Mechanical Stimulator for *In Vitro* Skeletal Muscle Modeling

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

Authored by:

Alex Barat
Randy Chin
Alan Gribble
Jacob Konowitch

Advisor:
Professor Raymond Page, Ph.D.

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Abstract

The use of an *in vitro* skeletal muscle model has the potential to transform clinical research such as drug screening for muscular myopathies. Current techniques are not suitable due to the lack of homology between their models and native skeletal muscle. These methods are limited based on the use of exogenous scaffolds and the difficulty to transfer tissue between cultures and the testing device. The need that this model must address, is a model that accurately mimics native skeletal muscle. Our group developed a modular and sterilizable, polycarbonate culture dish with polydimethylsiloxane (PDMS) posts and agarose molds to improve upon cell density and cell self-assembly. The dish has the ability to be connected to a linear actuator, providing axial stimulation in both the negative and positive direction. The hope is that stimulus will lead to the maturation of tissue into levels of strength and function similar to native muscle. This device can provide a better model that, if testing is confirmed, may lead to a clinical model in the future.

Executive Summary

Since skeletal muscle tissue is so prevalent in the body, the failure of skeletal muscle tissue severely limits the functionality of organs through loss of elasticity and muscle movement, which can lead to death. One contributor to the loss of skeletal muscle function is Duchenne Muscular Dystrophy (DMD). This genetic disease mutates the dystrophin gene, which leads to muscle degeneration and can eventually lead to death. Once skeletal muscle degenerates, motor function ceases, which inhibits the body from moving and leads to organ failure and death [25].

Skeletal muscle is an essential component of the body that allows the skeletal infrastructure to move voluntarily under the control of the somatic nervous system. *In vivo*, skeletal muscle tissue is subjected to strains, which align fibers and influences muscle weight and length [27]. The mechanical loading of the tissue facilitates the actin and myosin alignment with actin connecting to the extracellular matrix (ECM). This alignment of the muscle fibers and the connection of actin to a functioning ECM allows for the formation of native skeletal muscle [37]. Additionally, during embryogenesis and muscle organogenesis, passive strain is applied to the muscle via bone elongation and spontaneous twitch, allowing for fiber alignment and strengthening of muscle tissue [27].

The bodies of healthy mammals have the ability to heal small wounds and damaged tissue, naturally. Unfortunately, there is some damage or disease, such as DMD, that is too great for the body to overcome. DMD affects one in every 3,500 males born in the United States [25]. DMD degenerates the tissue over time through a hereditary defect in the gene responsible for dystrophin production. The decreased production or lack of dystrophin prevents the binding of actin to dystrophin-associated glycoproteins (DAGs) [30]. These glycoproteins attach muscle cells to the extracellular matrix and without their

presence, the muscle cells are constantly undergoing inflammation and degeneration. This constant inflammation, leads to an unsustainable amount of muscle repair which ultimately leads to the inability to heal and regenerate muscle at a fast enough rate [28]. Since, skeletal muscle is required for many basic functions, the symptoms that result from degenerated muscle can eventually cause death. These genetic mutations are spontaneous in nature and the exact cause and prevention of such mutations has yet to be assessed [25].

The work by Powell et al, has been useful in presenting the need for mechanical stimulation on developing tissue. The studies performed did not produce tissues that fully mimic natural skeletal muscle. Their lab has produced results of tissue that have lower contractile forces as well as smaller fiber size than native muscle [27]. Additional labs, such as the Matsumoto lab have produced fibrin gels seeded with myoblasts. Added strain on these myoblasts leads to the initiation of fiber alignment [19]. This work failed to meet the need of an *in vitro* muscle model because the use of a scaffold (ie, the fibrin gel) does not mimic native tissue formation.

Therefore, a skeletal muscle model that is structured like tissue found in the body and does not use an exogenous scaffold, provides usefulness in the field of *in vitro* clinical studies as well as drug development. Models like this are needed because there is not sufficient treatment to heal damaged or diseased tissue. With the use of a device that can create a muscle that closely mimics native tissue, experiments can be done to damage the construct and test healing capacities of new drugs.

The important aspects of developing a muscle model are the ability: to mechanically stimulate tissue, to maintain sterile environment, easily seed cells within

the system and to reproduce results. Thus, these objectives were used to produce a device that could improve upon previous work within this field.

Design

The design that was decided on for this project was a culmination of various original design alternatives. These alternatives were tested for feasibility to create a system that could linearly actuate a muscle tissue to simulate native skeletal muscle growth for *in vitro* testing. While many studies have attempted to create a system to provide strain to a tissue, most are created within a static petri dish. The design produced in this project created a modular, two-piece petri dish with an external actuation system to stimulate the dish.

The actuating petri dish is comprised of two halves. Half of the dish is anchored and the other half is movable to allow for axial stimulation.

A tissue mold was designed as well to reside within each chamber and help form the tissue as the cells differentiate and begin to fuse to each other. The system delivers the ability to support the cells while providing strain. The mold contains a base PDMS piece that has a tear dropped shaped post to reduce the formation of necrotic tissue. Two PDMS pieces with cast 2% agarose in cell culture media can be placed around the post to hold the tissue during differentiation and strain application. The design was minimized to address the risk of necrotic tissue formation.

The final aspects of the design were the components and casing for our linear actuator. The components of this linear actuator, included a stepper motor, programmable card, coupling and bolt attachment. This bolt pulls or pushes the petri dish in the desired precise motion in the negative and positive direction.

Results

A set of tests were run in order to determine the effectiveness of the design. The progression of testing was broken up into three sections: the sealing ability of the polycarbonate enclosure, the linear actuator's ability to exhibit 5%, 10%, 15% strain with less than 1% error, and the ability for the pooling system to contain media and the seeded tissue construct.

A leak test was conducted to determine the sealant of the polycarbonate enclosure device. The test filled 950 mL of water dyed with trypan blue into the enclosure system on a sheet of paper and checked after 24 hours. The test showed that the enclosure system was properly sealed as no leakage occurred over 24 hours. A humidity test over 24 hours at one hour humidity reading increments was conducted on the internal space of the enclosure system. The humidity levels fell within the operating humidity range for both the stepper motor and the Raspberry Pi microcontroller.

The set of tests for the linear actuator showed that actuating a set of elastic bands in both the positive and negative direction can be done at 5%, 10%, and 15% strain +/- 1%. This was measured by taking an image of the band at no actuation and then taking images at subsequent strains and analyzing through ImageJ software. The results showed that the actuator can actuate the band 5%, 10%, and 15% for both negative and positive strain.

The pool system with the agarose mold and post was tested to ensure that the tissues can be flooded with media even when linear actuation is occurring. This was tested by linear actuation of the modular portion of the pool system over a 24 hour

period. A paper towel was placed under the pool system and it was shown that there was no leakage of media.

Conclusions

Current models of skeletal muscle tissue engineering use exogenous scaffolds, i.e. fibrin microthreads or a collagen sponge. This project created a system that replicates native embryogenesis by producing constant strain on tissue constructs. Another improvement made upon previous MQPs is a completely self-contained actuation system, which is operational inside the incubator. Along with this improvement, the designed system allows for cell seeding, differentiation, and tissue formation to be contained within one dish, which eliminates the transfer of tissue samples. Tissue sample transference in current models mechanically weakens the sample, which this model eliminates.

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Authorship

All writing and editing of this report was split evenly throughout the group. Cell culturing and device viability testing was completed by Randy Chin and Jacob Konowitch. Rapid prototyping and design modeling was produced by Alan Gribble. Mold fabrication and manufacturing of design parts was accomplished by Alex Barat.

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Chapter 1 Introduction

Skeletal muscle is an essential component of the body that allows the skeletal infrastructure to move voluntarily. Since skeletal muscle tissue is so prevalent in the body, the failure of skeletal muscle tissue severely limits everyday movements and the ability of the body to regulate homeostasis [29]. One contributor to the loss of skeletal muscle function is Duchenne Muscular Dystrophy (DMD). This genetic disease, mutates the dystrophin gene, which leads to muscle degeneration and can eventually lead to death. Skeletal muscle repair and regeneration eventually ceases with fibrotic tissue and weakening of muscles inhibiting respiration and leading to death [22].

Currently, research on how to replace damaged tissue is done by conducting *in vivo* experiments on small animals or through *in vitro* muscle models [25]. Inevitably, the cost associated with conducting *in vivo* research is too high for it to be a viable way to gather data on dealing with Muscular Dystrophy patients. Additionally, mouse physiology does not properly imitate the human body. A more efficient, cheaper, and quicker way in developing a method to research and determine how to repair or replace damaged tissue is to culture cells *in vitro* in an environment that mimics native skeletal muscle environment in the human body. While conceptually, the idea of *in vitro* mimetic skeletal muscle tissues would be ideal, the current methodology involves the use of a synthetic scaffold to support growth by providing mechanical stability and promoting fiber alignment [15]. The problem that arises in these models is the lack of similarity to native human muscle tissue. Thus, a method that does not require a scaffold and does not rely on *in vivo* testing is an important step in the research of damaged muscle tissue.

In addition to developing a system without the use of a synthetic scaffold, mechanical stimulation is needed to produce a design for a skeletal muscle tissue model. A stimulus applied to an *in vitro* tissue, replicates *in vivo* stresses and strains. This stimulus helps to align cells, which leads to a muscle tissue that closely represents native tissue.

The cells used to mimic skeletal muscle tissue *in vitro* are C2C12 cells (from mice), thus the results of creating an *in vitro* skeletal muscle tissue are viable for a mouse's skeletal muscle tissue and not a human's skeletal muscle tissue. The reason for using C2C12 cells is because their ability to differentiate and proliferate is better than human muscle cells [39]. C2C12 cells have a doubling time of about 24 hours and can fuse into tissue within days of adding medium supplemented with 10% adult horse serum [39]. For human mesenchymal stem cells (HMSCs), it can take up to a few weeks to develop a tissue construct [26]. Cells are cultured in an environment that is aseptic or capable of being sterilized in order to maintain usefulness of the system without introducing outside factors that can contaminate cells.

The following sections, outline the literature of how skeletal muscle functions and will help to understand the proposed project goal. The clinical importance of the *in vitro* skeletal muscle tissue is very essential as its value to the medical field is quite beneficial to the continuing research on muscle tissue failure and the testing and validation of drugs to combat such diseases as DMD. Additionally, the objectives to be met in an improved design need to be addressed and outlined. Therefore the goals of the project are to design a device that can mimic native tissue of skeletal muscle through the addition of *de novo* factors such as mechanical stimulation while maintaining physiological morphology.

Chapter 2 Background

2.1 Clinical Significance

The bodies of healthy mammals have the innate ability to heal wounds and damaged tissue. Unfortunately, there is some damage that is too great for the body to heal naturally. Large muscle injuries like tears and strains are prevalent to the muscular structure as a result of physical trauma. Skeletal muscle injuries make up 55% of athletic injuries [17]. Additionally, traumatic accidents can involve extreme skeletal muscle damage or impairment. While small tears can heal over time, more severe injuries can be too large for the body to fix by itself and can result in scarring and loss of function. Tears that do not fully heal, do not regain full strength [17].

Conversely, people may have an impaired immune system or genetic defect that inhibits the healing capacity of muscles. Muscular diseases can affect functionality of skeletal muscle and cause mobility issues for patients. These muscular diseases are called myopathies and they have long term effects that damage muscle tissue [21]. One group of myopathies in particular is Muscular Dystrophies, which can be damaging to the tissue repair process in skeletal muscle [21]. As mentioned earlier, a specific type of Muscular Dystrophy based on a large genetic mutation, is Duchenne Muscular Dystrophy (DMD). DMD affects one in every 3,500 males born in the United States [25]. DMD degenerates muscles over time through a hereditary defect in the gene responsible for dystrophin production. The decreased production or lack of dystrophin prevents the binding of actin to dystrophin-associated glycoproteins (DAGs) [30]. These glycoproteins attach muscle cells to the extracellular matrix (ECM). Thus, without the presence of DAGs, the muscle cells do not attach, which causes an influx of Ca^{2++} into the muscle

cells. The excess amount of Ca^{2++} activates proteases which “snip” muscle fibers and create cyclic repair and regeneration [29]. The constant repair and regeneration causes fibrosis of muscle tissue and loss of elasticity and function [28]. Many times, the damage results in scoliosis and ambulatory loss which inevitably leads to the weakening of muscles that contribute to inspiration and expiration [22]. As a result, most patients with DMD die from respiratory failure or cardiac problems caused by a reduction of myocardial function [22]. Thus, a skeletal model to assess the cause and prevention of this disease is important in the continuing growth of medical research.

A skeletal muscle model that is structured like tissue found in the body, provides usefulness in the field of *in vitro* clinical studies as well as drug development. Models like this are needed because there is not sufficient treatment to heal damaged or diseased tissue. With the use of a device that can create a muscle that closely mimics native tissue, experiments can be done to damage the tissue and try to heal it with new or current drugs. The development of this system will also enable a large throughput and a shorter time period for the experimental process as compared to the *in vivo* alternative.

2.2 Skeletal Muscle

The clinical significance of this project is understood by knowing the basic physiology and anatomy of human native skeletal muscle. Native muscle tissue is produced and repaired within the body of a human, compared to implanted synthetics or man-made alternatives. The skeletal muscle group helps provide movement for the body and is comprised of bundles of tightly packed and highly oriented fibers [30]. These fibers are made up of multi-nucleated cells originating from myoblasts [2].

2.2.1 Muscle Embryogenesis

Muscle formation begins when fertilization of the egg forms a zygote and initiates embryonic development. The formation of somites results in the origination of skeletal muscle tissue [2, 3]. Development of the myotome from somites begins when mesenchymal cells recruit muscle progenitor cells through the c-Met-HGF pathway. This pathway is delineated with the interaction between the tyrosine kinase receptor c-Met and hepatocyte growth factor (HGF) [2, 3]. These progenitor cells subsequently activate myogenic regulatory factors (MRF), which cause them to differentiate into myoblasts. The differentiated myoblasts then form myocytes, which align and fuse to form muscle fibers or myofibrils [2, 3].

2.2.2 Skeletal Muscle Structure and Function

A skeletal muscle fiber is a bundle of several myofibrils, which are themselves packets of actin-myosin bridge containing myofilaments. The ends of each of these bundles of myofilaments are aligned uniaxialy [41]. The macroscopic breakdown of muscle fibers can be compared to that of fiber optic cables; several filaments bundled together to form a large fiber with several large fibers incorporated into another bundle to form the muscle. In Figure 1, the structure of skeletal muscle is highlighted as well as the

different layers that build up the fiber.

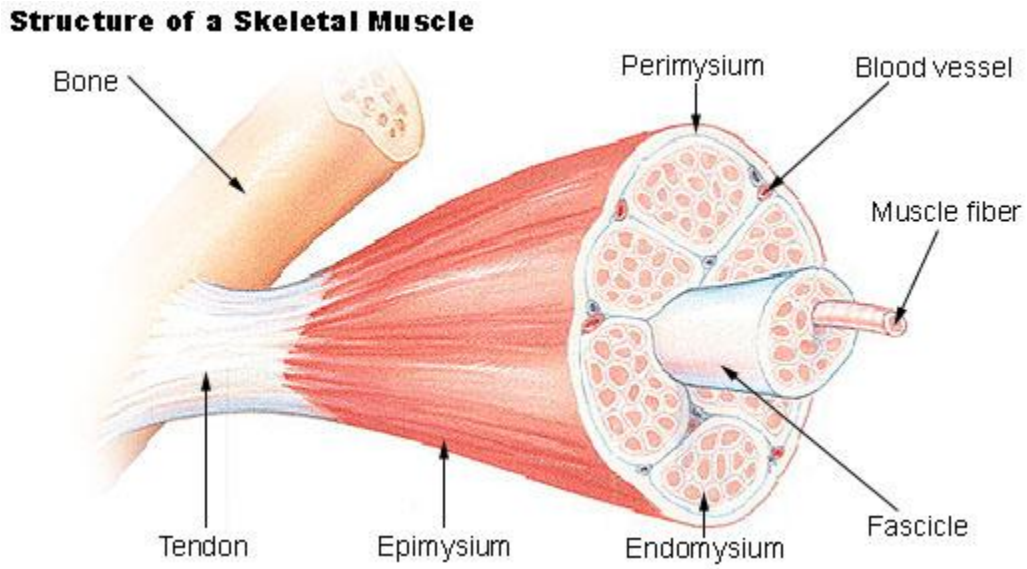


Figure 1: Structural diagram of skeletal muscle showing the different layers of ECM [30]

Also in Figure 1, the layers of the extracellular matrix (ECM), including the epimysium, perimysium and endomysium, can be seen. The ECM, which is an important aspect of skeletal muscle formation, is made up of glycoproteins and polysaccharides (specifically collagen Type IV and basal laminina), both of which are secreted by the cell itself. The ECM provides scaffolding or structural support for the cells to migrate and differentiate at the site of muscle repair and development. The ECM promotes cell aggregation and provides a substrate for cells to attach. ECM receptors, such as integrins in Figure 2, control processes of the cell and control binding of cells for support while muscles are being strained. Additionally, the ECM provides morphological direction through the binding of growth factors and maintains homeostasis within the cell [11].

