all functions considered to be necessary to the success of this project:

- Allows for and sustains tissue growth and differentiation
- Mechanically strains tissue
- Electrically stimulates tissue
- Allows for analysis of tissue construct
- Supports myoblast and fibroblast co-culture to allow for natural ECM formation

Further, the following functions were identified with this design to be 'wants' that are not critical to this project's success but that would add more to its functionality and accomplishment:

- Accurately measures stimuli applied to tissue

The functions listed in the needs column are deemed as such because they are vital to this project's success. It is necessary that this design be able to allow for cellular growth and formation into a viable piece of skeletal muscle tissue. In order to allow for cell alignment and maturation, mechanical and electrical stimulation are needed. While it is expected that the design be able to illustrate the strains and impulses imparted upon the tissue, it is not thought to be essential that these numbers be conveyed in real time. As a means of determining the efficacy

of the tissue formation, analysis, especially through immunohistochemistry, is required. The extracellular matrix is an important consideration in developing a truly biomimetic construct, and as such it is important that myoblasts and fibroblasts are able to be co-cultured in this system so that the fibroblasts may generate this ECM. Finally, it was deemed a desirable but not necessary function to be able to accurately measure the stimuli applied to the tissue. Especially in the case of stress, it would be incredibly challenging to accurately measure such small amounts of stress in the sterile, aqueous culture environment. While some stimulus parameters may be able to be controlled and measured, it is not feasible within the constraints of this project to expect that for all factors.

4.3 Functions-Means Analysis and Design Specifications

Another organized brainstorming technique that the team utilized was a Functions-Means chart. This allows the team to pair the necessary functions with many potential viable solutions. The output of this brainstorming exercise is viewable below, in Table 2.

Table 2: Functions-Means Chart

Function	List of Means				
Tissue Shape Design	Tissue Ring	Rectanglular tissue	Dogbone Mold		
Sterile Environment for Samples	Petri Dish	6-Well Plate	Custom-Made Rectangular	Accordion Petri Dish	
Anchors Tissue	Hydrogel Posts	Metal posts	Polymer posts	Hooks from top	
Apply Mechanical Strain	Reciprocating Cam	Hydraulic Piston	Rack & Pinion	Stepper Motor	Magnets
Stimulate Contraction	microelectrodes	Co-culture w/ neurons	Chemically Induced (Glutamine)		
Prevent Cell adhesion to surface	Free-floating in media	Agarose gel	PDMS gel		
Measure Contractile Stress/Force	Force Transducer (LabView)	Spring of known k-constant			
Measure Proliferation/Cell Counting	BrdU Stain	Nuclear Stain			
Measure Strain/Contraction Displacement	Calipers (Digital)	Marked Lines	Image J		

Using this chart, different means for each function could be combined to create designs that would sufficiently meet the requirements of the client. Following this, designs could be compared to determine which would meet the client's requirements most accurately and

completely. A tissue shape would need to be chosen and kept consistent between samples to reduce variance in testing. Consistency between samples will be vital to the collection of usable data and to the overall success of the device. This tissue sample would need to be kept in a safe and sterile environment that is able to maintain media and other materials necessary for tissue culture.. A surface that does not promote cell adhesion should be chosen as adherence to the surface would prevent cohesive fiber formation. Finally, the tissue would need to be anchored to provide tension during formation and to permit stimulation, which would allow for testing to be performed on the sample.

In order to test the tissue samples, mechanical strain will need to be applied and the overall length of the tissue sample will need to be stretched to 10% strain. This specification was chosen for all of our designs due to the large number of previously conducted studies proving successful cell alignment, proliferation, and specialization using this level of strain. In addition to strain testing via linear actuation, the tissue will also need to be stimulated in order to contract as normal muscle tissue does. Once again, measurements will need to be taken to learn more about the tissue sample during testing, and more functions involving data collection are required. These functions include the ability to determine the displacement occurring from contraction as well as the contractile force produced by the tissue. Finally, after this testing is complete, the tissue will need to be observed to measure changes in proliferation, cell specialization, as well as any changes in general tissue structure.

By creating a list of the functions required for a successful design, multiple designs could be brainstormed effectively. The creation of design alternatives is crucial to preventing design failure and ensuring that the design chosen is both feasible and produces the best results. In the following section, each of the design alternatives are discussed in further detail.

4.4 Design Alternatives

The following four designs were created and analyzed for their ability to satisfy the objectives and constraints given by our client. These four designs include a moveable magnet, a hydraulic syringe pump, a moveable lid, and a lever attached to a moveable plate.

4.4.1 Design Alternative 1 - Magnet

Last year, the two project teams that attempted to tackle this same problem attempted it by way of magnetic connections between the inside and outside of the culture dish (Aschettino et

al 2012; Gunnell et al 2012). Thus, the first proposed design alternative this year uses the same techniques and attempts to expand upon those designs. This proposed design may be seen in Figure 1 below.

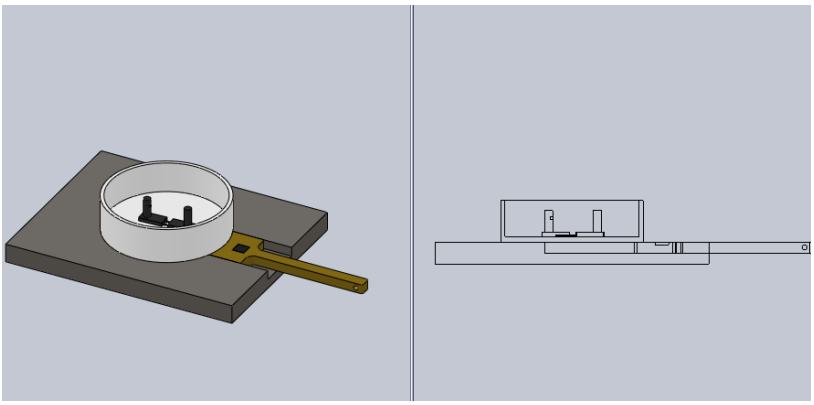

Figure 7: Magnet Design

In this design, a standard petri dish is used as the culture vessel. Two fixtures are placed within the dish, one fixed to the dish bottom and the other able to move. In the supporting base beneath the dish, a cutout allows for placement of a mechanical arm with a magnet insert. The mechanical arm is attached to a motor (not pictured) that will move it back and forth and as the magnet moves, the articulating plate inside the dish will follow.

Whereas neither of last year's designs incorporated mechanisms of electrical stimulation into the mechanical stimulation device, this proposed device hopes to do just that. An electrode has been inserted into the base of the dish to transmit electrical currents through the media. A wire will be run from the post to outside of the dish where it may connect to a device such as the BioPac system, controllable via the LabView software, to generate electric pulses.

The greatest benefit to this conceptual design is in preservation of sterility. Using magnetic connections to control the mechanical stimulation mechanism prevents physical connections between the sterile internal environment of the dish and the non-sterile exterior environment. This benefit is somewhat lost however when connecting the electrode wire to the external electrical system.

Also not pictured with this device is the molding system for the tissue. Keeping in mind that the muscle tissue formed in the course of this project is expected to completely self-assemble, the cells will not be seeded into a scaffold. Instead, the proposed culture technique

would involve using an agarose gel mold to direct the tissue formation. Utilizing a "dogbone" shape as seen in Figure 2, the gel mold will consist of a linear central region as well as two rings at the ends that surround anchoring posts to hold the tissue in place. Tissue will not adhere to the agarose gel, so it is a viable option to allow for the cells to form a cohesive tissue construct. Once the tissue has formed the gel mold may be easily cut away leaving just the tissue construct anchored to the posts that will be used to stimulate the tissue.

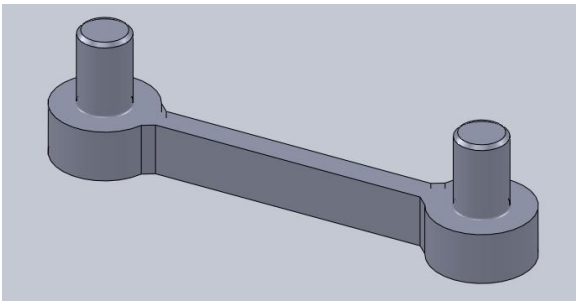

Figure 8: Dogbone Tissue Anchoring system with Anchoring Posts

Though this design offers a number of attractive features, namely in regards to sterility, it has some serious drawbacks as well. Using magnets to control mechanical strain is not ideal as they are very imprecise. There is not a direct correlation in movement between two magnets, especially in cases of small-scale movement such as that used in this design. Also, as was seen in last year's design, the mechanical components required to make this design function were fairly large, meaning that a larger culture dish was needed to house it all. This led to the use of much more media than would be otherwise needed to feed a muscle fiber, and the design ultimately proved to be somewhat cost-inefficient.

4.4.2 Design Alternative 2 - Hydraulic Syringe Pump

The Hydraulic Syringe Pump, below in Figure 3, utilizes a sterile liquid to adjust the stretching distance of the mechanical device. When the syringe is depressed, water fills a tube, which causes the movable tissue post to push itself closer to the fixed post. This is the relaxed position where the muscle remains when not being exercised. As soon as the protocol calls for muscle exercise, the syringe will be retracted and allow the liquid to return to the syringe barrel. The moving tissue post will slowly return back to its original position with assistance from a spring pushing against it. This process can be repeated as many times as needed until the spring loses its mechanical properties. Electrical stimulation would be included in the two tissue posts.

An electrode on each of the posts would directly provide an electrical signal to the tissue to provide this electrical stimulation.

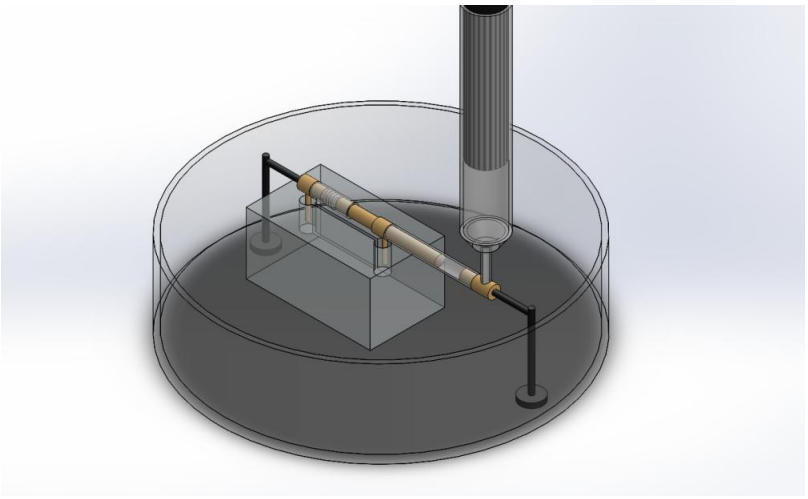

Figure 9: Hydraulic Syringe Pump

This design is convenient for tissue transfer because the posts sit vertically in the dog-bone-shaped agarose mold. Once the tissue has differentiated properly, the mold can be cut away from the post, and the tissue stretching can begin. Contamination is easily prevented as well because the syringe can be easily connected to the device inside a dish in a sterile manner.

Unfortunately, this design requires coordination between a lot of different parts, and they all must function perfectly to allow the device to work well. There is concern that this design is too intricate to work with the scale of this project, which could prompt imprecise results. Electrical stimulation might also be an issue in this design because there is no convenient way to set up the electrodes if direct stimulation is being used.

4.4.3 Design Alternative 3 – Moving Lid

The 'Moving Lid' design attempts to increase the device's efficiency by utilizing a six-well plate with wells that have a diameter of 35 millimeters as the environment for the cultured tissues. By utilizing a smaller well, less media and agarose will be needed to fill the environment, reducing the overall cost of tissue culture; in addition, using a six-well plate allows the user to apply mechanical and electrical stimulation to six times as many tissues. This six-well plate is secured into a base that has set screws on two sides to fixate the plate's location prevent sliding when mechanical forces are applied. In addition, a hinge is located on the end of the base,

allowing for an attachment to be added which would prevent the lid from lifting upwards off of the 6-well plate during testing.

In this design, the tissue is formed in a dogbone-shaped mold created with agarose gel. The tissue sample is formed around two glass posts, which will serve as a means of anchoring the tissue so that it may be strained mechanically. Each of these posts will be fixed to different surfaces. One post will be fixed to the bottom of the well, which will remain stationary; meanwhile, the second post will be fixed to the lid of the six-well plate. Glass posts were chosen for this design because glass has the ability to be heated and shaped into hook-like features which will be useful in ensuring the tissue sample does not fall off of the anchoring system.

