BioMimeticSkeletal Muscle Tissue Model

A Major Qualifying Project submitted to the faulty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Bachelor of Science

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ABSTRACT

In vitro tissue modeling of skeletal muscle has a potential role in understanding muscle tissue physiology. Current systems contain limitations including the presence of extracellular matrix and diffusion limitations. Our team developed a system that allows for sterile 3D tissue development, mimics *in vivo* attachment points, has a scaffold-less construction, and can mechanically stimulate developing muscle tissue *in vitro*. C2C12 cells were characterized using BrdU and myosin staining to find the optimal time point for seeding and to confirm the maturation of the formed myofibers. A dog-bone shaped negative mold was used to form an agarose mold into which cells were seeded. Mechanical stimulation plates were created to enable uniaxial strain and tissue anchorage. A mechanical stimulation regime was developed and tested to better understand the factors leading to muscle growth. We believe this *in vitro* tissue model will not only allow a better understanding of developing muscle tissue, but can also be used as a screening modality for evaluation of future therapeutic treatments for muscle diseases and volumetric muscle loss.

EXECUTIVE SUMMARY

A reliable *in vitro* skeletal muscle tissue model could facilitate the understanding of the development of muscle tissue *in vivo* and aid in the testing of novel drugs and therapeutics to combat muscle disease. While *in vitro* skeletal muscle tissue models exist, their current limitations include 2D monolayer cell culture, complicated assemblies, and high costs. Our group's goal was to design a 3D skeletal muscle tissue model that could test the effect of mechanical stimulation on maturing skeletal muscle while being easy to use and manufacture at a relatively low cost.

Research has indicated that applying cyclic uniaxial strain to developing muscle tissue can increase myofiber diameter and alignment and aid in the overall maturation of the tissue. Most literature suggested that a strain of 10% was optimal to promote nuclear relocation and myofiber maturation.

Working through the design process, our team developed a cell culture preparation method and mechanical stimulation design to accomplish the objectives of our project. Mouse myogenic C2C12 cells were seeded into a dog-bone agarose gel to form a 3D tissue. This gel was anchored to two mechanical stimulation plates manufactured from high density polyethelene (HDPE). The plates consisted of a stationary plate and an articulating plate that controls displacement. A magnetic CAM and dish holder assembly was designed to allow mechanical stimulation in a closed system to maintain sterility. At days four, five, and six of tissue development, the tissue was mechanically stimulated for one hour at 1Hz every 12 hours. Both non-stimulated and stimulated muscle tissue were then compared via confocal microsopy.

METHODOLOGY

MECHANICAL STIMULATION APPARATUS

In order to create a mechanically conditioned skeletal muscle tissue, a mechanical stimulation device was designed and built. High density polyethylene (HDPE) blocks were used to machine the magnet arm, base plate, petri dish holder as well as the stimulation plates. Acrylic was laser cut to appropriate dimensions for the motor arm and lastly the motor cog was machined out of aluminum. A simple synchronous gear motor turned the motor cog, reciprocating the motor arm and magnet arm. This back and forth motion actuated the stimulation plates through magnetic connections on the external petri dish holder and internal mechanical stimulation plates in contact with the cultured tissue. Using this device, the stimulation plates were actuated uniaxialy to achieve 1.0 mm or 10% strain.

AGAROSE GEL PREPARATION

A 2% agarose gel was selected as our tissue seeding mold based on the stiffness experienced with 2% agarose gel. The mold negative used to form the agarose tissue mold was made from ABS plastic using a 3D rapid prototyping machine available in Higgins Laboratory. Limitations of prototyping resolution required further corrections of the dimensions by hand to modify the mold negative via 120 grit sandpaper to the correct dimensions. Molten 2% agarose gel was prepared with DMEM cell culture medium and sterilized by a 120 min autoclave cycle reaching a maximum temperature and pressure of 121^{o}C and 15 psi respectively. The 2% agarose gel mold was prepared by pipetting 7 ml of molten, 85^{o}C, sterile agarose through the gel ports in the inverted mechanical stimulation plates.

The gel cooled and set for 10 minutes. Then the stimulation plates were inverted, and the mold walls and negative mold removed. This procedure produced a sterile 2% agarose gel on the mechanical

stimulation plates. The agarose gel has a dog-bone shape comprised of two circular indents connected by a triangular channel into which myogenic cells were seeded.

Cell Seeding Preparation

Myogenic C2C12 cells were cultured in proliferation medium containing Dulbeccos Modified Eagle Medium (DMEM), 10% fetal bovine serum, 1% Penicillin Streptomycin, and 1% GlutaMAX. Once the cells reached 60% confluence, they were trypsinized and resuspended in differentiation medium. Differentiation medium contains 50/50 DMEM and Ham's F12, 2% horse serum, 1% insulin, 1% Penicillin Streptomycin, 1% GlutaMAX, and 1% Fungizone (Amphotericin B). Three million cells were seeded in the agarose gel mold. Once seeded, 20 mL of medium was added to the tissue culture plates.

Mechanical Stimulation Regiment

After the tissue had self-assembled and formed in the gel mold for four days, the stimulation regiment began using a protocol adopted from Powell et al. and Kumar et al; which involved stimulating the tissue at 10% strain, at 1 Hz for 30 minutes every 12 hours for four days.

Results

Device Results

Magnetic connection of the motor arm to the articulating plate through the sterile petri dish allowed for a sterile method of mechanical stimulation of the tissue. The mechanical stimulation plates and base plate were designed to fit the 150 mm diameter petri dish precisely to allow for exactly 1 mm of displacement or 10% stain to the tissue per minute. This apparatus was successful in mechanically

stimulating the self-assembled C2C12 tissue for 30 minutes every 12 hours for up to four days without failure of function.

Biological Results

A skeletal muscle tissue construct was created after seeding 3 million C2C12 cells using the methods described above. The mold allowed for a very thin myofibril, approximately 160 µm in diameter, to develop within the v-shaped channel due to cellular aggregation 24 hours after cell seeding. This tissue was mechanically stimulated with the regimen specified in the methodology section beginning at Day 4 of development and ending at Day 8. It was observed that the tissue lost integrity during mechanical stimulation as seen through the loss of the myofibril formation in the center of the gel mold. Another tissue sample was mechanically stimulated 5 days after seeding and it was observed to retained continuity.

Conclusion

A number of objectives were accomplished throughout this project. A method was developed to produce a 3D tissue construct that enabled formation, differentiation and maturation of engineered skeletal muscle tissue *in vitro*. An agarose gel mold was designed to aid in the self-assembly process and support the tissue throughout its development. Finally, a device was created that allowed for the sterile mechanical stimulation of engineered skeletal muscle tissue.

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CHAPTER 1.INTRODUCTION

Skeletal muscle loss directly targets all age groups, both old and young. Those over the age of 30 are threatened by declining muscle mass known as sarcopenia. Sarcopeniais directly correlated with aging and affects up to 50% of people over the age of 80. Muscular dystrophy, the weakness and loss of muscles, is one of the most common diseases in young children. Muscular dystrophy affects 1 in every 3,500 people (Hoffman, 1987). As there is no cure for this disease, those affected are continually burdened by the symptoms of their disease (Emery, 1993). Muscular atrophy, diseases, and injury can result in serious muscle damage. In these cases, scar tissue may form and restrict the muscle from regenerating completely. Unless the scarred fibers are replaced, permanent decreases in muscle contraction and elasticity can occur.

Although the body naturally regenerates damaged muscle tissue via satellite cells, this process is time consuming and can only fully repair small-scale injuries. Repair strategies to large scale muscle injuries have been developed seeding myoblasts onto microthreads using a rotational seeding system. Unfortunately, the cells on the surface of the microthreads are often unable to proliferate through to the center of the microthread. Thus, the structures of the muscle fibers are not adequately replicated and this process cannot be used clinically. As a result, it would be advantageous to create a mold for tissue growth that has a channel guiding cells into the correct alignment to form replications of natural muscle fibers. The mold can also allow for control of muscle fiber length and width, for the creation of uniform mechanical properties throughout the myofiber, and for an increase in the potential success of mechanical function after growth. The cells will be mixed in a natural gel matrix before being seeded onto the mold. The gel will help with cell migration and proliferation and will be formulated to degrade at a controlled rate. The degradation rate will be set so that the load on the matrix can be

transferred to the new muscle tissue at a rate comparable to the growth of the new muscle tissues extracellular matrix (ECM). Anchoring posts will provide support and tension for the muscle tissue as it differentiates and matures. Engineered tissue rings on the anchoring posts will provide for easy removal of the tissue from the mold for testing post-culture and post-mechanical stimulation.

The following chapters of the project report contain a literature review outlining current techniques for skeletal muscle tissue engineering as well as the advantages and disadvantage of the materials used; a project strategy including objectives, functions and means the project team used to create their designs; evaluation of the designs and a determination of which design best meets the criteria set forth by the client and user; design verification, including test results and all findings; and finally discussion and conclusions including future recommendations that the project group have made.

CHAPTER 2.LITERATURE REVIEW

This project explored strategies for designing a device to mechanically stimulate skeletal muscle tissue *in vitro* while maintaining sterility and biocompatibility of the tissue. This chapter evaluates background information and pertinent literature on skeletal muscle tissue, muscle loss and regeneration, current gel and scaffold designs for growing skeletal muscle, different means for mechanical and electrical stimulation of tissues, and different tests for myocyte culture.

2.1 MUSCLE AND MYOBLAST PHYSIOLOGY MUSCLE AND MYOBLAST PHYSIOLOGY

Three muscular systems are responsible for the many functions of the body, including locomotion, facilitating digestion, and blood flow. These systems are the skeletal, smooth muscle, and cardiac muscle systems. All of the muscle systems contain myocytes or muscle fibers; however, their physiology and control mechanisms vary. For smooth and cardiac muscle tissue, contraction is involuntary. For involuntary contractions, no cognitive motor control is administered to the tissue. On the other hand, skeletal muscle tissue has voluntary contractions and controls of the cognitive motor control required for locomotion and movement of the skeletal system.

2.1.1 SKELETAL MUSCLE HIERARCHY

The anatomy of a skeletal muscle consists of a decreasing hierarchal model. The top-most layer is the fascia, a fibrous connective tissue composed of mostly collagen, which anchors the muscle to the bone. Beneath the fascia, the muscle is covered with a collagenous epimysium (Figure 1). Under the epimysium, the perimysium surrounds the fascicles, bundles of muscle fibers, which are divided into individual myocytes, or muscle fibers, by the endomysium. Within each fascicle, there are bundles of myofibers which run the entire length of the muscle. The myofibril is the actual contractile unit of the

muscle fiber (Shier, 2009). A myofiber is one myocyte or one muscle cell that contains the contractile protein filaments.

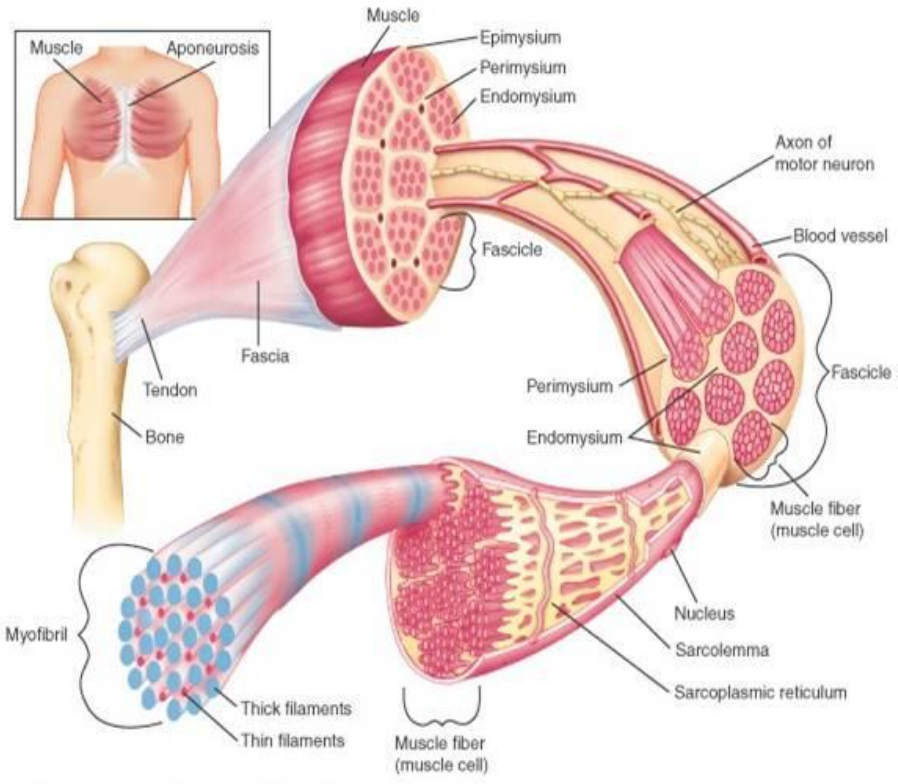


FIGURE 1 SKELETAL MUSCLE HIERARCHY

2.1.2 Myocyte Structure and Function

Myocytes are multinucleated cells controlled by a single motor neuron axon located at the motor end plate. The outsides of myocytes are covered with a network of tubes called the sarcoplasmic reticulum (Shier, 2009). The sarcoplasmic reticulum is responsible for translating the neuro-electrical signal from a motor neuron into a chemical gradient signal of acetylcholine for muscle contraction. Within a myococyte there are contractile units called sarcomeres. A sarcomere contains thick and thin filaments overlapping each other (Figure 2). When the myocyte is stimulated by calcium ions from the

sarcoplasmic reticulum, the thick and thin filament's motor proteins begin to contract and pull the Z lines of the sarcomere closer together resulting in movement (Figure 2) (Shier, 2009).