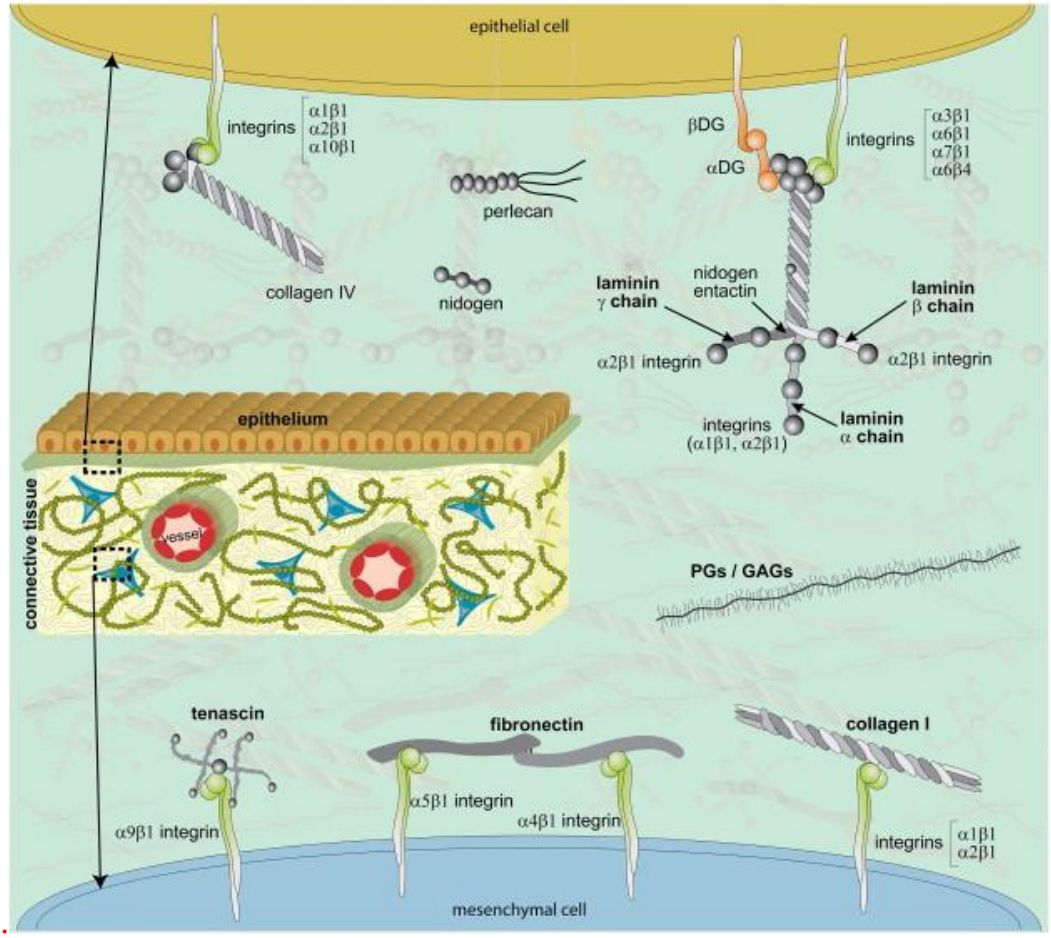


Figure 2: Diagram of the ECM and the surrounding molecules [33]

The structure of skeletal muscle can be further broken down in descending hierarchy. The outermost covering of an individual skeletal muscle is called fascia. This layer is composed of fibrous connective tissue and it separates each muscle from the others while also maintaining its shape [30]. Underneath the fascia and directly surrounding the muscle is another layer of connective tissue called the epimysium. Covered by the epimysium is the perimysium, which surrounds sections of bundles of muscle fibers called fascicles. Within the fascicles, the individual muscle fibers are surrounded by another layer of connective tissue called endomysium. The separate layers

of connective tissue allow for each section of muscle to move individually. Inside each myofibril there are myocytes, which are the contractile unit of a myofibril [30].

The myofibrils are long, thin strands that run the length of the muscle. These myofibrils consist of two protein filaments, one thick and one thin. The thick filament is myosin and the thin filament is actin. These two proteins form the striations in muscle fiber and in turn form a repeating pattern unit called a sarcomere. The two proteins work together to provide movement for muscle fibers [30]. Myosin is a thick filament with extended cross-bridges projecting outward. Actin is a helix structure that provides the myosin cross-bridges a binding site. The thin actin filament is surrounded by two inhibitors of the binding site, troponin and tropomysoin [30]. In order for contraction to occur, calcium ions must be released by the sarcoplasmic reticulum. This occurs when the cell membrane depolarizes and releases sodium. Potassium ions then repolarize the area causing an action potential [30]. This action potential causes the release of calcium ions which bind to troponin and make the tropomysosin vacate the area of myosin cross-bridge binding. Once the binding site is available, adenosine triphosphate (ATP) is hydrolyzed by myosin into adenosine diphosphate (ADP) and inorganic phosphate (P_i) and used as an energy source, loading the actin and myosin filaments like a gun. The ADP and P_i release and allow for relaxation through binding of another ATP molecule [29].

2.3 Skeletal Muscle Repair

When skeletal muscle is injured the healing process follows a consistent pattern. To recover functionality of the muscle, cells undergo regeneration and remodeling. The process begins with inflammation then goes to tissue repair and remodeling.

2.3.1 Inflammation

Inflammation of the muscle tissue is an important factor as it is the body's first response to any trauma the body experiences. The speed of inflammation of the muscle depends on how vascularized the injury site is at the time of trauma. The first step in the inflammation process is the collective migration and activation of neutrophils to the injured area to clear debris tissue [34]. Neutrophils and macrophages exist mostly in an inactive state, consuming dead cells and non-infectious debris. At the point of injury, they migrate from the nearby blood vessels through the ECM to the injury site. Usually chemical signals to recruit phagocytes are relayed through interferon gamma but in the case of a muscle tear or injury the phagocytes migrate through the area and engulf the debris from the injury. Macrophages are useful in the initial inflammatory stage but are also capable of changing from a pro-inflammatory phenotype to anti-inflammatory once the regenerative response has begun to take place. Macrophages also play a role in the integration of growth factors into the injury area to promote muscle regeneration [24]. Once regeneration begins to occur, macrophages in the area release cytokines and various growth factors. The release of cytokines not only promotes proliferation and differentiation of cells in the area but also promotes vascularization and inhibits cellular apoptosis [24].

2.3.2 Tissue Repair and Remodeling

In healthy tissue, fibroblasts are responsible for providing collagen and maintaining cellular structure. When a tissue is damaged, the initial inflammation cascade begins and the phagocytes clear any debris. Satellite cells are activated, then migrate through the

basement membrane, begin to proliferate and, with the help of fibroblasts, begin to form new tissue. The satellite cells function is activated through growth factors like HGF, FGF and IGF [7]. The fibroblasts initially release a network of fibrin (an ECM protein) into the wounded area, which causes clotting at the wound site to stop bleeding. The fibroblasts then release collagen and other proteins, which combine to form scar tissue. While the collagen fills in the wound the fibrin is broken down. Once the collagen has filled in the wounded area it also breaks down. In healthy humans, the breakdown rate is equal to the rate of cell proliferation and maturation in the area [28]. In humans with defects or impaired immune systems, dynamic reciprocity can be uneven and cause more breakdown of tissue than repair.

Human skeletal muscle has yet to be successfully modeled *in-vitro*, therefore, research has been done to create tissue models, mimetic to human skeletal muscle, to test potential therapies for deficiencies of fully functioning muscle, as described above.

2.4 Three Dimensional (3D) Tissue Culture

An appropriate cell type to develop an *in vitro* engineered skeletal tissue model would be human mesenchymal stem cells (HMSCs mentioned earlier). HMSCs have the ability to differentiate into many different parts of the human body, including the ability to form into skeletal muscle fibers [5]. Thus, if research is to be done on the effects of certain drugs or certain damage to human muscle, it would make sense to use a model that mimics the human body. Unfortunately, for this application, studies have shown that in culture, *ex vivo* human stem cells, such as bone marrow stromal cells, differentiate into a wide variety of cellular types including hematopoietic cells [30]. The problem with this

is that the cells may differentiate into cells that are of no use (ie. bone where muscle is needed). Additionally, the use of hematopoietic stem cells, results in a lack of satellite cells, which are essential in skeletal muscle repair and regeneration [5]. Another reason that HMSCs are not used is the period of time at which they proliferate and differentiate, as mentioned previously. The research using HMSCs for skeletal muscle tissue growth is improving, but many researchers have shifted their focus to using a different cell type, which allows for more rapid growth.

An alternative cell type used by many skeletal tissue models is the C2C12 cell line. C2C12 cells were developed by David Yaffe through passaging of mouse myoblasts that were cultured from C3H mice [39]. These cells are used because of their rapid rate of proliferation and their capacity to differentiate swiftly. Additionally, the media in which they are maintained is relatively easy to formulate at a low cost [39]. Since the goal is to generate a tissue model close to native skeletal muscle tissue, the cell needs the ability to generate a cell line of satellite cells that has the ability to be specifically modified to suit the need of experimentation [2]. Additionally C2C12 cells have been used to form myotubes and allow for contractile function to the structure while still maintaining its integrity after mechanical stimulation [2]. This leads to C2C12 cells being chosen for primary use of tissue engineered skeletal muscle *in vitro* modeling.

Cell Self-Assembly

The ability for cells to self-assemble into a three-dimensional structure around a post allows for the creation of molds that can be seeded with cells. This also eliminates the need for any exogenous matrix providing structural support, and more closely mimics embryonic myogenesis. Gwyther et al., showed that smooth muscle cells can be seeded

in agarose ring wells and cultured. At the conclusion of culture of the rings, the harvested rings yield tensile strengths of 100-500 kPa in testing [12]. This study demonstrated the ability to seed cells in a mold and produce tissue with a cell density more analogous to native tissue without the use of an exogenous scaffold.

2.4.1 Culture environment

Scaffold-less tissue formation

One of the methods to seed cells in a 3D environment to recreate an *in vivo* skeletal muscle is to use scaffolds. The main use of these scaffolds is to become the artificial replacement for natural ECM. The ECM provides the cells mechanical support so they can formulate into skeletal muscle tissue in a 3D environment. Another method is to make a muscle tissue without the use of a scaffold. The use of fibroblasts in culture with the C2C12 myoblasts allows for differentiation and production of ECM, similar to *in-vivo* processes. Dickinson et al., showed fibroblasts co-cultured with mouse myoblasts had the ability to form 3D structures. The proliferation of myoblasts and fibroblast was done separately and then differentiation of the mouse myoblasts was initiated with the addition of fibroblasts. After 10 days, the cell layer lifted up and began to form a ball, called a myoid [8]. Myoblasts cultured without fibroblasts aligned into myotubes but did not form a 3D structure by rolling into a myoid. This result emphasizes the principal that co-culturing myoblasts with fibroblasts has the ability to provide an ECM into which muscle can mature and potentially become contractile [8]. This experiment, as well as the use of fibroblasts with myoblasts in MQP's by Almeida et al. and Fidanza et al., asserts the ability for cells in a scaffold-less environment to form an ECM structure system[1,10].

2.4.2 Stimulation of tissue

In vivo, skeletal muscle tissue is subjected to strains and stresses, which aligns fibers and atrophies muscle through increasing fiber size [25]. The mechanical loading of the tissue facilitates the actin and myosin alignment and the creation of ECM. The alignment of the muscle fibers and the production of a functioning ECM allows for the formation of healthy skeletal muscle [37]. With this knowledge, many models of tissue engineered skeletal muscle incorporate mechanical, electrical or both types of stimulation to help with the alignment and strength of the *in vitro* tissue and improve models to the point of mimicking native tissue. For example, a mechanical stimulator called the Mechanical Cell Stimulator (MCS) was created in order to mimic *in vivo* mechanical motion and therefore align fibers in the muscle construct [25].

The results shown from the cyclic mechanical loading on the material demonstrate that, over time, the area of the myofibrils and the average diameter of the fiber increase [25]. The results showed an average fiber diameter of about 5 μm, which is much less than the adult muscle fiber diameter range of 10-100 μm. Although this result is much smaller than native skeletal muscle it does show that increased stimulation leads to increased fiber size [25]. Another observation is that the elastic modulus of the mechanically conditioned tissue model stays consistent over a week span while the tissue modeled without the mechanical conditioning has an increased elastic modulus over time [25].

Elastic modulus is the relationship of stress over strain and thus with an increasing modulus, either the stress for a given strain increases or the strain experienced from a given stress decreases. This observation shows that a lack of stimulation can lead

to tightening and a decreased flexibility in muscles. This result along with the previously mentioned phenomenon of increasing fiber size, shows the mechanical loading due to stimulation creates tissue with similar properties and elasticity of native tissue.

Additionally, Moon et al. subjected skeletal muscle precursor cells to cyclic strain of $\pm$ 10% [23]. The results from this study showed maturation of muscle cells, through unidirectional alignment as well as the expression of muscle marker proteins and presence of ECM. Controls in this study were done using samples that were not strained. These controls, showed little alignment and did not show muscle maturation [23]. Therefore, it can be seen that mechanical stimulation allows for tissue engineered skeletal muscle models to achieve mechanical properties that are similar to native tissue.

2.4.3 Limitations

With the challenge to find an effective model for *in vitro* tissue engineered skeletal models, there are many different methods that have been generated in order to attempt to mimic native skeletal muscle tissue. While each method has their advantages, there are associated negatives that prevent the model from accurately mimicking native tissues that includes lack of cell density, inability to withstand mechanical loading, and degraded debris from scaffolds and hydrogels. An accurate, *in-vitro*, skeletal muscle model could potentially be used as a screening method for possible muscular dystrophy therapies. However, to test such therapies, the tissue model must be of an appropriate cellular density analogous to native skeletal muscle. Therefore the goal of our project is to improve the mechanical actuation of the tissue engineered skeletal muscle and to improve the density of the cells when seeded onto the mold design created.

Chapter 3 Methodology

The aim of this project was to create a mechanical stimulation system that could be used with C2C12 cells to culture, align, and grow an *in vitro* skeletal muscle mimetic model. This chapter shows how the various objectives are prioritized, constraints have been faced with, and functions the design must achieve.

3.1 Initial Client Statement

Below is the initial client statement the group received after meeting for the first time with the client, Professor Raymond Page.

> *"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."

Once this initial client statement was received it was analyzed, broken down, and eventually revised in order to create a list of constraints and objectives to help as guidelines.

3.2 Objectives and Constraints

After carefully studying the initial client statement the group decided on a few key objectives as desirable outcomes for the project. The group also determined constraints to be guidelines as the project progresses.

3.2.1 Objectives

The group organized an objectives tree from the objectives determined to be necessary and ranked them in a hierarchy that shows broad topics towards the top as well as more specific goals at the bottom. Each objective is necessary for various reasons and therefore can be ranked.

The system needs to grow tissue in a seeding chamber in order to develop a shape that can be mechanically stimulated without destroying the tissue sample. The designed shape of the tissue promotes tissue formation and integrity. Once the C2C12 cells have been cultured, differentiated and seeded into the mold, they begin to contract to create a mimetic muscle fiber. During growth, this muscle tissue must attach around an anchor(s) to provide stability of the cells mimetic of native skeletal muscle anchorage [30].

The tissue structure is very important to create a model that replicates the parameters of skeletal muscle as closely as possible. Axial alignment of the myoblasts into multinucleated myofibrils as well as proper cell density of the tissue is important aspect in creating native skeletal muscle. The mechanical integrity of the muscle is also important so that no part of the muscle tissue tears during testing due to an unaligned fibers, i.e. weakened structure.

The reproducibility of the cultured muscle fiber is integral to consistent results. Conducting tissue growth multiple times will allow for the measurement of the

muscle fiber and then allow for statistical analysis. This ensures that the grown muscle fiber would fall within a 10% radius and would give a more consistent tissue when utilizing the mechanical actuation system.

Quantification of the induced mechanical stimulation is of great importance, and can be achieved by observing the strain on the tissue sample. Strain is defined as the change in length divided by the original length of the sample. The tissue should contract, and that contractile force will be measured using LabVIEW software in order to determine how consistent the tissue can contract. In order to produce an *in vitro* model of a skeletal muscle tissue, objectives need to be quantified in order to ensure the design will succeed as Figure 3, below, outlines.