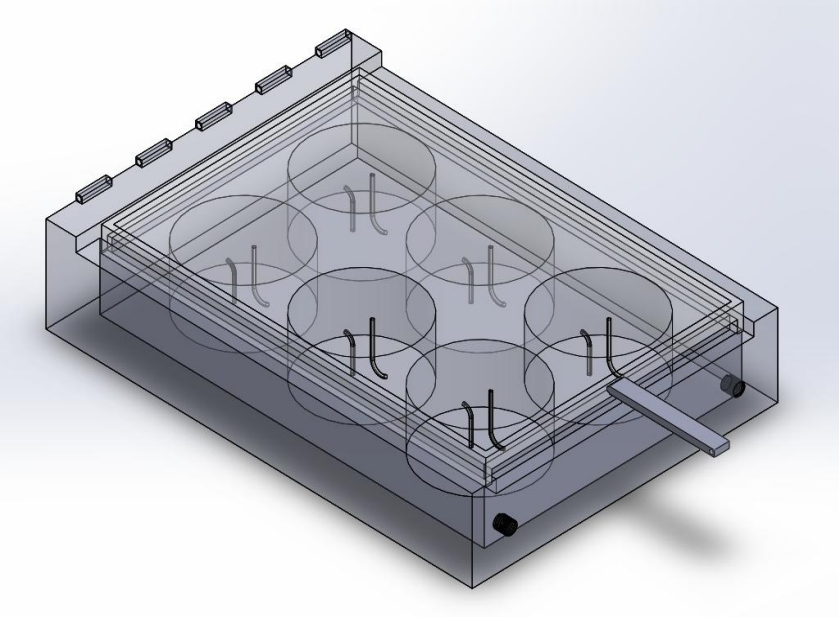

Figure 10: Movable Lid Design

Figure 4, above, shows how the base, 6-well plate, and the lid interact with each other. In addition to this, a stepper motor will be attached to the lid's protrusion. The stepper motor was chosen because it has the ability to perform cyclically, as well as be programmed to stop and strain the material statically. This motor will be fixed to the base of the device, and connected in series to a force transducer at the lid's protrusion, allowing for the forces exerted on or by the tissue sample to be accurately determined.

A single electrode was chosen for this design, it would be located along the upper hook, and be connected directly to each of the tissue samples. The location where the electrode's wire enters the sterile environment would be sealed with a septum in order to prevent contamination.

During electrical stimulation, the lid would have the ability to move freely without interference with the stepper motor.

While this design successfully allows the culture and testing of multiple samples simultaneously while preventing the environments from becoming contaminated, it does have a number of major drawbacks. With this design, tissue samples would need to be cultured within the device, as tissue transfer is extremely difficult with this design, simultaneously transferring multiple samples and anchoring them between the lid and plate could lead to tissue samples which are destroyed during transfer or possible contamination as the lid is removed. In this design, the agarose mold would need to be cut away after culture is complete and it becomes time to begin straining and stimulating the samples.

4.4.4 Alternative Design 4 - Lever Design

The Lever Design consists of two dish inserts with posts for the tissue to attach to and develop within a 35 mm petri dish. The two plates overlap and are aligned on a track allowing for uniaxial movement. One of the inserts is fixed in place through a press fit into the dish, while the other is attached to a lever that extends out of the petri dish. The lever is passed through a one way rubber septum to a stepper motor, allowing the design maintain sterility. In-direct electrical stimulation is present by two electrodes which protrude in a sterile manner through the top of the petri dish and into the media. This design is pictured below in Figure 9 without the agarose mold which can later be cut away once proper fiber development has occurred..

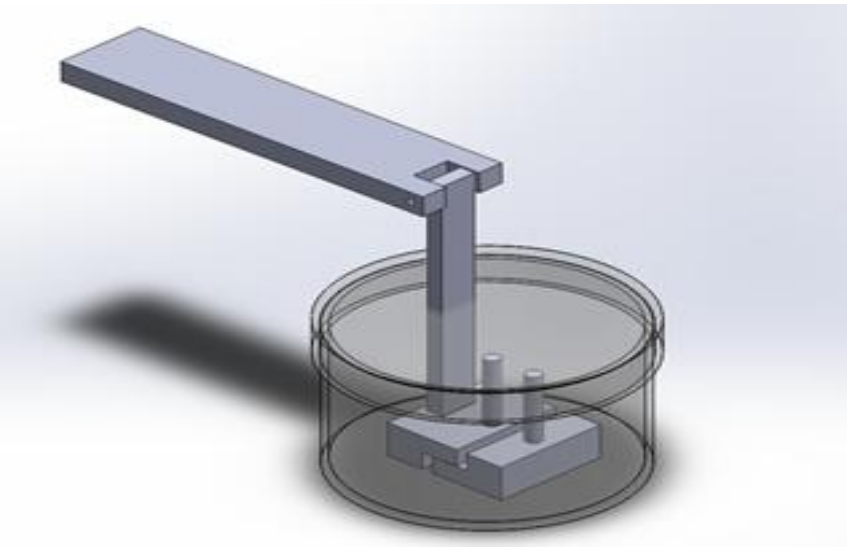

Figure 11: Lever Design

4.5 Final Design

After creating the alternative designs the group performed a design comparison. This was done by numerically evaluating each design in order to determine the degree to which objectives were satisfied. The design comparison is shown below in Table 3.

Table 3: Design Comparison

Multiplier	Objectives	Magnet	Lever	Syringe Pump	Moveable lid
N/A	**Fits in Incubator**	Y	Y	Y	Y
N/A	**Biocompatible/Sterilizible**	Y	Y	Y	Y
N/A	**Safe for Tissue**	Y	Y	Y	Y
4	**Electrical Stimulation**	2	2	2	2
4	**Mechanical Stimulation**	1	3	2	3
5	**Sterile Enviroment**	3	3	2	3
3	**Measure Stress and Strain**	1	2	2	2
1	**Time/Cost Effective**	2	3	2	2
5	**Tissue Transfer**	1	3	3	1
2	**Ease of use**	2	3	1	2
	Total	**41**	**65**	**51**	**52**

After comparing the designs and adding all the values up the lever design was picked to be our conceptual final design. It had the highest score and satisfies all the constraints.

4.6 Feasibility Studies

In order to verify the chosen preliminary design a series of feasibility studies must be conducted to determine various components and the optimal cell culture environment for the design to function. These studies include the shape of the agarose mold, the co-culturing of fibroblasts, and the post shape.

4.6.1 Co-Culturing with Fibroblasts

To create an in vitro muscle fiber that most clearly resemble an *in vivo* muscle fiber fibroblasts and myoblasts must be used. The two cell types will be cultured separately and subsequently combined to create the muscle fiber. This will be attempted to see if co-culturing with fibroblasts is possible. This is an important consideration for tissue development because

fibroblasts are primarily responsible for production of the extracellular matrix that supports the tissue. If the fibroblasts can be combined successfully with the myoblasts this technique will be used in all testing of any designs created; if it does not work only myoblasts will be used to create a muscle fiber.

4.6.2 Anchoring Posts and Mold Shape

The post shape of the mold and the well shape of the mold was chosen according to feasibility studies to be performed later on in the project. These studies were intended to be done to identify the best anchor for the muscle fiber. Several different shapes were chosen as potential options, however it was decided that a cone shaped mold anchor, followed by a circular actuating anchor would be the best choice. The cone shaped mold anchors are shown below, in figure 10, provides a less dramatic transition from a round shape to a linear shape. In creating a cone-shaped anchoring post we hope to avoid tissue necrosis at the point of intersection between the linear and anchored regions of the tissue that may otherwise occur in tissues without such a transition. This shape is only present in the mold, and not on the device's posts due to manufacturing constraints. Ideally, the cone-shaped development in the mold will allow for less of a chance for the fiber to break upon mechanical stimulation.

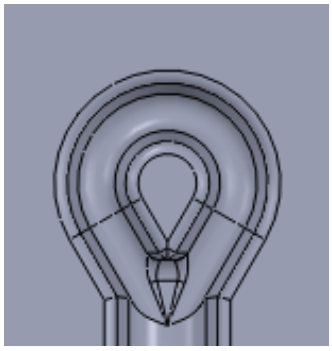

Figure 12: Cone Shaped Post Design

Well shape will also be tested to determine the optimal design. A v-shaped design and a u-shaped design were both developed to determine if there is a benefit to having a rounded or pointed bottom. These two well designs are somewhat narrow to constrict the culture surface area and completely direct tissue construct formation. A third mold well design was also developed based on the presumption that the cells align during differentiation and fusion without specific guidance. In this design, the two anchoring posts still remain but the surrounding well is open so that the cellular behavior will dictate tissue formation. Each of these three designs will

be tested to determine which provides the best results for forming a cohesive tissue construct. Conceptual models for the mold designs may be seen in figures 13 and 14 below.

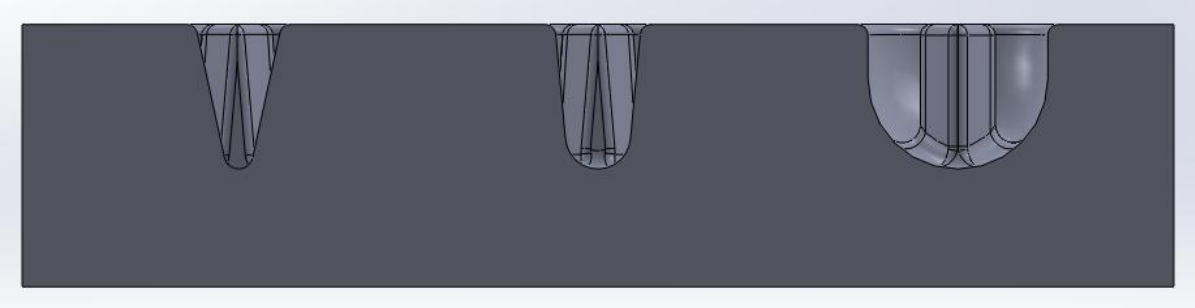

Figure 13: Front View of Well Shapes

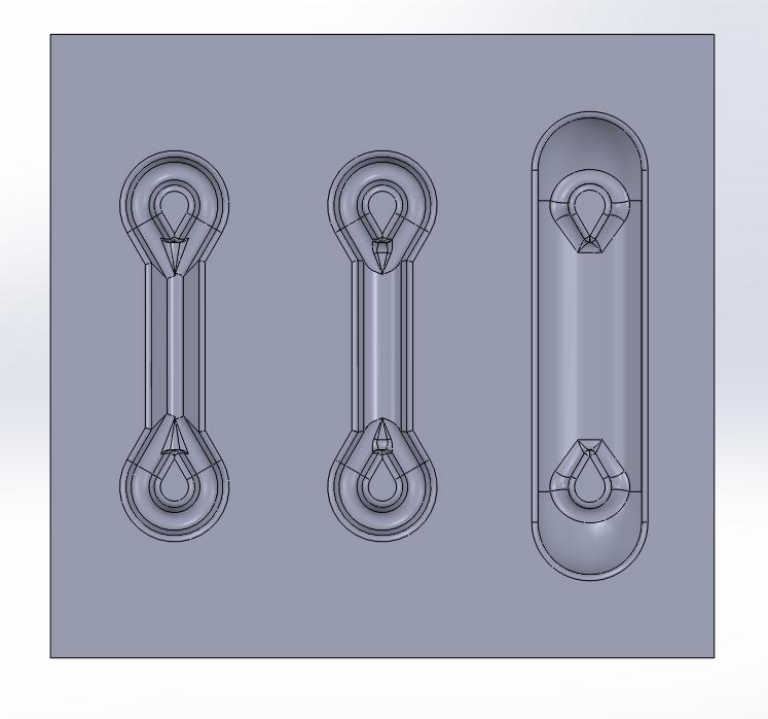

Figure 14: Top View of Well Shapes

The final mold design includes the three conceptual models to be tested and may be seen in figure 12 above. This is a double negative of the final mold we hope to create; a prototype of the finished product, seen in figure 13 below, will be cast in polydimethylsiloxane (PDMS), the mold of which will then be cast in agarose gel to create the final working tissue mold.

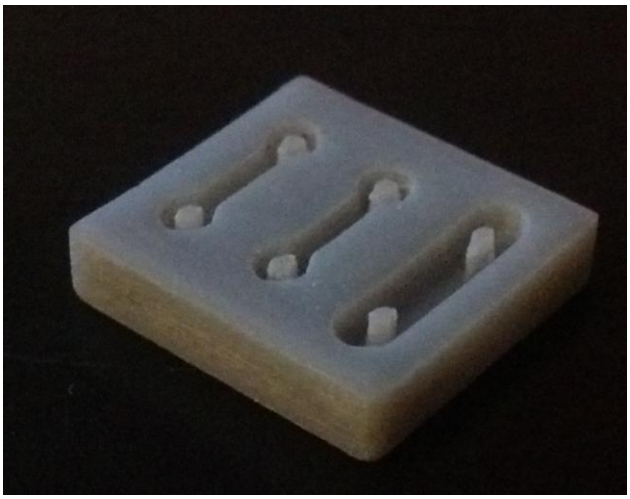

Figure 15: Manufactured Mold for PDMS Casting

4.7 Preliminary Data

Before choosing a design, preliminary data was considered in order to appropriately choose a final design. The following categories were looked at carefully while making alternative designs. Each of these subsequent items will all need data to justify their use in this design.

4.7.1 Cell Culture Standards

The cells seeded into our tissue mold must be able to successfully differentiate and fuse to form muscle fibers without adhesion. For this reason, agarose was chosen as the mold for tissue growth. Agarose can also include media and growth factors to further encourage cell growth and differentiation.

A three-dimensional tissue construct should be able to be created and it should be able to be transferred from mold to device without any tearing. For this reason the v shaped well was chosen due to its believed ability to direct tissue growth better than the other two mold shapes. Previous project work has successfully demonstrated that this works in developing a 3-D tissue construct (Ohlson, 2011). Cells will be stained and viewed using standard immunocytochemistry protocols to determine if they have successfully differentiated. Observing the tissue construct under a microscope will also prove whether or not a successful 3-D construct has been developed.

4.7.2 Strain Distance

This device should stretch the muscle to at least 10% strain. However, an adjustable device would be desired in order to be able to experiment with optimal strain. Therefore, we can observe the distance of strain with either a close up camera shot and Image J software, or by using Calipers. If the device can successfully stretch to 10% of the original standard length we decide on, the strain distance has been successfully developed.