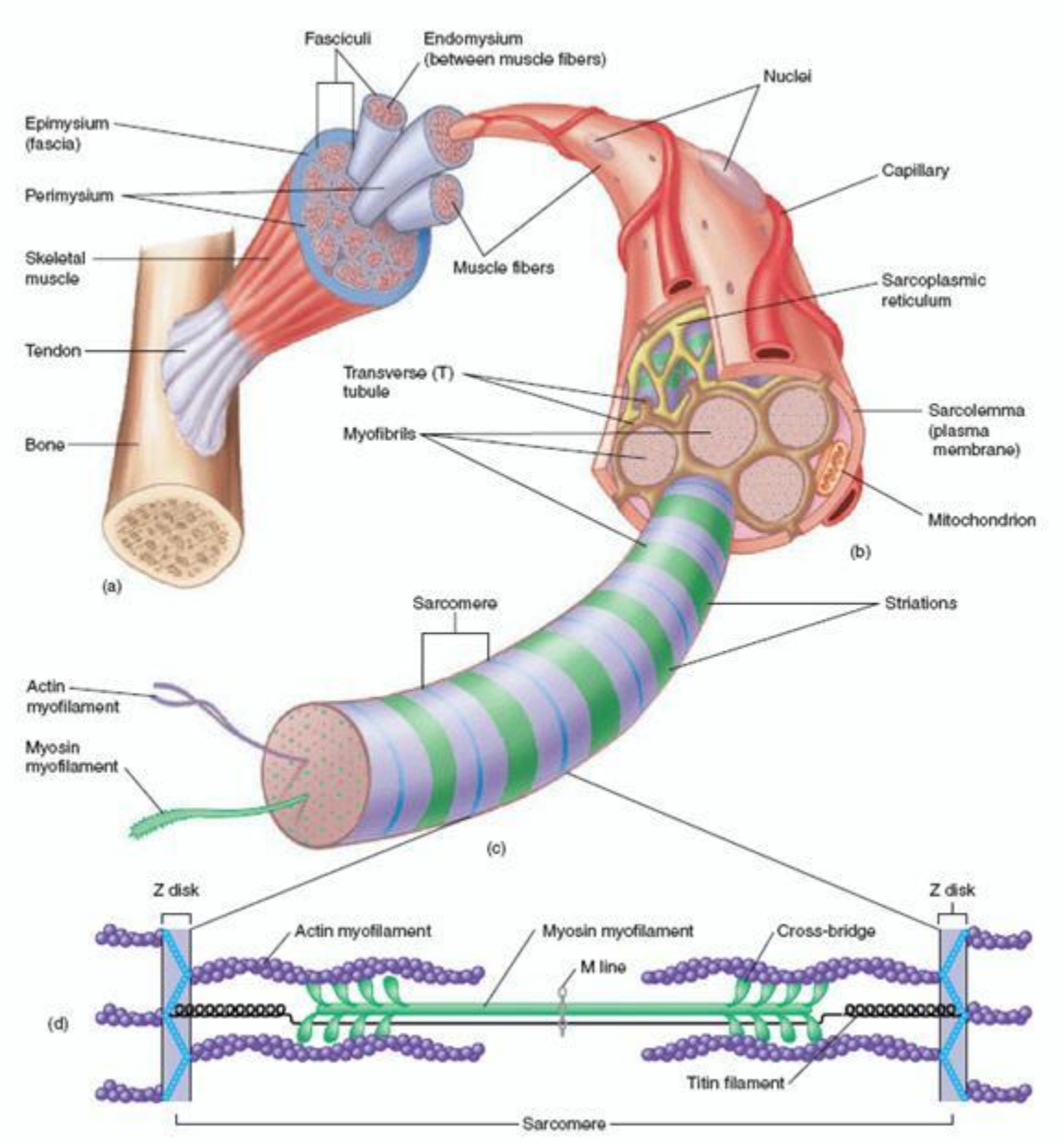


FIGURE 2: MYOCYTE AND SARCOMERE STRUCTURE

2.1.3 Regulatory Process of protein Contraction

There are two proteins involved in the contractile function of the muscle, actin and myosin. These two proteins act in conjunction to move sliding filaments across one another, resulting in contraction. This process is depicted in Figure 3.

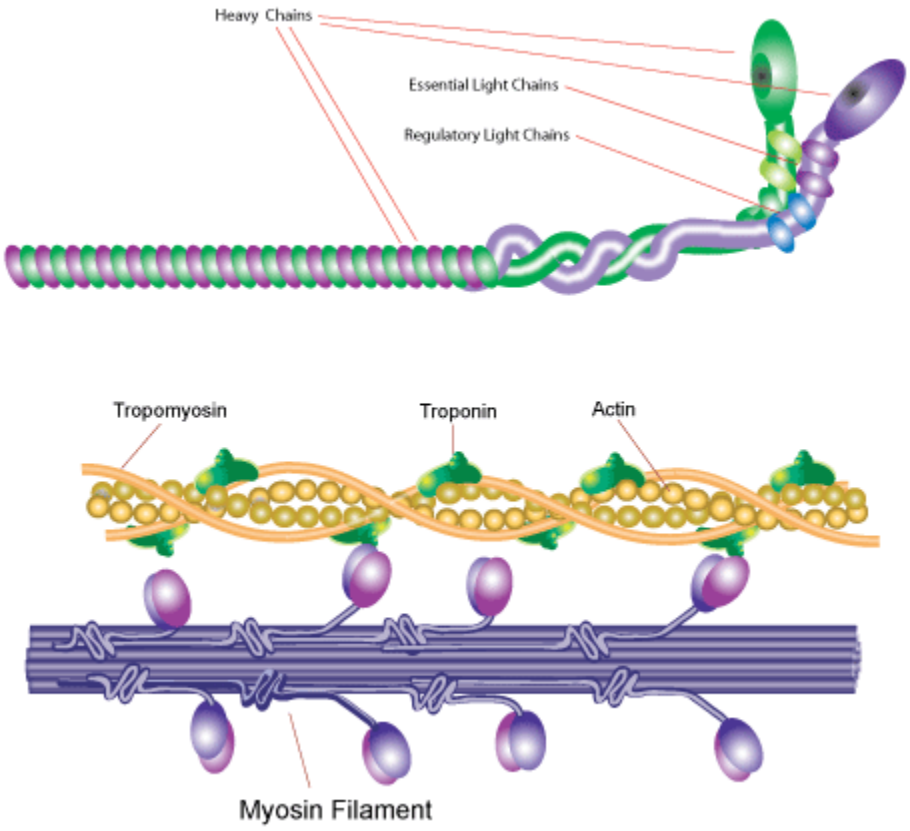


FIGURE 3: CONTRACTILE PROTEINS

The regulatory process for contraction includes the proteins tropomysoin and troponin. Tropomyosin is a competitive inhibitor of the binding site of an actin filament preventing the myosin cross-bridge binding (Shier, 2009). When calcium ions are present in the cell, released by an action potential from the sarcoplasmic reticulum and terminal cisternae, they bind to the protein troponin. This binding creates an allosteric change in structure of the tropomyosin and exposes the binding site of

the actin filament (Figure 3). This initiates an ATP dependent step, where the actin and myosin filaments bind and contract in the power stroke creating sliding filaments (Shier, 2009).

2.2 CLINICAL SIGNIFICANCE:

Skeletal muscle loss is a pressing issue facing doctors today. It comes in many different forms and has many different causes, but the common trend among all of them is the lack of effective treatment. Skeletal muscle loss begins in most individuals after the age of 30, as a result of aging. This decline of muscle mass, called sarcopenia, occurs at a rate of about 1% per year and is present in 5%-13% of those aged 60 to 70 years and 11%- 50% of those aged over 80 (Morley, 2011). Sarcopenia can affect a patient's ability to walk or even to stand. Currently, there is no satisfactory solution on the market to help sarcopenia patients.

Another form of skeletal muscle loss affecting people is muscular dystrophy (MD). MD is caused from an abnormality in the genetic code for certain muscle proteins (Bushby, 2000). The primary characteristics of MD involve onset of muscle weakness and loss in early childhood that progresses with age. One in every 3,500 people is affected by MD. Symptoms include drooling, eyelids drooping, frequent falls and problems walking, and general loss of strength and muscle size. There is no known cure for MD and the only type of treatment available is attempting to treat the symptoms through physical therapy (Muscular Dystrophy, 2010).

One of the most common causes of skeletal muscle loss is rhabdomyosarcoma, a cancer that forms in the soft tissue of striated muscle. Rhabdomyosarcoma makes up 50% of all soft tissue sarcomas in children. (Andrassy, 2002) The first response to rhabdomyosarcoma is to treat the patient with radiation and chemotherapy, but often times this is not enough, and surgical removal of the sarcoma is necessary. This is a highly invasive procedure as it involves removing all of the surrounding tissue and

muscle to ensure no cancerous cells remain. Frequently a second reconstructive surgery is necessary to rebuild the muscle cells that were removed. (Soft Tissue Sarcoma, 2011)

Compartment syndrome is a type of muscle loss that has a quick onset and must be dealt with in a timely manner for patients to recover normal function. Compartment syndrome is characterized by swelling or bleeding in a muscle. This creates pressure within the muscle and its surrounding tissues as well as halts blood flow to the area which results in tissue necrosis (Hargens et. al, 1981). Typically, some of the tissue can survive for four hours without blood flow. However, after six hours the use of the tissues is uncertain, and by eight hours the damage has become permanent (Whitesides, 1996). Compartment syndrome is often caused by a serious injury or fracture and cannot be ignored, but even if it is caught quickly, surgery is still required and any muscle loss has to be repaired.

In the case of traumatic injury, the muscle cells are damaged beyond repair, but the injury itself is not severe enough to require limb amputation. This often occurs in military personal, as 25% of injuries sustained in combat consist of large muscle defects such as lacerations [Owings et al. Orthop]. Muscle loss due to traumatic injury can be treated with tissue transfer from another source, but great difficulty is associated with procuring donor tissues and with donor site morbidity. Therefore, for traumatic injury muscle loss and a great number of other muscle loss cases, an option for tissue engineered skeletal muscle would be highly beneficial.

2.2.1 Need for a skeletal muscle tissue engineering model

Though many different tissue engineering methods have been developed and tested, there is still no gold standard method for 3D skeletal muscle tissue engineering. Our group hopes to solve this problem through designing a simple, cost effective method for developing 3D skeletal muscle in vitro that gives scientists the ability to test the capability of various cells to form skeletal muscle as well as

provide in vitro testing for the study of different therapeutic treatments for genetic diseases such as muscular dystrophy.

Currently it is known that skeletal muscle has regenerative properties (Tedesco et al, 2010). However, the exact mechanism behind this regeneration and how these properties can be harnessed in order to stimulate regeneration in vivo is not known, due to the expansive signaling system cells are exposed to in vivo. Through creating a definitive model for 3D skeletal muscle tissue engineering in vitro, scientist will be better enabled to study the mechanism behind skeletal muscle regeneration and the onset and progression of skeletal muscle disorders such as muscular dystrophy. Doing so will aid in the development for next generation treatment of large scale skeletal muscle injuries and detrimental genetic anomalies that prevent skeletal muscle repair.

2.3 Muscle Regeneration

Unlike many tissues in the body, muscle tissue naturally regenerates (Tedesco et al, 2010). Its regenerative properties give it potential for many therapeutic applications in the future. However, there are no ongoing clinical trials involving tissue engineered skeletal muscle implantation in humans.

2.3.1 Skeletal Muscle Regeneration in Native Tissue

Satellite cells found between the sarcolemma and basal lamina are responsible for the regenerative properties of skeletal muscle. These cells were discovered in 1961 by Alexander Mauro (Tedesco et al, 2010). Satellite cells (SCs) are activated in vivo through mitogenic signals released during injury resulting in asymmetric cell division where Pax7, MyoD, and Myf5 are expressed in surrounding differentiating cells (Tedesco et al, 2010). MyoD and Myf5 are basic helix-loop-helix (bHLH) transcription factors. In humans Myof5 and MyoD genes exist on different chromosomes but are both transcribed only in skeletal muscle cells. Myf5 RNA transcripts are created before MyoD in muscle tissue (Zweigerdt,

1997). On the other hand, the transcription factor is expressed in quiescent satellite cells and has been found to be necessary to preserve the stem cell population (Yi, 2011). SCs encompass 2.5%-6% of the nuclei in an average adult muscle fiber (Tedesco et al, 2010). If muscle injury occurs, SCs are activated via cellular HGF signaling and begin to proliferate and form myoblasts. Activation of SCs and their differentiation is controlled by regulated expression of myogenic regulatory factors including Myf5 and MyoD (Tedesco et al, 2010) as can be seen in Figure 4. This process results in fusion of regenerating fibers. Since they are expressed only during myoblast differentiation, myosin is used as markers of mature muscle formation (Zweigerdt, 1997).

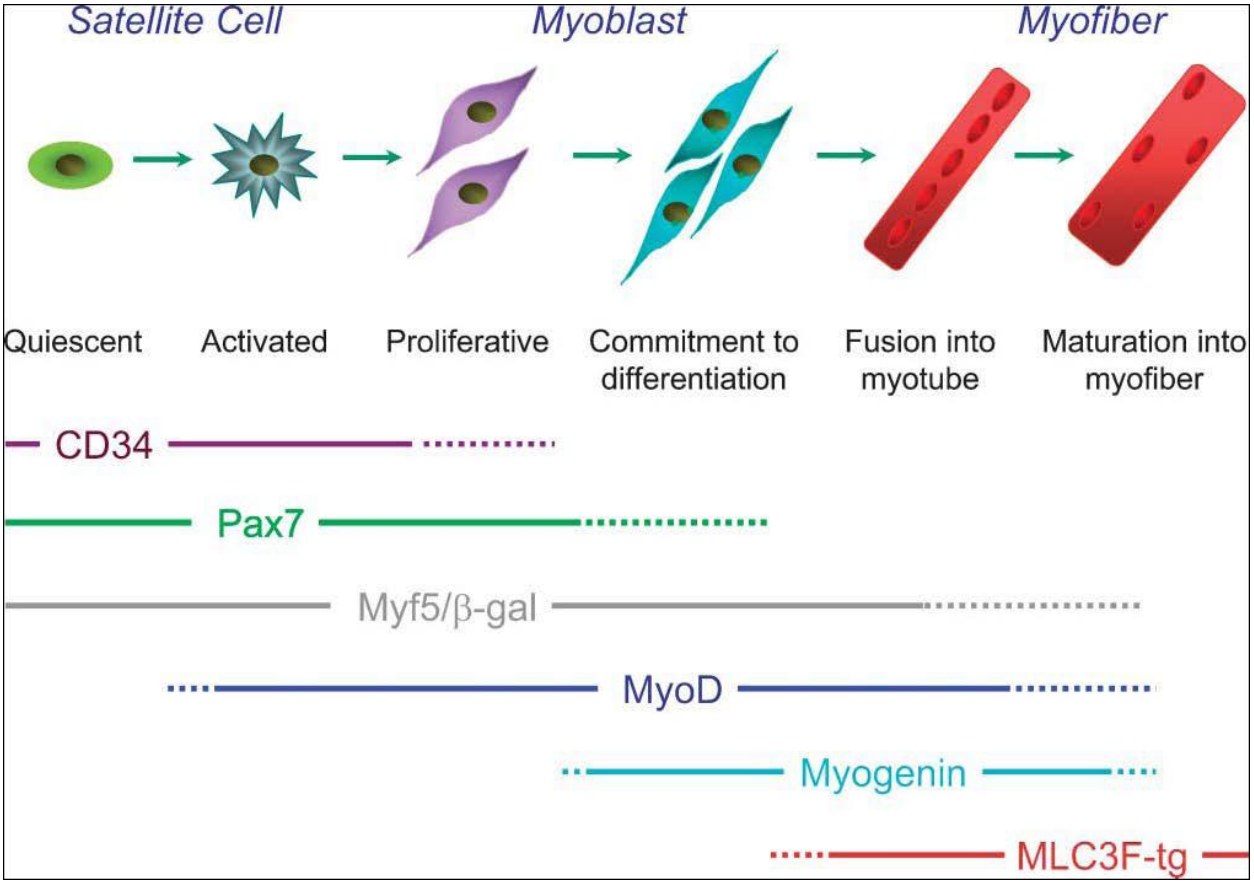


FIGURE 4: SATELITE CELL DIFFERENTIATION

2.3.2 C2C12 Cells

The C2C12 cell line is a myoblast cell line derived from mice that is capable of becoming muscle fibers after differentiation. The C2C12 cells originated in 1977 through the serial passage of myoblasts obtained from the thigh muscle of C3H mice. Yaffe and Saxel were the two scientists who isolated these cells and displayed their capacity to differentiate. C2C12 cells are useful for modeling non-muscle cell to skeletal muscle cell differentiation. For best results, the C2C12 cell line is grown in DMEM with 10% fetal bovine serum (FBS) ("C2C12," 2011). When the C2C12 cells leave the proliferation cell cycle due to low mitogen concentrations they irreversibly enter an apoptosis resistant phenotype state. This also causes the cells to express certain genes and mature into myotubules (Rios, 2000).