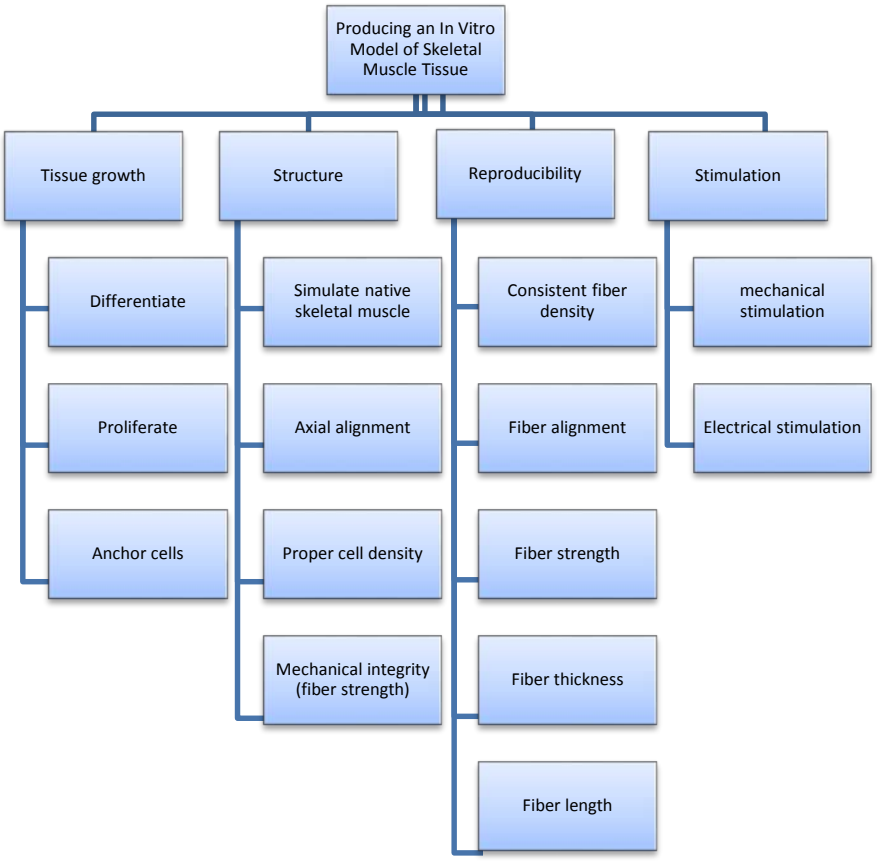


Figure 3: Objectives Tree

From these four main objectives the team ranked them in a pairwise comparison chart, seen below. A pairwise comparison, in Table 1, showed that the most important objective for the system was stimulation. Stimulation was determined to be the most

important objective due to the need to strengthen and align muscle fibers to produce a viable tissue. However, another objective of great importance is the ability to form the tissue itself. Stimulation is a large aspect of this project, but the formation of a tissue must be achieved before it can be stimulated. Reproducibility and tissue growth were determined to be of equal importance as the device must be able to produce viable tissue repeatedly. Structure was determined to be the least prioritized objective because the testing model will not have the same chemical structure as a native tissue but will need to behave like a native tissue.

Table 1: Pairwise Comparison Chart

	Tissue Formation	Structure	Reproducibility	Stimulation	Final
Tissue Formation		.5	.5	1	2
Structure	0		1	.5	1.5
Reproducibility	1	1		0	2
Stimulation	1	1	1		3

3.2.2 Constraints

The constraints serve as guidelines during the design process were determined by the team and the client using meetings between the client and team as well as the initial client statement.

Skeletal muscle model system design must:

- Be compatible with an *in-vitro* tissue environment (37°C, 5% CO2, 95% humidity)
    - Mechanical parts must withstand incubation conditions
- Not exceed 60% to 70% confluence of C2C12 cells in 2-dimensional culture
- Able to be used in aseptic conditions

The actual muscle fiber should be a size that does not require blood flow, therefore no vascularization of the tissue. The percent functionality should be high enough to be able to accurately create a model to compare to native skeletal muscle. However, the tissue should have a high percent reproducibility. With high reproducibility, the tissue can be optimized with higher cell counts or various growth factors.

This system also has some limitations as well. While adult mesenchymal stem cells would be ideal, they would take too long to culture for this project. Therefore, C2C12 cells are used, despite the fact that they are mouse myoblasts that do not replicate human tissue. The project team is also limited to a budget of $156 per team member.

3.3 Revised Client Statement

After carefully evaluating the initial client statement and after deliberations between the team and the client, a revised client statement was created to accurately describe the project in as concise a manner as possible.

> *"This project is to design and optimize an in vitro system to model skeletal muscle that improves contractile function from existing models by improving cell alignment and density. This system used C2C12 cells and no exogenous scaffold. The system withstood the conditions of an incubator and remained sterile. The*

cells within the system were able to be stimulated to increase cell alignment and strengthen the model muscle tissue."

3.4 Project approach

After considering all project constraints and objectives, the team decided on a step-by-step process to get to the final design model. The first step was to culture cells from a frozen state. The cells were plated at approximately 100,000 cells per plate and supplemented with a proliferative complete media mixture (see Appendix D). Cells were cultured until they reached 60-70% confluence. Cell passaging was required to make sure the cells were viable and the cell density was appropriately reached (see Appendix E for passaging steps). Once this is accomplished initial molds will be tested for viability. Current models exist designed to seed cells in a ring formation for the eventual purpose to becoming tissue engineered blood vessels [12]. To accomplish mold viability, cells will be seeded on the preliminary molds and observed for tissue formation based on previous studies for tissue engineered blood vessels [12]. The third test will be used to quantify fiber viability and cell density.

Once these factors were considered design of a tissue-seeding construct in order to formulate a shape for a skeletal muscle tissue was started. This design is a six piece break away mold that was capable of actuating the tissue while it was forming around tear drop shaped PDMS posts to minimize necrosis. Then, once the tissue had fully formed, the mold breaks away, leaving the posts and base layer. The following chapter goes into more detail surrounding each design.

Chapter 4 Alternative Design

4.1 Introduction

While the revision of the client statement aided in further clarifying the objectives of this project; a single solution to fulfill all of the objectives is not easily obtainable. There are a multitude of designs that fulfill portions of the objectives with strengths and weaknesses that are associated with them. In order to select a final design that meets the objectives, the project group generated many alternative designs and compared how their functions fit within the scope of the project. In the following section, this paper outlines the needs analysis of the designed device.

4.2 Needs Analysis

The objectives outlined in the previous section were taken into account when brainstorming ideas to generate a design alternative. Also due to the time constraint of 28 weeks, the feasibility of the functions were taken into account and ranked based on the necessity to ensure that the device solves the client's objectives.

A broad list of functions are outlined below and taken into consideration when developing an alternative device:

- Mechanically actuate tissue
- Electrically stimulate tissue
- Promote growth of tissue and allow for differentiation
- Allow for the ability for extracellular matrix formation to occur
- Maintain a sterile environment
- Allow for easy access to tissue for analytical and reproducible purposes
- Withstand incubator environment conditions

The functions outlined above were kept in mind when generating a list of design alternatives. It is vital that the design can support the growth of the C2C12 cells and the

formation into skeletal muscle tissue. As a corollary, the design must be able to withstand mechanical stimulation that a native skeletal muscle tissue would be subjected to. These two functions would result in a better cellular alignment and maturation that would further improve the model's representation of a skeletal muscle tissue.

The major function that the design should produce, is the ability to accurately modulate different percentages of strain that could be reliably reproduced with consistent data. The formation of the tissue is another vital function that is taken into account when generating a design. The use of immunohistochemical staining is necessary to determine if the tissue formation on the design is viable or not. Phalloidin was used to stain for actin and myosin as well as a myosin antibody to stain for myosin and the Hoechst stain were be used for nucleus identification.

Finally, the design must be able to function in a sterile environment and have the tissue easily accessible to conduct testing or change sample for reproducible testing. The most difficult challenge that the design faces is the ability to measure small amounts of strain from a minimally actuated device in a sterile, aqueous environment. Additionally, the ability to easily reproduce the testing of new samples while still maintaining the sterility and viability of the tissue is necessary. All of these functions are taken into account when brainstorming to develop our device.

4.3 Functions Analysis / Design Specifications

Taking into account all of the functions necessary to fulfilling the objectives of the client statement helped the group in generating device specifications to determine if the design would be a viable solution. In order to systematically generate solutions for the

necessary functions of the device, a Functions-Means chart was compiled. The results from the brainstorming are detailed in Table 2 below.

Table 2: Functions-Means Chart

Function	Means		
Mechanical Actuation	Electromagnet	Stepper Motor	Syringe Pump
Anchor Tissue	Agarose Post	Metal Post	PDMS Post
Microcontroller Protection	Polycarbonate Box	Polystyrene Box	Steel Box with cement coating
Tissue Containment	Corrugated petri dish	Pool System	Static petri dish
Tissue Formation	Fibrin microthreads	Teardrop Dogbone mold	
Measure Contractile Force	Force Transducer	Post Deflection	

The chart lays out varying means to satisfy the criteria of the functions outlined can be used to generate a design that would best fulfill the requirements of the client.

The generated tissue must have a size and shape consistent throughout the entire sample to ensure accurate quantitative results. The device must be able to withstand and function in a sterile environment, which will be standard incubator conditions. The formulated tissue needs to have an anchor for tension generation. This tension can be manipulated to accomplish one of the main objectives of the project.

The function of stimulating the tissue construct is broken up into mechanical and electrical stimulation. For mechanical stimulation, the device must be applied on the tissue construct modulating the length of the tissue to 10% strain, with this amount of strain based off previous MQP work and research [1][20]. When the tissue is subjected to this amount of strain, the resulting tissue would lead to better cell alignment and proliferation that has been observed in previous studies. The direction in which the design will be stimulated will be in a uniaxial direction but would be able to modulate between both positive and negative strain that native skeletal muscle exhibits. The other function that is vital in our design is the ability to electrically stimulate the tissue construct leading to a contraction phenomenon that can be observed and measured. These functions must

be measured during the stimulation and actuation process to ensure proper data is collected. The collected data for these functions will be the contractile force of the tissue. Along with data collected on the displacement of the tissue along with the strain percentage over a period of time.

The list of functions that were generated, ensures that a design can be generated effectively. The generation of many design alternatives will help optimize the proper device that would best perform the necessary functions in a feasible and accurate manner. The following section outlines a set of design alternatives that were developed in order to fulfill the required functions for the client's objectives.

4.4 Design Alternatives

The following section details design alternatives that were generated and that fulfill different functions necessary to achieve the objectives laid out by the client. First, there are two design alternatives for developing a mechanical actuation device that would maintain accessibility and sterility. The design alternatives are a motor/piston system and a hydraulic pump system that is external to the seeding plate. The next set of design alternatives will detail the system components in which the tissue constructs will be seeded and actuated. These designs include a corrugated plate, an external modular electromagnet, and an internal hydraulic pump system.

4.4.1 Modular Plate

Many previous attempts at actuating skeletal muscle grown *in vitro* to form models (including many MQP's) has been done by penetrating the vessel that the tissue is cultured in as a direct linkage to the tissue.. While this can prove useful, putting holes

into a Petri dish can create a variety of issues ranging from fracturing Petri dishes, to lack of consistency in holes, to issues properly sealing the hole.

Given these complications, a design was created that would take the concept of a regular Petri dish in two separate pieces, connected with an impermeable membrane, so the entire container could be externally actuated. There would be two halves composed of polystyrene with a sheet of butadiene rubber connecting the two sides. The design, shown in Figure 4, would be constructed in such a way that the rubber sheet would have enough range of motion that when the dish was actuated it would simply use the slack in the sheet to expand. The design picture shows the design halves without the butadiene rubber sheet between the two. They are aligned in such a way that one side could be anchored and the other could be mounted on a moving part that could actuate steadily at set rates.

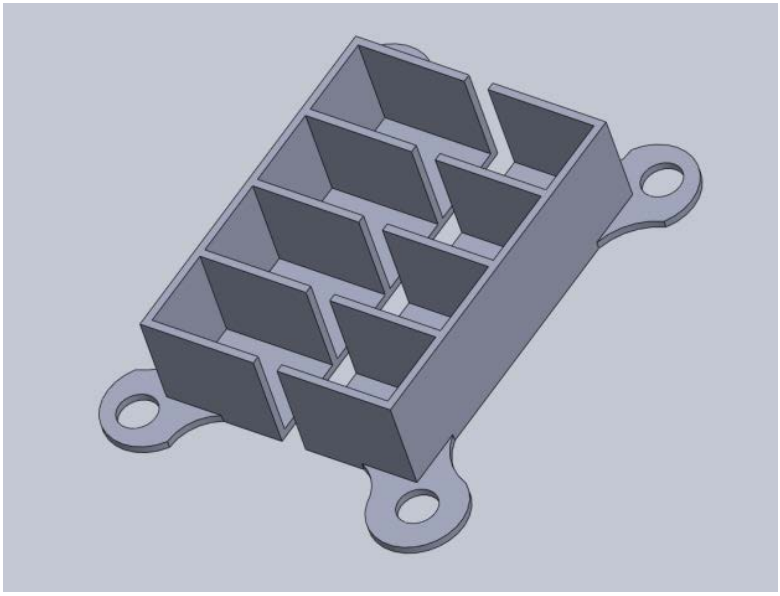


Figure 4: Modulating culture dish

Using this system allows for the attachment of devices externally. This means that keeping sterility will be less difficult and the results can be more easily

reproduced. The way the system is modulated allows for multifunctionality, as well. As the mold is rather small, the dish can be designed much wider than needed and multiple compartments can be utilized to actuate and test multiple tissue models simultaneously allowing for more data to be collected in a smaller window of time. It also means each tissue in the set will have been subjected to identical strain rates which results in tissue of similar strength. This design possesses a much higher throughput than current systems. Using this it will be possible to determine what could have caused any faults in tissue during testing. If one tissue model fails very early in the process but none of the others do, it is possible that human error in the cell seeding process was the cause. However, if all samples in a set are torn or experience some sort of fault it would imply that there was a miscalculation in the applied strain rates.

While this design offers many benefits, there are a few potential issues that, if not addressed properly, would result in poorly managed tissue models. Since one side is anchored and the other modular, the system could three dimensionally move against the tissue, resulting in either torn or poorly aligned tissue. Two issues could arise that are equally problematic if there is not enough slack in the rubber sheet. The force applied mechanically to the modular side of the dish could not be enough to stretch the rubber resulting in a skeletal muscle model that was not properly actuated during culturing. The second option would be that the force applied to the modular end could be enough to overcome the sealant binding the butadiene rubber to the polystyrene resulting in a leak affecting the systems sterility.

4.4.2 Agarose Mold

This project group has chosen to have the tissue samples possess a dogbone shape. This design is similar in concept with a few modifications to address issues faced

by previous projects. The device shown in Figure 5 and 6 is an agarose mold in a dog bone shape however it is broken down and is composed of six pieces.

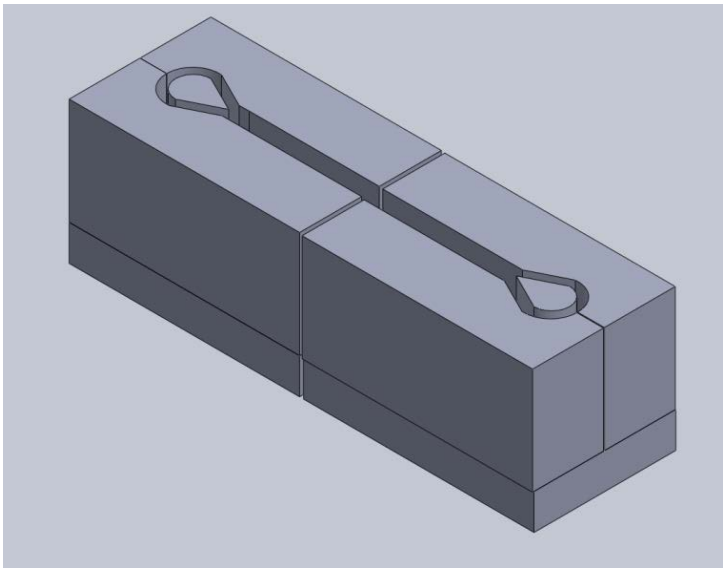


Figure 5: Dog bone molding system

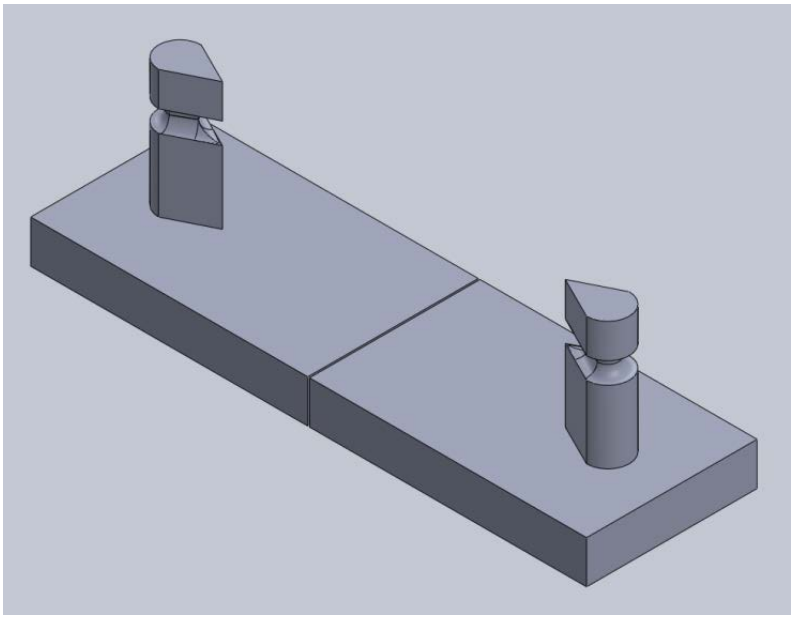


Figure 6: Inner mold with tear drop posts

The top of the design shows the agarose mold with the seeding channel connecting the two posts. The seeding channel is 100 μm in radius so that when feeding cells, the media will be able to diffuse all the way through the tissue resulting in reduced

necrosis in the middle of the tissue [1,10]. The agarose mold has two holes, which are occupied by PDMS posts protruding from the base of the design. The base is a flat piece of PDMS with a post on each side. These posts are in a teardrop shape to avoid necrosis on a cylinder that was experienced in past MQP's [1,10]. The posts also have a channel longitudinally in them to keep the tissue in place.