4.7.3 Sterility

Cell culture and testing requires all parts involved to be sterile to prevent contamination. Therefore, cell cultures will be grown in the device before mechanical and electrical testing in order to ensure there is no contamination. Several of the design alternatives involve moving parts that could potentially bring contamination into the system. However, after growing several cultures and testing for contamination, we should know which aspects cause the design to become contaminated or whether it can maintain a sterile environment.

This system must also be easy to sterilize so that it can be easily reused. In order to allow for easy sterilization, it was decided that all parts must be able to be autoclave compatible. However, in cases that this was not possible, ethylene oxide would be an acceptable form of sterilization. Ideally, any part that would be in contact with cells would need to be sterilized in the autoclave because in some cases, ethylene oxide can cause cell death.

4.7.4 Materials

Several different materials will be used in this design, and each potential material must be tested for its desired use. For instance, metal electrodes must be integrated into each design in order to electrically stimulate the muscle tissue. However, if these metals oxidize easily, they will not be able to sustain themselves in the cell culture media. In turn, they will not be feasible to use in the design. It is for this reason that the material and mechanical properties of each different part are appropriate for their intended use. Not only must they accomplish their predetermined job, but they must not cause a problem anywhere else in the design.

Chapter 5: Manufacturing

In order to ensure that the device was both effective in its function and easy to manufacture, the strengths and limitations of CNC manufacturing processes were acknowledged when conceptualizing the structure of the device. The 3-dimensional models of some components were modified to reflect these criteria. The following explains the manufacturing process in detail and the modifications that were made to the design along the way.

5.1 Final Design and Manufacturing Processes

The final design chosen was the stepper motor-actuated lever design. This design utilizes a 35mm diameter petri dish, in which the tissue is cultured on the agarose mold. Inside the petri dish, two plate-post constructs are used to mount the tissue, as well as apply strains. Although overall design is the same, the dish insert was altered to improve the function of the entire device. One plate will be fit to the inner diameter of the petri dish, and would not require any external forms of fixation. In addition, the heights of the two plates are now staggered to allow the plates to move without interference. These dish inserts can be seen in the figures below.

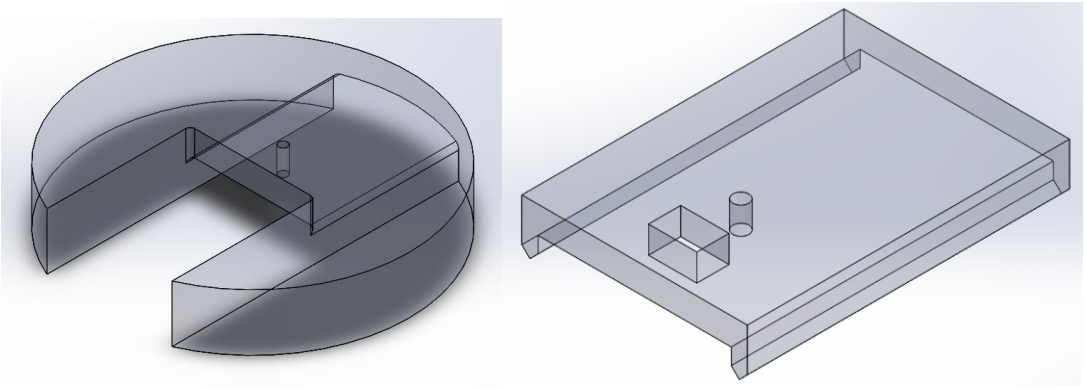

Figure 16: Pictured on the left is the stationary dish insert, and pictured on the left is the moving dish insert.

Other additions to the design included a base construct that has the ability to hold the petri dish in place, as well as attach to the stepper motor. This fixation ensured that forces applied during actuation do not cause the entire petri dish to move, but only the actuating dish insert. This base fixture is approximately 60mm wide in each direction, and was manufactured from a ½" thick polycarbonate sheet. This design also utilized a total of four M4 x 0.7mm

tapped holes. This included two holes to adjust the tightness of the petri dish fixation based on screw tightness, and a second set of threaded holes allows for the attachment of the stepper motor, which is approximately 54mm wide. A top view showing the placement of the tapped holes, signified by the red dots, is displayed in Figure 10.

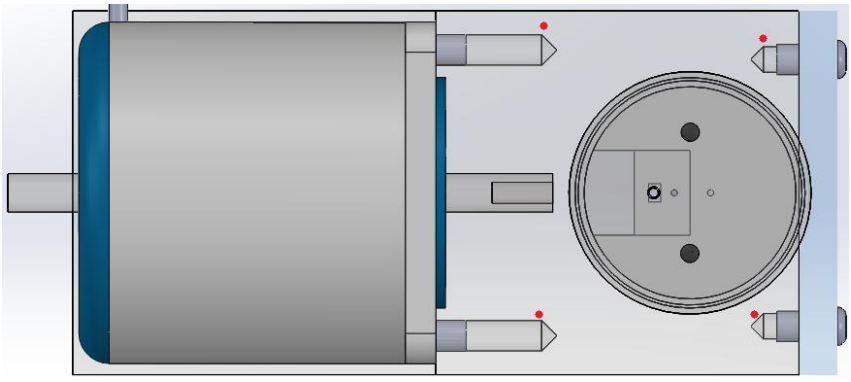

Figure 17: Translucent Top View of Full Design

The placement of the motor allowed for a direct connection of the stepper motor's actuation shaft to a lever arm via a single connection point. The lever arm will cross through the cover of the petri dish, through a hole fit with a rubber septum. This septum will applies enough of a holding force to hold the lever at a specified height, and will serve as the lever arm's fulcrum point. This lever arm will be manufactured using a lathe from polycarbonate rods, much like the circular petri dish insert.

The last element of this design is the removable electrode system. The electrodes connect to copper wire, which conducts currents through the system. Metal screws act as the electrodes and will penetrate the cover of the dish. A soldering iron was used to create the holes in the dish cover to avoid cracking. These screws were trimmed to an appropriate length and a pipette tip was threaded onto screw on the inside cover. Platinum-iridium wire was then used as the tip of the electrode, which will connect to the screw inside the pipette tip.

All of these parts were assembled to create our final design. The stepper motor was purchased and polycarbonate components were manufactured using the aforementioned processes. The manufacturing of the polycarbonate components was either performed by or supervised by appropriately trained manufacturing students, depending upon whether the use of the lathe or mill is required, respectively. The final design appeared as the model developed in Solidworks. An isometric view of this model is viewable in Figure 2, below.

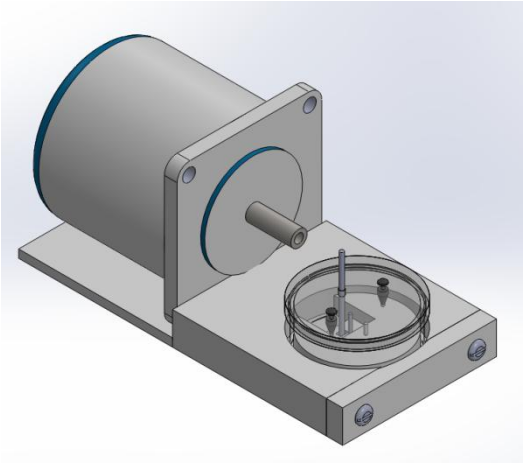

Figure 18: Model of Complete Design

5.2 Material Choices

As previously stated, the final design is composed of multiple components in order to accomplish the desired product. The working parts include a stepper motor for mechanical actuation, platinum and copper wire for electrical stimulation, medical adhesive, septums, and polycarbonate to make the device. Table 4, shown below, is our bill of materials thus far with the specifications, price, and amount.

Table 4: Bill of Materials

	Specifications	Amount	Price	Ordered From:
Stepper Motor	29 oz. (200 steps/rev, threaded shaft)	1	29.95	Sparkfun
Platinum Wire	99.95% platinum	3ft	140.54	Tritech research
Easy Driver	N/A	1	28.07	Sparkfun
Precision Seal Rubber Septum	7mm O.D.	10	12.90	Sigma Aldrich
Polycarbonate rod and sheet	10 ft	1	40	McMaster
Screws	M3 .05 40mm length Metric Pan Head Phillip Drive	100	14.22	Amazon

This particular stepper motor was chosen because it is made for linear actuation and has 200 steps per revolution. Because of these specifications, it is very precise and accurate, and can be used for micromotion in the dimensions needed. This is advantageous for our device because our 5mm tissue will only be strained 10%, for a total of only 0.5mm. The platinum was chosen because it is a great conductor and has a higher resistance to corrosion than other metals. Because it is a very pure alloy, 99.95% platinum, it can produce a better electric field. A strong

and consistent electric field is important because the electrodes are stimulating the media, which will become our electric field. The media needs to have consistent current and voltage throughout in order to ensure the field reaches the tissue evenly and symmetrically. Silicone glue was deemed necessary to seal the electrode screws to prevent contamination, while still being able to be sterilized. It is inexpensive and able to be autoclaved, making it the perfect choice. The Precision Seal Rubber Septum will be put in the top the petri dish the actuation hinge goes through in order to keep everything sterile. This particular septum was chosen because it is low cost and does not touch the muscle or media and therefore can be sterilized using ethylene oxide. Finally, polycarbonate was the material of choice for most of the device because it can also be autoclaved, it is low cost, and easy to manufacture.

5.3 Stepper Motor Modification

The chosen stepper motor was selected primarily for its ability to actuate linearly as it rotates, allowing for accurate and controllable movement through a controller. To ensure that the motion created by the motor is applied with precision, the motor was modified to enable it to be fixed to a stainless steel back plate. The modification to the motor involved removing the four screws around its perimeter, and replacing the original screws with longer M3 screws with the same pitch. Removing the threads within the motor's face will prevent cross threading and misalignment when fixated to the threaded back plate. This process was completed by hand with a battery-powered drill.

Figure 19: Stepper Motor Modification

5.3.1 Back Plate

Stainless steel was chosen as the material for the back plate of the device due to its ability to resist deflection, and therefore prevent any unwanted micro-motion. To manufacture this component, the stainless steel stock was cut to the desired width of the device, and to a height that would ensure that the motor's actuation remained at an appropriate height above the dish. Next a series of holes were drilled into the plate which included two loose-fitting M5 through-holes, four evenly spaced, tapped M3 holes, and a central hole to allow the passage of the actuating shaft. A HAAS vertical mini-mill was chosen to create this component, guided by code generated using Esprit.

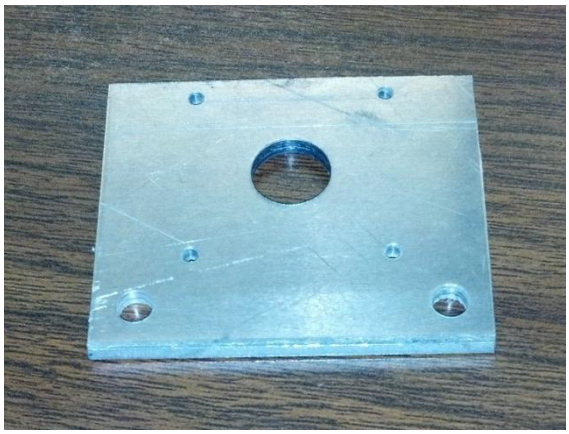

Figure 20: Stainless Steel Back Plate

5.4 Base

The base design involves two manufactured components which were created from 6" x 6" x ½" polycarbonate stock. The purpose of the base is to fixate the petri dish in place, and ensure it does not move while the motor is in use. Additionally, the base utilizes an interference-

fit locking mechanism with thumbscrews to ensure that dish placement and removal is easy for the user and does not require additional tools.

The first step in the manufacturing process was cut the base plate of the assembly to our specifications and mill a circular hole that would allow for the placement of our petri dish. For this step, an endmill was used to face the material in a HAAS minimill. Next, four M5 tapped holes were drilled into the sides of the base: two 20mm deep holes on the posterior for back plate anchorage, and two 10mm holes for the locking mechanism on the anterior of the device.

The locking mechanism of this design uses differing radii to introduce interference when the second part of the base is fully tightened into place. This interference prevents the top of the dish from moving during actuation, which allows the dish top to serve as a fulcrum point in the lever design. To create this component, polycarbonate sheet stock was cut to size and an endmill was used to remove material within the given radius. Loose-fitting M5 through-holes were then drilled through the side of the part. The components that make up the completed base can be viewed in figure 23 below.

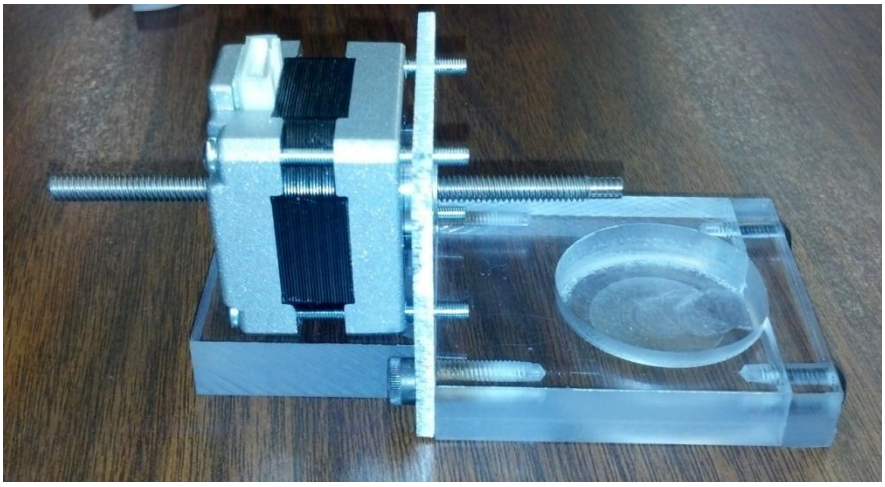

Figure 21: Fixated Motor and Base

5.5 Posts

Myoblast cells are anchorage dependent cells, and muscle tissue requires points of anchorage in order to properly align, contract, and function. In the design of this device, posts were conceptualized as an easy and effective means of anchoring the tissue within the mold. Unfortunately, the extremely small diameter of the posts made the manufacturing of these posts using a lathe impossible due to large amounts of deflection. To overcome this impediment, to simplify the production of the posts, a minimill was used with an Esprit code that cleared out a

circular channel. The process left 1mm section of material intact in the center of the cut, acting as the tissue posts. Posts can be easily removed from the polycarbonate stock using hemostats and trimmed to the proper height. These posts were then press-fit into the holes on the sliding dish inserts.