2.3.3 Current Skeletal Muscle Tissue Models and Limitations

One of the first models for tissue engineering of skeletal muscle was the culture of avian myoblast monolayers (Liao et al, 2009). After this initial success, other groups moved onto cell-in-gel techniques such as the culture of myoblasts in a Matrigel™ matrix. Other scientists evaluated cell culture without a scaffold (Strohman et al, 1990). This was first accomplished by myoblast aggregation on top of a Saran Wrap membrane. These cells were observed to form myotubes and display contractile activity. Another form of 3D tissue culture developed was laminin-coated Sylguard® plates which produced a cylindrical tissue structure in addition to tissue contraction. The laminin coating provided a means of cell attachment and mobility and the Sylguard® enabled the connection of two sutures to the dish. These sutures helped guide the cells into forming a tubular structure. However, this technique required 35 days of culture and fibroblasts cells were needed in order to produce the proper ECM to support a contractile 3D tissue (Liao et al, 2009). Building off of this method for 3D muscle tissue culture, Huang et al, (year)used a biodegradable fibrin gel seeded with myoid muscle cells to replace the fibroblasts and

laminin of the previous model. The fibrin gel contained anchoring points which seeded myoid muscle cells adhered to and formed a contractile tissue in seven to ten days (Huang et al, 2005). The use of fibrin was particularly advantageous as a matrix for skeletal muscle tissue as a result of its degradability which encourages cells to develop their own ECM over time (Liao et al, 2009).

Another approach for 3D culture of skeletal muscle tissue is to use a polyglocolic acid (PGA) polymer scaffold mesh seeded with neonatal rat myoblasts. This method provided a vascularized muscle tissue six weeks after implantation into rats (Liao et al, 2009; Higgins, 2001). In addition, acellular matrices of intestinal tissue seeded at a cell density of $80\times10^6/cm^2$ with rat myoblasts have been used as a matrix for 3D cell culture (Conconi, 2005). Although this method requires a high volume of cells and is dependent on obtaining bladder and intestine tissue which can be decellularized using detergents, this construct has shown promising vascularization and muscle structure preservation at months one and two when transplanted into rats for the repair of oblique abdominis muscle (Conconi, 2005; Liao et al, 2009). However, neither of these skeletal muscle modeling systems have been used for in vitro studies.

Phosphorous based glass fibers have been used as a scaffold for craniofacial skeletal muscle. In one particular study by Shah et al, human masseter-derived cells were seeded onto glass fibers in various arrangements. The 3D mesh arrangement proved to be optimal for cell attachment and proliferation. Matrigel™ and insulin-like growth factor-1 (IGF-1) were also used to support the cells and increase muscle fiber formation in this model (Liao et al, 2009). Capitalizing on the idea that skeletal muscle forms from the fusion of parallel clusters of myotubes, another study fashioned silicone wafers, micro patterned with 6mm long lines, to produce aligned skeletal muscle tissue. They concluded that the micropatterning guided the cytoskeletal and morphogenic organization of myoblasts as they differentiated into myotubes. Other models have used low shear wet spinning of polycaprolactone (PCL) seeded with fibroblasts and C2C12 myoblasts to obtain a 3D skeletal muscle tissue. The construct was

reported to reach cellular confluence after four days, and the orientation of the fibroblasts and myoblasts was found to be guided by PCL fiber alignment. Also PCL fibers coated with gelatin provided increased cell attachment. However, no mention of full contractile tissue was made (Liao et al, 2009; Williamson, 2006). Similar results were found for electrospun degradable polyesterurethane seeded with C2C12 cells for skeletal muscle tissue scaffolds. Cell attachment and alignment was characterized, and cellular orientation was found to be directed by electrospun fiber alignment (Riboldi, 2005).

Though many diverse methods of skeletal muscle tissue formation in vitro have been explored, recent studies have indicated the need for mechanical stimulation of skeletal muscle tissue in order to promote myofibril alignment, differentiation, and in particular maturation. Though some groups such as Matsumoto et al have recognized the need for mechanical stimulation and integrated it into their myoblast culture scheme, no definitive method of muscle stimulation has been accepted by the scientific community (Matsumoto et al, 2007). Many skeletal muscle engineering methods exist. However, each model has their own limitations such as high cost; extensive culture time; or lack of innervation, vascularization, cell contraction, and/or muscle fiber alignment within the skeletal muscle tissue.

2.4 GELS

The use of gels in tissue engineering is becoming more common as the advantages of a gel scaffold over a traditional scaffold, such as the scaffolds made of metal alloys, polymers, or medical grade ceramics (Jones, 2002), have become more apparent. Because gels are a viscous material, they can fill any defect and are not confined by a rigid shape. Also, gel scaffolds allow for the addition of therapeutic agents such as growth factors to be present during the proliferation of the tissue. The traditional scaffolds also may contain cytotoxic residual solvents leftover from their manufacturer,

which are not present in gel scaffolds (Gutowska, 2001). All of these factors, along with the excellent biocompatibility of gels, led the design team to decide to use a gel for seeding cells.

2.4.1 HYDROGELS

Natural hydrogels are the most common choice for tissue engineering because they are structurally similar to components in the body and have adequate biocompatibility for tissue engineering. There are several different types of natural hydrogels, including collagen, gelatin, fibrin, alginate, agarose, and chitosan; all of which have their unique advantages and disadvantages. Collagen for example has a highly porous lattice that is useful for growth of many types of tissues, and it has also been developed to incorporate differentiation inducing materials to help stimulate differentiation (Glowacki, 2008) but collagen has poor mechanical properties and is ineffective in the management of infected sites (C. Lee, 2001). Gelatin too is too weak to be used for skeletal muscle tissue growth applications despite its tendency to gel easily. Alginate has a low cost, but its degradation rate is uncontrollable and unpredictable. Thus, it cannot be used for studies that require specific degradation (Lee, 2001).

2.4.2 FIBRIN

In the end, fibrin was chosen from a plethora of hydrogel options. Fibrin was chosen because it has nontoxic degradation, which is essential for tissue engineering, since release of toxic byproducts in the body is becoming an increasing problem (Gutowska, 2001). Also, fibrin causes no inflammatory response in vivo. This is a criterion that must be met for any tissue engineering application. Controlled degradation is another advantage of fibrin. Knowing the degradation rate can ensure that the load will be transferred to the new tissue at a manageable rate (Hoque, 2005). Fibrin promotes cell migration and proliferation, both of which will ensure the cells seeded grow into a complete tissue. Also, fibrin typically

has a lower mechanical strength, but since the gel is going to be used as a suspension to support the cells, the effect of its mechanical properties is not significant.

2.4.3 Gel Mold Materials

A gel based mold was deemed necessary for the experiment. Criteria similar to that used for the evaluation of various gel scaffolds were used to determine which gel would form the best mold. There was a choice between agarose and collagen gel mold. Both gels would allow for cell detachment in culture, however agarose is easier to handle in the fact that it does not denature or crosslink in heat, pH, or UV light affecting adhesion index. In the end, agarose was chosen since agarose inhibits cell attachment and can be sterilized using an autoclave. Thus, in using agarose, the myoblasts will stick to the fibrin gel and not adhere to the agarose mold. This is important because if the myoblasts adhere to the agarose mold they will not be able to be removed from the mold and this will inhibit mechanical stimulation. Studies have shown that cells cultured in molds made of 1% agarose gel retain their shape better than higher percentages and do not buckle as a result of radial growth (Gunja, 2009). It will be important for the tissue grown in this experiment to maintain its shape so that it can be both mechanically and electrically stimulated.

2.5 Scaffolds

A scaffold is an internal structure that supports cells as they proliferate and organize them into a mature tissue structure. The best scaffolds are resorbable and degrade at approximately the same rate that the cells produce their own native ECM for support. In some cases scaffold-less tissue structures, which allow for cell self-aggregation, have shown some experimental promise (Dean, 2007).

2.5.1 BIOGLASS

Bioactive glasses are nontoxic, biocompatible, and composed of SiO_2, Na_2O, CaO, and P_2O_5 (Rezwan et al, 2006). These substances release ions of calcium, sodium, silicone, and phosphorous when they degrade. When osteoblasts within the body are surrounded by certain concentrations of these ions, bone mineralization occurs. Thus, bioglasses promote osteoblast proliferation and differentiation when used as scaffolds through increasing the metabolic activity of cells in local tissue (Rezwan et al, 2006). These ions could have positive proliferative effects for skeletal muscle since concentrations of these ions could up regulate the gene expression of growth factors that aid in skeletal muscle proliferation or differentiation. Additionally, bioglasses are an attractive scaffold option as they support enzymatic activities, vascularization, and cellular adhesion (Rezwan et al, 2006). Bioglasses can be easily processed to include different ionic concentrations, diverse shapes, and various porous structures.

Currently bioglass is used almost solely for orthopedic applications (Rezwan et al, 2006). Myocytes do not perform the same functions as osteoblasts within the body. Thus, it is unknown whether the ionic byproducts produced by bioglass during resorption will aid or hinder the development of myoblasts into functional skeletal muscle tissue in vitro. In addition, the rigidity of bioglass may inhibit or alter the extracellular matrix produced by the myocytes in vitro (Rezwan et al, 2006). The greatest issue in using bioglass as a scaffold for skeletal muscle tissue engineering is its slow degradation rate (Yao et al, 2007). Borate bioactive glasses have the swiftest degradation rate out of the bioglass family. Borate bioactive glass can be altered to degrade faster in order to match the bone tissue regeneration rate (Yao et al, 2007). Since a simple fracture takes 6 weeks to heal, the degradation rate of a borate bioactive glass would be equivalent to this time frame (Rezwan et al, 2006). Thus, if implantation of a long term scaffold in the body was the primary objective of the research, this approach would prove useful. However, as the goal of the study is to model the skeletal muscle tissue and its mechanics in vitro

within a week long time frame, the scaffold might not degrade rapidly enough for skeletal muscle tissue to be appropriately tested.

2.5.2 ELECTROSPINNING

In order to increase the degradation rate of bioglass, electrospinning of the bioglass was considered. The fundamental process of electrospinning involves creating a viscous polymer solution. This solution is then passed through a distribution needle and negatively charged at a high voltage of around 15kV (Li, Dan and Xia, Younan, 2004). Below the dispensed solution is a grounded rotating mandrel. The electrostatic attractions between the negatively charged solution and grounded mandrel create spider web-like nanofibers that coat the rotating mandrel to create the desired scaffold or material construction. Conditions such as voltage, mandrel size, rotation speed of the mandrel, infusion rate of the polymer, type of polymer, and spin distance away from the mandrel can all be altered to tailor the electrospinning process to different applications (Li, Dan and Xia, Younan, 2004).

Electrospinning increases the surface area to volume ratio of polymers through its production of fibers 10-100nm in diameter (Li, Dan and Xia, Younan, 2004). These conditions allow for excellent cell adhesion. As a result, electrospinning is an ideal way to process polymers for use as scaffolds in the body. The rise in the surface area to volume ratio of the polymer also increases the degradation rate of the polymer when it is exposed to physiological fluid (Li, Dan and Xia, Younan, 2004). However, as bioglass degrades between two to six months, electrospun bioglass would take at least weeks to degrade (Yao et al, 2007; Rezwan et al, 2006). Since skeletal muscle tissue used for in vitro modeling takes only seven to ten days to form, the electrospun bioglass scaffold, still intact after seven to ten days, would inhibit the group's ability to test skeletal muscle tissue in vitro (Liao et al, 2009). Electrospunbioglass could be useful as an implantable scaffold for skeletal muscle tissue regeneration in the body as the scaffold would degrade at approximately the same rate as new tissue is remodeled.

However, it would not be an optimal material for the culture of a functional skeletal muscle tissue model in vitro.

2.5.3 Polyglocolic Acid (PGA) Scaffolds

PGA is a synthetic, biocompatible, biodegradable polymer used widely in tissue engineering. Like bioglass and other polymers including polyethylene (PE), polycaprolactone (PCL), and polyurethane (PU), it can be electrospun into a biomaterial scaffold for tissue engineering (Subbia et al, 2005). However, PGA holds a few advantages as a biomaterial scaffold over these other polymers. Unlike PE and PU, PGA is biodegradable. Moreover, unlike PCL, which as a melting point of 60˚C, PGA has a melting pint of 230˚C, maintains its structural integrity, and does not become soft and malleable under biological conditions (Debenedetti, 2001; Labet, 2009). As a polymer it is easy to process into numerous shapes as well as different porosities and can be formulated to achieve various degradation rates. This is accomplished by altering the chemical composition, crystallinity, and molecular weight of the polymer (Armentano et al, 2010). The porosity of the polymer allows for cellular infiltration and attachment while the degradation of the polymer allows for the cells to support themselves with their own extracellular matrix over time.

Though the degradation of PGA is one of its advantages, the acidic byproducts produced during degradation are a disadvantage. These byproducts decrease the pH of the local tissue area, resulting in alterations in the native cell differentiation as well as ECM production and orientation. Specifically, in a study for vascular tissue engineering, PGA degradation products were linked to increased dedifferentiation and decreased mitosis of porcine smooth muscle cells (SMCs). The negative influence of PGA on SMC differentiation was done by Western blotting experiments for calponin, a differentiation marker for SMCs (Higgins et al, 2003). Despite the limitations, there have been some studies that recorded success in using PGA as a scaffold for skeletal muscle tissue engineering. In one study,

myotubes were observed aligning with the PGA fibers in the biodegradable scaffold (Langelaan et al, 2010). In another study, myoblasts were seeded onto a PGA mesh and implanted in vivo. Vascularized muscle tissue formed after 6 weeks (Saxena et al, 2001). However, the risk of acidic byproducts has deterred researchers from using biodegradable, aliphatic polymers such as PGA as scaffold materials for in vitro tissue culture.

2.6 Mechanical Stimulation in Developing Tissues

The connective tissues in the body – blood vessels, tendons, ligaments, muscle, etc. have viscoelastic mechanical properties and structural organization on the cellular level that is specific to that tissue. The mechanical properties of a tissue depend on a variety of things including individual cell features, ECM properties, the organization and interaction of different cell types, and its surrounding three-dimensional environment. When producing an engineered tissue, it is important to try to mimic the muscles' natural mechanical properties during development. By doing this, one can promote ECM development as well as the organization and assembly of the cells within the tissue (Vandenburgh et al, 1991). There has been a variety of research performed in the area of dynamic and static mechanical stimulation of developing tissues. Many researchers believe that mechanical stimulation of developing tissue can induce the alignment of cytoskeletal proteins and ECM components parallel to the axis of applied strain (Powell et al, 2002). This is due to cells adhering to a matrix of extracellular proteins and transmitting the force to the cytoskeleton. This sequence of events shows parallels to growth factor signaling pathways and changes in cell behavior including proliferation and differentiation (Moon et al, 2008).