PDMS was used so that once the tissue has fully formed and can be tested; the agarose mold can be removed leaving the posts and base. The tissue can then have a current applied to induce contraction. Knowing the tensile stress of PDMS and using ImageJ to measure deflection of the posts, the contractile force of the tissue per gram of muscle tissue can be calculated.

Before using this system, there are two complications that would have to be addressed. Firstly, the agarose mold has a crease in the center through the seeding channel that could affect tissue formation. Secondly, the ability of the PDMS posts to calculate contractile force is effective but if not adhered properly, the back of the base PDMS will lift when the tissue contracts.

4.4.3 External Modular Electromagnet

This design will utilize an electromagnet that would be placed underneath a polystyrene tissue culture plate. Within the culture plate, shown in Figure 7 and 8, two posts made of PDMS would be placed 15mm apart. One post would contain an embedded magnet that would interact with the external electromagnet. The goal of the magnet interaction is to modulate the external magnet, which would pull the magnetized post and create tension for the attached tissue. The external electromagnet would be connected to a mechanical

actuation system that is powered using a stepper motor and rod. The rod connected to the electromagnet would modulate back and forth allowing the post to move in both the positive and negative direction. The external magnet would be placed in a controlled channel that would prevent the magnet from extending too far and tearing the cultured tissue construct.

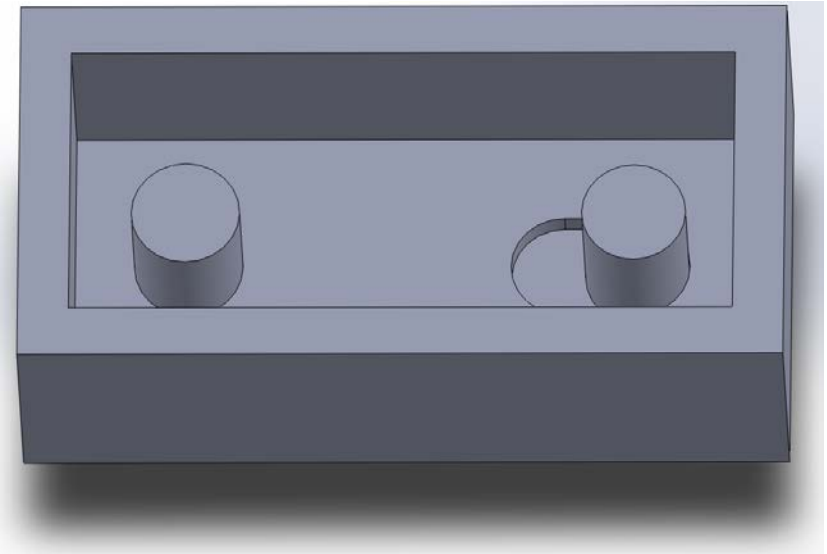


Figure 7: Top view of electromagnet dish

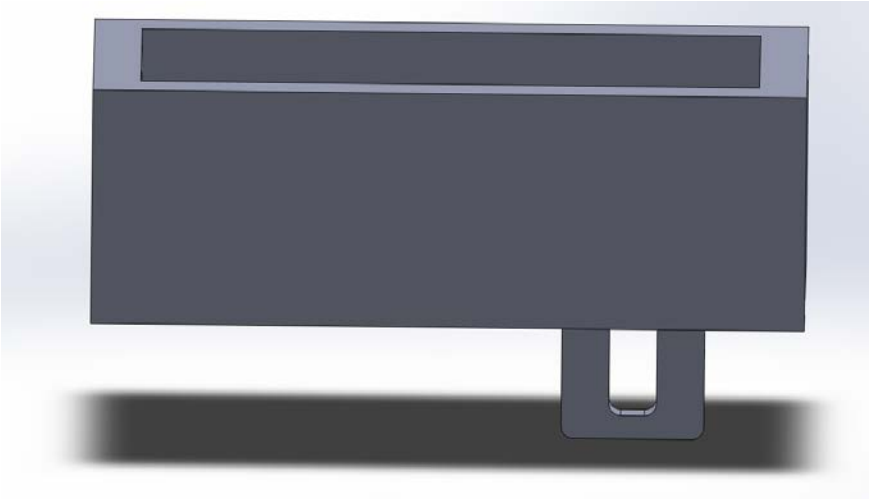


Figure 8: Side view of electromagnet dish

One of the major disadvantages with using magnets to modulate a post is the potential delay that is associated with the interactions between two magnets. For instance while the external electromagnet is being displaced by the actuation system, the magnetized post would need to fight against friction as the magnetic forces attempt to displace the post. Another issue that arises is that one post being dynamic and modular might prevent a sound structure that allows the tissue construct to be placed on and prevent any tension from occurring before testing. These factors prevent the design from being able to gather viable mechanical stimulation data due to the inability to mitigate the amount of dynamic variables in the design.

4.4.4 Internal Hydraulic Pump

This design will utilize the modular plate that was discussed in section 4.4.1 along with a hydraulic syringe pump system. The mechanism of the device would utilize the compression of a syringe that uses pressure to push an incompressible fluid through a tube into another syringe. A liquid such as mineral oil is an acceptable option, as it is commonly used in hydraulic systems [35]. Bubbles in the system increase the potential for errors and inconsistencies in the displacement of the modular plate as these bubbles can compress and realign themselves in various ways affecting the pumps movement. This causes the syringe to fill up with the liquid and will facilitate movement of the plunger. The plunger head would be attached to another syringe, which is connected to the modular plate. This action will modulate the components of the plate separating the posts with the tissue mold being subjected to, and removed from tension. The modular plate will have one end anchored to a platform, which will allow the

modulated syringe to move one post while keeping the other stationary. This stimulation will allow the tissue to displace in both a positive and negative direction.

One concern is the proper translation from the actuation of the syringe to the modulation of the modular plate. This inconsistent amount of modulation could prevent a proper reproducibility of mechanical stimulation results. Additionally, the attachment of the pump to the base of the plate makes sterilization processes harder. If the plate needed to be moved into the biological safety cabinet for culturing, the tubing connection to the syringe would need to be removed. The removal and subsequent re-attachment of the tubing can cause air bubbles and differences in modulation, but can be removed by degassing the system and bleeding the air out. Therefore, while the concept of the design will ensure the tissue be maintained in a sterile environment, the potential inconsistencies in the mechanical data could potentially render the design ineffective to produce mimetic tissue. These imperfections could also change the application of strain throughout testing exposing different sets of tissue to different conditions, affecting experimentation reproducibility.

4.4.5 Push Arm

This design was created to address the issue of the hydraulic pump interface discussed previously. When using a syringe pump to actuate the tissue it can be an issue to detach the system to test in a sterile environment and then reattach the system without creating any air bubbles in the tube when you return it to the incubator. This system allows for a syringe head to fit into the slot at one end of the rod arm, in Figure 9, while the other end can be bound to a culture plate to actuate it. The syringe head then moves

the rod arm linearly, which in turn actuates the tissue. The end that can be bound to a plate, as seen below, is capable of being screwed on and off in order to fit it into systems with small holes to accommodate a system similar to this.

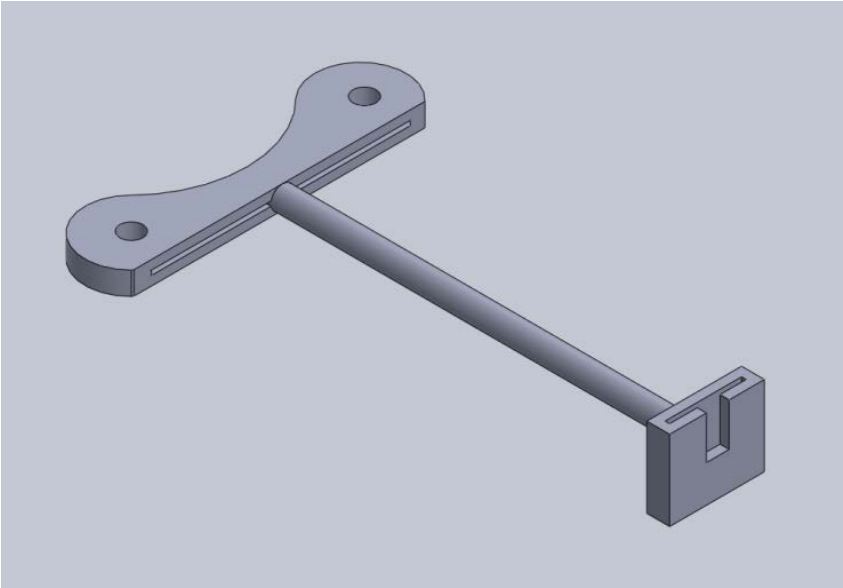


Figure 9: Push arm for connection with syringe pump

This design allows for much easier transfer between hydraulic pump modulation systems to the testing of tissues in a sterile environment. The easy detachment means that air bubbles in a pump will be much less likely to form and actuating the tissue will be just as consistent.

If not properly guided along a linear path the rod arm could pull in an unexpected direction. Another disadvantage is that the rod arm's length, if not supported, will create a lever on one end with the tissue being lifted into the air. To test for movement inconsistencies, a camera could be set up viewing the system for 1 hour from different angles. A team member could analyze the footage to determine any nonlinear movement that should be adjusted. From there a support structure could be created to address

whatever situation that is encountered. As the slightest misalignment could cause tears in the tissue all potential for alignment complications should be accounted for.

4.4.6 External Container

This design allows access to the expandable petri dish while keeping the whole system sterile and supporting the culture plate to keep both halves level at all times. This system would be used in concert with the push arm. The container, in Figure 10, is large enough to allow the system to actuate and it also has a hole in the side to allow access to the unanchored half of the plate by a syringe pump or mechanical arm. This whole can be sealed using a septum or rubber cork to maintain sterility.

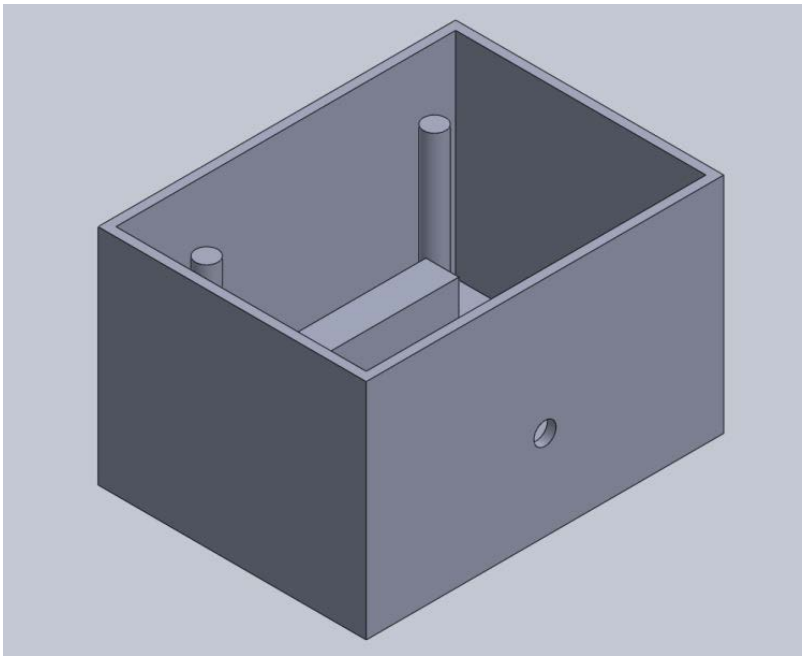


Figure 10: External container for use with push arm

The greatest benefit to this design is that using posts to anchor the plate, and used with the expandable culture plate, the system can actuate the tissue in only uniaxial direction. This would then prevent tears in the tissue from unexpected two or three dimensional movement.

While this design was created to solve other complications it does have limitations of its own. A proper seal with the opening in the container must be maintained for sterility. Also if the design is made too large it will be too bulky to transport between the incubator and the sterile testing environment and require too much media to be feasible, however if it is too small it will be too difficult to access internally to test and make any modifications to the setup such as tissue mold realignment or situations of a similar manner.

4.4.7 Stepper Motor Linear Actuator

This design utilized a variable stepper motor that was connected to threaded rod. A sliding tube with a slotted head was attached to the threaded rod of the stepper motor which allows for mechanical positive and negative displacement to occur. The threaded rod is manufactured using a bolt head which was turned off using a lave machine in the manufacturing labs at Worcester Polytechnic Institute. The threaded rod is then attached to a spider coupling that has two sides with a set of set screws. One end slots onto the threaded rod, while the other side slots onto the stepper motor head. This design was shown to be the most successful which heavily influenced the parameter of the final design.

4.5 Preliminary Data

Before finalizing a design, the project team needed to perform experiments and collect data pertaining to certain criteria of the project. The following sections outline these criteria and detail the experiments performed to choose a final design.

4.5.1 Tissue Formation and Mold

For optimal tissue formation, a material with low cell adhesion properties is ideal. Polydimethylsiloxane (PDMS) was chosen as the material that would comprise the base and posts that the cells would be seeded around. The elastic modulus PDMS can also be controlled through varying the ratio of elastomer base to curing agent. This property makes PDMS an ideal material for this application, because depending on the observed strength of the tissue sample, the PDMS composition can be altered for following iterations of testing.

The tissue-seeding channel must also have non-adhesive properties, so agarose was chosen. Agarose also has the added benefit of potentially formed with various growth factors and cell culture media to help promote tissue formation.

4.5.2 Mechanical Actuation Device

There were several methods of displacement that were investigated by the project team before a final design was selected. The mechanical actuation device must be consistent for the duration of tissue stimulation. Some preliminary designs, such as the electromagnet and hydraulic pump systems, were deemed too inaccurate with variables that were too large for the overall success of the design. An electronic stepper motor controlled by a Raspberry Pi microcontroller was chosen because of its ability to be precisely programmed.

Having electronic components incorporated into the design required one of two things; either device would have to transverse the incubator wall or there would have to be protection from the humidity and increased temperature inside the incubator. Initially, it was thought that using a hydraulic system through the wall of the incubator would

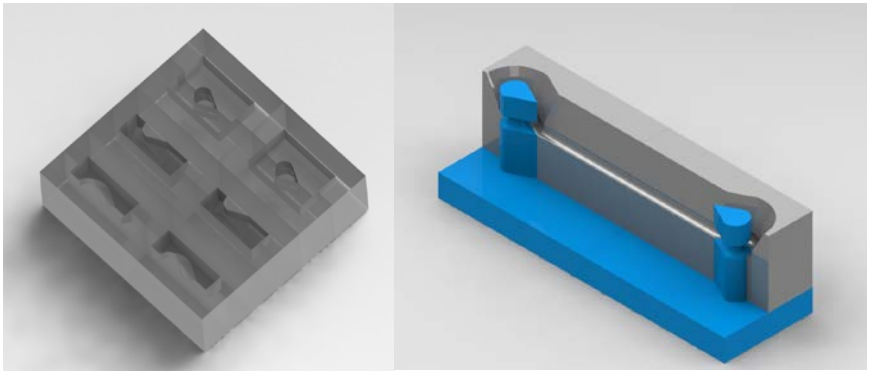
work, however, the project team chose a polycarbonate box to house the stepper motor and microcontroller due to inconsistencies with the syringe pump used to power the hydraulic system.

Chapter 5 Final Design

The final design chosen by the project team has several different components, each of which possesses a unique and necessary purpose and function. This chapter outlines each part of the final design and the method of its production.