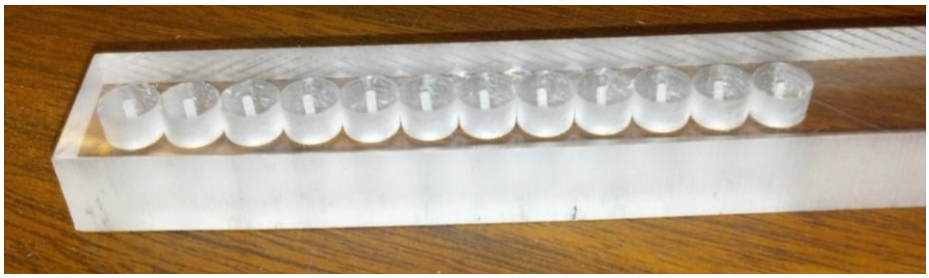

Figure 22: Anchoring Post Production Method

5.6 Dish Inserts

The next step was the creation of the stationary and actuating dish inserts, described in Section 5.1. The stationary insert was created from a 2.5"polycarbonate rod stock, and was first placed in a lathe. Using the lathe, the diameter of the rod was reduced to tightly fit within the petri dish, and then cut to a height to allow the piece to be well gripped and further modified in a minimill. In the minimill, two main cuts were made. First, an endmill was used to face the bottom of the component to create the channel that served as an actuating track for the moving insert. Next, the part was repositioned and the endmill was used again to make the second cut and create the plate's "U shape". A smaller diameter tool was used, in addition to a smaller cutting depth for each interval.

In order to ensure that the polycarbonate inserts do not interfere with the hanging electrodes, two additional holes were drilled vertically through the sides of the insert. These holes will create small wells to be filled with media and allow for the tips of the electrodes to be completely submerged and bring current to the tissue sample.

The second movable dish insert was also created using a HAAS minimill. A small endmill was used to cut the stock to size, and to create the center channel of the plate. This center channel was created to allow for space for the rubber insert to fit without dragging along the bottom of the disk, and to reduce the surface area in contact between the part and the bottom of the dish. Two holes were drilled into the actuating plate, a 4mm diameter hole for the rubber insert, and a .0040" hole, which will be used to press-fit the 1mm posts.

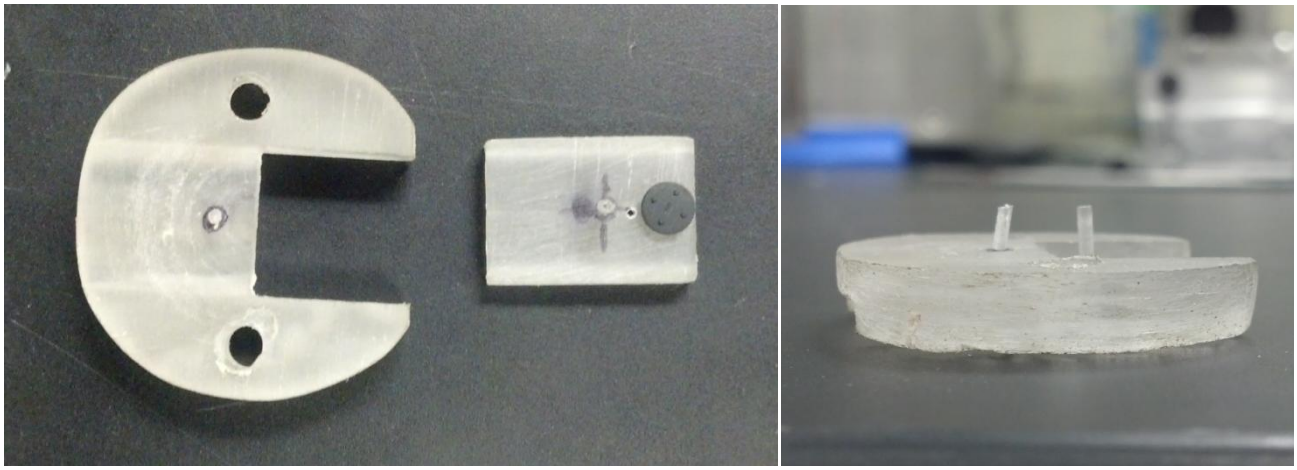

Figure 23: Fully Assembled Dish Inserts with Posts

5.7 Dish Cover Modifications

To enable entry points for both mechanical and electrical stimulation, the cover of the petri dish was modified. Using a Bunsen burner, metal pieces of 6mm and 2mm diameter were heated and used to create the septum hole and electrode holes, respectively. A 7mm septum was press fit into its respective hole and trimmed of excess rubber, and two screws were placed through the electrode holes. These components were then adhered to their positions using silicone adhesive that preserved the enclosed sterile environment during use. Finally, the tip of a 100mL syringe needle was used as a lever and was placed through the septum and into the rubber component of the actuating dish insert.

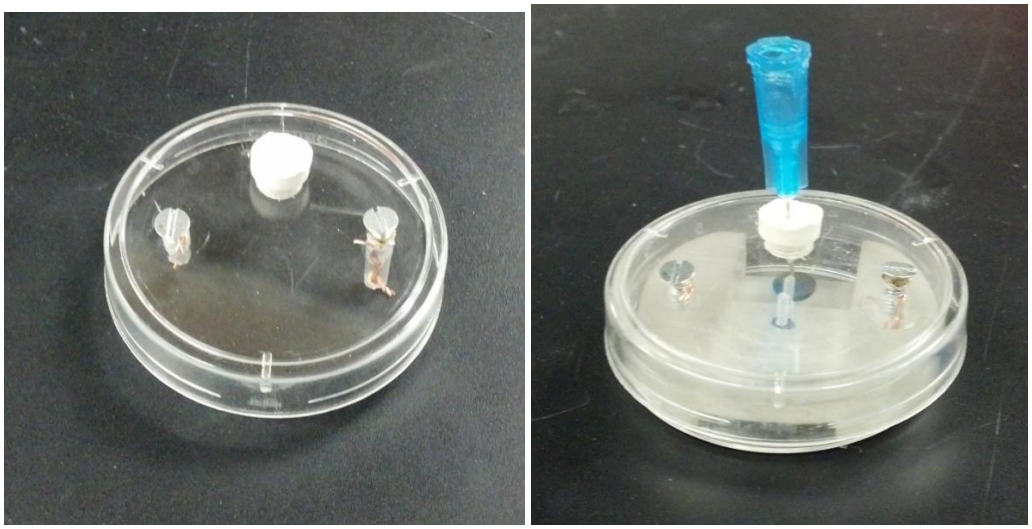

Figure 24: Modified Dish Cover and Full Dish Assembly

5.8 Electrodes

The intent for the design of the electrodes involved keeping them modular, for sterilization purposes, while also ensuring strong connection between the power source and the electrode tip. A screw, which penetrates the dish cover, is the connection point for the power source as well as the electrode itself, and carries the current into the dish. The modular section of the electrode tip was created by crossing a small platinum piece of wire with copper wire of similar diameter, and winding the platinum wire around itself until secure. Copper was wound within the inner diameter of a pipette tip cut to size. Screws were then used to coax the copper wire into a helical shape. A small amount of silicone adhesive was added within the pipette tip to hold the assembly in place without permanently attaching the tip to the screw.

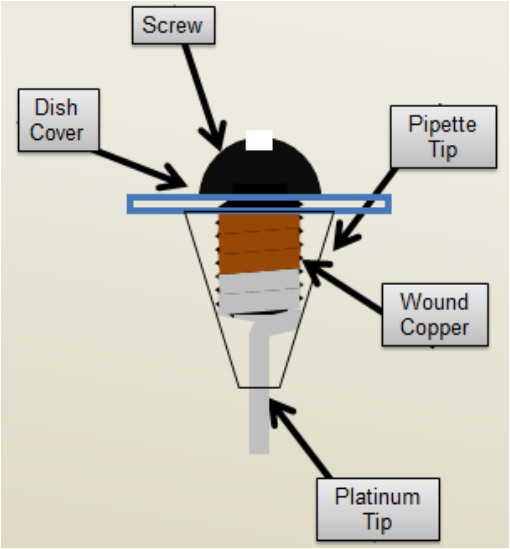

Figure 25: Electrode Assembly Diagram

5.9 Lever-Motor Connection

The final component necessary in ensuring that the linear motion created by the stepper motor effective reaches the tissue is the connection between the lever and the actuating shaft. A modified 22-gauge needle was used as the actuating arm and was connected to the motor drive shaft using two washers. The washers were screwed onto the shaft and the top portion of the needle was fitted in place securely between the two. The rear washer, closer on the shaft to the motor itself, was fixed in place on the shaft using a silicone adhesive. The full assembly may be seen in Figure 28.

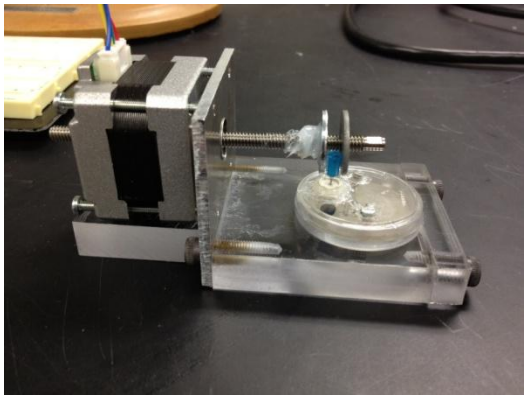

Figure 26: Full Mechanical Actuation Assembly

Chapter 6: Motor Wiring and Programming

A mechanism of mechanical actuation for the tissue constructs was implemented into the system and driven via a linear actuating stepper motor. This motor was connected to an EasyDriver Stepper Motor, which was then powered and controlled by a connection with an Arduino Uno microcontroller. The motor was connected to a twelve volt power supply from the EasyDriver, which itself was wired to a five volt power supply. The wiring diagram for the stepper motor may be seen in Figure 28 below.

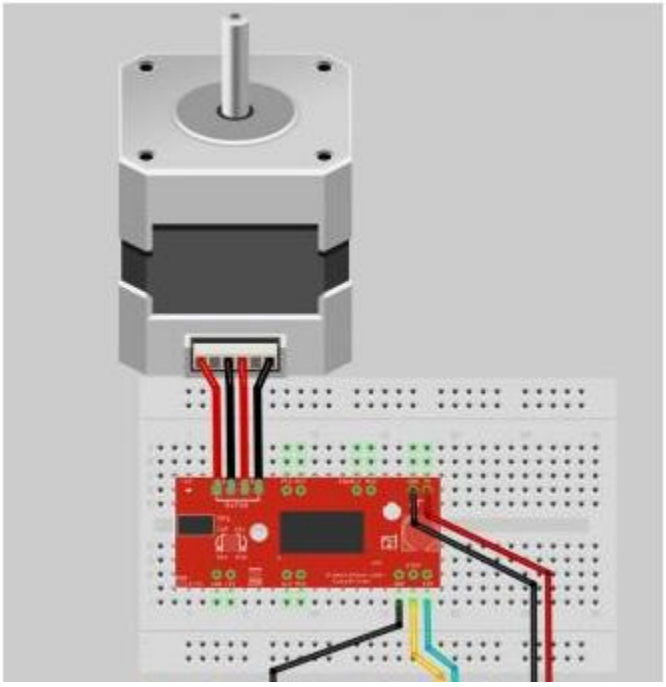

Figure 27: Motor WIring Diagram (Stepping Up to the Challenge, 2013).

The motor system was controlled and powered by the Arduino Uno microcontroller. The controller had both an AC output for power and a USB output to allow for connection to a computer. Arduino uses a very simple coding language to program its systems; this allowed for parameters that could be easily tailored according to the needs of project, and as such the amount and rate of strain were easy to control and modify. Coding instructions for the microcontroller may be found in Appendix G.

Chapter 7: Methodology

The following section outlines progress made for our MQP project. We have worked to finalize our design, choose appropriate materials, conduct preliminary cell culture work, and conduct testing procedures

7.1 Preliminary Muscle Cell Culture

For the purposes of growing and forming the mature muscle tissue, the C2C12 cell line was used. This is an immortalized mouse myoblast cell line that serves as progenitors to the mature muscle cells. These cells may be maintained in an undifferentiated state or induced to enter terminal differentiation and form mature muscle cells based on the culture medium and treatment conditions used. Further details and step-by-step explanations of each cell culture procedure are provided in Appendix D**.**

7.1.1 Cell Culture

Cells must be cultured in a liquid culture medium in order to survive and remain viable. Culture medium contains the metabolites, proteins, growth factors, and nutrients cells need to live; it is also made at a pH of 7.4, and typically contains an indicator that causes the color of the medium to change as the pH level changes. As cells undergo replication and other normal processes, they will take in these nutrients and proteins from the medium and release waste products, causing a change in the pH. Medium in the normal pH range will appear red but as the cellular processes persist over time the medium tends to become more acidic and thus takes on a yellow hue.