Mechanical stimulation is especially applicable to the engineering of muscle tissue in vitro. It has been hypothesized that to develop a mature functional muscle tissue, one must apply mechanical

stimulation to promote the alignment of myoblasts and differentiation of these cells into myofibers. Mechanical stimulation will not only help the differentiation of the tissue but will result in an overall stronger engineered tissue by producing more actin and myosin fibers as seen in Figure 5. (Moon et. al, 2008)

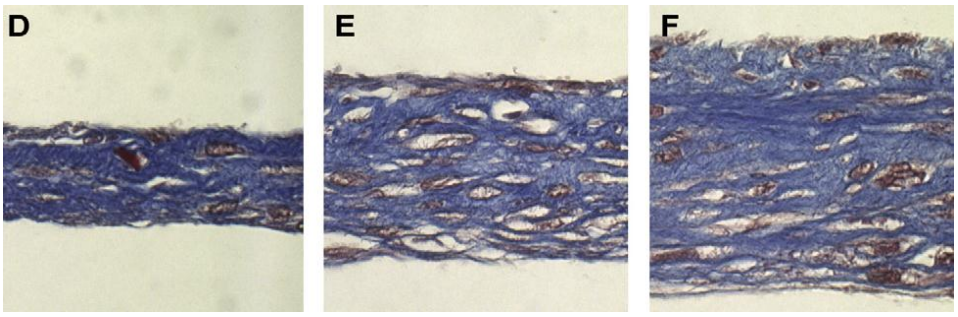
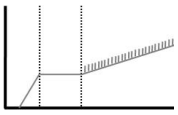


FIGURE 5: INCREASED THICKNESS DUE TO MECHANICAL STIMULATION IN DEVELOPING TISSUE (MOON, 2008)

Although there is not a full understanding of the effect of mechanical stimulation on tissue growth, comprehending the interactions between cells, ECM and mechanical stimulation and how they influence tissue growth and organization is of the utmost importance for the design of load bearing engineered tissues. After a review of a number of different scientific papers pertaining to mechanical stimulation and muscle tissue growth, it is apparent that the effectiveness of the mechanical stimulation depends on a variety of factors including the stimulation regiment and amount of strain applied. Table 1 summarizes a group of studies that focused on using mechanical stimulation to mature developing tissues. In all but one study, mechanical stimulation of the cells aided in the orientation of myotuble during muscle development. This increased the muscle tissue's mechanical properties. Unfortunately, there is not yet a definite correlation between a specific mechanical stimulation regime and optimal tissue development. Static mechanical stimulation promoted alignment and fusion of myotubes (Kurokawa et al, 2007), while dynamic stimulation resulted in proliferation and differentiation of myblasts (Grossi, 2007). From these studies, it is also apparent that the success of mechanical stimulation on developing tissue is dependent on the amount of strain applied to the tissue. The applied

strain should be tuned to the desired effects and properties of the developing muscle tissue. In past studies, the amount of strain used to stimulate the developing tissue ranged from 5% to 25%. The best results were seen at 10% strain.

TABLE 1: SUMMARY OF MECHANICAL STIMULATION STUDIES USING DIFFERENT CELL TYPES AND STIMULATION REGIMES

Reference	Cell Type	Strain	Duration	Main results
Vandenburgh et al. 1991	Embryonic avian myoblasts (3D)	25% 	24 days	Oriented myotubes, stimulated muscle development
Tatsumi et al. 2001	Rat satellite cells (2D)	25%	12-sec intervals for 1.5 days	Increased satellite cell activation
Powell et al. 2002	Human myoblasts in collage/Matrigel (3D)	5-15%	15x stretch relaxation, followed by 28 min rest (8 days)	Increased elasticity, myofiber diameter, and myofiber area
Kumar et al. 2004	C2C12 (2D)	17%	0.5 sec strain, 0.5 sec relaxation for 1h per day, 5 days	Inhibited MHC expression and formation of myotubes
Kurokawa et al. 2007	C2C12 (2D)	10%	1, 3, and 5 days	Promoted myoblast differentiation
Grossi et al. 2007	C2C12 (2D)	10%	2 sec strain, 2 sec relaxation for 1 h per day	Induced proliferation and inhibited differentiation
Moon et al. 2008	Human MPC's in acellular tissue scaffolds (3D)	10%	5 min/h for 5 days-3 weeks	Higher titanic and twitch responses post implantation

Overall, the results of mechanical stimulation studies seem to contradict one another. These contradictions could be a result of differences in the time and environment in which the first stimulation occurred. In general, cells cultured in a 2D environment react less to mechanical stimulation than those cultured in a 3D environment due to the way the cells attach to one another. This will most likely have an effect on how force is transduced through these cells, and therefore change the results of a mechanical stimulation protocol.

2.6.1 Anchoring Systems

To apply mechanical stimulation to a developing tissue one must design an apparatus that can anchor the tissue and provide and environment conducive to cell growth. The challenges of developing an anchoring system include adhering cells to a surface that can be mechanically manipulated with a consistent and static or dynamic stress. Although there are a number of apparatuses and tissue culture plates that have been designed to apply strain to a developing tissue (Grossi, 2007), a vast majority of them are directed to 2D tissue culture. The team has decided to grow and develop the muscle tissue in a 3D environment using a gel mold system and thusly needs a 3D mechanical stimulation anchoring system. A number of these 3D anchoring systems have been developed previously, but almost all have been used in conjunction with a bioreactor. The project will aim to grow muscle tissue in vitro without the use of a bioreactor and therefore will need an anchoring system designed specifically for the team's needs.

There are two main ways tissue is anchored in a 3D cell culture environment. One method is to promote cell adhesion to a surface that one can then pull on and create a strain within the tissue. Figure 6 shows this anchoring system mounted in a bioreactor. The cells have adhered to a tissue paper material on both ends. A metal wire is then attached to the paper so that it can be mechanically stretched to stimulate the tissue. The second method, seen in Figure 7, consists of getting the tissue to

form around two posts by making a “dog-bone” mold. This method is fairly simple and works as long as the tissue develops uniformly throughout the mold and around the two posts.

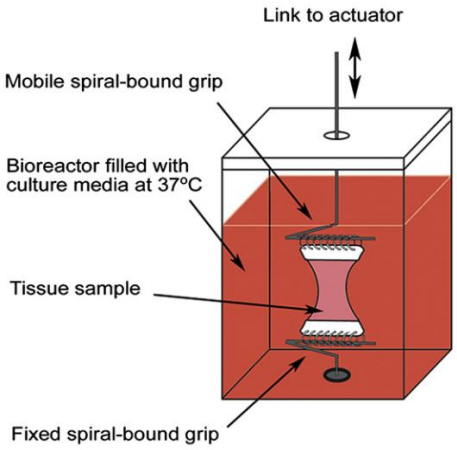


FIGURE 6: CUSTOM BUILT CELL ADHESION ANCHORING SYSTEM DEVELOPED FOR BIOREACTOR

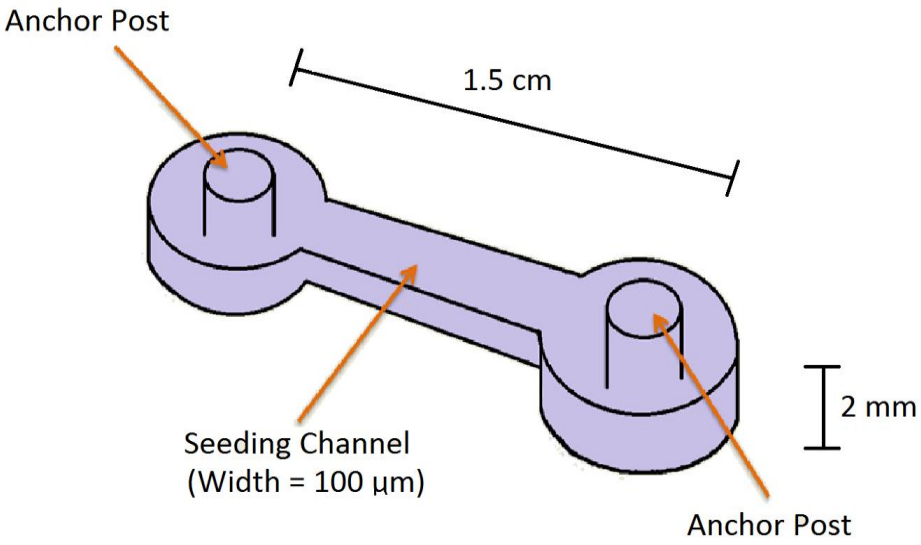


FIGURE 7: "DOG-BONE" MOLD ANCHORING SYSTEM

In both methods, the anchored tissue must then have strain applied to it by a mechanically controlled means. Typically, this is done by linking the anchoring system to an actuator or step motor controlled by a computer program or physical engineering controls. From here, a specific mechanical stimulation regime can be designed for the desired tissue development.

2.7 Electrical Stimulation in Developing Tissues

Recently electrical stimulation of developing muscle tissue has been an avenue of research that tissue engineers have been interested in, since neuronal activity has proven paramount during myogenesis *in vivo*. It has been proven that the stimulation of skeletal muscle is necessary for the proper maturation and prolonged life of muscle tissue (Thelen et al. 1997). Without proper electrical stimulation, muscle will atrophy and die. When growing tissue *in vitro,* the contraction of a muscle tissue due to neuronal activity can be mimicked my applying an electrical stimulus. It has been shown that causing muscle fibers to contract promoted the differentiation of myotubes and the development of the sacromeres (Fujita et al. 2007). In one study, early electrical stimulation accelerated the maturation of the tissue causing cross striations whereas cultures without electrical stimulation were slower to develop (Langelann et al, 2010).

Much like the mechanical stimulation of developing tissues, the regime of electrical stimulation is important for the proper maturation of muscle tissue. Factors such as duration, voltage, amperage, and timing all play an important, but not completely understood, role in muscle differentiation. An electrical stimulation protocol has already been developed, but there has not been a study to identify the ideal electrical stimulation regime for the maturation of muscle tissue. Table 2 identifies a number of studies using electrical stimulation for the maturation of muscle tissue and their respective results. All the factors that combine to form an electrical stimulation regime must be delicately balanced to ensure optimal maturation of muscle tissue.

TABLE 2: SUMMARY OF ELECTRICAL STIMULATION STUDIES

Reference	Cell Type	Electrical Field	Pulse Frequency (Hz)	Pulse Duration (ms)	Duration	Main Result
Wehrle et al. 1994	Rat Satellite cells (2D)	4-8 mA	40	250	13 days	Increased expression slow MHC
Thelen et al. 1997	C2C12 (2D)	3 V/cm^3	2	6	2 days	Reduced fast type Ca2+-ATPase expression
Radisic et al. 2004	Rat cardiomyocytes	5 V/cm	1	2	5 days	Timing of electrical stim. with differentiation process is delicate
Pedrotty et al. 2005	Rabbit myoblasts in PGA (3D)	0.6 V/cm	0.5-10	0.5-250	1-14 days	Increased proliferation, no effect on differentiation
Fujita et al. 2007	C2C12 (2D)	6.7 V/cm	0.1-10	24	1-9h (8 days after differentiation)	Accelerated *de novo* sacromere assembly
Yamasaki et al. 2009	C2C12 on/in collagen (2D-3D)	8.3 V/cm	0.5-10	10	80 sec (6 and 12 days after differentiation)	Contractile performance similar in 2D and 3D

Not only can electrical stimulation aid in the progression of skeletal muscle development in vitro, it also provides an easy and noninvasive way to assess the functionality of the tissue. If the tissue is mature, it should be able to propagate a charge down the length of the fiber in the form of a contraction. By causing the muscle fiber to contract, one can measure the force generated by the engineered tissue and compare it to that of native skeletal muscle. To date, the best engineered muscle tissue can only generate about 2%-8% of the force of native skeletal muscle (Yamasaki et al. 2009). This is not satisfactory and more research must be done in this field.

Although the stimulus must be carefully controlled so not to damage the tissue, the stimulus can be caused by attaching a wire lead to opposite ends of the tissue as seen in Figure 8. The team had a BioPac computer controlled system available in the laboratory to apply a controlled electrical stimulus to the tissue. Through this computer controlled system, all the factors of the electrical stimulus can be

monitored and controlled while the response to the stimulus is recorded. This provides a simple way to assess the functionality of the tissue.

FIGURE 8: ELECTRICAL STIMULATION OF MUSCLE IN "DOG-BONE" MOLD

2.8 Skeletal Muscle Tissue Requirements and Techniques

Functional, tissue-engineered skeletal muscle for regeneration must have parallel myofibril alignment with actin and myosin filaments. Also, acetylcholine receptors, which create direct forces within the tissue, must be expressed and intracellular calcium storage must be available to allow for contraction of the cells (Liao et al, 2009). The tissue itself, if prepared for implantation, must be biocompatible and needs to integrate with native cells in order to begin regenerating lost muscle tissue. Additionally, similar to any cell based tissue, skeletal muscle tissue used for regeneration must become vascularized and innervated (Liao et al, 2009).

In terms of modeling, the cell culturing technique must be cost effective, quick, simple, and replicable. The tissue must be formed from established cell lines such as C2C12 to limit the variability that occurs through the isolation of primary cells (Liao et al, 2009). Doing so allows for the ability to test alterations in the gene function of the tissue. The model also needs to be mechanically and electrically stimulated.

Two primary techniques have been used for tissue regeneration. One involves culturing the tissue *in vitro* through incubation or use of a bioreactor. This new tissue is then implanted into the body after differentiation has occurred (Liao et al, 2009). The other technique used in an *in vivo* approach where satellite cells are obtained, expanded through *in vitro* culture, and then seeded onto some sort of natural, synthetic, or biological matrix which allows for the cells to be transported and implanted into the body. Once in the body, the cells will ideally differentiate into myotubes under the support of the scaffold material (Liao et al, 2009). Obviously, in terms of modeling, an in vitro tissue culture would be an ideal tissue engineering approach as differentiated tissue would be necessary.

2.9 Tests for Myocyte Culture

It is necessary to characterize cells in term of their viability, differentiation, and cytoskeletal arrangement in order to accurately access the functionality of the tissue. The following tests can be used to evaluate the above parameters.

2.9.1 Live Dead Assay

This fluorescence assay is used to determine cell viability after culturing. There are two components to this assay; determining live cell count and dead cell count. The live cell count is determined by counting fluorescent cells. Live cells fluoresce because the intracellular esterase converts calcein acetoxymethoyl into calcein which is highly fluorescent. The dead cells fluoresce a different color using ethidium homodimer because this molecule enters the ruptured membrane and becomes fluorescent when it binds to nucleic acids. The ethidium homodimer only makes dead cells fluoresce because it is not membrane permeable. The results of this assay are typically displayed as percent live cells and percent survival. These data points will be useful in indicating if the culture techniques are successful in providing enough viable cells.

2.9.2 Actin Fluorescence Staining

Actin stains are used to determine the structure of the cellular cytoskeleton of the tissue post culture. In skeletal muscle, it is ideal for actin filaments to display alignment. Most actin stains identify F-actin via electron microscopy using fluorescent phalloidins. Phalloidins contain phallotoxins. The phallotoxins are derived from Amantaphalloides mushroom. Phallotoxins are water soluble and stain both large and small actin filaments equally. Additionally, the contrast between stained and unstained regions is significant. Furthermore, actin binding proteins, such as myosin, can still bind to actin after phallotoxin treatment as the particles are extremely small, 12-15 angstroms in diameter. As phalloidins are fluorescent, it is advised to keep fluorescently labeled specimens during tissue analysis protected from light ("The Molecular Probes," 2010).

2.9.3 Myosin Heavy Chain Marker

The use of myosin markers allows researchers to determine undifferentiated myoblasts from myoblasts that have differentiated into myotubes. Only skeletal muscle myotubes will fluoresce when stained for myosin heavy chain. As a result, indirect immunofluorescence with anti-myosin heavy chain (MF-20) antibody can be used to determine which myocytes have differentiated into myotubes. After use of an anti-myosin heavy chain antibody, a secondary antibody such as Alexafluor-488 can be used to allow the cells that stain positive for myosin heavy chain to fluoresce ("Myosin Heavy Chain Immunocytochemistry Protocol," 2011).

2.10 Computer Aided Manufacturing (CAM)

In many cases engineers need a way to manufacture or fabricate their designs with high precision and accuracy. CAM allows the user to reproduce a solid model that has been generated on a

computer program. A computer controlled machine then produces the solid model by cutting away stock material or (in some cases) printing the material layer by layer. Both of these techniques allow for a design to be manufactured quickly and efficiently with high accuracy. During our MQP project the team used two varieties of CAM; a HAAS MiniMill and a Rapid Prototyping (RP) 3D printer. While both machines utilize CAM technology, they approach the problem in two very different ways to provide a finished product in a timely manner.

2.10.1 HAAS MINIMILL

The HASS MiniMill is a computer controlled mill that carries out cutting operations on stock material to obtain a desired part. Using rotating cutting tools, the MiniMill removes material from a clamped stock material. The tool path is controlled by a program file that is created from computer software such as ESPRIT. ESPRIT is an easy to use program that allows the user to create cutting operations from a previously made solid part file. Once programmed correctly, the HAAS MiniMill runs through a series of commands to change to the appropriate tools and make the necessary cuts to manufacture a part. The user can control a number a parameters during manufacturing including cutting speeds and feed rates to customize the way their part is manufactured. The duration of the manufacturing process depends on the complexity of the part. On the WPI campus there are currently five HAAS MiniMills available for student use.