5.1 Agarose/PDMS Molds and Seeding Channel

The final design incorporates a previously described post system comprised of PDMS cast over a negative mold made of Veroclear, a rapid-prototyping material. This negative mold was designed in SolidWorks, and subsequently 3D-printed. The images below show the negative mold and a SolidWorks drawing of the post system.



Left: Veroclear negative mold. Right: Representation of post and seeding channel system. Blue: PDMS post and Grey: agarose channel

In the final design the PDMS posts were re-dimensioned to be smaller in order to require less cells and therefore less culture time before testing. There was a 4 mm gap between posts, a 73% decrease in length. The post was downsized to 1.5 mm wide and 2.25 mm long. The post heights were decreased to 2.1 mm high. The PDMS posts and agarose seeding channel have a height of 2.1mm and the channel has a 65° chamfer. A tissue formed within this mold would have an 8mm length and a 100μm radius. The design takes into account the potential of the tissue forming, contracting and popping off of the posts and prevents this by having a notch in each post to allow the tissue to settle in

there. There is a break in the base of the post system to allow for movement back in forth inspired by the mechanical actuation system.

5.2 Pool System

This aspect of the design acts as a housing for the molds and tissue culture materials, and connects the dynamic portion to the mechanical actuation system. The image below shows the pool system portion of the design. The final design of the actuating plate underwent repeated changes to address issues faced in order to get it to the final state. The fixed half is a 4 chamber dish with each chamber being 14.5 mm by 10 mm, combining with the other half to make a total chamber dimension of 19 mm by 10 mm. The plate is connected to a dish that is slightly wider than the plate in order to accommodate the other half of the actuating system and keep it in track allowing for only one dimensional linear movement. The fixed half is elevated 3 mm above the fluid reservoir so that the movable half and fixed halves' chambers are level. The moveable half has identical dimensions in chamber size and height. The only difference is a slot on its rear face. This slot allows for the connection of the head of the linear actuator to the modular portion of the actuating plate

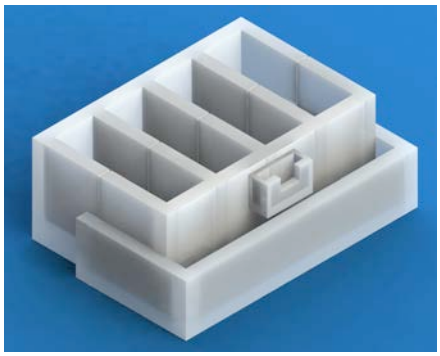


Figure 11: 2-Piece pool system with slot for attachment to mechanical actuator

The pool system, similar to the negative post and seeding channel mold, was drawn out in SolidWorks and rapid-prototyped out of Veroclear material. The heat deflection temperature of this material is 50°C, which, with thick enough walls, can withstand several cycles of sterilization in a steam autoclave. Taking into account the size of the skeletal muscle tissue, this design has the capability to allow for at least 10% strain to the sample, and more.

5.3 Linear Actuator

The linear actuator portion of the final design has, in itself, many components. This aspect of the design is where the motion that causes the strain on the tissue sample.

5.3.1 Stepper Motor and Coupling

The stepper motor incorporated in this design allowed for very controlled movements and mechanical stimulation through precise numbers of rotations. This particular motor has 1/64 steps per rotation, and is connected to a coupling, which links the stepper motor to a threaded bolt. Using a lathe machine, the project team shaved much of the bolt head off so that it might fit in the coupling and complete the system. The bolt had a 3/8” diameter and a 0.7mm per revolution pitch.

5.3.2 Raspberry Pi Microcontroller

Having a stepper motor as the source of motion for this design is advantageous because of the very precise movements that it can create. However, these movements and rotations must be programmed first, which is what the RaspberryPi Microcontroller functions as. The code for the design can be found in the Appendix F.

5.4 Polycarbonate Enclosure

One of the design stipulations that the project team wanted to abide by was complete enclosure within an incubator. This meant that there had to be some sort of protection for the stepper motor and microcontroller from the incredibly high humidity of the incubator. Sheets of polycarbonate were ordered from Piedmont Plastics, and assembled into a 6x6x3" box and sealed with Gorilla Glue and bathroom caulk which can be seen in the figure below.

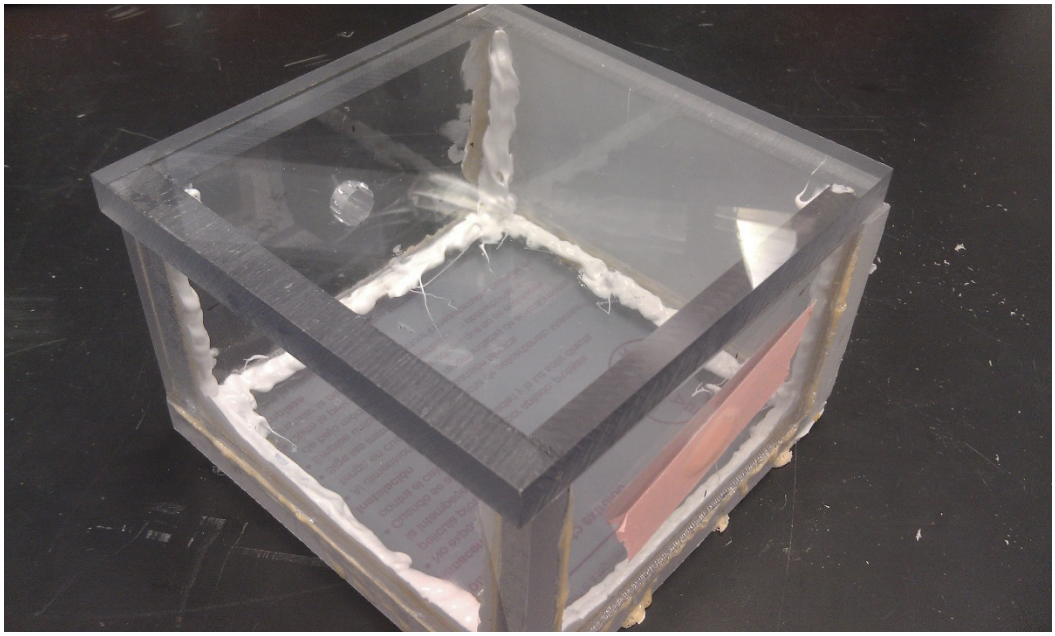


Figure 12: Polycarbonate Enclosure Box

To allow for a power source and extension of the linear actuator, two, one inch, holes were drilled in opposites sides of the box and both sealed with a septum and caulk around the essential equipment.

5.5 Final Construct

Once all of the components were created, the final assembly was generated in order to fulfill the objectives laid out within the project. As seen in the figure below, the full design construct was comprised of a fully sealed polycarbonate enclosure system that was connected to a stepper motor linear actuation system.

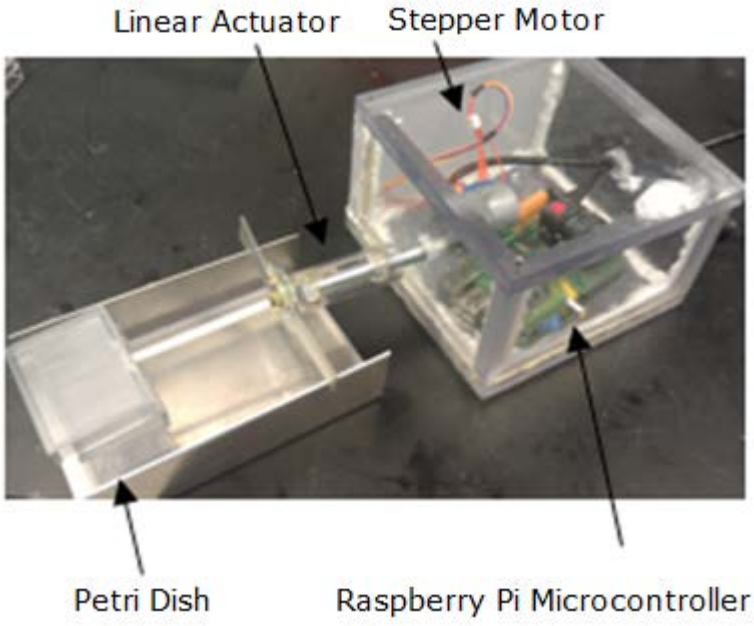


Figure 13: Final Design Components

The advantage of this actuation system was that its completely independent from the modular petri dish that housed the tissue mold constructs. This was important as that meant that while the whole the whole device can operate within the incubator, there was no need to sterilize the system with autoclaving. The only component that needed to maintain sterility and be autoclaved was the modular petri dish. The modular petri dish will only contained the C2C12 tissue construct, the PDMS posts, and the agarose mold construct which can all be properly autoclaved without issue.

With these specifications of the final design, the next section delved into the process in which the designed was manufactured and how it was validated to meet the objectives of the project.

Chapter 6 Final Design Methodology

After taking into consideration all of the design alternatives and the results from its validation; various changes were made to finalize a design. The following section details out the specification and material selection that were used in the final design along with the procedures that were used to test the design.

6.1 Veroclear Negative Mold

In order to create a post construct that could be used to seed cells to formulate tissue in a cost efficient manner; the base was created using a negative mold that was created out of a plastic material that was generated using the rapid prototyping machine in Higgins Laboratory at Worcester Polytechnic Institute. The Solidworks model file was submitted and the mold was printed and received in roughly a week. The material that the negative mold was printed with was Veroclear which is able to withstand the conditions of autoclaving. This allows the negative mold to be sterilized before casting PDMS to create the post construct.

6.2 PDMS Post mold

The next step is to cast a PDMS mold from the negative mold made of Veroclear. In order to create the PDMS positive mold, an elastomer base needed to be mixed with curing agent in a 10:1 ratio. So when creating the PDMS positive mold, 10 grams of elastomer was mixed with 1 gram of curing agent. The resulting product was then casted over the negative Veroclear post mold placed in a weigh boat. The construct was then placed in a degasser for one hour in order to purge the PDMS of air bubbles. Then once removed from the degasser, the product was placed in the 60°C oven for around 24 hours. The product is removed from the oven and the excess PDMS was cut away using a

scalpel leaving the positive mold of the PDMS post. The resulting PDMS post was sterilized using autoclaving to be used to seed the cells onto.

6.3 Agarose

The other component was the agarose construct that was the seeding channel that the cells were seeded onto. A negative mold template consisting of 4 components of the seeding channel was made using the rapid prototyping machine in Higgins Lab. The material for the negative mold was Veroclear similar to the negative mold of the post. The negative mold was then sterilized using the autoclave and placed under a cell culture hood. The agarose mold was constructed using 2 grams of agarose powder mixed with 100mL DMEM. The mixture is placed into a container and autoclaved for 30 minutes. The product was casted over the Veroclear seeding channel negative mold in cell culture hood using a micropipette. Shortly after, the agarose sets onto the negative mold and hardens. Then using a scalpel, the excess agarose was cut off leaving the positive seeding channel molds that the C2C12 cells were seeded on.

The agarose mold was altered to incorporate all changes that had been made to the PDMS base in order to remain as one collective mold. The one change made to the agarose mold that did not deal with complications faced by the PDMS piece as well was a chamfered seeding channel. This chamfered seeding channel allowed for much more efficient use of the seeding channel as well as provided a less even surface when casting the molds which resulted in less adhesion between the mold and its negative mold resulting in less tearing when the mold was removed.

6.4 Actuating plate with media pool

The final design of the actuating plate underwent repeated changes to address issues faced in order to get it to the final state. The fixed half is a 4 chamber dish with

each chamber being 14.5 mm by 10 mm, combining with the other half to make a total chamber dimension of 19 mm by 10 mm. The plate is connected to a dish that is slightly wider than the plate in order to accommodate the other half of the actuating system and keep it in track allowing for only one dimensional linear movement. The fixed half is elevated 3 mm above the fluid reservoir so that the movable half and fixed halves' chambers are level. The moveable half has identical dimensions in chamber size and height. The only difference is a slot on its rear face. This slot allows for the connection of the head of the linear actuator to the modular portion of the actuating plate.

6.5 Manufacturing

In order to create a device to stimulate an *in vitro* skeletal muscle tissue model, the creation of a mechanical actuation system was needed. The parameter that the device must fulfill was the ability to actuate the tissue construct in a controlled and precise manner for a period of three to five days. The device must also not affect the tissue construct or the incubator environment in any way. After verification of the alternative designs in the previous chapters; the group decided to utilize a stepper motor to exhibit linear actuation that can be controlled to modulate the tissue from 5-20% strain. The following section explains the manufacturing process of the mechanical actuation system and the modifications that were made along with the design's specifications.

6.6 Mechanical Actuation Controller

In order to generate a mechanical stepper motor linear actuation system, one of the essential components was the central processing system that housed the code and controller for the actuation. That component was a Raspberry Pi single-board computer that was created by the Raspberry Pi Foundation stationed in the United Kingdom. The

specific model used for the creation of the mechanical actuation system was the Raspberry Pi B 756-8308 Model (Figure 15) which contained two Universal Serial Bus (USB) 2.0 inputs, one High-Definition Multimedia Interface (HDMI) input, one Secure Digital (SD) slot, one microUSB slot, and one 10/100 BaseT Ethernet input. The dimensions of the Raspberry Pi were 3.37’ x 2.21’ x 0.83’ (85.6 x 56 x 21 mm) with a small extrusion created from the insertion of a SD card. The Raspberry Pi also weighed roughly 45 grams. The Raspberry Pi device was powered by a 5v source, which outputs 700-1000mA.

Figure 14: Raspberry Pi B Model

The purpose that the Raspberry Pi served was the centralized controller containing the stepper motor actuation code written in Python computer language. The Raspberry Pi was installed with the Operating System (OS) Raspian, which was taken from the image file provided by the Raspberry Pi website (raspberrypi.org) and inserted in the SD card. This allowed the Raspberry Pi to install all of the drivers necessary to operate any device that was placed in the USB outputs, HDMI output, and Ethernet connection. The HDMI output allowed the group to display the Graphic User Interface (GUI) for the Raspbian OS in order to develop the necessary code the actuation of the

stepper motor. The program for the stepper motor was written to the file Stepper_Motor.py (Appendix F), which is a python executable program that was run by the Raspberry Pi in order to control the stepper motor.

The Raspberry Pi itself did not immediately interface with the stepper motor but instead required another interface in order to connect to the stepper motor. That device was the Raspberry Pi Face Digital Interface Expansion board (Figure 16) that connects to the General-purpose input/output GPIO header connection point that was on the Raspberry Pi module.

Figure 15: Raspberry Pi Face

This allowed the Raspberry Pi Face to communicate with the Raspberry Pi central controller. The Raspberry Pi Face allowed for the controlling of the stepper by having two relays, eight inputs, and eight outputs that was controlled with Python coding. The size of the Raspberry Pi Face was analogous to the Raspberry Pi as it inserted right above the Raspberry Pi module. In order to utilize the Raspberry Pi Face with the Raspberry Pi, there were packages of drivers and applications that were installed using the Raspbian OS repository accessed from the terminal controller. Once the connection between the

Raspberry Pi and the Raspberry Pi Face was set up, the Stepper_Motor.py python file was applied to the expansion board, which connected to the stepper motor.

The stepper motor that was utilized in our linear actuator device was the 28BYJ-48 DC 5V 4-Phase 5-Wire Arduino Stepper Motor (Figure 17). The specifications of the stepper motor were a speed variation ratio of 1/64, frequency of 100 Hz, and four phases. The size and weight of the motor is 3.7 x 2.0 x 0.9 in and 1.1 ounces respectively. The speed ratio of 1/64 meant that the motor takes 64 steps to complete one revolution. These parameters of the stepper motor were critical in ensuring that the actuator could actuate the construct up to 5-20% strain.

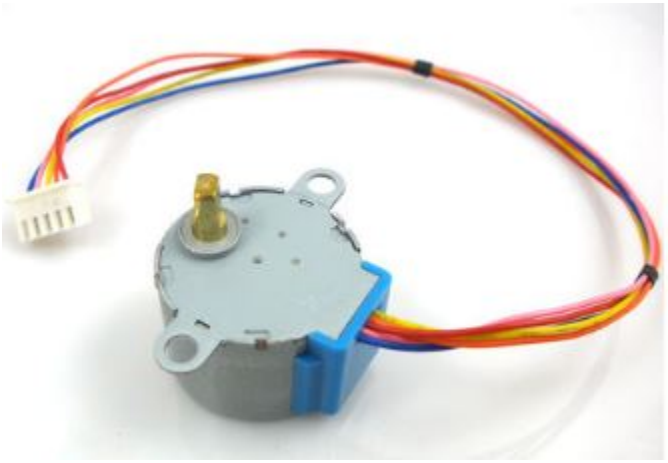


Figure 16: Stepper Motor

The stepper motor contained one shaft, which was where the linear actuator component was inserted on. The stepper motor has one set of wires, which powered the motor. This connection point was connected to the Raspberry Pi Face using a set of jumper wires. This allowed the stepper motor to both be powered and controlled by the Raspberry Pi board. The stepper motor has the ability to be controlled for clockwise and counterclockwise rotation as dictated by the Stepper_Motor.py program (Appendix F).