Many different supplements may be added to the culture medium as well to elicit different cellular behaviors. Common examples of these supplements include glutamine, animal serum, and penicillin/streptomycin. Glutamine is an amino acid that improves the growth and performance of cells. Animal serum, commonly fetal bovine serum, is the plasma portion of blood from the source animal that remains after coagulation has occurred following death. Fetal bovine serum contains many different growth factors that are very beneficial in cell proliferation. Another commonly used serum is horse serum; in this instance, the serum is derived from the adult animal and thus contains proteins more tailored to this mature organism that may direct progenitor or stem cells to differentiate. While animal sera are very beneficial in culture work,

there are numerous ethical issues associated with their use, which have lead to the development of synthetic substitutes to serve in the same applications. Penicillin/streptomycin, commonly abbreviated as pen-strep, is an antibiotic that fights infection by foreign contaminants that may be accidentally introduced during culture work. Pen-strep is not always included as a supplement to culture medium as it tends to mask but not eliminate bacterial contaminants, but it may be included in such circumstances as when the researcher is less experienced or when the lab space is shared by multiple groups and the potential for accidents is increased.

For the purpose of long-term storage, cultured cells may be held indefinitely in liquid nitrogen for cryopreservation. To begin working with these cells, they must be removed from liquid nitrogen storage and thawed. Cell thawing is a fairly rapid procedure that begins when cells are transferred from liquid nitrogen to a water bath maintained at 37 °C. The cryovial is submerged in the water bath for approximately one to two minutes, until there is only a small crystal of frozen suspension remaining. It is then rapidly transferred to the culture hood and combined with the normal culture medium. This suspension is then centrifuged to isolate the cell pellet and then re-suspended in a new volume of medium before being added to culture flasks and incubated.

Once the cells have been added and supplemented with the necessary volume of medium (10 ml total volume in a 75 cm^2 flask is the standard for this work), they are moved to an incubator set to optimal growth conditions. Mammalian cell culture incubators are set to a temperature of 37 °C with a 5% carbon dioxide concentration in the ambient air and between 90 and 100% humidity. To preserve the cells in an undifferentiated state of proliferation, the standard proliferation medium used consists of a 60:40 (by volume) ratio of Dulbecco's Modified Eagle Medium to Ham's F12 medium supplemented with 10% FCIII (a fetal bovine serum substitute) and 1% Glutamax (a glutamine substitute). When terminal differentiation and mature muscle formation is sought, the choice of medium is changed to a differentiation medium. This consists of a 60:40 (by volume) ratio of DMEM to F12, supplemented with 2% horse serum, 1% Glutamax, and 1% insulin-transferrin-selenium, an additive that works in a similar manner to and bolsters the effects of the horse serum.

If allowed to become overly confluent, C2C12 cells will exhibit signs of contact inhibition and may begin to differentiate. This is due to the fact that when cells do begin to contact each other they will arrest proliferation. To circumvent this issue, cells must be passaged

and added at lower seeding densities to new culture vessels to maintain their integrity. Many lines of cultured cells, including C2C12 cells, will adhere to the bottom of the culture vessel as a result of protein interactions between the cell and dish surface. Thus, the first step in passaging requires breaking apart these interactions and removing the cells from this adherent state. The culture medium is removed and trypsin, a protease, is added for a short period of time to disrupt these protein interactions. Culture medium is then added back to the dish as the animal serum contains protease inhibitors that will stop further destructive actions of trypsin. The suspension is then transferred to a centrifuge tube and centrifuged to isolate the cell pellet. Old medium is removed and new medium is added to the pellet to re-suspend the cells, a count is conducted to approximate the total number of cells present within the suspension, and the cells are added to new culture plates and incubated. These cells should be cultured to no more than 60-70% confluence before being passaged and added to new plates at lower seeding densities.

When it is desired for stocks of cells to be frozen for later use, similar steps are followed as for the passaging procedure. However, once the cell count is determined, an appropriate number of cells is instead moved to a freezing medium stock. This medium consists of DMEM supplemented with 20% dimethylsulfoxide, a cryoprotectant that prevents cell death during freezing, and 10% FCIII. The suspension is added to the cryovial, placed into an insulating freezing container, and moved to a -80 °C freezer. In contrast to the thawing process for cells, freezing is a slow procedure in which the cells are expected to cool to frozen very gradually so they are not damaged by shock from the cold. After spending roughly 24-48 hours in the -80 °C freezer, cells will typically be transferred directly to liquid nitrogen storage for indefinite preservation; however they may be held in this -80 °C freezer for several months without issue.

7.2 Mold Creation and Validation

An integral part of the device being able to function as it is intended is proper muscle fiber development. After the time spent developing the mold shape, the mold was created and tested to assure that it would function as designed.

7.2.1 ABS Plastic Mold

In order to produce our mold in a sterile, but also cost effective manner, we decided to first make a negative mold out of an inexpensive plastic material. Originally, our team decided to use the 3-D printer in Higgins Labs at Worcester Polytechnic Institute because it is an easy

machine to use with a quick manufacturing turnaround. Once the design was submitted and the product was received, we observed the mold under a microscope. Unfortunately, the 3-D printer had very low definition that created visible cavities in the mold. These cavities presented a problem because the PDMS mold, once poured, would adapt this same texture, eventually transferring it to the agarose mold as well.

ABS plastic was the next option explored. This mold was created with the objet machine located in the same Higgins manufacturing facility. The first mold created had well walls that were extremely steep, hoping that steep wells would be better for fiber formation. Because of the extremely small size, the posts on the mold could not be manufactured without breaking. Therefore, it was decided to decrease the pitch of the walls in order to aid in the manufacturing process. A glossy finish was also chosen to allow for easier removal of the PDMS by decreasing the surface roughness of the mold. This final mold can be seen in the figure below.

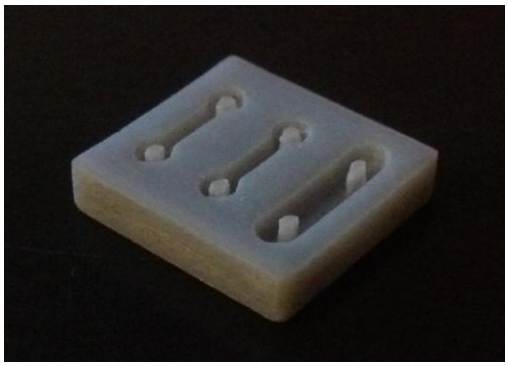

Figure 28: ABS Plastic Double Negative Mold

7.2.2 PDMS Mold Process

Upon receiving the third mold, the PDMS positive mold could be created. After several trial runs, it was found that 5 grams of elastomer base to 0.7grams of curing agent created a flexible but structurally sound mold that would be able to model the correct shape for the agarose. The mixture was combined in a 35mm petri dish and thoroughly mixed for at least 2 minutes. Once the composition was completely combined, the ABS plastic mold was added to the mixture and completely submerged. All bubbles created from mixing were removed through a 20 minute vacuum chamber. The petri dish and mold submerged in PDMS solution was then placed to cure in a 60°C oven for one hour. After the hour, the PDMS was checked to make sure it was solid, and then removed from the oven.

To remove the PDMS mold from the ABS mold, excess PDMS was cut away slowly with a scalpel around the ABS mold. Once the excess was removed, the PDMS was very slowly peeled from the mold. This was a slow and careful process to ensure all details were preserved. Finally, once the mold was completely peeled away from the ABS plastic, the PDMS mold was sterilized by autoclave in order to prepare it for sterile agarose mold production.

7.2.3 – Agarose Mold Construction

The agarose mold that was needed to guide fiber development needed to be constructed in a sterile manner in order to ensure the cells were not contaminated. To do this, the already autoclaved PDMS mold, along with a sterilized wall to surround the mold was placed into a sterile hood along with sterile petri dishes, sterile pipettes, and sterile forceps.

Sterile 2% agarose solution was created by adding 1gram of agarose powder to 50mL of DMEM solution. It was important that simply the base solution of DMEM was used without any extra serum because this could potentially cause adverse reactions with the agarose powder. Once the mixture was added to the media, the bottle containing the mixture was sealed. The top was not completely screwed on to ensure that when autoclaved, it would not get stuck shut. Autoclave tape was used to mark the bottle, and the bottle of media and agarose was autoclaved for 30 minutes to boil the solution in a sterile manner.

Once the autoclave cycle was complete, the bottle was transferred to the sterile hood. The wall to surround the mold was installed with sterile forceps, and the gel was pipetted into the mold. About 800-900 mL of the solution was pipetted into the mold to achieve the desired thickness. After the gel was solid, it was removed by pushing the bottom of the PDMS from the wall surrounding the gel and slowly separating the PDMS and the agarose. The agarose mold was then placed in a sterile petri dish, labeled, and set aside for later experimentation. This process was repeated several times to create multiple molds.

7.3 Proof of Concept Testing

Before cell seeding and final validation, the individual functions of the device were tested and validated to ensure proper function.

7.3.1 Mechanical Proof of Concept

The mechanical actuation system that was developed to provide for uniaxial linear strain in the tissue involved a stepper motor driving an actuating lever arm that attached to one of the two dish inserts within the stepper motor. The lever was attached to the moving insert, which was capable of moving along one axis within the tracks of the larger fixed dish insert. Two tissue anchoring posts were included in this setup, with one post on each dish that would hold the tissue in place. These posts served to sustain the tension that the tissue exerted during development and later allowed for actuation as a means of mechanical stimulation. In order for this system to be validated it was necessary to confirm that the polycarbonate tissue posts were capable of sustaining the tissue's tension and that the motor could successfully be used to actuate this system with a tissue construct anchored to it. This was done by wrapping a dental orthodontic rubber band around the two anchoring posts, and observing the effects in the petri dish

7.3.2 Electrical Proof of Concept Testing

To validate the electrical stimulation of our device, media was put into the petri dish and the electrodes were hooked up to an electrical source, the Agilent 33220A. It was set at a frequency of 7 Hz and amplitude of 10 volts. To make sure the correct electrical signal was being sent, the leads from the Algilent 332200A were connected to an oscilloscope and the correct signal was verified. The leads were then attached to the electrodes and the media began to bubble, thus verifying that an electrical field was created within the media. Below is a figure demonstrating our experimental process.

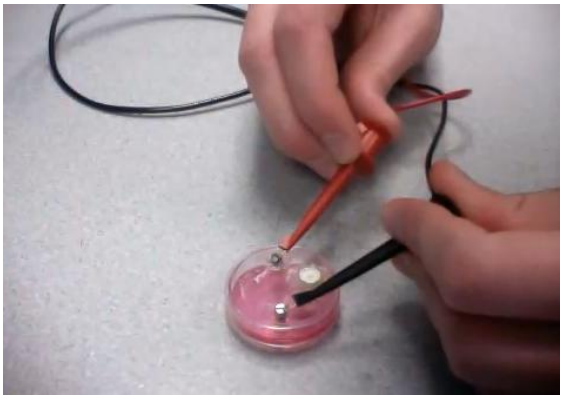

Figure 29: Electrical Stimulation Proof of Concept Testing

7.4 3D Tissue Construct Development

Once the culture techniques had been verified in 2D culture, the team was ready to attempt to seed cells into the agarose mold to test for tissue formation and prepare for stimulation of the mature construct. Preparation for seeding was done by changing the cell culture medium in both the C2C12 myoblast cells and the CRL 2097 fibroblast cells to differentiation medium approximately twenty-four hours prior to seeding. This allowed the cells being used to begin adapting to the new growth factors and exit the cell cycle before seeding began so that differentiation and tissue formation could begin as soon as the cells were seeded together.

At the point of cell seeding twenty-four hours later, the tissue mold was placed on top of the dish inserts, all of which was completed inside the 35mm petri dish. The mold was then flooded with cell differentiation medium and incubated for a period of at least fifteen minutes in order to condition the environment for the introduction of cells. Cells should then be trypsinized and counted to allow for proper seeding densities. Recommendations from a department graduate student stated that the fibroblast seeding density should be 25% of the myoblast seeding density, and this value was used as a baseline. The utilized myoblast seeding count was one million cells and the fibroblasts in turn were seeded at an approximate density of 250,000 cells. The total volume held within the channel was measured at 20ml. It was important to consider uniform seeding across the entire mold, and so an effort was made during seeding to evenly distribute the cell suspension as evenly as possible between the central linear region of the mold and the circular regions surrounding the tissue anchoring posts.

Chapter 8: Testing and Validation

The final design required testing following assembly to ensure that the system was in workin order and could accomplish the establish goals of the project. This was first accomplished by testing each of the individual components of the system before finally attempting to operate the entire system in unison.

8.1 Mechanical Stimulation Proof of Concept

By wrapping a dental orthotic rubber band around the two anchoring posts and powering the motor, it was proven that the system was capable of operating properly and actuating the elastic band. The device functioned as designed, fully actuating it outwards and then back inwards at a controlled pace. As intended, the posts proved to be slightly flexible so that they could give with the contractile stresses in the muscle tissue construct, but did not break due to these stresses.