2.10.2 RAPID PROTOTYPING 3D PRINTER

A rapid prototyping (RP) 3D printer is a machine that creates a three dimensional construct. The finished product is generally made out of a plastic material. The 3D printer consists of a computer controlled robotic arm with an extruder attached to it. While the user interface is much simpler than that of the HAAS MiniMill, the RP 3D printer can create complex parts in a relatively short amount of

time. A solid part file can be loaded and programmed into the machine allowing for a smooth transition from a SolidWorks file to a working prototype. The RP printer creates the part by printing each layer of the component from bottom to top. While this machine is a great piece of technology it does have some limitations. The 3D printer has a limited resolution and can only print features that are larger than one square millimeter. Additionally, the 3D printer can only create parts that are within the machines 13”X13”x6” size constraint. These limitations did not affect our project drastically seeing that our parts are very small and do not require too small of a resolution. Fortunately, WPI has the convenience of having a RP 3D printer on campus and can produce parts at a very low cost to students.

CHAPTER 3. PROJECT STRATEGY

The goal of this project was to design and model a scaffoldless tissue mold system and mechanical stimulation platform in order to culture myogenic cells into aligned myotube tissue constructs. A three dimensional mold system composed of agarose gel was used to form the tissue during culture. The addition of a mechanical stimulation platform allowed for the gel to be removed before axial cyclic mechanical stimulation occurs without moving the tissue. These two systems were necessary to fulfill the goals and objectives of the project. This chapter outlines steps taken to prioritize which objectives, functions and constraints were essential for the project's success. Lastly a project approach section outlines the step-wise methods taken to create the three dimensional tissue construct.

3.1 DESIGN

As an engineer, it is extremely important to understand every aspect of the problem in the form of a client statement. The design process provides the ability to break down a complex problem into simpler parts to gain an improved perspective on the goals and objectives of the project. It also allows for the objectives, functions, means, and constraints of the device to be determined through a logical progression of team discussions and well-informed decisions. The project approach and future designs are then built from these fundamental principles to reach a desired goal. The section below describes the team's design process and the path that was taken to revise the initial client statement and to determine and prioritize the objectives, functions, means and constraints. The design process helped the team design an apparatus for growing and mechanically stimulating muscle tissue *in vitro.*

3.2 Initial Client Statement

The first task the group completed was to revise the initial client statement. Often times, a client is not sure exactly what they want and therefore asks for a solution to a very broad, unfocused problem. As a design team, it was the group's responsibility to ask questions that clarified the client's needs and wants so that the problem could be fully understood. From there, the client statement was revised into just a few sentences that capture the problem and its desired solution. The initial client statement was provided by Dr. Raymond Page and read:

> **"Currently, the laboratory uses extruded fibrin microthreads with human skeletal muscle derived cells seeded onto the surface and transplanted into SCID mouse skeletal muscle injury models to study the effect of various cell derivation and culture methods on functional tissue regeneration. The use of animals is time consuming and costly which severely limits the number of parameters that can be evaluated. Currently, the microthreads are produced first and then cells with myogenic potential are seeded onto the microthreads using a rotational cell seeding system. The limitations of this system include the ability to only achieve a cell density limited to the surface area of the microthreads and the system is not compatible with long term culture to evaluate the differentiation potential of the cells in vitro. For cylindrical tissue such as skeletal muscle fibers to form, the cells must degrade the microthread material and proliferate and migrate into the core. The proliferation phase of the cell cycle is not compatible with the quiescent phase required for cell fusion and matrix synthesis needed for skeletal muscle tissue formation. This could to lead to premature breakdown of the tissue structure before the seeded cells can synthesize new matrix. An optimal situation would involve a system where cells could be seeded at the density required for cell fusion and tissue formation. However, the current microthread production process involves a stretching and drying step to produce axially aligned fibers, which is not compatible with seeding the cells within the microthreads at the time of formation.**
>
> **A tissue engineered skeletal muscle system would enable the study of skeletal muscle tissue formation, maturation and the potentiality of cells entirely in vitro that could be used to approximate the utility of their use for the replacement of lost or damaged skeletal muscle tissue. The goal of this project is to design and produce a system that recapitulates skeletal muscle fiber structure into which myogenic cells can be seeded such that skeletal muscle tissue is formed. The system must be either produced aseptically or must be sterilizable and fit into an incubator in order to permit study of live cultures over time. The engineered system should**

further be amenable to the study of effect of mechanical strain and /or electrical stimulation on muscle fiber maturation and contractile function."

After reading the initial client statement, the team developed a number of questions to ask at subsequent design meetings with the client. These questions helped clarify the problem so that the team could continue with the design process.

The next step was to decide who the stake holders would be for the final product. Determining the stakeholders of a project is a small but important precursor to the design process. Knowing who the designer, client, and users of the product will be helps the team fully understand the scope of the project and may identify objectives that were not previously discussed or thought of. Dr. Raymond Page is the main client for the product; other potential clients of this device would be muscle tissue engineering researchers, those researchers working on muscular dystrophy, and drug companies for testing and screening drugs on muscle tissue *in vitro*. The users of this device are future MQP teams and graduate students at WPI who will be involved in tissue engineering muscle. The design team consisted of Michael Aschettino, Steven Delfosse, Katherine Larson, and Caitlin Quinn, who all worked to refine the problem to satisfy the needs of both the client and users.

3.3 Objectives, Functions,Constraints

After the initial client statement was completely understood, the project goals were defined, and the stakeholders were identified, the next step was to begin to break down the goals into objectives. Objectives are the tasks that the product should be able to perform to make a quality product; most of the project objectives should be met with the final design. The team also had to identify the project constraints which are conditions the product must meet to satisfy the client. Finally, functions were determined, which are the means in which the objectives and constraints are met. Below are the objectives, functions, and constraints determined for this project:

Objectives:

- Use a scaffold-less construction for gel mold.
- Create 3-D tissue construction of muscle fiber.
- Provide mechanical stimulation for aligned myotubs.
- Use electrical stimulation to test contraction of the tissue.
- Allows for cells in culture to create their native ECM.
- Produce a procedure to allow for reproducible results.
- The total cost of product and procedure should be minimal.
- The material and equipment needs to be nontoxic and sterilizable.
- Ensure that the elapsed time of the experiment is within industry standards.
- Use biocompatible materials for the device.

Functions:

- Allows for cellular differentiation in the gel mold.
- Mechanically stimulates cells to improve mechanical strength and myofiber alignment.
- Electrically stimulates cells to test contractile functionality.
- Provide myocyte alignment similar to native in vivo tissue.
- Allow for easy removal of tissue from gel mold for testing.
- Control cell density seeding to ensure optimum tissue formation.
- The design will be able to produce myofibers.
- Provide rigid structure for the tissueto anchor to during culture.
- Control myofiber dimensions through the design of the gel mold.

Constraints:

- The design and materials must be aseptic and sterile for cell culture.
- The budget consists of $624.
- There is a limited time period of 25 weeks.
- The general size of tissue culture is on the millimeter scale and manufacturability may not have a small enough resolution.
- The project is limited to C2C12 cells until concept can be proven.
- Sterilizable or made from presterilized parts and assembled aseptically

TAILORED OBJECTIVES

After creating general lists of the objectives, functions, and constraints as well as a pair wise comparison chart, the design team combined all of the detailed information into a more comprehensive list of tailored objectives. The tailored objectives were completed for the top six objectives as

established by the pair wise comparison chart and meeting with the client. The tailored objectives include details on how each of the top objectives will be achieved.

1. Scaffold-less construction
    - Agarose mold
2. Create 3-D construct
    - Model 3-D mold in CAD
    - Design of mold
    - Mold Hull
    - Cell seeding density
3. Mechanical stimulation
    - Step motor: tensile and axial strain
    - Anchor posts
4. Electrical stimulation
    - Anchor posts
    - Appropriate voltages, intervals,
5. Biocompatibility
    - Non-toxic materials
    - Use of tissue culture hydrogels
    - Sterility of equipment
6. Model skeletal muscle *in vitro*
    - Aligned myotubes
    - Contraction on open electrical stimulation
    - Actin and myosin formation orientation
    - Cell proliferation and differentiation
    - Allow cell to create native ECM

By establishing a defined set of tailored objectives, the project group had a detailed outline for the steps required to create a design to satisfy the client.

3.3.1 Quantitative Analysis

After establishing objectives, it was helpful to create visual models for the design team to consult. There are many different types of models, ranging from comparison charts used to determine which objectives hold the most weight, to objectives trees that determine a hierarchy of design goals. The following are examples of some of these visual models.

PAIRWISE COMPARISON CHART

TABLE 3: PAIRWISE COMPARISON CHART

Goals	Scaffoldless	3D	Mechanical	Electrical	Cell ECM	Anchors	Reproducible	Cost	Safe	Time	Biocompatable	Score
Scaffoldless Contruction		0	0	1	0	0	0	1	0	1	0	**3**
Create 3-d Construction	1		0.5	1	1	1	1	1	1	1	0.5	**9**
Mechanical Stimulation	1	0.5		1	1	1	1	1	1	1	0.5	**9**
Electrical Stimulation	0	0	0		0	0	0	1	0	1	0	**2**
Cells Create Native ECM	1	0	0	1		0.5	1	1	0	1	0	**5.5**
Anchor Fibers	1	0	0	1	0.5		1	1	0	1	0	**5.5**
Reproducible Results	1	0	0	1	0	0		0	0	1	0	**3**
Cost Effective	0	0	0	0	0	0	1		0	1	0	**2**
Safe	1	0	0	1	1	1	1	1		1	0	**7**
Time Efficient	0	0	0	0	0	0	0	0	0		0	**0**
Biocompatable	1	0.5	0.5	1	1	1	1	1	1	1		**9**

Based on the pairwise comparison chart shown above, the design team determined that creating a 3-D construct, mechanical stimulation of the muscle fibers, and the biocompatibility of the construction materials with skeletal muscle are the most important design objectives to meet. Cells creating a native ECM and ensuring the fibers are anchored were also all determined to be important objectives. The rest of the objectives were deemed less important and will be integrated into the design based on convenience.

OBJECTIVES TREE

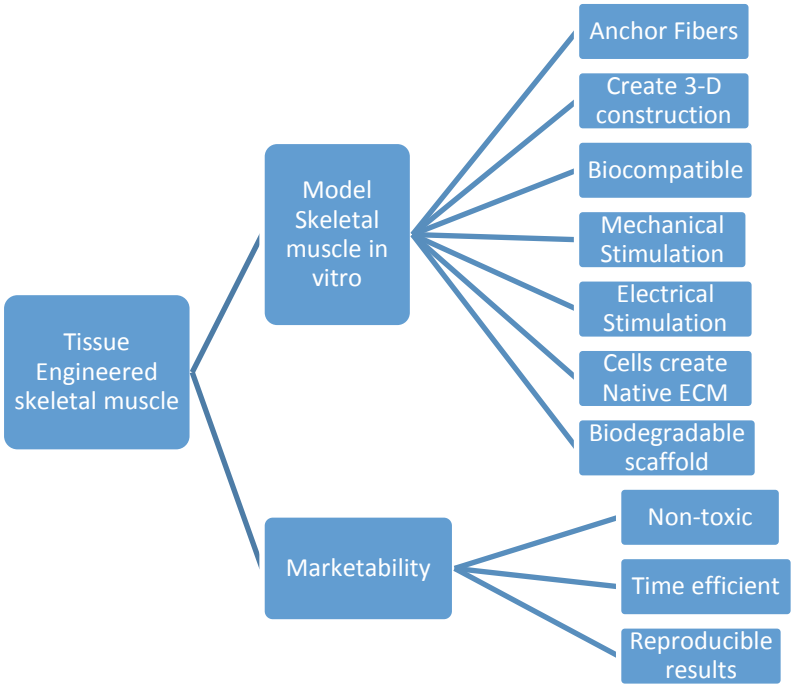


FIGURE 9: OBJECTIVES TREE

The Objective tree is a visual model of the main design objectives and their hierarchical structure. As can be seen in Figure 9, the marketability is independent of modeling the muscle *in vitro*, but both design aspects support many sub-objectives that are necessary for the project's success.

FUNCTION MEANS MATRIX

A function means matrix provides a visual representation of the means that are necessary for each function to be accomplished. The function means matrix for this project can be seen below in table four.

TABLE 4: COMPREHENSIVE LIST OF FUCNTIONS AND THEIR MEANS

Functions	Means
Allows for cellular differentiation and myofiber formation in the gel mold.	Small channels for self-aggregation and presence of differentiation media during culture
Mechanically stimulates cells to improve mechanical strength and myofiber alignment.	Mechanical stimulation apparatus via magnetic actuation
Electrically stimulates cells to test contractile functionality.	Use of electrically charged wire to contractile function
Provide myocytealignment similar to native in vivo tissue.	Uniaxial mechanical stimulation of tissue via mechanical stimulation apparatus
Allow for easy removal of tissue from gel mold for testing.	Rapid prototyped tissue rings
Control cell density seeding to ensure optimum tissue formation.	BrdU staining to obtain information on best myocyte formation
Control myofiber dimensions through the design of the gel mold.	Gel mold creation and design
Provide rigid structure for the tissue to anchor to during culture.	Posts on mechanical stimulation apparatus and tissue rings

3.4 Revised Client Statement

After meeting with the client and considering the objectives, functions, and constraints a revised client statement was created. This statement accurately describes the final design as well as the expected outcome of the tissue culture process.

> *"The goal of this project is to design a novel, biocompatible gel mold capable of being sterilized onto which myogenic cells can be seeded to form skeletal muscle fibers. This scaffold structure must have the ability to be incorporated into a device that provides mechanical stimulation to assess the role of this parameter on tissue engineered skeletal muscle maturation".*

3.5 Project Approach

The project team has devised a simplified five step plan to take the procedure from cell seeding to final tissue construction. These steps represent the major requirements for skeletal muscle tissue culture. They include culturing cells, preparing agarose gel mold, pipetting fibrin gel solution into the mold, allowing for the tissue to form, and lastly proof of concept testing.

1. Cell Culture

This step includes taking the frozen cells and seeding them into well plates at 1.5 million cells per well at the cell density used. The cells should be cultured for a period of time until they read 50-60% confluence. This step is required to ensure the cells are healthy and the proper cell density is available for seeding. After the cells have been cultured but before seeding, the cells must be placed in differentiation media to induce differentiation (see Appendix G). The cells are left in this media for two days before they can be seeded onto the gel mold.

2. Preparing Agarose Gel mold

The agarose gel mold is the structural component of the design which is intended to shape the myogenic cells into the desired tissue form. The agarose at 2% and DMEM culture media was prepared by pipetting the liquid agarose into the negative gel mold previously prepared and sterilized. The agarose gel is allowed to cure under sterilized conditions. Then, once cured, the gel is removed from the negative mold and placed in a sterile cell culture dish.

3. Pipetting Cell Solution

Once the gel mold is created and differentiation has been initiated, the cells need to be seeded onto the gel mold at a specific density and volume. The gel remains in the petri dish and the dish is filled with differentiation medium up to the bottom surface of the agarose mold.

4. Allowing For The Tissue to Form

The mold will be left in the incubator for two days or until sufficient tissue formation has taken place. Then, the petri dish with its contents will be placed in the hood with the mechanical stimulation apparatus and the mechanical stimulation regime will be completed on the tissue for the next two days. Finally, the tissue construct will be removed from the agarose gel and prepared for proof of concept testing.