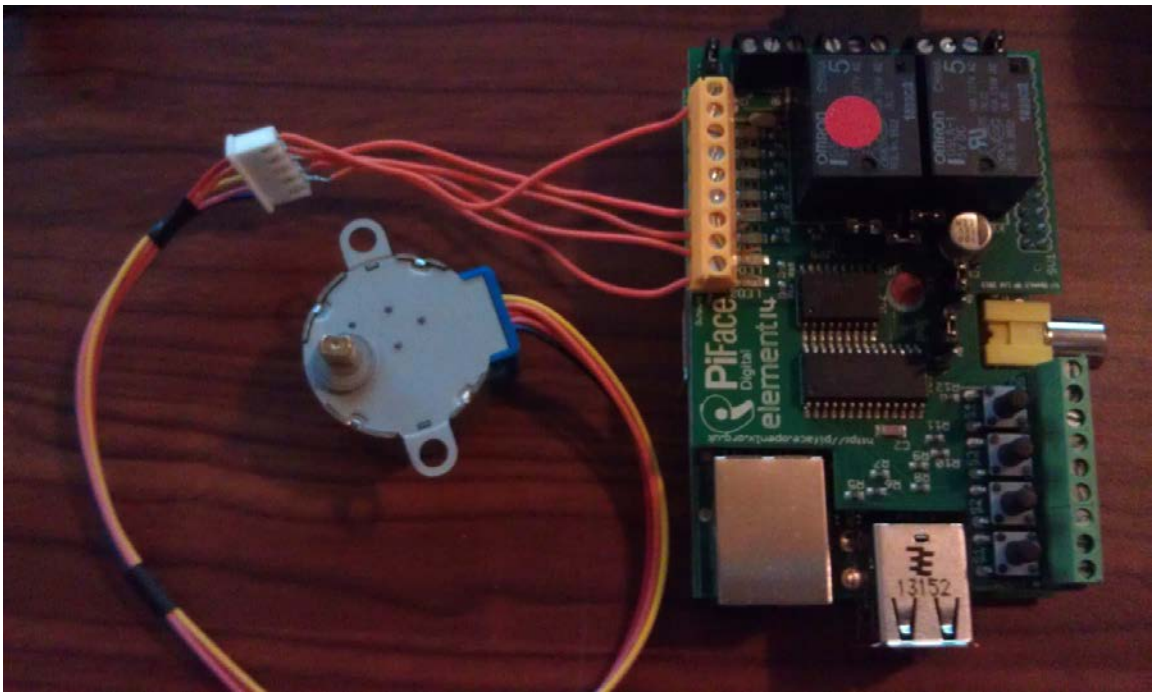


Figure 17: Full Construct of Mechanical Actuation Controller and Stepper Motor

The Raspberry Pi, Raspberry Pi Face, and stepper motor components (Figure 18) constituted the mechanical actuator controller that was used to modulate the linear actuator. These components, due their inability to withstand either a high temperature or high humidity environment, led to the creation of an air tight enclosure that would protect the internal components from the environment present within the incubator that houses the tissue construct chambers.

6.7 Mechanical Actuation Controller Enclosure

With the intentions of placing the mechanical actuation device into the incubator in which the tissue constructs were housed, the necessity of the protection of the internal components was paramount. The incubator environment ran at 37°C, 95% humidity, and 5% CO_2, which was not conducive to the Raspberry Pi interface. Therefore an enclosure system was being created in order to ensure that the internal components can be utilized without failure. A box was constructed using the material Polycarbonate, which exhibited

thermal properties that shielded the internal environment from the humidity and temperatures of the incubator. Sheets of Polycarbonate were purchased and cut into different pieces that would be sealed together using water based silicone glue. The dimensions of the device was 6 inch width x 6 inch length x 3 inch height. Two sheets of Polycarbonate had 2 circular holes cut in order to allow for a power source wire to be fed through. This hole was sealed using silicone based glue that surrounded the hole and wires which created a seal to protect the internal enclosure system from the incubator environment. The other circular hole was where the linear actuator is put through in order to connect the stepper motor shaft to the linear actuator apparatus. A rubber septum was attached to the linear actuator shaft to seal the hole in the polycarbonate box that the linear actuator uses to interface with the stepper motor. The sheets of polycarbonate was glued together with silicone glue both on the outside and inside to prevent any leakage of the incubator environment into the device. The top cover of the polycarbonate was not silicone glued to the rest of the box but instead sealed using silicone vacuum grease. In the end, the enclosure had two sealed output holes and houses the Raspberry Pi, Raspberry Pi Face, and the stepper motor connected to the linear actuation device which was capable of being placed in an incubator environment.

6.8 Linear Actuator

The final component to the creation of a mechanical actuation device is the linear actuator itself. Since the only function this device needs to do is modulate the tissue construct chamber in one coordinate system in both the positive and negative direction; the use of a traveling nut linear actuator on a threaded bolt was used. The principle of the linear actuator was that the head of the bolt would be placed on the shaft of the stepper motor through the Polycarbonate enclosure. The head of the bolt was turned off using a

lave milling machine in order to fit onto to the stepper motor by connection of a spider coupling component. The spider coupling works by having two components with existing set screws connect to the shaft of the stepper motor and the shaft of the cut bolt. Now when the stepper motor was rotating in both the clockwise and counterclockwise direction; the bolt also rotated at the same ratio as the stepper motor. The other component of the linear actuator would be the nut component that sat on the threads of the bolt. A sliding tube that was connected to the nut, which allowed for the tube to displace as the nut was modulating along the threads of the bolt. The end of the sliding tube has been designed to have a groove that can be slotted into the rectangular slot that was present on the modular component of the chamber. The sliding tube also contained two extrusions that rested on a platform. The reason for the resting extrusions on the platform was so when the actuation occurred, the nut did not also rotate along with the shaft. Thus when the system ran, the nut moved along the threads in a positive and negative direction which in response modulates the sliding tube attached to the modular chamber back and forth. This process linearly actuated the skeletal muscle tissue that the group had created.

The advantage of this linear actuation system was that the whole component is completely separate from the skeletal tissue construct. Therefore, the components that were used to create this linear actuator device does not need to be able to withstand autoclaving as it does not even need to be autoclaved.

Chapter 7 Final Design Validation

The final design, after being fully assembled, needed a series of testing that ensured that the design fully accomplished the objectives of the project.

7.1 Mechanical Actuation Proof of Concept

In order to test the ability of the final linear actuator to achieve 5%-20% strain in both the positive and negative direction, a set of test were run using orthodontic elastic bands. The 4mm diameter elastic bands were attached to the PDMS post that were on both the stationary plate and the modular plate. The modular plate was placed as close to the stationary plate as possible and then was actuated using the linear actuator which was connected to the modular portion of the pool system. As seen in the figures below.

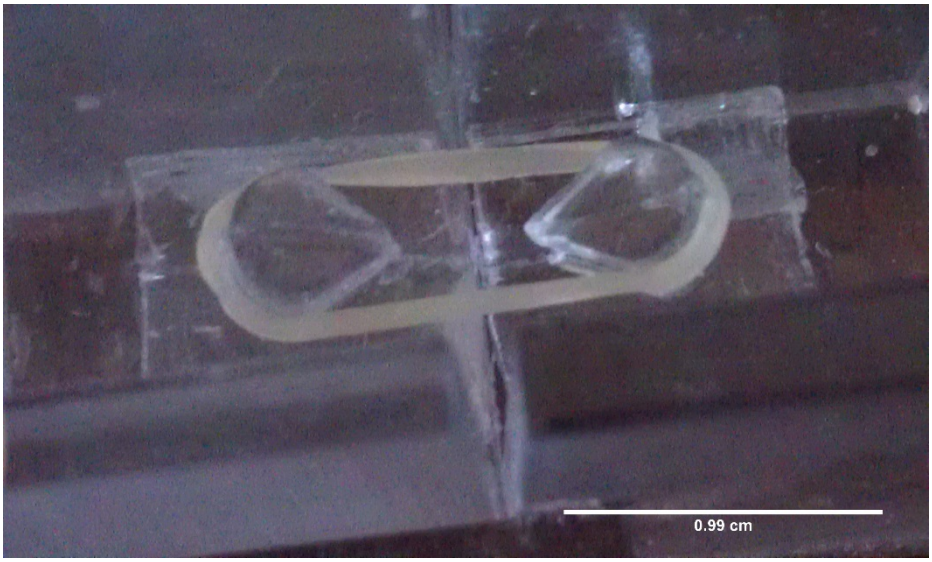


Figure 18: Rested Elastic Band

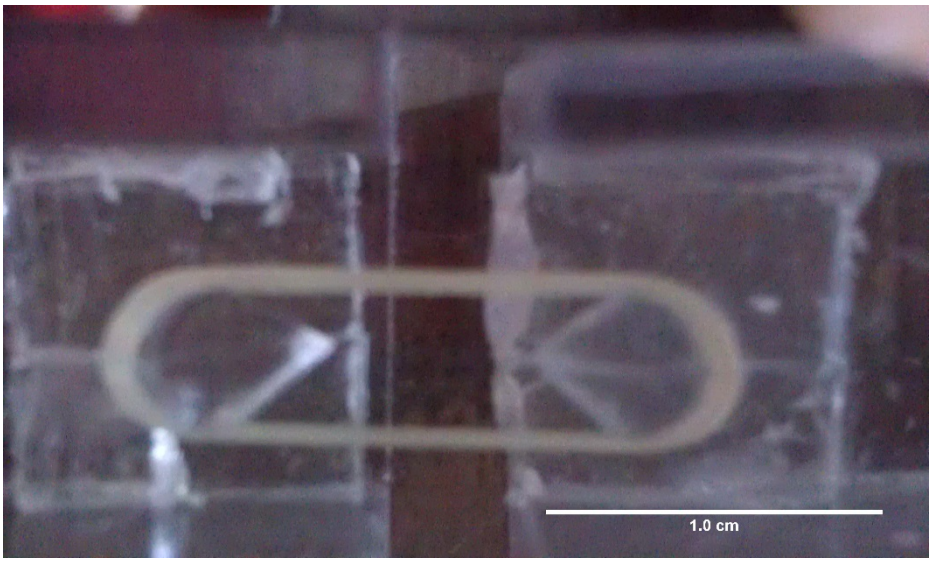


Figure 19: Stretched Elastic Band

The actuation worked just as intended as the orthodontic band was able to stretch back and forth at a controlled rate. The PDMS post also was shown to be flexible which allows analysis of the potential stress generated from a muscle tissue. The following table shows the rotation and speed in which the linear actuator operated in order to achieve the desired mechanical strains that would capitulates what native physiological skeletal muscle experiences.

Table 3: Linear Actuator Data

Strain	Length(mm)	Steps	Rotations	Strain Rate (mm/min)
0	15	0	0	0
5%	15.75	69	1.07	0.15
10%	16.5	137	2.14	0.3
15%	17.25	208	3.218	0.45
20%	18	274	4.281	0.6

This shows that the linear actuation's parameter in order to actuate the orthodontic elastic band to the desired percent strain. While this validates the ability for the linear

actuator to achieve the optimal amount of strain for an elastic band; the ability of generating the proper amount for a tissue construct needs to be further tested.

7.2 Polycarbonate Enclosure Box Validation

In order to achieve the objective of allowing the system to withstand an incubator environment, the validation of the final design of the enclosure system was necessary to ensure no contact was made between the internal components and the incubator conditions. The first test to ensure the viability of the enclosure system was an internal humidity test. A hygrometer which displays both the temperature and relative humidity of the surrounding environment was placed into the incubator alone in order to validate that the hygrometer was accurate. The resulting readings after leaving the hygrometer inside for 24 hours was 95% humidity and 37°C which is consistent to the standard environment conditions of an incubator. After validating the hygrometer, it was placed inside the internal portion of the polycarbonate enclosure system. The polycarbonate box was then placed into the incubator and the relative humidity and temperature reading were taken over a 30 hour span. The results from the hygrometer readings can be seen in the figure below.

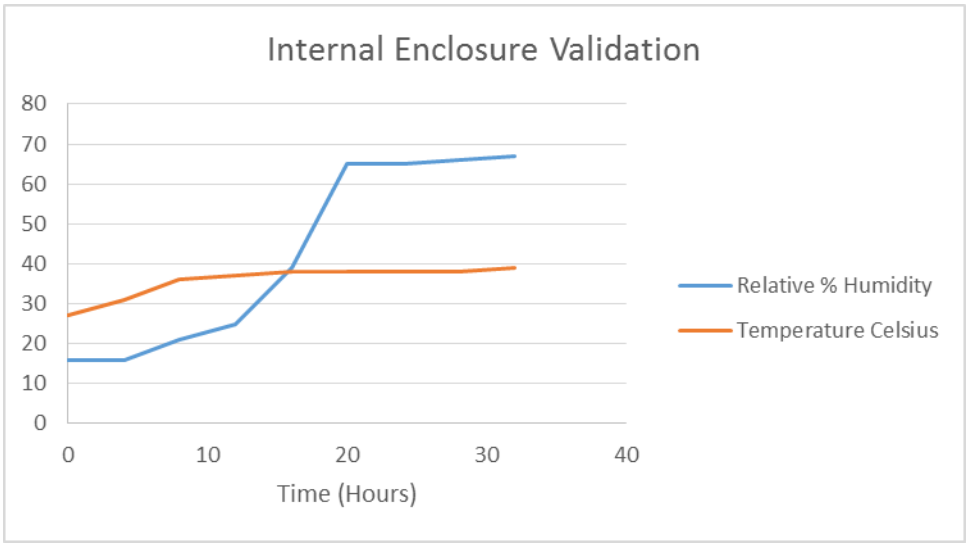


Figure 20: Hygrometer Reading Data

The relative humidity within the polycarbonate box showed a gradual increase over the first 20 hours but tapered off in the remaining 10 hours around 60-70% relative humidity. The temperature also showed a marginal increase but stabilized at around 38°C. These two parameters fall within the standard operating temperature and humidity of the Raspberry Pi microcontroller and the associated stepper motor.

The other validation test that ensured the sealant of the polycarbonate box was a water leak validation test conducted over a 24 hour timeframe. The polycarbonate box was filled with 950mL of water dyed with trypan blue. The whole system was then placed onto a paper towel which can be seen in the figure below.

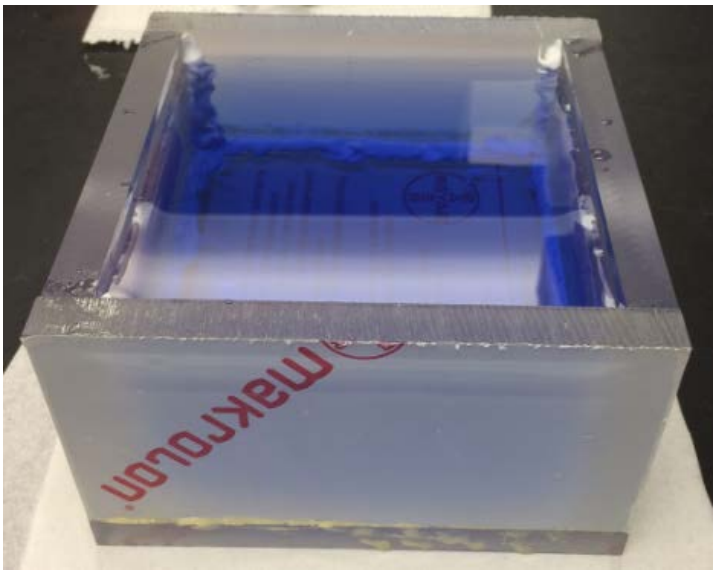


Figure 21: Leak Validation Test

After 24 hours, the device was observed and no leakage was observed on the surrounding paper towels. If there were any leakage, the surrounding paper towel would have been dyed with a blue color but none of that was observed. Therefore, the polycarbonate box lacked any potential leakage which validates that the device can properly seal the microcontroller and the stepper motor system.

Chapter 8 Discussion

This chapter highlights the goals and objectives that were met through this project. This include a design of an innovative culture dish for actuation as well as new molds to help prevent necrotic tissue from forming. Additionally, the use of a linear actuator device inside an incubator was utilized to stimulate the modular culture dish.

8.1 Culture Dish

The goal of the culture dish was to create a system that could fully encapsulate actuation without having to remove tissue when it needed to be cultured. An objective of this project was to develop and maintain tissue growth. Through the use of a movable culture dish, the system was able to perform actuation as well as maintain the environment in which the tissue could survive without having to move between systems. The dish was developed to incorporate both the concept of actuation, as well as the use of a sterile and maintained environment. Thus, the dish helps to accomplish two of the objectives: stimulation and tissue growth. The modular petri dish had four chambers that allowed for four concurrent testing to occur per test. This allows for more data to be collected as well as maximize the usage of media placed in the pool construct. Since there is a modular portion of the pool construct, the entire system needs to be filled with media. If there was only one test occurring; that would be highly inefficient use of that amount of media which can be used for more samples. Based off the results of the linear actuation of the modular component in the positive and negative direction, there was no spillage of the media caused from the actuation meaning that the petri dish is fully contained.