8.2 Electrical Stimulation Proof of Concept

After preforming the aforementioned electrical validation procedure, the electrical current was not only read successfully through the oscilloscope, but it was also successfully producing small bubbles in the media due to the electrolytes present in solution. This proved that electrical current was passing through the device, and that the electric field would be propagated to the cells once seeded in the assembly.

8.3 Sterilization

Sterilization of all components and maintaining a sterile environment for the cells is a key concern in conducting cell culture work. Using a rubber septum as an interface between the internal environment of the culture vessel and the external surroundings and system components allowed for maintenance of this sterile environment. The needle that served as the actuating lever arm was the only component that bridged the two sides. This needle was sterilized with ethylene oxide treatment prior to introduction into the system and held separately and sterilely until it was used. Following introduction through the septum in remained in place as much as was possible or was thoroughly re-sterilized if removal became necessary.

The remaining components of the system were similarly treated with ethylene oxide sterilization prior to use to ensure their sterility. The system was assembled in a largely aseptic manner and it was thus essential that the components be sterilized once built. Polycarbonate, which was used for the dish insert material, is able to be autoclaved safely, however several other components of the system, namely the polystyrene cell culture petri dish, may not be subjected to such high temperatures safely and thus all components were sterilized under ethylene oxide treatment. For subsequent trials after initial sterilization, the components were soaked in 70% isopropyl alcohol and subjected to UV light exposure for several hours following extensive rinsing with sterile PBS to sterilize.

Penicillin/streptomycin is a commonly used antibacterial reagent in cell culture work to prevent contamination. It is generally avoided if possible, however, because of a number of known problems associated with its use. Pen/strep is generally known to mask the presence of bacterial contaminants present at low concentrations and it further may induce the development of antibacterial-resistant strains of bacteria. That being said, in the context of this project it became necessary to use pen/strep to ensure that results were obtained. Working in high-volume lab spaces such as the lab available to the team and working under time constraints where contamination may seriously mar the end results mandated the use of pen/strep, at a concentration of 1% by volume, within the culture medium.

8.4 Bromodeoxyuridine Staining for Cell Proliferation

As was previously mentioned, the C2C12 muscle cells are cultured in proliferation medium for continued growth and transferred to differentiation medium for terminal differentiation and fusion to occur. The different behaviors of the cells under varying treatments may be visualized and quantified using bromodeoxyuridine. BrdU is a thymidine analogue nucleoside that will incorporate itself into cellular DNA during the S phase of the cell cycle, and therefore may be used to mark cells that are undergoing division. Fluorescently tagged antibodies may then be used to target the cells containing BrdU, allowing for imaging under a fluorescent microscope. A full procedure for BrdU staining may be seen in Appendix F.

An equal number of cells were added to 6-well plates in three replicates of proliferation and differentiation medium. Last year's project team confirmed that 2% horse serum supplementation in the medium provided for optimal differentiation (Aschettino et. al., 2012),

therefore all three differentiation wells made use of this concentration in the medium. BrdU was added to the wells approximately fifteen hours prior to analysis. At the fifteen hour time point, the cells were fixated in methanol to prepare for staining. They were then permeabilized with hydrochloric acid to allow for the subsequent penetration and binding of antibodies. The primary antibody, raised against BrdU, was added to the wells and incubated to allow for binding to the cells that had previously incorporated BrdU. Fluorescently-tagged secondary antibodies raised against the isotype of the primary antibody were then added to stain these same cells. Finally, cells were counterstained with Hoechst 33342 dye, a blue fluorescent dye that tags cellular DNA and marks all cells in culture.

Immunocytochemical analysis of cells cultured in proliferation medium may be seen in Figure 18 below.

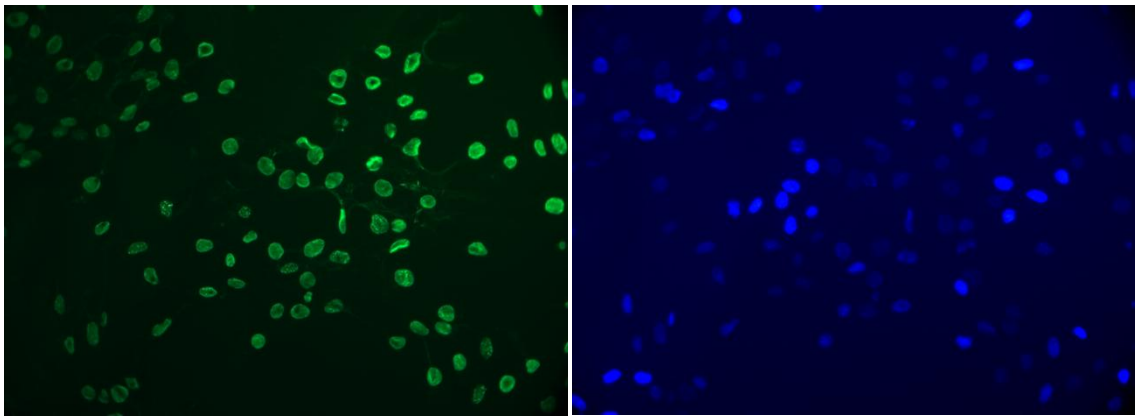

Figure 30: BrdU Staining of C2C12 Cells Cultured in Proliferation Medium

Similar ICC analysis of cells cultured in differentiation medium is seen in Figure 19.

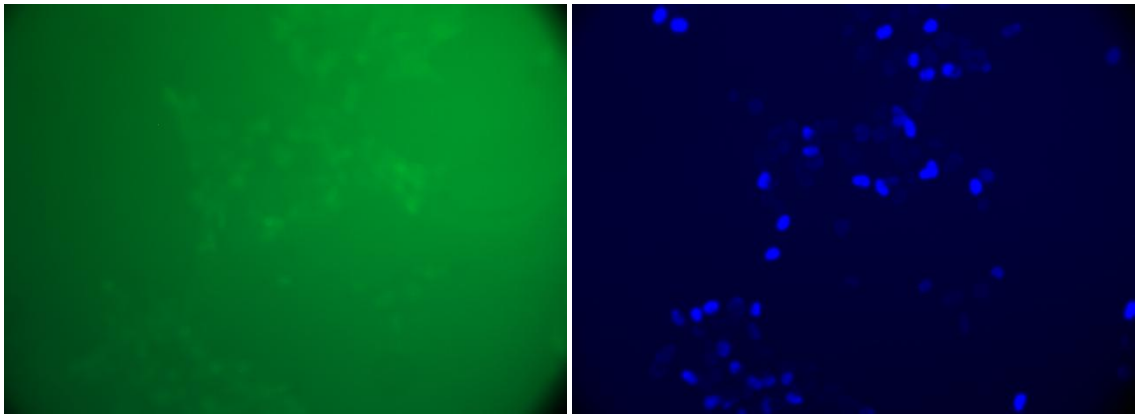

Figure 31: BrdU Staining of C2C12 Cells Cultured in Differentiation Medium

As the images above show, the cells cultured in the proliferation medium demonstrated almost complete uptake of BrdU, marked with the green dye. Cells cultured in the differentiation medium showed little to no uptake (the deeper and more intense green seen in the second image is due to overexposure of the plate by the camera, as no BrdU was detected in the image at baseline).

When viewed under phase contrast microscopy, these same images showed striking differences between the two sets of cells as well. Proliferating cells were more isolated and spread out across the plate, whereas differentiating cells had already begun to join together in preparation for fusion. Representative images of these phenomena may be seen below in Figure 20.

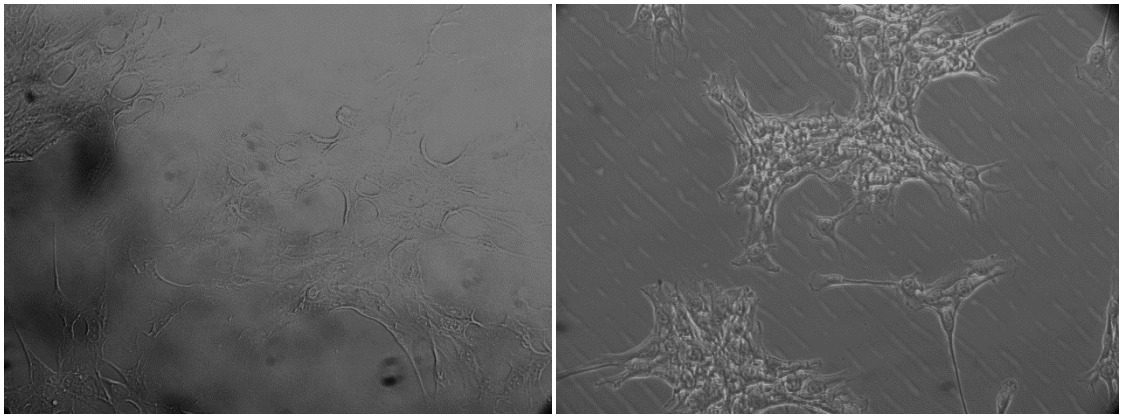

Figure 32: Phase Contrast Microscopy of Proliferating (left) and Differentiating (right) C2C12 cells

While this assay was mostly in line with the expected results, it was not without error. A number of cells in specific regions of two of the wells showed a fair amount of BrdU uptake.

This result may be accounted for in that, due to time constraints, the cells were only introduced to differentiation medium roughly twelve hours prior to the addition of BrdU. This would not necessarily allow for all of the cells to respond to the new proteins and growth factors and enter a phase of terminal differentiation, and it is instead likely that some of the cells in the population were still within the cell cycle when BrdU was added. Nonetheless, if given more time, it is fully expected that there would be a complete correlation for cells in proliferation medium incorporating BrdU as well as no BrdU uptake in differentiating cells.

8.4 Cell Seeding in Agarose Tissue Mold

Moderate tissue formation was seen after approximately four to five days in the agarose mold. However, it is at this point that the team encountered the largest issue in its design. The agarose mold could not easily be removed from the dish as intended without damaging or losing the tissue that it had previously been supporting. Several attempts at this proved unsuccessful and, due to the time constraints of this project, redesigns could not be completed to attempt to improve the design and allow for a fully operational system.

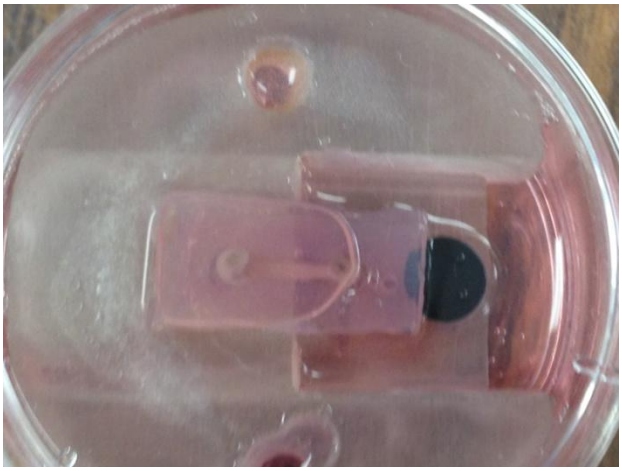

Figure 33: Tissue in Dish 12h After Seeding

Chapter 9: Project Impact

9.1 Manufacturing

Modeling the entire system in a computer aided design software program was the first step toward producing a final working product. Solidworks 2012 was utilized for this means. Each individual component of the system was modeled in this program separately and then combined to form an assembly, which was seen previously in figure 17. The base component for which we designed around was the 35mm petri dish, and the remaining parts were built to house and fit around this dish. Solidworks allows for a fairly simple method of developing and accurately modifying dimensions and tolerances in components and also permits these geometric models to be exported as files for manufacturing.

The base material for manufacturing was polycarbonate, which is a rather inexpensive material that is easy to work with. Using only the resources available to the team on campus, the dish inserts and petri dish base plate were able to be manufactured exactly to specification.
The stainless steel plate to support the stepper motor was also manufactured using the on-campus resources.

9.2 Sustainability

Certain considerations were input into the design process of this system in an attempt to limit the environmental impact of its manufacture and use. This is a common consideration that engineers must make in product development as conservation of resources is both cost-effective and important to a cleaner future. For purposes of sustainability, this device was designed to operate within a 35mm petri dish. In using such a small dish size, cell culture media and reagents were spared as larger sized dishes require several milliliters more media to completely fill. All of the individual components were designed to be reusable. The wires associated with the electrode design may need to be replaced after numerous uses due to the potential for corrosion, though a limited number of resources were used for this and so the waste level is low. Further, a platinum tip was chosen to extend from the plastic electrode casing into the media as the only metallic component in contact with this liquid; culture medium is rich in electrolytes

and so a non-corrosive material such as platinum is able to withstand this environment well and corrosive byproducts are not expected to be overly present.

9.3 Economics

Biological and biomedical research tends to be quite expensive. In 2012 alone, $124 million was spent by the NIH on research surrounding various forms of muscular dystrophy; $4.4 billion was spent on bioengineering and regenerative medicine research; and $14.2 billion was spent on clinical research and trials (cumulatively, extending across many more areas of research than musculoskeletal disorders) (Estimates of Funding, 2013). A major contributor to this number is the need for a platform of testing for various proposed therapies. In the bench-top setting, 2D monolayer cultures as well as 3D cultures developed within scaffolds may be used to conduct preliminary testing on these therapeutic efforts at muscle regeneration. However, neither of these platforms are a very close approximation of natural muscle tissue, therefore extensive *in vivo* testing must be conducted to thoroughly validate the efficacy of these treatments. In developing an accurate and reproducible model of skeletal muscle tissue the need for animal and human testing is somewhat reduced and overall project spending could therefore be decreased. This project does offer the potential to provide this platform in the future.