5. Proof of Concept Testing

There were several tests that needed to be run to ensure the tissue that was formed is alive and contains the correct proteins and cytoskeleton matrix. Firstly, a Live or Dead assay was completed to determine percent live cells and percent survival. An actin stain was used to determine the cytoskeletal structure using fluorescent microscopy with and fluorescent labeled phalloidins of F-actin proteins. Lastly, Myosin heavy chain markers were inspected by fluorescence. The presence of these indicated that there was skeletal myotubules and provided indication of how the fibers were aligned in the tissue.

CHAPTER 4: ALTERNATIVE DESIGNS

Design alternatives are an essential part of the design process as they provide valuable design considerations that may be overlooked if alternatives were not considered. Therefore, the project group outlined multiple variations of different aspects of the design, including the mold, the mechanical and electrical stimulation apparatus, and the tissue rings. Design alternatives are an integral part of design failure prevention.

4.1 MOLD ALTERNATIVES

When trying to grow any tissue *in vitro* it is important to decide what environment the tissue will be cultured in. For simplicity, often times tissue culture is done in a 2D environment by growing the tissue on a culture plate or dish. Many researchers argue that to produce a tissue *in vitro* that is similar to the native tissue in vivo, the environment that the tissue is developed in must be mimicked. Due to this, many studies are using 3D scaffolds and gel molds to mimic the three-dimensional environment that all tissues are developed in within the body. The team has decided to culture muscle tissue in a 3D environment and will be improving upon a gel mold design that a previous MQP team had devised.

The dog-bone gel mold design, seen in Figure 11, is designed to allow muscle tissue to be cultured in a 3D environment while giving the tissue two anchor points at either end. The overall tissue length (post to post) will be one centimeter while the width of the seeding channel will be sanded to a fine point to permit the control of the tissue diameter by changing the volume of cells that are seeded. This should promote the production of myofiber and myotube formation by mimicking the native size and environment of developing muscle tissue in vivo. One significant change made to the gel mold design is the shape of the seeding channel. Previously the seeding channel had a flat bottom which

allowed cells to disperse across the bottom. This is not advantageous for the development of a uniform round fiber. The team decided to create a V shaped seeding channel to allow the aggregation of muscle cells in the bottom of the seeding channel promoting a more uniform tissue formation. An example of the previous seeding channel and the new seeding channel can be seen below in Figure 10.

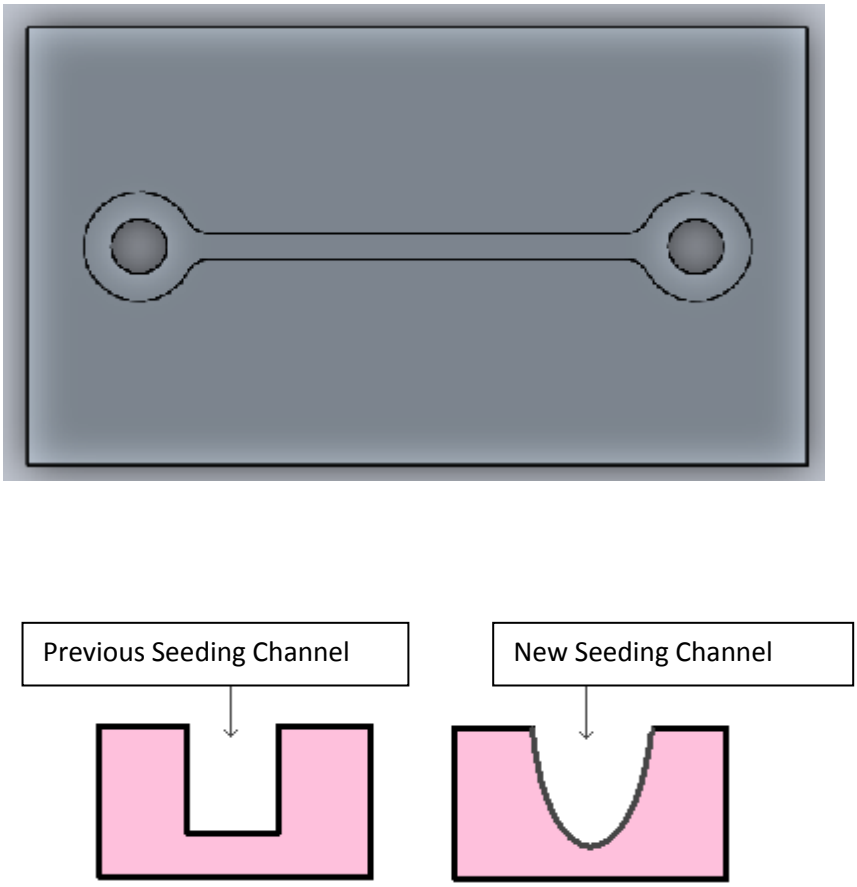


FIGURE 10: EXAMPLE OF PREVIOUS AND NEW SEEDING CHANNELS

The material chosen for the mold was agarose gel. Not only does agarose gel prevent cell attachment to its surface, it not cytotoxic, thus no deleterious effect on the developing muscle tissue. Another advantage of using agarose gel as our mold material is its ability to permit diffusion of growth media through its pores. This will allow proper nutrition and growth of the developing muscle tissue while still providing a 3D environment for tissue development.

4.2 Mechanical and Electrical Apparatusalternatives

The next portion of the team's design consisted of an apparatus that could contain the agarose gel mold, anchor the muscle tissue, and promote fiber maturation through mechanical or electrical stimulation. It was decided that this apparatus would remain attached to the muscle tissue as it cultured in a petri dish.

The main objective of the apparatus being designed is to have the ability to provide a stimulus that promotes the development of the tissue and myofiber maturation. From the team's previous research, it was determined that both mechanical and electrical stimuli play an important role in tissue maturation (Moon et. al, 2008).

4.2.1 Mechanical Stimulation Apparatus

The first design proposed was an apparatus that could apply a dynamic uniaxial strain to the developing tissue. As seen in Figure 11, the team's basic mechanical design consists of one plate that is stationary and fixed to the petri dish while the other plate is moved by a motor arm. Both these plates contain tissue posts that are initially 15mm apart and a rail system that allows for aligned movement. When a mechanical stimulus is desired, the articulating plate is pulled apart from the base plate, applying 10% strain to the tissue. This is typically done using a computer controlled stepper motor or actuator to control the displacement of the plate.

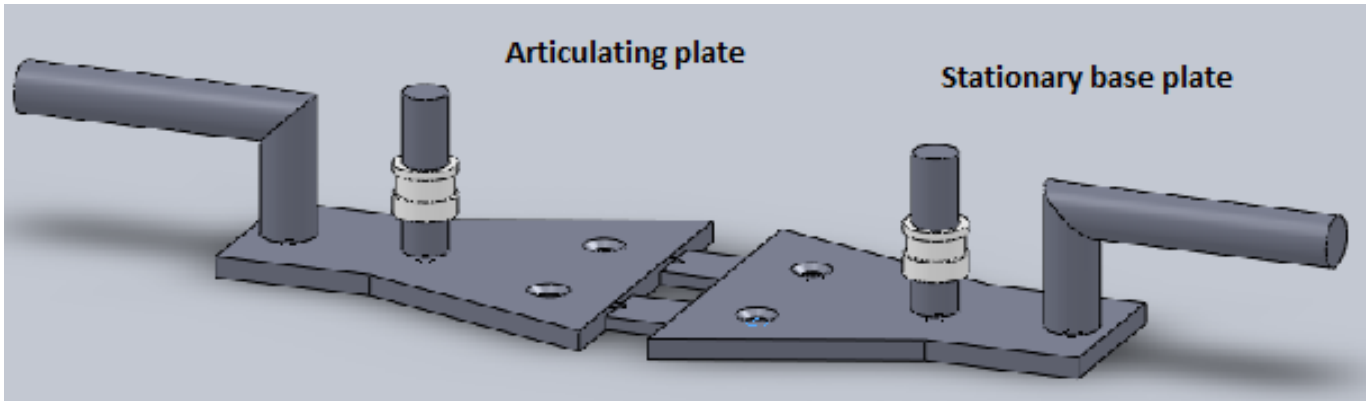


FIGURE 11: FIRST ITERATION OF MECHANICAL STIMULATION DESIGN

4.2.1.1 LOCOMOTION

After review, the team proposed a system in which the strain applied to the developing tissue could be controlled. Seen in Figure 12, the motor and cam system used in our designs operates at one revolution per minute and provides 1.5mm of displacement between the two anchor posts via a cam system. The team also left room for adjustment of the displacement. By replacing the motor cog attachment a specific displacement can be customized for different applications. The next design aspect that needed to be considered was the attachment point of the motor arm to our mechanical stimulation apparatus. The team developed two design alternatives that could meet this design consideration.

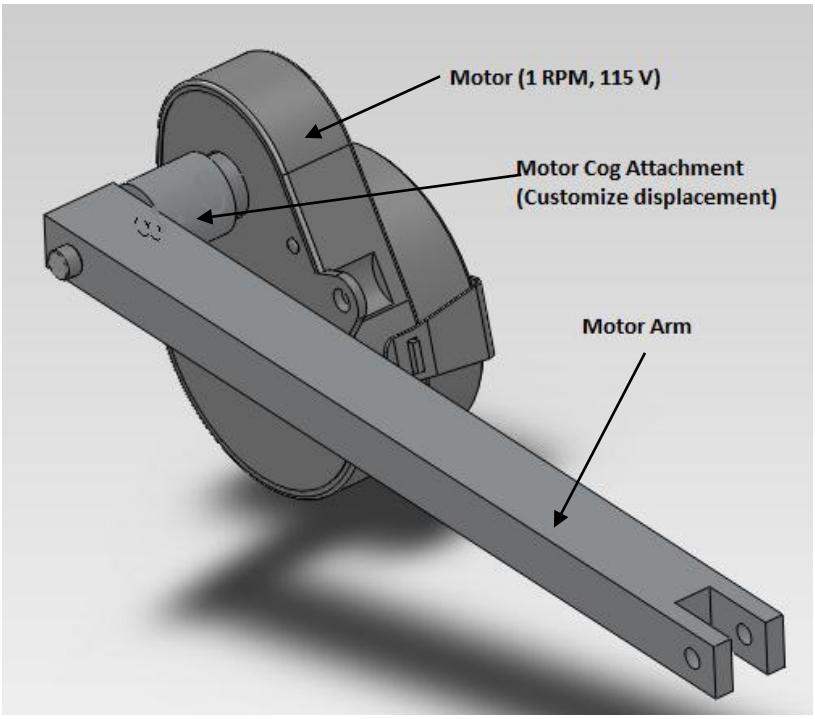


FIGURE 12: MOTOR AND CAM SYSTEM

4.2.1.2 PRELIMINARY DESIGN

The first design the team proposed consisted of a physical attachment point between the motor arm and the mechanical stimulation apparatus. Our preliminary design can be seen in Figure 13. The advantage of a physical attachment point to the motor arm is the ability to have more precise control over the displacement of the plates. However, allowing there to be a physical attachment point to the motor arm compromises sterility. There is no feasible way to keep the entire design sterile while mechanically stimulating in this manner because the petri dish would have to remain open inside the incubator. The team's preliminary design is anchored to the petri dish to allow for precise movement of the articulating plate. A sterile camp would be used in this design to keep the base plate in place while stimulation occurs.

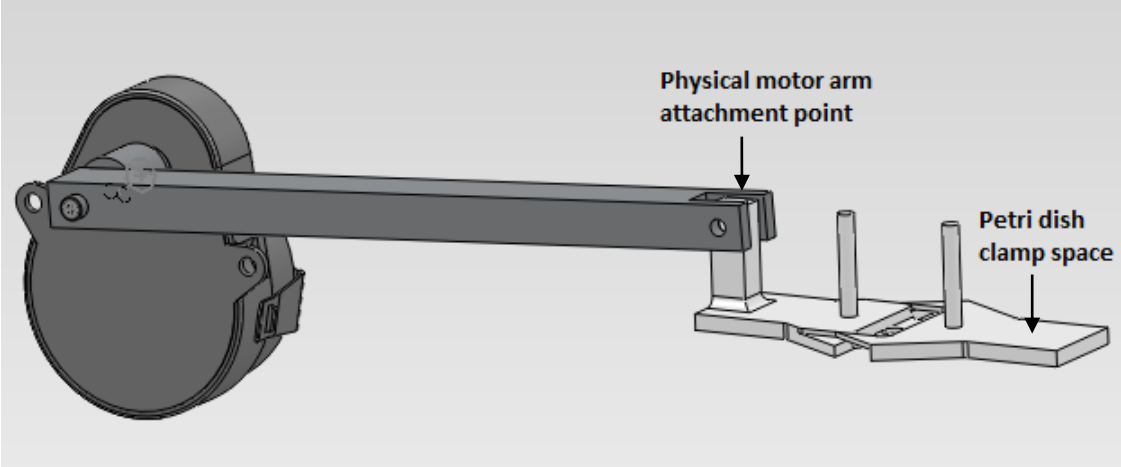


FIGURE 13: PRELIMINARY DESIGN, PHYSICAL ATTACHMENT AND PETRI DISH CLAMP

After careful consideration there were a number of necessities that our first design did not meet. The team's preliminary design made the placement of the agarose gel around the stimulation posts difficult due to a lack of accessibility. The team also felt that our preliminary design lacked adequate fixation and could potentially move during stimulation.

4.2.1.3 FINAL DESIGN

After some modification, the group proposed a refined mechanical stimulation platform that solved the problems of our preliminary design. As seen in Figure 14, the team's revised design allows the petri dish to stay closed while being mechanically stimulated. Because there is a magnetic connection between the motor arm and the stimulation plate, one simply has to turn on the motor to apply mechanical stimulation. A magnetic connection will also be used in this design to anchor the base stimulation plate. The team also incorporated a petri dish contour and two anchoring magnetic connections to the base stimulation plate. This ensured that our tissue construct would stay stationary during mechanical stimulation, only allowing movement in a uniaxial direction. Two additional gel pouring holes were added to the stimulation plates to aid in the agarose gel pouring process. In this design the molds could be attached to the stimulation platform while agarose is poured through the gel pouring holes. The gel could set around the stimulation posts allowing for a simple procedure for creating a sterile gel mold.

Our refined design allowed the team to further develop our mechanical stimulation apparatus. As seen in Figure 14, a dish holder was designed to support the petri dish and the motor arm during mechanical stimulation. The dish holder was machined from a block of HDPE using a HAAS MiniMill.