8.2 Linear Stimulation

The proof of concept of the mechanical system of the linear actuator has been validated to ensure that the system worked as expected. The linear actuator has the ability to be modulated and modified in order to fit the need of the construct needing to be actuated.

Once the linear actuator was completely assembled, the device has tested to ensure that the design can mechanically stimulate from 5-20%. The test that was conducted was straining test that used orthodontic elastic bands surrounding the PDMS posts in the pool system. The results showed that the orthodontic band attached to the post at 15mm can be actuated 5%, 10%, 15%, and 20%. This corresponds to 15.75mm, 16.5mm, 17.25mm, and 18mm that the orthodontic elastic band was stretched. This validated that the linear actuator could generate the proper amount of strain that is necessary in skeletal muscle development. Unfortunately, this proper amount of strain was not tested with skeletal muscle tissue as the tissue construct did not formulate enough to gather viable strain measurements that would be mimetic to the strain native tissue would experience. The revision of the seeding process of the cells onto the mold construct would allow for better tissue constructs to be formulated to withstand strains from 5% to 20%.

8.3 Molding

The new molds that were created were developed for the purpose of growing tissue that did not present necrosis along the post which they were seeded. The teardrop shape presented the ability for cells to be seeded and not form in a ring. The teardrop shape allows the cells to form into a myotendinous junction of native tissue without

forming at too sharp an angle that minimizes the amount of necrosis. These new molding systems are important in accomplishing the given objectives because they allow cells to align around the post in a way which helps to properly grow a tissue construct. With necrotic tissue in previous designs (see [1]), the tissue construct did not hold long enough to be mature through stimulation. Thus the new molds was expected to achieve the structure, tissue growth and potential reproducibility required for the project. The C2C12 cells were seeded onto the agarose seeding channels that were created using the rapid prototyped negative molds. After four to five days after the cells were seed onto the mold, there was only a small amount of tissue formation observed. One of the design's largest shortcomings was that the separate agarose pieces led to the leakage of media that made the cell seeding onto the construct difficult to complete. Another large shortcoming of the agarose design was that the size of the construct was small and required the need to cut away the extraneous agarose. This caused some disturbance to the tissue construct which contributed to the lack of mature tissue development.

8.4 Incubator Stimulator

The stimulation system that was developed has the goal of being feasible within the incubator. The use of a standard stepper motor within the incubator was not possible because of the large amount of humidity and temperature strain that it placed on the electrical components. This problem resulted in the proposal of a waterproof box or containment system that can be configured. The final linear actuator system was enclosed in a polycarbonate box sealed using silicone glue. The hole that the linear actuator exited was sealed with a rubber septum that encapsulated the linear actuator threaded shaft. The other exit point for the Raspberry Pi microcontroller electrical wiring was sealed using silicone glue. The top lid of the polycarbonate was sealed using silicone vacuum grease.

The resulting system was tested to ensure validity of components in an incubator environment. The tests ran were a leak validation and a timed humidity test. The lack of any leakage of water from the leak validation test ensured that the internal components did not make contact with the incubation environment. This was further validated with the humidity test using a hygrometer. The results showed that while the internal humidity rose over the first 20 hours, the rise in humidity tapered off and stayed roughly in the 60-70% range. This falls within the standard operating threshold for the Raspberry Pi microcontroller that has an allowable maximum range of 70% humidity. These validations test ensured linear actuator was able to withstand the incubator environment while conducting the necessary linear actuation needed to formulate mimetic skeletal muscle tissue.

Chapter 9 Project Impact

The results and design with the completion of this project has significant implications in how the *in vitro* skeletal muscles models are mechanically actuated and tested.

9.1 Manufacturing

The multitude of components necessary for the function of the final working device required the aid of a computer aided design software program. Solidworks 2014 was used in order to model each component's parameters and material. The components were individually generated and then put together into one coherent assembly that comprised the final design. The use of Solidworks allow for accurate dimensions and tolerances to be set in order to be exported for manufacturing purposes. The components were brought to the manufacturing resources provided on Worcester Polytechnic Institute's campus. The modular petri dish was rapid prototyped using veroclear material from the rapid prototype machine. The PDMS post mold and agarose base mold templates were also rapid prototyped using the rapid prototype machine exactly to specification.

The linear actuator component were manufactured with the aid of the machine shops on Worcester Polytechnic Institute's campus. The machine shop allow for the processing of the bolt to fit onto the stepper motor shaft with the aid of a coupling component. The machine shop also aided in processing the sheets of ½ inch polycarbonate sheets to form the polycarbonate enclosure box and the two output holes that allowed for the linear actuator and electrical wiring to protrude from the enclosure.

9.2 Societal Influence

There are societal implications that may stem from the use of *in vitro* models of native skeletal muscle. For instance, the current models to test for behavior of muscular diseases are animal models that are not physiologically analogous to human physiology. *In vitro* models will allow for the utilization of human mesenchymal stem cells to create skeletal muscles. This leads to a more predictive model of diseased skeletal muscle when the model is subjected to muscular diseases. Thus the ability to create a mimetic skeletal model influences the foundation of skeletal muscle disease research.

9.3 Ethical Concern

When utilizing any living organism; the concern of ethics arises due to the nature in which the organisms are treated. In the case of this project, the use of cell culturing of C2C12 cells were supplemented with various animal serums and growth factors. The question of where the components of cell culture were harvested may be asked. Though, the positives of creating an *in vitro* skeletal muscle will in the long run eliminate the need to use animal models to test the effects of debilitating diseases that would inevitably kill the animal models. Instead the use of mimetic skeletal models will allow for many samples to be made which also uses human mesenchymal cells that better emulate how human interactions with muscular diseases occurs.

9.4 Health and Safety Issue

The issue with health and safety are a legitimate concern when conducting proposed experiments to understand the effects of different muscular diseases. While current methods used *in vivo* animal subjects to test treatments to diseases, the risk of exposure is high due to the unpredictability of the animal's effect to the disease or treatment. In order to better control the response to illness and treatments, the use of *in*

vitro model allows for a more controlled environment. Also the use of *in vitro* models provides better data to be collected as the muscle tissue is the only component in the system. The hope is that eventually *in vitro* models will be effective enough that using human mesenchymal stem cells to formulate native skeletal tissue can be used to generate mimetic models to human skeletal muscle. This would help expedite the process of creating drugs and other various treatments for muscular deficiencies that currently affects many lives presently.

9.5 Sustainability

When determining the material to use to generate the final design; one of the large considerations taken was the environmental impact that the material and manufacturing process has on the device. The components that were created to limit the use of resource as well as being cost-effective. The petri dish construct was maximized to utilize only the necessary amount of material to contain both the proper amount of media and the tissue mold construct. The components that are reusable as the material allows them to be autoclaved repeatedly without any deformation. This ensured that the constructs can be used repeatedly over many samples of testing that minimizes the use of material and manufacturing that needed to be done. Also the linear actuator was developed to be a modular device that can easily replace individual components if necessary. The linear actuator along with the polycarbonate box was designed to be reusable and not require any additional manufacturing. These factors taken into account during the project allows the device to be as sustainable as it possibly can.

Chapter 10 Conclusions and Recommendations

10.1 Recommendations

With the completion of this project, there were a number of successful progresses made on the tissue mold structure, tissue dish holder, and mechanical actuation system. By conducting validation studies on this device, a set of observations led to several recommendations that can be factored in for future projects.

10.2 Tissue Formation

The formation of the tissue was shaped using an agarose mold from a design template created out of PDMS. One of the larger issues was since the order of size of the agarose mold was quite small, the ability to cut the agarose into a usable shape was quite difficult to do. A way to combat this issue the group believes is to create a template rack out of PDMS that can be used to quickly and neatly make agarose molds that can be easily cut and be used to seed cells on for tissue formation.

10.3 Mechanical Actuation System

With the mechanical actuation system being immersed into the incubator for the duration of the testing, the ability to regulate and monitor the temperature and humidity of the internal components is necessary. Therefore, utilizing the Raspberry Pi technology, a humidity tracker can be programmed in order to alert the experimenters of any potential harm to mechanical actuation system. Another recommendation is that the utilization of a camera inside the incubator would allow for the measurement of the displacement of the tissue to be measured real time. This will allow for a relationship between tissue formation, time, and displacement speed to be developed in order to get a better understanding on how mechanical stimulation can induce better skeletal muscle tissue formation. Since the incubator environment lacks any lighting source, one option might

be to fluorescently tag the post so that the camera can tag and focus on the post to measure the displacement of the post over a period of time. These recommendations are strongly encouraged for future projects in order to improve the monitoring and formation of skeletal muscle tissue growth *in vitro*.

10.4 Conclusions

At the conclusion of this project, a device that is able to facilitate *in vitro* tissue engineered skeletal muscle was designed. In order to achieve this goal, the device specifications were broken up into three separate components, the tissue molds, the mechanical actuation system, and the tissue dish holder. The key component was the ability for the mechanical actuation system to accurately modulate the tissue construct in a consistent manner. The tissue construct and the tissue dish holder were all created to be used in a sterile environment and the components besides the cells could be autoclaved to ensure sterility. Since the mechanical component was separate from the tissue construct, the necessity for the device to survive autoclaving and sterility does not have a factor in the viability of the tissue. While there were many limitations that prevent the mechanical actuation system to fully be mobile and small enough to be taken in and out of the incubator with ease; the results from this project shows great potential that can be further tested and modified in subsequent projects. The end result of the project showed that a mechanical actuation system does not need to be bulky and convoluted while being able to withstand incubator conditions. The mechanical actuation system was validated to produce strains from 5-20% that can be translated to a tissue mold construct. This project has improved on previous research and designs for actuating *in vitro* skeletal muscle tissue and generated a template for future projects.

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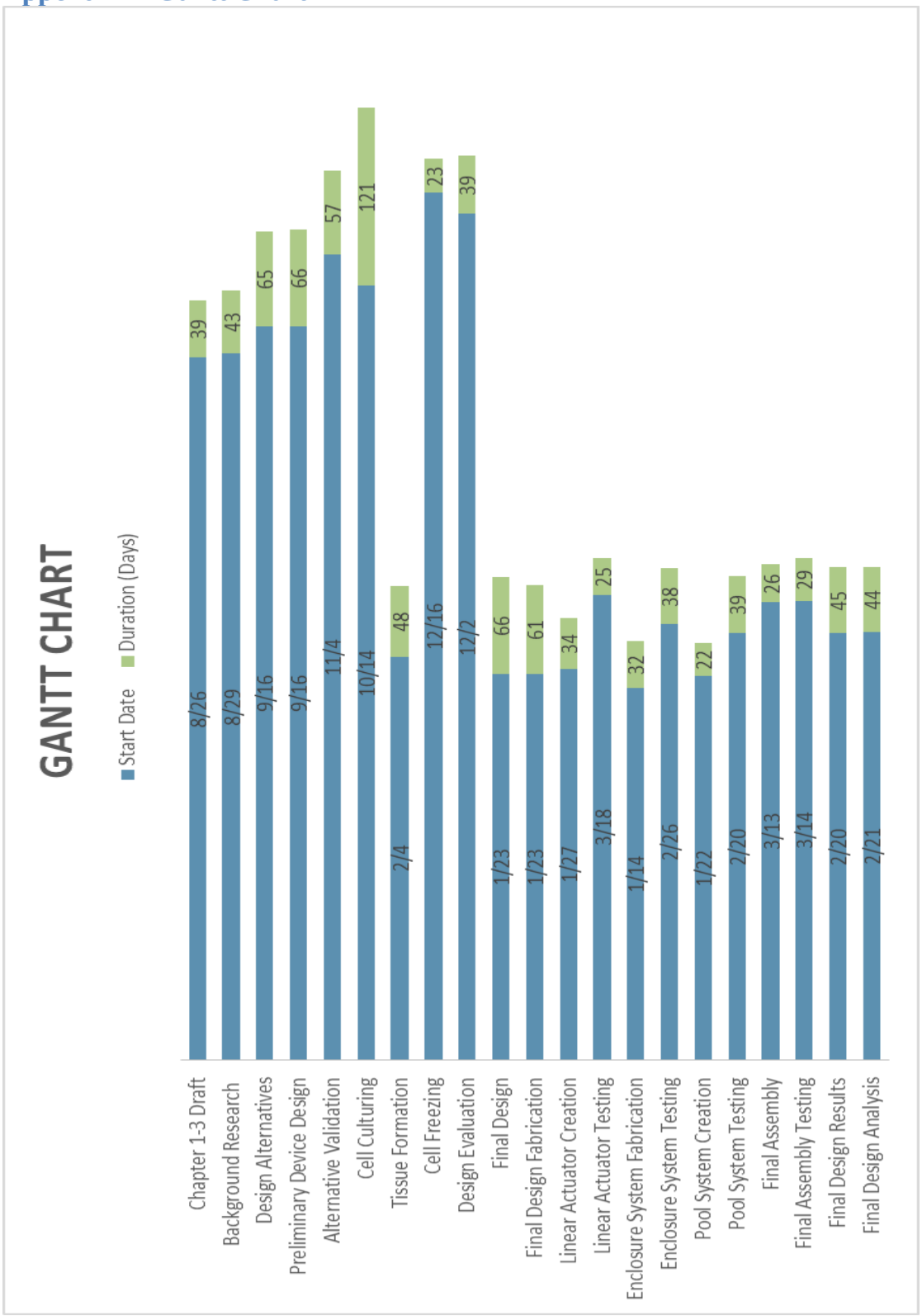
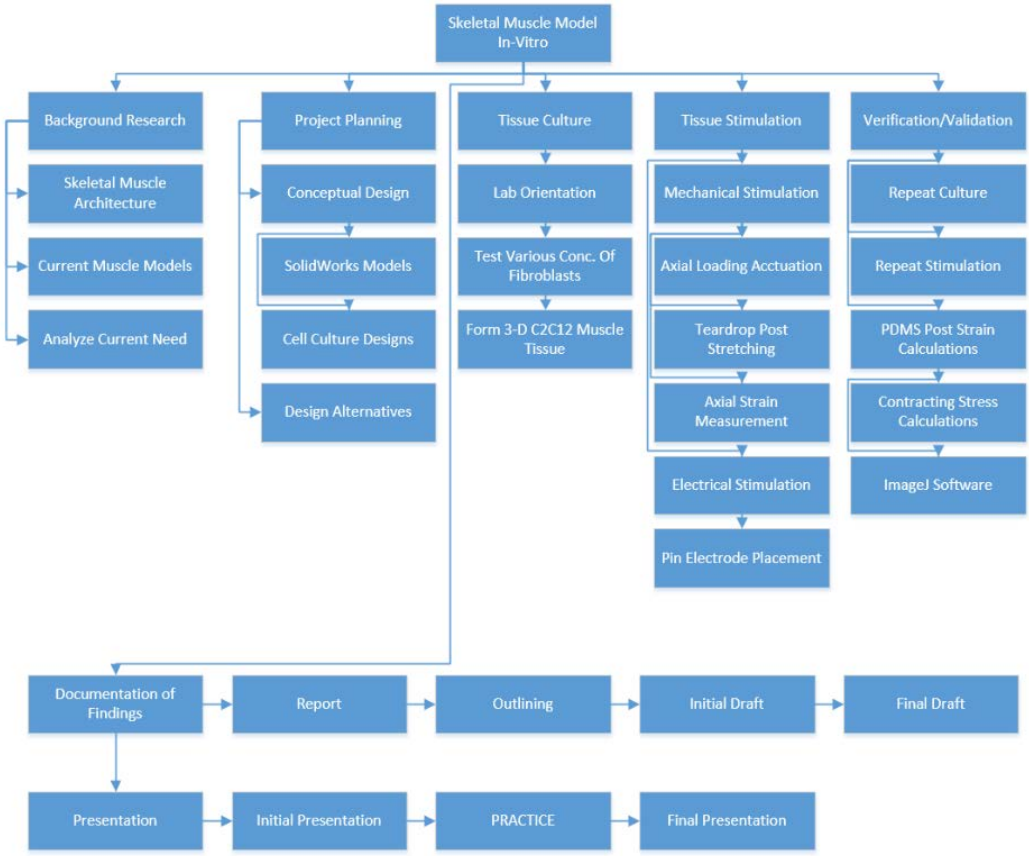
Appendices