9.4 Ethics

The cell culture work in this project utilized animal serums within the culture media for the growth factors they contain. This is sure to raise some ethical concerns due to the manner in which the sera are obtained. However, the grand scope of this project is to aim for the development of a product that will ultimately reduce the number of animals and humans required as testing platforms for clinical therapies. Thus the benefits of a successful device are likely to far outweigh the ethical costs associated herein.

9.5 Health & Safety

Conducting *in vitro* testing of proposed clinical therapies permits determination of the safety of this therapy prior to introduction into human or animal subjects. These subjects are thereafter put at less risk of injury or illness as a result of treatments at the trial phase. A determination of functionality may be studied on an in-depth basis through real-time analysis of

their effects on the tissue. It is hopeful that this could provide as well for a quicker output of drugs and treatments that could save numerous lives once on the market.

Chapter 10: Discussion

The system described in this project report met a number of the established goals on an individual basis. However, there were a number of design shortcomings that ultimately did not allow the system to work as well as intended on a full scale. Nonetheless there are a number of implications toward future works in skeletal muscle tissue engineering associated with this project.

10.1 Stimulation Mechanisms

Proof of concept testing on the mechanical and electrical stimulation mechanisms independently verified that the two systems worked as intended. Each could be easily programmed and modified according to the individual needs of the product, and each mechanism could be carried out in a manner that preserved the sterility of the system.

When skeletal muscle cells begin to fuse and develop tissue constructs, contractions will begin sporadically as the tissue contracts upon itself. Should the anchoring posts not have been able to flex under this tension while continuing to hold the tissue in place, the tissue would rupture. Fortunately, the polycarbonate posts that were used in the system were proven sufficiently flexible to sustain this tension and maintain the tissue integrity during formation. Validation tests further illustrated that the motor would later have the ability to successfully apply strain to the tissue; given the 5mm length of the central linear region of the tissue mold, the goal of 10% strain was demonstrated to be possible within this system as the proof of concept test that was run using an elastic band reached strains greater than 0.5mm, the target level. This was unfortunately not confirmed with a mature tissue construct as the tissue did not survive to this point but assuming revisions to the design were made to better facilitate this aspect of the project it is anticipated that this would be possible.

Using a previous MQP report (Dao et. al., 2012) as the basis for our own design, electrodes were designed that could be integrated into this design and emit electric pulses to the tissue in a manner mimicking motor neuron function. Cell culture medium is a liquid rich in electrolytes, which means that it will conduct electricity well. Therefore, the electrodes were aligned perpendicular to the tissue to generate the electric field through the petri dish. This

system was demonstrated effective in practice, as electric pulses were demonstrated to be conducted through the medium. This was again not able to be verified in tissues but it is estimated that it would continue to work as designed in final practice.

10.2 Tissue Formation

Agarose was chosen as the hydrogel material to be used as the tissue mold for a number of reasons. It is first and foremost a biocompatible material and will thus interact well with the cells. Further, the cells do not adhere to the surface of the gel, which is an important consideration. When cultured in standard petri dishes, proteins facilitate contact between the cells and the polystyrene dish and the cells adhere, forming a monolayer; the cells are then able to migrate about the dish surface but do not detach and cannot form three-dimensional constructs in this manner. Using a non-adherent surface with a defined channel shape directs the cells to fuse with each other and in the shape provided by the mold channel.

Roughly four to five days after the cells were seeded into the mold channel, there was moderate tissue formation witnessed. Utilizing a co-culture system, the fibroblasts would be able to generate supporting extracellular matrix proteins as the myoblasts fused to form tissue. It was at this point however that the device's greatest shortcoming was elucidated, however. Whereas the design was developed with the vision that the tissue mold could be fairly easily cut away using a sterile scalpel, this did not prove possible, at least within our means, without damaging the tissue or pulling it away and losing it along with the extracted mold. It is also a distinct possibility that the tissue was simply not provided with enough time to mature into one unified construct and that further time was required for development of the tissue so that it would be sufficiently strong to withstand the process of gel extraction.

10.3 Overall Design

Several issues were encountered in testing and validation of this tissue engineering system that precluded the successful attainment of all goals. As previously referenced, the greatest issue that the team was not able to overcome was the proposed method of removing the agarose tissue mold from the petri dish and leaving the tissue intact, which was proven unsuccessful in multiple attempts. Another factor that may well have contributed to this was the size of the petri dish. Previous MQP projects attempting to approach the same topic utilized 100mm petri dishes (Aschettino et. al., 2012). It was indicated that the consumption of resources

in this design was high and that a smaller dish could be used. Given the scope of this project and the sought-after development of a tissue 5mm in length, 35mm was a viable option for the size of our petri dish. However, this limited to some extent the ease with which the system could be manipulated. It also left very little clearance between the top of the mold and the underside of the petri dish lid, which made submerging all of the components of the dish challenging.

The connection point between the motor shaft and the lever arm proved sufficient for our purposes, but could have been improved upon. The mechanism of locking the lever in place consisted of two washers fitted firmly on each side of the lever top at the point it contacted the motor shaft. Given that this was a threaded shaft the options for connection mechanisms were limited, though this could have been explored more in depth to determine alternative means.

In order to incorporate the mechanical and electrical systems into the petri dish, holes were placed in the petri dish top. The parts were placed in the lid and then sealed using silicone adhesive. The presence of all of these components as well as the sealing adhesive material utilized much of the central lid space and it thus became difficult to conduct full analysis of the forming tissue using microscopy. For this reason the cells were generally cultured using a normal lid before changing to the sterile modified lid after several days in preparation for stimulation.

Chapter 11: Recommendations and Conclusions

Certain successes were seen within each of the individual components of this system, being the tissue mold system, the mechanical actuation system, and the electrical stimulation system. Based upon observations of some of the limitations and how they could have been overcome, the team has developed several recommendations for future attempts at this project.

11.1 Tissue Mold System Recommendations

Agarose mold extraction from the petri dish, as previously stated, proved to be the greatest obstacle in this project. As this gel is relatively easy to cut through, it was anticipated that a scalpel could be used to precisely cut out the mold while leaving the tissue intact. This did not prove to be the case however and it is recommended that options be explored to improve upon this. We felt that it would be in our best interests to form the tissue directly upon the mechanical actuation system as it would not require the tissue to be transferred from one vessel to another and would ideally have resulted in the least handling of the tissue possible. However, alternatives should be considered from both approaches to consider which is safest for tissue development.

Figures 11 and 12 of this report illustrate the molding system developed for this project. Three separate mold channel designs were devised, which were to be tested to validate which optimized tissue formation. Due to time constraints this could not be accomplished, and the U-shaped channel was utilized throughout. It is recommended that each of these different mold channel designs be tested and compared, along with other potential designs, to confirm which promotes the best tissue development.

Cell seeding in this project was conducted at a standard of 1 million myoblast cells and 250,000 fibroblast cells. Different densities, which were not tested in this project due to limited resources and time, should be experimented with to determine what density provides the best results. Different mold depths could also be experimented with to permit and analyze varying tissue diameters.

11.2 Motor Recommendations

The motor that was selected for use in this project met a number of our design requirements. However, one need in particular that was not met by this motor was compatibility with the environment of the incubator. Incubators operate at high levels of humidity, typically close to 100%. The motor that was used in this design would not have been able to withstand long periods of time within this environment. Waterproof motors were found to be by and large outside of the project budget and thus not feasible to incorporate. For that reason, it was our primary aim to develop a system that would simply be capable of applying mechanical strain before we considered testing with prolonged periods of strain in an incubator environment. For more permanent and stable iterations of this project a more high-performance motor compatible with the incubator environment should be selected.

Our motor was further complicated by utilizing an irregular drive shaft for linear actuation. The shaft was of an M5 (5mm) diameter, of which the industry standard dictates has a 0.8mm pitch and therefore the majority of the associated mechanical components for M5 diameter parts consist of 0.8mm pitches. This shaft, on the other hand, was threaded with a 2mm double-started pitch, for which no stock parts could be found to match. This made the connection point between the lever arm and the motor shaft difficult to perfect. Slightly modifying M5 washers allowed them to be screwed onto the shaft where they held the lever in place. This screw system anchored the lever tightly enough that it could be accurately controlled and would not slip out of place. However, the system could have benefitted from a more static and stable connection point, one that likely would involve a direct connection between the two components.

11.3 Petri Dish Recommendations

The chosen 35mm petri dish limited the available working space for the tissue, mold, and physical inserts. Though it was beneficial in conserving resources, the interior components were too large to integrate well. It is recommended that this design be scaled up to work with a 60mm petri dish if possible as this would provide more overhead clearance between the top of the mold system where the tissue is formed and the interior side of the lid. This would also ideally permit the development of more intricate dish inserts that might operate more favorably than the ones developed in this project. Finally, using a larger dish should allow for a greater spread of the

inserts across the lid, meaning that the view into the interior of the dish would be less occluded by peripheral pieces and that greater tissue visualization and analysis would be possible.

11.4 Conclusions

Overall, a device was developed to permit the *in vitro* formation of tissue engineered skeletal muscle. The three major components developed therein were the tissue molding system, the mechanical actuation system, and the electrical stimulation system. Each of these systems was validated on an individual basis as tissue was successfully formed within the agarose hydrogel mold and the mechanical and electrical systems were successfully programmed to work as desired. The entire device was further developed in such a means that that sterile environment for the tissue was preserved so that safe and healthy development could be seen. Some limitations in the final assembly prevented all of it from working properly as the device was intended to, however the findings of this project show promise for future attempts at this project. The final device in this project was intended to serve as a prototype for a more adept variation that could be used many times over as a reliable source for pre-clinical testing of piloted therapeutic treatment options, and the components of this system may be adapted to a device that will successfully promote the *in vitro* development of biomimetic skeletal muscle tissue.

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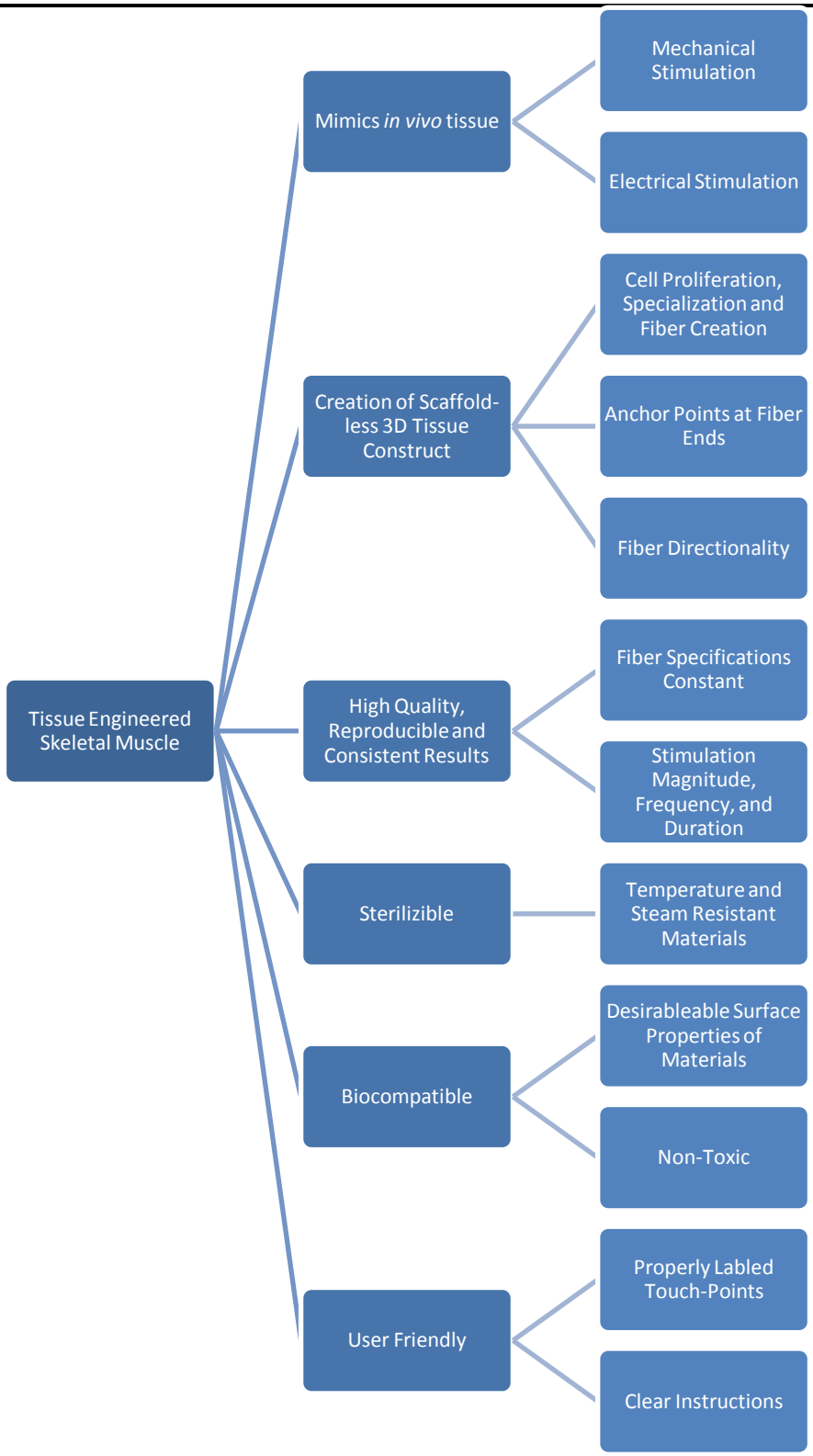
Appendix A: Pairwise Comparison Chart