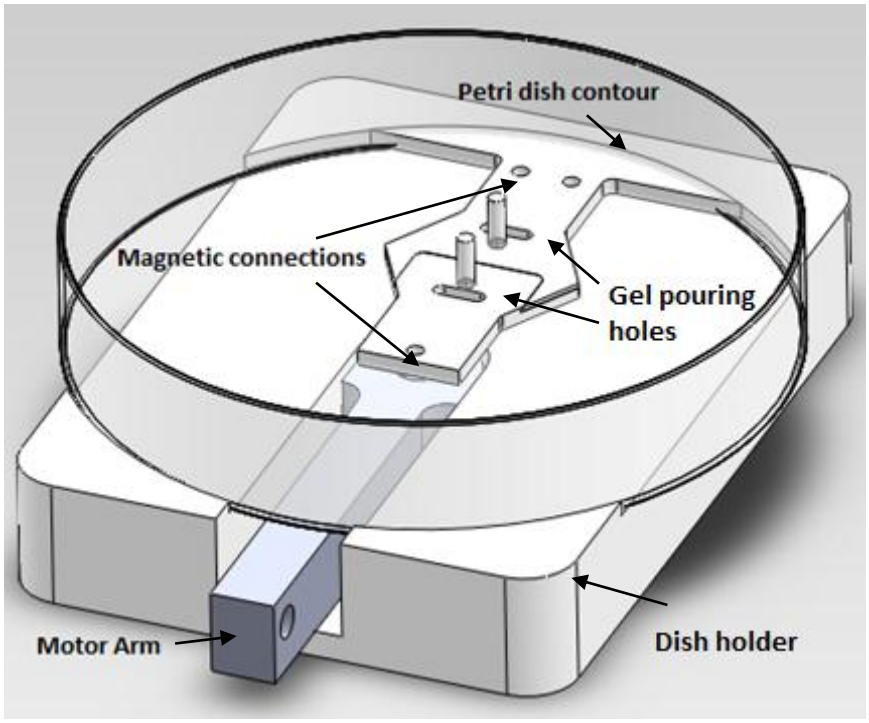


FIGURE 14: FINAL DESIGN, MAGNETIC CONNECTION TO MOTOR ARM AND PETRI DISH

4.2.2 Electrical Stimulation Apparatus

The second major design proposed was an apparatus that anchored the tissue while applying an electric field throughout the tissue medium and the agarose gel. A constant electrical stimulus promotes the cell communication necessary for contraction. The electrical stimulation design seen in Figure 15 provides two anchor points for the developing tissue and applies an electric field through the those anchor points into the medium. The electrical stimulus, however, is never in direct contact with the tissue due to the tissue rings. The stimulus would be delivered by the BioPac system found in the laboratory which is able to deliver a controlled electrical stimulus.

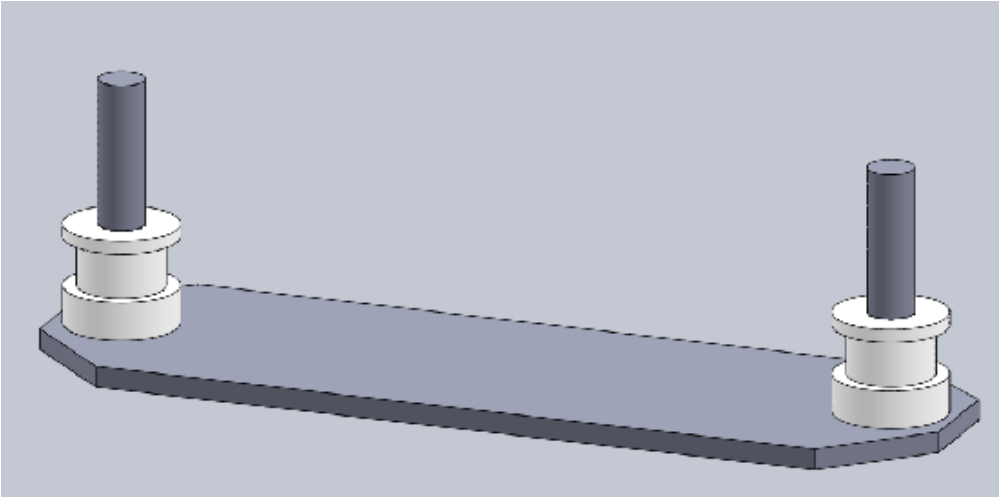


FIGURE 15: ELECTRICAL STIMULATION PLATFORM

4.2.3The Material

It is important to carefully consider the type of material that will be used when working with tissue culture. Many times materials can cause cytotoxicity and kill cells. The materials that were considered in the design of our cell maturation apparatus were 316L stainless steel and high density polyethylene (HDPE). Both materials are biocompatible and have enough mechanical strength for this application. Keeping the apparatus clean and free of bacteria is paramount while trying to culture a

tissue to avoid contamination. While Stainless steel is autoclavable, HDPE is not. However, HDPE can be sterilized via Ethylene Oxide sterilization. Table 5 shows a comparison between the two materials.

TABLE 5: COMPARISON OF STAINLESS STEEL AND POLYPROPYLENE

Characteristic	**316L stainless steel**	**HDPE**
Biocompatible	Yes	Yes
Sterilizable	Autoclave	Ethylene Oxide
Young's Modulus (E)	~250 GPa	~2 GPa
Density	8.03 g/cm^3	0.95 g/cm^3

After thoroughly comparing the two materials, the team decided to use HDPE. The deciding factor for the team was the fact that HDPE is much less dense than stainless steel. Having a lighter material inside the petri dish will help with the overall ease of use when it comes to transport and mechanical stimulation. Secondly, HDPE is much easier to machine than stainless steel. Machinists can use much slower cutting speeds and a variety of different cutting techniques with HDPE that they cannot with stainless steel. This allows us to machine our entire stimulus apparatus out of one solid sheet of HDPE. Finally, HDPE costs much less then stainless steel.

4.2.4 Choice and Reasoning

Ultimately the team decided to compromise on a hybrid of both the electrical and mechanical stimulation designs. By weighting our objectives, we were able to determine that a mechanical stimulus is more important during cell development than a constant electrical field. Because of this, the mechanical stimulus design is the basis for our engineered tissue apparatus. However, the team will manually apply an electrical stimulus at the end of tissue development to test the functionality of the muscle fiber. Although there will not be an electrical field present while the tissue is being formed and cultured, we feel that the electrical stimulus functionality test is a fair compromise due to our budget

and time constraints. Another reason behind not having a constant electric field is safety. The team may be at risk when testing or transporting the tissue if there is a constant electrical field in the culture medium and agarose gel mold. The final design of the mechanical stimulation platform will allow for a sterile and simple process to stimulate a developing muscle tissue.

4.3 Tissue Ring Alternatives:

The primary purpose of the tissue rings is to provide attachment points for skeletal muscle tissue growth and enable easy removal of the tissue from the mechanical stimulation apparatus for testing. The thicker bottom half of the tissue ring will provide a bridge support over which the tissue rests. This will prevent the tissue from adhering to, being pinched by, or letting native ECM production be affected by the polymer base of the mechanical stimulation structure. The smaller rim of the polymer tissue ring keeps the tissue from removing itself from the gel during culture. The ring will be made of HDPE as this polymer is inexpensive, sturdy, and easily processed to create a thin structure onto which skeletal muscle tissue can adhere. PP can also be sterilized via ethylene oxide (ETOH) gas, autoclaving, or alcohol. A scaled-up, preliminary design of the tissue ring is seen below in Figure 16.

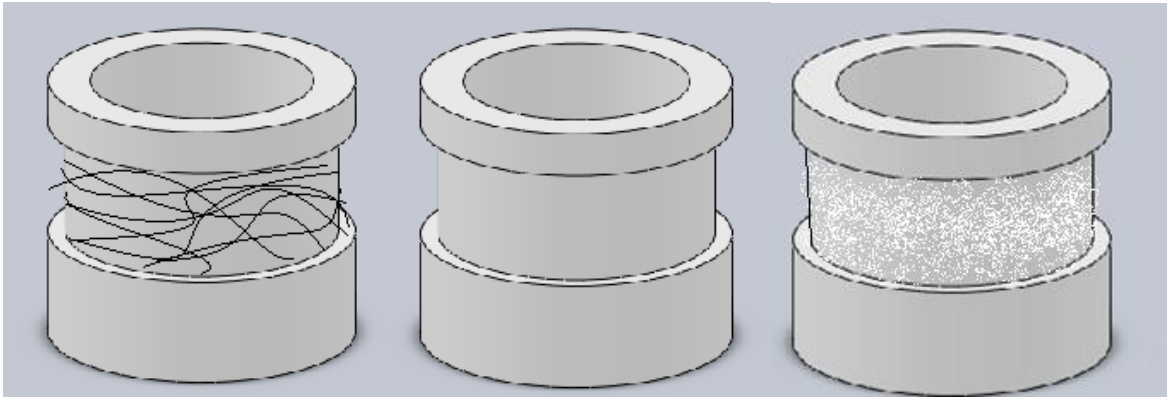


FIGURE 16FROM LEFT TO RIGHT: ELECTROSPUN PE/PU, BARE PP, AND HYDROXYAPATITE COATED TISSUE RINGS

4.3.1 Electrospun PE/PU onto PP ring:

The electrospun PE/PU coating contains fine polymer nanothreads, as seen in Figure 14, which will allow for cells to better adhere to the tissue rings. With the electrospun coating, instead of fibrous encapsulation of the bare PP tissue ring, the cells can grab onto the nanofibers in the PE/PU coating. This in-growth will allow the rings to better support the skeletal muscle tissue when it is removed from its mechanical stimulation apparatus to be tested for tensile strength, cell differentiation, cell vitality, and cyctoskeletal organization.

4.3.2 Hydroxyapatite:

Hydroxyapatite is a bioactive and osteoconductive material (Cook et al, 1992). It is commonly used in clinical applications as a fixation device suitable for cellular in-growth and attachment (Cook et al, 1992). The advantage of using hydroxyapatite for fixation is that it would better simulate muscle on bone fixation seen in native skeletal muscle tissue. Hydroxyapatite can be purchased as a nanocrystaline paste or spray on powder for coating purposes from companies such as Fluidinova ("Fluidinova SA," 2011).

4.4 Sterilization alternatives:

Sterilization is defined as "the process by which living organisms are removed or killed to the extent that they are no longer detectable in standard culture media in which they previously had been found to proliferate" (Block, 2001). Sterilization is important because microorganisms can easily be transferred from one cell to another, and it is these easy transfers that result in the wide-spread diseases, sicknesses and deaths every year. With the advances in medical technology, sterilization is becoming even more important. Therefore, different means of sterilization were researched to

determine which would be most effective for this project. The first was NaOH. NaOH is a very caustic and corrosive form of sterilization but has been found to be very effective for inactivating proteins and many medical device manufacturers recommend NAOH as an effective form of sterilization. Typically, devices sterilized with NaOH need to soak for approximately 1 hour before use. The greatest disadvantage of NAOH sterilization is that, especially when the NaOH is heated (autoclaved), the NaOH can be overly corrosive and destroy the device (Block, 2001). Another type of sterilization that may be used is ethylene oxide (EtO) gas sterilization. In this type of sterilization gas infiltrates the product to kill any organisms left on it after production (Ethylene, 2005). EtO sterilization has been a common sterilization method for medical devices since 1928. Some of the disadvantages of this method include EtO is a toxic gas and therefore has the potential to leave behind toxic residue on the material being sterilized. For this reason high levels of precaution need to be taken in association with this EtO sterilization (Pandya, 2011).

CHAPTER 5: DESIGN VERIFICATION

5.1 CULTURING OF C2C12 CELLS:

In order to obtain a cell population large enough for tissue formation, C2C12 cell culture is necessary. Cell culture is also needed in order to acquire populations of cells for C2C12 characterization in terms of optimal cell seeding density for myotube formation and optimal media content for cellular differentiation.

5.1.1 MAKING MEDIA:

As cells cannot maintain their viability without an environment containing appropriate pH, metabolites, and growth factors, media is needed for cell culture. There are many different types of media including high serum, low serum, serum free, and freezing media. Media may also contain antibiotics in some cases. However, it is normally advised to culture without this additive as it can mask bacterial contamination in cultures ("Making media", 2010). In our project a proliferation medium with a base of 88% Dulbecco's Modified Eagles Media (DMEM), 1% glutamax, 1% penn strep, and 10% fetal bovine serum (FBS) was used for basic cell culture. Differentiation of C2C12 cells was obtained through the use of differentiation media containing 48.5% F12 and 48.5% DMEM, 2% Horse Serum and 1% penn strep Freezing media containing 70% DMEM, 10% FBS, and 20%DMSO was used for cryopreservation of cells("C2C12 differentiation," 2011;"Cell culture manual-Detailed", 2011). Note that antibiotics were used for the proliferation and differentiation media due to the high traffic environment within the laboratory which increases the risk of contamination. The freezing medium, however, contains no antibiotics. Procedures for the creation of all of these media types are given in appendix G.

5.1.2 Thawing Cells:

As freezing cells is the best way to preserve their functionality and viability, thawing cells is an important procedure for beginning the culture of new cell lines. As the DMSO used for freezing cells is toxic to the cells, cells must be thawed quickly but delicately as the cells are in a fragile state ("Thawing cells", A2010). The process for cell freezing is described in appendix D.

5.1.3 Subculturing Adherent Cell Lines:

Cells must be subcultured periodically in order to prevent cells from becoming over confluent and losing their functionality. In the case of C2C12 cells, if cells become 90% or over, they can begin to differentiate into myotubes and the myoblasts tend to lose their ability to proliferate. If C2C12 cells reach over confluence, populations of cells high in fibroblastic nature can occur which results in issues with myotube formation when cells are seeded to create a tissue *in vitro*. Thus C2C12 cells were kept at a confluence of around 60-70% before subculture in T75 flasks ("Trypsonizing and plating of adherent cells", 2010). The process for subculturing adherent cell lines is described in appendix F.

5.1.4 Counting cells:

Cell counting and viability testing is an important practice in cell culture as it allows the experimenter to determine the number of live cells per mL that they have in a particular cell suspension. Through this knowledge cell numbers needed for particular experiments can be appropriately obtained and distributed. There are many ways in which to count cells including an MTT cellular proliferation assay. However, the most common and least expensive method is through use of a hemocytometer ("Cell counting", 2010 & Freshney, 1994).

A hemocytometer is a small glass device with a 0.1mm deep channel. The hemocytometer has a large glass base and a thin coverslip which lies on top of the device as seen in Figure 17. A 10 µl volume

of cell suspension is pipetted into each side of the hemocytometer for counting. The glass base contains two sides, each with a 3X3 square grid. Each of the 9 major squares within the 3X3 grid is 1mm by 1mm. The cells within a certain number of the 9 major squares are then counted and the number of cells per square averaged. Using this number and the dimensions of the hemocytometer, the number of cells per mL can be calculated ("Cell counting", 2010 & Freshney, 1994). The process and calculations for determining the concentration of cells per mL in a particular suspension using a hemocytometer is given in appendix H.

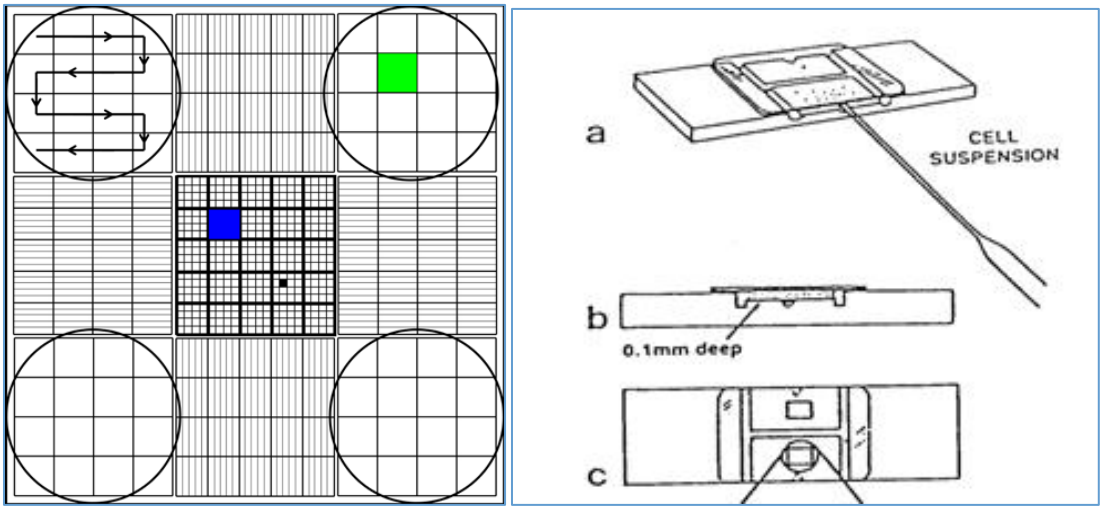


FIGURE 17: IMAGE OF HEMOCYTOMETER DEVICE (LEFT) AND 9X9 GRID (RIGHT)

5.1.5 CRYOPRESERVATION:

Cells must be maintained periodically. In order to store cells for long periods of time or transport them to other locations, cells must be cryopreserved. Cells from primary lines will also reach senescence after a certain number of passages. Thus, cells are preserved at a low passage number to preserve their proliferative capacity. Normally this process begins in a -80 °C freezer and ends with long term cell storing in liquid nitrogen. For our purposes, cells were cryopreserved at a lower passage in order to preserve the myocyte population of the C2C12 cells. Cells were also cryopreserved as stock for culturing

after term breaks. Cells are frozen slowly at 1˚C/minute in order to prevent the cells from going into shock (Cryopreservation, 2010). The process for cell freezing is described in appendix E.