Appendix A Gantt Chart



Appendix B Work Breakdown Structure



Appendix C Functions Means Chart

Function	Means		
Mechanical Actuation	Electromagnet	Stepper Motor	Syringe Pump
Anchor Tissue	Agarose Post	Metal Post	PDMS Post
Microcontroller Protection	Polycarbonate Box	Polystyrene Box	Steel Box with cement coating
Tissue Containment	Corrugated petri dish	Pool System	Static petri dish
Tissue Formation	Fibrin microthreads	Teardrop Dogbone mold	
Measure Contractile Force	Force Transducer	Post Deflection	

Appendix D Proliferative Media

Complete Media for Proliferation

DMEM (Dulbecco's Modified Eagle's Medium)	243 ml
Ham's F12	162 ml
HyClone™ FCIII (FetalClone™ III Serum)	45 ml
Glutamax	4.5 ml

(Media "recipe" from Sakthikumar Ambady [18])

Appendix E Protocol for Passaging Cells

1. Confluence of 60-70% should be observed for passaging of C2C12 cells under a fluorescence microscope.
2. Aspirate the medium from the cell culture using a sterile pipet tip and vacuum pump.
3. Add 5 ml sterile Dulbecco's Phosphate Buffered Saline (DPBS) to the culture plate and swirl gently.
4. Aspirate DPBS and add 5 ml 0.05% trypsin +EDTA and place in incubator for 5 minutes.
5. After about 2-5 minutes, remove plate from incubator and view under a fluorescence microscope. Tilt plate back and forth to make sure that all the cells have lifted from the plate.
6. After confirming that the cells have lifted off the plate, add 5 ml of complete media (using preparation above).
7. Pipette cell and media mixture from the plate and place in 15 ml conical tube; remove 50 ul of mixture for cell counting.
8. Place conical tube in the centrifuge and spin for 5 minutes at 1200 rpm.
9. While cells are spinning, take a 10 ul sample from the 50 ul mixture and count the cells.
10. Place 10 ul of mixture onto the hemocytometer and count the 4 corner boxes.
11. Once counted, divide the total by 4 to get the average count of the boxes. Take that average and multiply by 10,000 and then by the number of ml that the cell mixture is suspended in (in this case, 10). This will determine how many cells are in the mixture of 10 ml.
12. Once the cells have finished spinning, there will be a pellet at the bottom of the conical tube. Aspirate the supernatant, or surrounding media, and make sure to keep the pellet intact.
13. Resuspend the cells in 1 ml of complete media.
14. Calculate the amount of mixture that needs to be plate by taking the cell count number before and the number of cells are desire and creating a relationship. For example if 500,000 cells are to be plated and $1*10^6$ were counted:

$$\frac{1 * 10^6 cells}{1\ ml} = \frac{500{,}000\ cells}{x\ ml}$$

x = 0.5 ml → 500 ul

15. From this, plate 500 ul of the cell mixture on a 100 mm plate. Add media to the plate to make the total volume 10 ml (ie. Add 9.5 ml of complete media).

Protocol taken from Dr. Sakthikumar Ambady's "Cell Culture Manual-Short" [35].

Appendix F Raspberry Pi Motor Controller in Python Programming Language

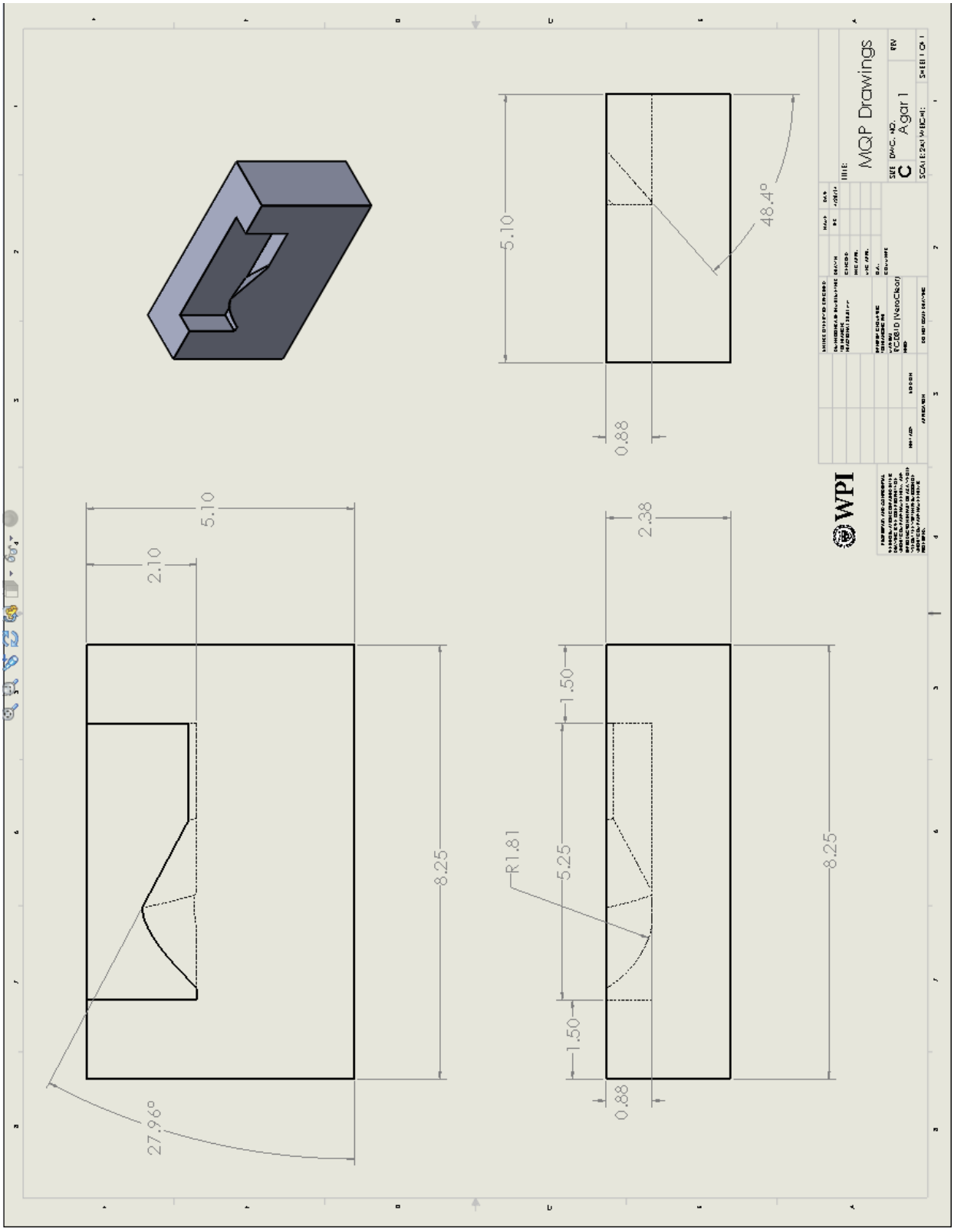
```
#Stepper Motor Actuation
  #Author: Alexander Barat, Randy Chin, Alan Gribble, Jacob Konowitch

# import time variable and assign it to variable 'pause'
  from time import pause

#import the parameters for the piface microcontroller module
  import piface.pfio as piface
  pfio.init()

#coding for negative actuation
  def negative(rotation, speed)
      slptime = 1
      float(speed)
#create a loop to initiate commands to the raspberry piface microcontroller
      for loop in range(1,int(512*float(rotation)))
          piface.digital_write(7,1)
          pause(slptime)
          piface.digital_write(4,0)
          pause(slptime)
          piface.digital_write(6,1)
          pause(slptime)
          piface.digital_write(7,0)
          pause(slptime)
          piface.digital_write(5,1)
          pause(slptime)
          piface.digital_write(6,0)
          pause(slptime)
          piface.digital_write(4,1)
          pause(slptime)
          piface.digital_write(5,0)
          pause(slptime)
      piface.digital_write(4,0)


#coding for positive actuation
  def positive(rotation, speed):
      slptime = 1
      float(speed)
#create a loop to initiate commands to the raspberry piface microcontroller
      for loop in range(1,int(512*float(rotation))):
          piface.digital_write(4,1)
          pause(slptime)
```

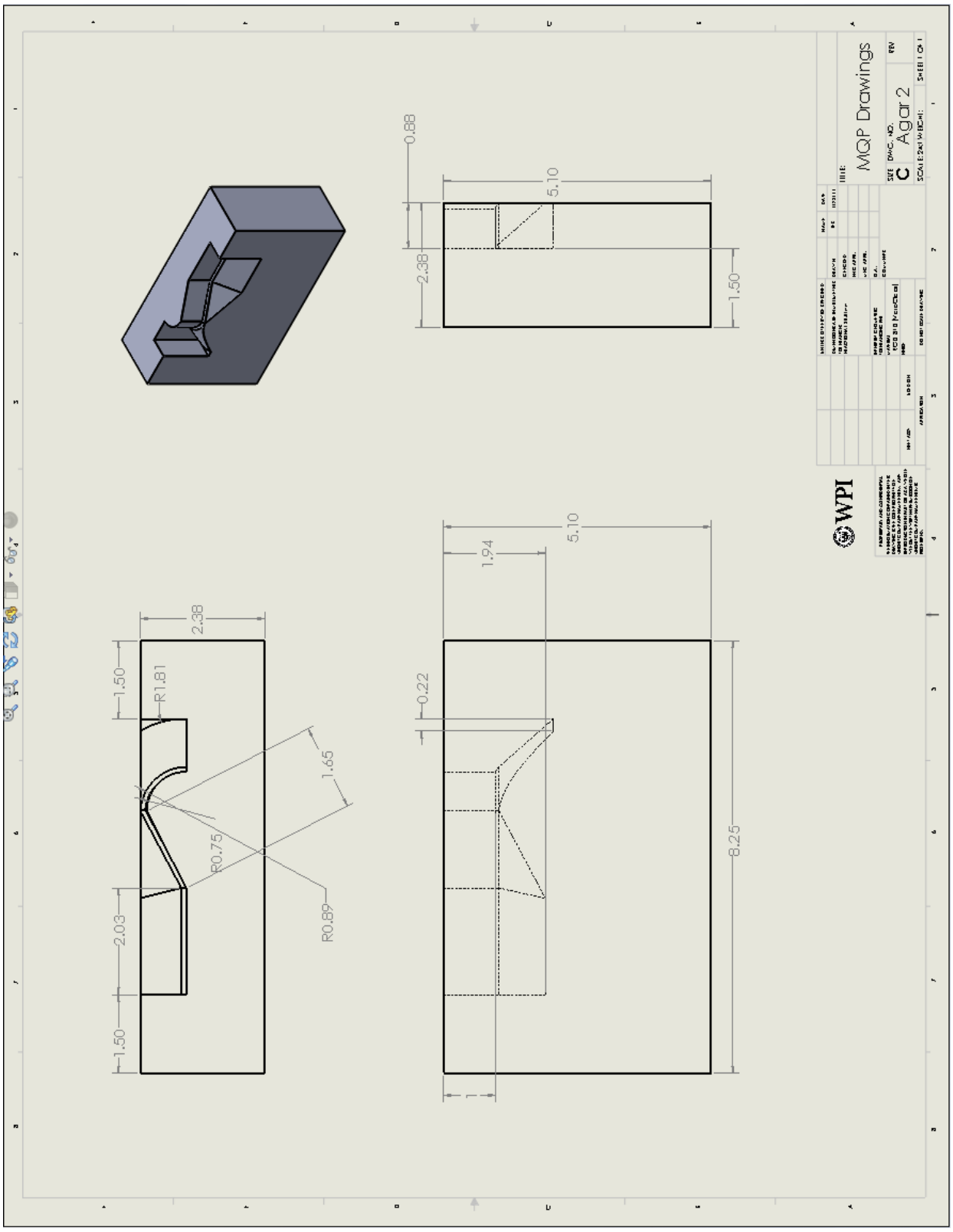
```
        piface.digital_write(7,0)
        pause(slptime)
        piface.digital_write(5,1)
        pause(slptime)
        piface.digital_write(4,0)
        pause(slptime)
        piface.digital_write(6,1)
        pause(slptime)
        piface.digital_write(5,0)
        pause(slptime);
        piface.digital_write(7,1)
        pause(slptime)
        piface.digital_write(6,0)
        pause(slptime)
    piface.digital_write(7,0)


#when program is initiated, the desired parameters for rotation and speed are inputted
  print"Type: lineardirection(rotation, speed)"
```

Appendix G Expense Sheet

Supplier	Material	Cost
McMaster-Carr	Spider Shaft Coupling Polycarbonate Silicone Putty	113.26
WPI-ME	Mold/Prototype	161.52
Amazon	Raspberry Pi Raspberry Pi - Pi Face Stepper Motor	141.40
Home Depot	Bolts Tubing Nuts Hygrometer Gorilla/Silicone Glue	80.77
Total		496.95

Appendix H: CAD drawings

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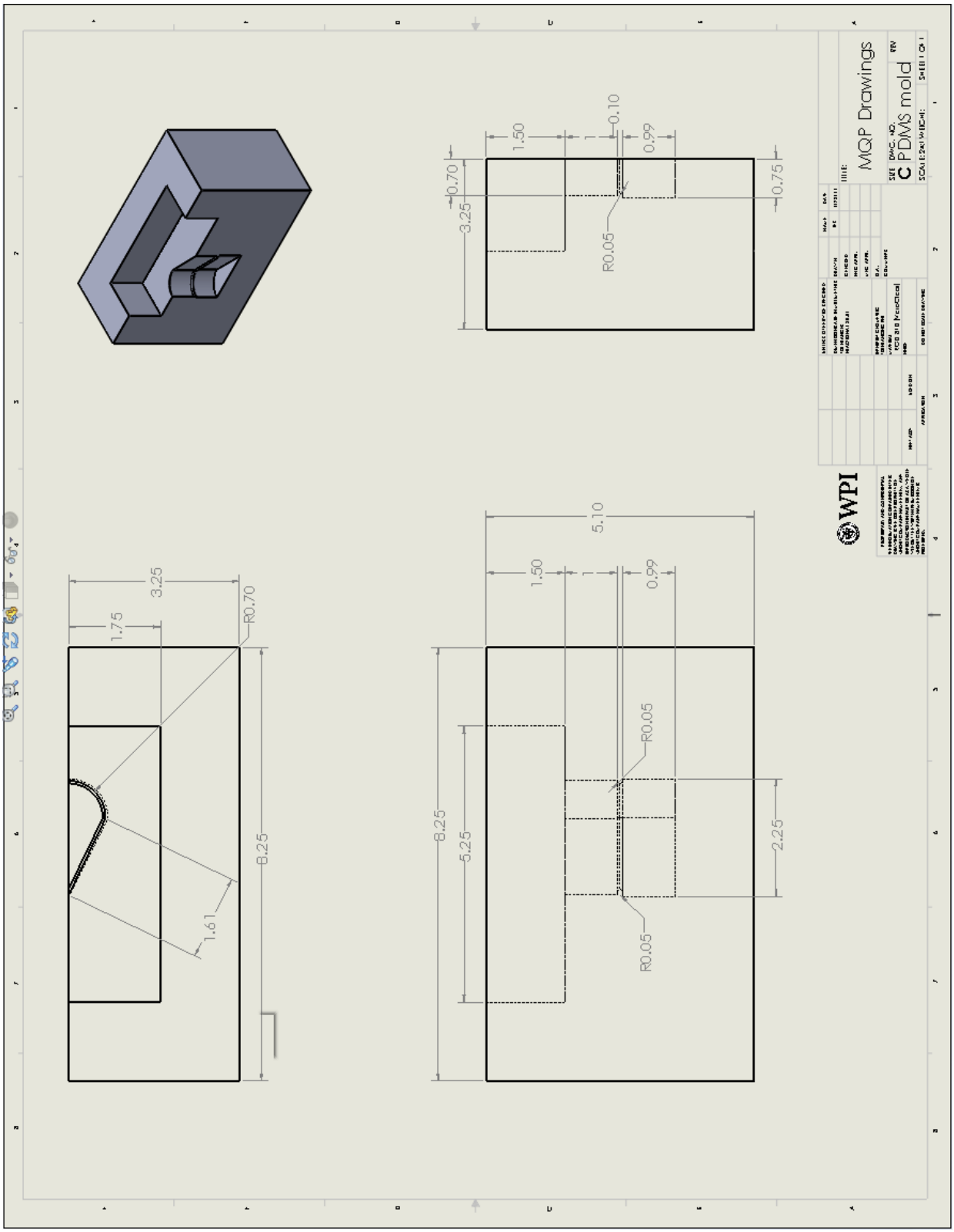
5.10
2.38
1.50
2.38
1.50
R1.81
1.65
R0.75
R0.89
2.03
1.50
5.10
1.94
0.22
8.25
1
WPI
MQP Drawings
Agar 2
C


MQP Drawings
C PDMS mold
WPI
3.25
1.75
R0.70
8.25
1.61
1.50
1
0.10
0.99
0.70
3.25
0.75
R0.05
5.10
1.50
1
0.99
8.25
5.25
R0.05
2.25
R0.05