	Electrical Stimulation	Mechanical Stimulation	Sterile Environment	Measure Stress and Strain	Time/Cost Effective	Tissue Transfer	Ease of use	**Totals**
Electrical Stimulation		.5	0	1	1	0	1	***3.5***
Mechanical Stimulation	.5		0	1	1	0	1	***3.5***
Sterile Environment	1	1		1	1	0.5	1	***5.5***
Measure Stress and Strain	0	0	0		1	0	1	***2***
Time/Cost Effective	0	0	0	0		0	0	***0***
Tissue Transfer	1	1	0.5	1	1		1	***5.5***
Ease of use	0	0	0	0	1	0		***1***

Appendix B: Objectives Tree



Appendix C: Budget

Supplier	Material	Cost
Sigma Aldrich	Septums	$24.94
McMaster-Carr	Polycarbonate	$62.60
SparkFun	Motor	$37.73
WPI-ME	Mold Prototypes	$7.02
Amazon	Screws	$14.22
Tritech Research	Platinum Wire	$140.54
Sparkfun	Easy Driver	$28.07
	Total	**$315.12**

Appendix D: Cell Culture Protocols

Passaging Adherent Cells

1. C2C12 cells should be passaged at 60-70% confluence to prevent contact inhibition and terminal differentiation
2. Aspirate the cell culture medium out of the flask using a sterile aspirator tip and vacuum tube
3. Add 5ml sterile Phosphate Buffered Saline (PBS) to the flask, swirl to rinse the entire adherent surface, and aspirate
4. Add 1ml pre-warmed 0.25% trypsin + EDTA, let sit for 30s-1min
5. Tilt flask back and forth and examine how many cells are still adhering to the bottom; tap sides of flask to try to loosen more cells
6. Once cellular monolayer is completely broken up, add 5ml DMEM+FBS, spread evenly over bottom of flask
7. Pipette cell suspension from flask and move to 15ml conical tube; centrifuge at 1200rpm for 5min
8. Aspirate media and resuspend in 5ml new media; pipette up and down to break up clumps and fully resuspend
9. Perform cell count to accurately determine number of cells present
10. Add $2.0*10^5$ cells to the flask and bring the total volume to 10ml, spreading the suspension evenly across the bottom of the flask
11. Label the flask and place in incubator
12. Aspirate any remaining unused cells and used media

Thawing Cell Stocks

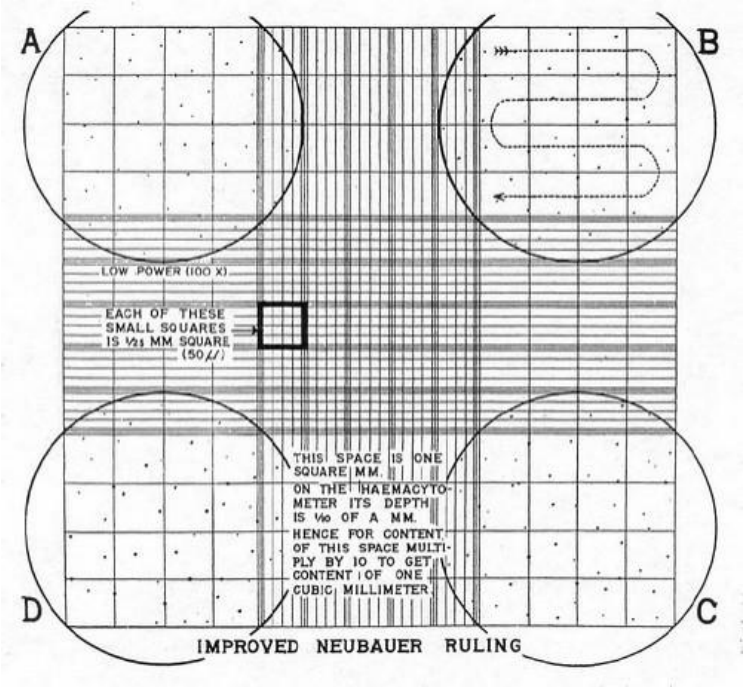
1. Place empty 15ml conical tube in hood
2. Remove crytoube of cells from cold storage and transfer immediately to 37 °C water bath
3. Hold cells in bath with forceps until mostly thawed (one small crystal remaining in the tube; 1-2min)
4. Remove tube from bath, wipe with 70% ethanol, and transfer to hood
5. Pipette cells gently in tube to resuspend, then add suspension dropwise to the 15ml conical tube
6. Add 1ml proliferation medium slowly to the cryovial, swirl, then pipette out and add dropwise to the 15ml tube
7. Add an additional 8ml prewarmed media dropwise to the 15ml tube, tapping or swirling the tube between drops
8. Invert tube slowly 2-3 times and centrifuge cell suspension for 5min at 1200rpm
9. Aspirate media from tube without disturbing cell pellet
10. Resuspend cells in 2ml proliferation media and perform cell count (if not already known)
11. Plate $2.0*10^5$ cells to a flask and bring final media volume to 10ml; ensure that suspension evenly and completely covers the bottom of the flask
12. Label the flask and place in incubator

Freezing Cell Stocks

1. Follow cell passaging protocol through step 7
2. Aspirate medium and resuspend in 2ml proliferation media; count cells
3. Transfer 2.0*10^5 cells (or other desired amount) to 15ml conical tube and centrifuge 5min at 1200rpm
4. Aspirate media and resuspend pellet in 1ml freezing media
5. Immediately transfer suspension to cryovial and place in Nunc cooler, then move to -80 ℃ storage overnight
6. If possible, transfer to liquid nitrogen storage the following day for long-term cryopreservation

Cell Counting Procedure

1. If dirty, clean hemocytometer and coverslip with 70% ethanol and Kim wipes, taking care not to scratch the glass surface; ensure both are dry and free of ethanol before using
2. Ensure cell suspension is homogenized, taking care to swirl or otherwise mix if not
3. Transfer 150µl cell suspension to 1.5ml Eppendorf tube and mix cells with pipette
4. Transfer 100µl of this suspension to a new Eppendorf tube and mix with 100µl Trypan Blue (1:2 dilution); pipette gently to mix entire solution
5. Add 100µl to each chamber of the hemocytometer and place under microscope to view
6. Count the number of living and dead cells in each $1mm^2$ area of the chamber
7. All cells contained entirely within the large squares are counted; cells touching the top and left lines of the square are counted, cells touching the right and bottom lines are ignored
8. Count all live cells from both halves of the hemocytometer, then divide by the number of $1mm^2$ regions counted to calculate the average number of cells present
9. Dead cells may be counted separately, if desired, for a viability count



http://cdnimg.visualizeus.com/thumbs/d2/80/medicine,pathology,reference-d28043c91644f1f790639d7819ce5362_h.jpg

Cell Counting Calculations

1. In a standard $1mm^2$ hemocytometer, each large square is $0.1mm^3$; the number of cells per square, *n*, is equivalent to $n * 10^4$/ml
2. Because a 1:2 dilution was conducted when Trypan Blue was added, the cell count must be multiplied by 2 for the total count

3. Thus, the total cell count $c = 2n * 10^4$/ml
4. Using this calculation, plate cells to new culture vessels at appropriate densities
5. As an aside, Trypan blue is cytotoxic and will kill cells if they are left on for too long; thus, longer waiting periods between adding Trypan blue and counting cells will lead to lower counts and lower perceived viability percentage

Appendix E: Cell Culture Medium Components

C2C12 Proliferation Medium

60:40 (v:v) DMEM:F12 + 10% FCIII + 4mM L-Glut/1% G-Max

Component	Vol per 100ml	Final Concentration
DMEM	53.4ml	89% (60:40 [v:v] DMEM:F12)
F12	35.6ml	
FCIII	10ml	10%
Glutamax	1ml	1%

CRL 2097 Proliferation Medium

DMEM + 10% FCIII + 1% G-Max

Component	Vol per 100ml	Final Concentration
DMEM	89ml	89%
FCIII	10ml	10%
Glutamax	1ml	1%

C2C12 Differentiation Medium

DF12 + 2% HS + 1% G-Max + 1% ITS

Component	Vol per 100ml	Final Concentration
DMEM	57.6ml	96% (60:40 [v:v] DMEM:F12)
F12	38.4ml	
Horse Serum	2ml	2%
Glutamax	1ml	1%
ITS	1ml	1%

2097 Differentiation Medium (24h prior to coculture)

DMEM + 2% HS + 1% ITS

Component	Vol per 100ml	Final Concentration
DMEM	97ml	97%

Horse Serum	2ml	2%
ITS	1ml	1%

Trypsin

C2C12 – 0.25% Trypsin + EDTA

2097 – 0.05% Trypsin + EDTA

Freezing Medium

70% DMEM + 20% FCIII + 10% DMSO

Component	Vol per 100ml	Final Concentration
DMEM	70ml	70%
FCIII	20ml	20%
DMSO	10ml	10%

Appendix F: Immunocytochemistry Protocols

BrdU Cell Proliferation Immunocytochemistry Assay

1. Add 1.0 µl BrdU stock solution/well at least 12 hours prior to assay
2. Aspirate culture medium and wash 2x with DPBS+
3. Fix cells in -20 °C methanol; add methanol to wells and incubate for 10 min at -20 °C
4. Aspirate methanol and wash with PBS
5. Add 1.5M HCl to wells and incubate at RT for 20 min
6. Aspirate HCl and wash 3x in PBS
7. Dilute anti-BrdU antibody 1:100 in PBS + 0.05% Tween-20
8. Add primary antibody solution to wells and incubate (covered) at RT for 30 min
9. Aspirate antibody solution and wash 3x in PBS
10. Dilute secondary antibody 1:500 in PBS + 0.05% Tween-20
11. Add secondary antibody solution to wells and incubate (covered) a RT for 30 min
12. Aspirate antibody solution and wash 3x in PBS
13. Dilute stock Hoechst 33342 (1 mg/ml) dye 1:2000 (final concentration 0.5 µg/ml)
14. Add Hoechst 33342 solution to wells and incubate at RT for 10 min
15. Aspirate Hoechst solution, wash with PBS, and add PBS
16. Cells may now be viewed under fluorescent microscope

Note: if cells were cultured in serum-free medium, they must be blocked using a solution of PBS + 5% FBS + 0.05% Tween-20 at RT for 15 min

Appendix G: Arduino Uno Microcontroller Programming

The Arduino Uno microcontroller makes use of a simple coding language. An example of code that we used may be seen below.

```
int stepDelay = 3;
```

This line indicates the elapsed time occurring between each step in the code. Time in the Arduino code is measured in milliseconds, meaning that in this instance 3 milliseconds elapse between each step of the code.

```
const int stepOutputPin = 3;
const int directionOutputPin = 2;
```

In this section of the code the output pins are established. Pin number 3 is associated with step output and pin number 2 is associated with direction output. These two lines must match the wiring of the Arduino to the EasyDriver.

```
void setup() {
pinMode(stepOutputPin, OUTPUT);
pinMode(directionOutputPin, OUTPUT);
}

void loop() {
spin(1000, 0);
delay(2000);
spin(1000, 1);
delay(2000);
}
```

This section dictates the movement of the motor. In a looped function, the motor steps 1000 times in the 0 direction (the forward and backward directions are indicated as 0 and 1, respectively); it then delays for 2 seconds (2000 milliseconds) before stepping 1000 times in the 1 direction; a 2 second delay initiates before the loop begins once more.

```
void spin(int steps, int direc) {
digitalWrite(directionOutputPin, direc);
for (int i = 0; i < steps; i++) {
digitalWrite(stepOutputPin, HIGH);
delay(stepDelay);
digitalWrite(stepOutputPin, LOW);
delay(stepDelay);
}
}
```

In this code, the motor is programmed to repeat forever, or until the power source is disconnected. However, it is also possible to program the controller to operate for a limited number of cycles. The code for this is given below.

```
int stepDelay = 3;
int iterations = 2;
boolean completed = false;
```

Using the 'iterations' and 'boolean' functions provides for a maximum number of loops to be completed. The code indicates that 2 loops are to be completed. If two loops have been completed (Boolean completed would equal true in this instance) then the motor will stop; if two loops have not been completed (Boolean completed = false) then the motor will continue until this condition is met. This section is supplemented below in the loop section.

```
const int stepOutputPin = 3;
const int directionOutputPin = 2;

void setup() {
pinMode(stepOutputPin, OUTPUT);
pinMode(directionOutputPin, OUTPUT);
}

void loop() {
if(completed == false) {
for(int i = 0; i < iterations; i++) {
spin(1000, 0);
delay(2000);
```

```
spin(1000, 1);
delay(2000);
}
completed = true;
}
}
```

In this section, subsequent to what is included in the first part of the coding, the iteration loop is established. At the beginning it poses that if the term of completion is not met it will run the loop as directed. Once the Boolean condition and the maximum number of loops is run the program will cease.

```
void spin(int steps, int direc) {
digitalWrite(directionOutputPin, direc);
for (int i = 0; i < steps; i++) {
digitalWrite(stepOutputPin, HIGH);
delay(stepDelay);
digitalWrite(stepOutputPin, LOW);
delay(stepDelay);
}
}
```

It is further possible to include notes within the lines of coding in the Arduino software. To include text that is omitted from the coding, a section is begun with " /* " and concluded with " */ " . Any text included within these characters is omitted from the coding and appears in grey in the text field.