5.2Bromodeoxyuridine (BrdU) Staining:

Bromodeoxyuridine (BrdU) is a nucleoside used to detect proliferating cells. It does so through incorporation intro just synthesized DNA which occurs during the S phase of the cell cycle. The BrdU will substitute for the thymidine during this DNA replication and is then identified using anti-BrdU primary and fluorescent secondary antibodies which cause the BrdU to fluoresce green under a fluorescent microscope (Lehner, 2011). The procedure for this stain is given in appendix E.

5.2.1 (BrdU) Staining For C2C12 Cells Exposed to Differentiation Medium with 2%, 1%, 0.5%, and 0% Horse Serum Concentrations:

An experiment was set up for the determination of the percent of horse serum at which DNA synthesis and cellular proliferation ceases. The information from this study will be used for the prevention of over proliferation of non-myogenic C2C12 cells during skeletal muscle tissue maturation. Non-myogenic cells prevent myotube formation and decrease the structural integrity of the fiber. Through changing the differentiation medium to either a lower serum percentage or a serum free medium after initial tissue formation, the structural integrity of the tissue throughout the maturation process should be preserved.

For the experiment at total of three 24-well plates (Plate #1 – Plate #3) with 5,000 C2C12 cells/well were cultured in differentiation media with 2%, 1%, 0.5%, and 0% horse serum or proliferation media with 10% FBS. Two wells worth of C2C12 cells were used for each of the different culture medias for a total of ten wells of C2C12 cells per time point. The cells were allowed to culture in an incubator under standard tissue culture conditions of 37˚C with 5% $C0_2$ in humid free air for 2 days (Plate#1), 3

days (Plate#2) and 4 days (Plate#3) where day 0 was the point at which the cells were first placed into the 24-well plates and exposed to their various differentiation media formulations or control media. At each time point the C2C12 cells were incubated with BrdU for four hours and fixed according to the BrdU protocol in Appendix. After all of the plates were fixed and stained for BrdU, they were observed using fluorescence microscopy where cells containing BrdU fluoresced green. BrdU images were compared against Hoescht stained cells using a dapi filter in which all cell nuclei fluoresce blue.

It was found that the 0% horse serum differentiation media contained no cells with BrdU expression at all time points (Days 2-4) indicating that no cells in the serum free media were proliferating. It was also found that C2C12 cells cultured in 2%, 1%, and 0.5% horse serum differentiation media had similar BrdU expression at all time points with a small number of cells still showing cellular proliferation at days 2, 3, and 4. The day 2, 3, and 4 controls with C2C12 cells cultured in 10% FBS proliferation media showed nearly 100% BrdU expression. This was expected as 10% FBS has been proven to enhance C2C12 cell proliferation. Overall, the expected control results as well as the lack of BrdU expression for C2C12 cells cultured in serum free conditions indicates that transitioning from normal 2% differentiation medium to a serum free medium after initial tissue formation could preserve the mechanical integrity of the muscle fiber during maturation.

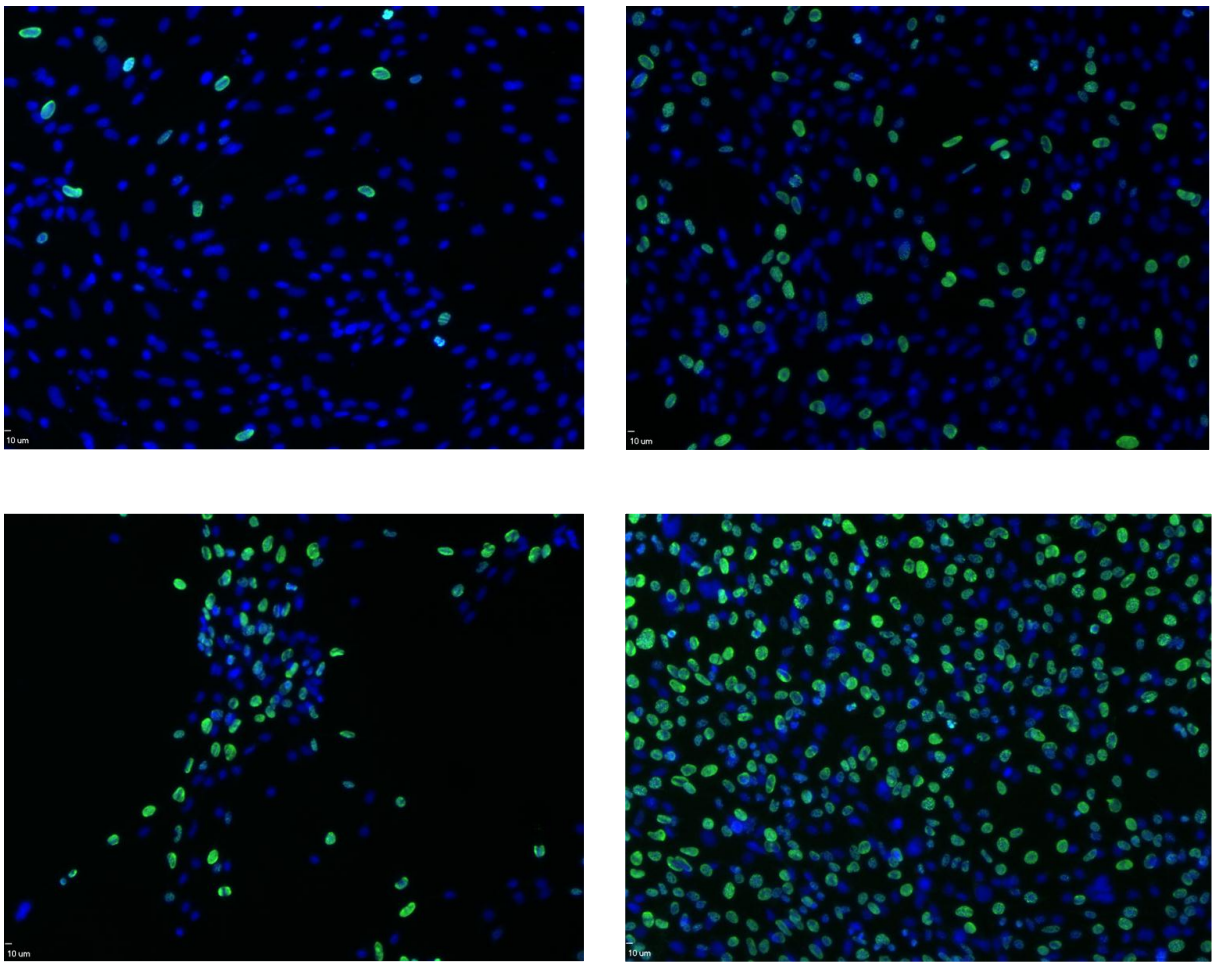


FIGURE 18 BRDU STAINED C2C12 CELLS IN 0% HS (TOP LEFT), 1% (TOP RIGHT), 2% HS (BOTTOM LEFT), AND PROLIFERATION MEDIA (BOTTOM RIGHT) AT DAY 3

The Figure 18 shows a BrdU stain 3 days after 10,000 cells had been cultured in differentiation medium containing 0, 1, & 2% horse serum. Proliferation medium with 10% FBS was used as a control. The green stained nuclei represent cells still within synthesis phase. The blue dapi stained cells indicate the total number of cells present in the image. In the lower right hand corner, nearly all of the dapi stained cells express BrdU indicating that approximately 90% of the cells are within synthesis phase of the cell cycle and are proliferating. This is expected for this image as these cells were cultured in 10% FBS proliferation media. However, only around 10% of the cells are within synthesis phase in the image within the upper right hand corner with cells cultured in 1% horse serum differentiation medium,

indicating that the other cells have exited the cell cycle and are capable of commitment to mature muscle tissue. This data indicates that even at day 3 cells are still undergoing a small amount of proliferation at a time point where the cells have been shown later in Figure 19 to be maturing into myotubes.

5.3 Myosin ICC Assay

Myosin Immunocytochemical (ICC) Staining is an assay used to detect the expression of myosin following differentiation of myoblasts into myotubes. Myosin is the most common protein in muscle cells and aids in the contractile properties of the cell.

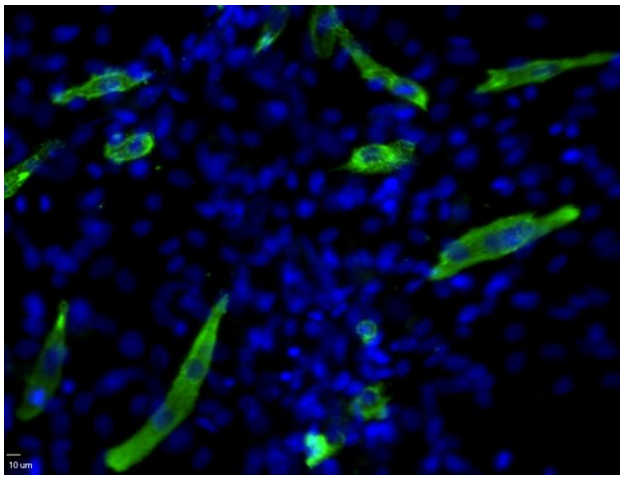


FIGURE 19 MYOSIN STAINED C2C12 CELLS AT DAY 3

A Myosin HC immunostain, whose protocol is specified in Appendix J, was performed on 10,000 C2C12 cells in a 24 well plate 3 days after culture in 2% horse serum differentiation medium. In Figure 19 above, the blue stained nuclei encompassed by fluorescent green myosin indicate the presence of myoblast fusion into myotubes at day 3 of culture. A very small percentage of cells, approximately 10%, are involved with myotube formation at this time point. This is a very small cell number and could be due to the tendency of the C2C12 line to form a fibroblastic vs. myogenic cell line if the cells reach a certain confluence over time. This will be further discussed in Chapter 6.

5.4 Agarose Gel

In order to create the agarose gels, a protocol adapted from the University of Liverpool was used (Lewis, 2001). The protocol calls for 5g of agarose and 50ml of distilled water for a 1% agarose gel. We modified the protocol to create a 2% agarose gel using culture media instead of water which was determined to be most effective for our mold system. Also, we determined that DMEM was a more effective liquid for the creation of the gel than distilled water because of the nutrients it will provide for the growing cells. Another adaptation that was necessary from the original protocol was the sterilization of the agarose gel. Therefore the agarose/DMEM solution was melted by autoclave sterilization for an hour at 120 C. For use in the mold, the agarose, with a closed cap to prevent contamination, was melted using a microwave oven for loosely 20 seconds. About 6 mL of the agarose liquid was pipetted aseptically into the negative mold using a biaxial gel ports. Before and after every reheating of the agarose, the sample was weighed so that any water boiled off during heating could be added back into the sample in the form of sterile distilled water. After the agarose was pipetted into the mold, the mixture must then be left to sit for approximately ten minutes to solidify before the negative mold could be removed.

5.4.1 C2C12 Tissue Formation Within The Gel

In figure 20, below is a complete image of a tissue seeded with 3 million C2C12 cells.

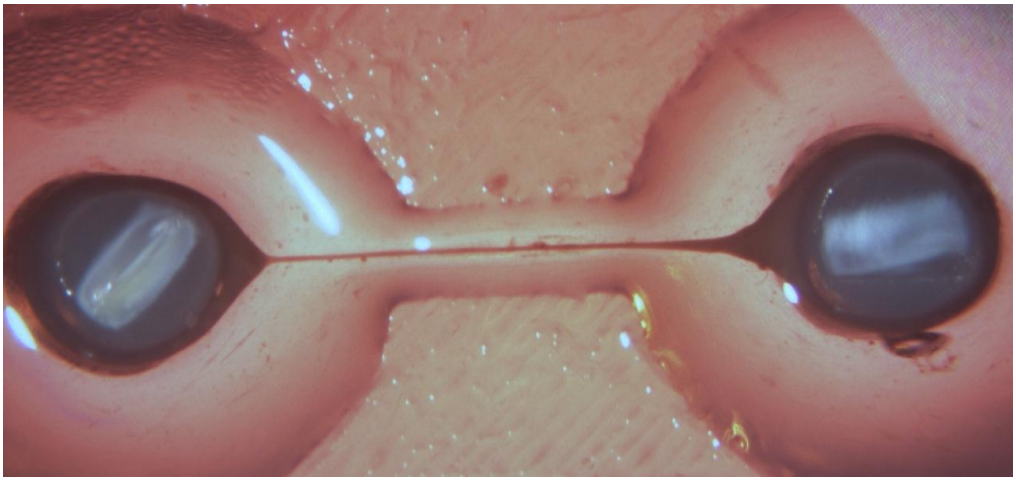


FIGURE 20: C2C12 TISSUE AT DAY 5 OF MATURATION IN STATIC CULTURE

After the cells had been seeded within the agarose gel mold the cells self-assembled within 24 hours to form a continuous 3D construct. The dog-bone shape of the tissue around the posts and within the V-shaped channel is clearly seen. Thicker tissue formation occurred around the posts enabling better anchorage of the tissue during mechanical stimulation. Additionally, within the V-shaped channel a thin myofiber approximately 16 cells or 160µm in diameter formed. This fiber diameter was calculated using Image J.

5.4.2 Static versus Mechanically Stimulated Tissue Evaluation

In figure 21below is an image of a static tissue at day 5 of maturation in differentiation media and an image of a tissue on day eight of maturation, four days after mechanical stimulation.

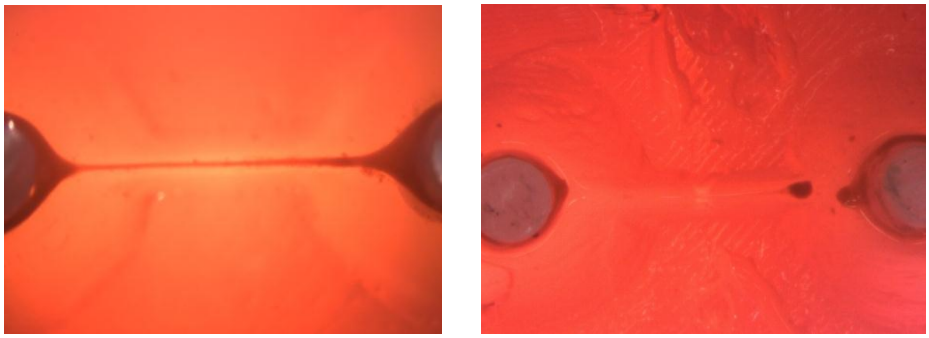


FIGURE 21: STATIC TISSUE (LEFT) AND MECHANICALLY STIMULATED TISSUE (RIGHT)

Both tissues were seeded with 3 million C2C12 cells. Before, mechanical stimulation the tissue was continuous and had matured for four days in differentiation media. It is evident that the static tissue maintained its continuity while the mechanically stimulated tissue lost its continuity during the mechanical stimulation process. It is hypothesized that the loss of continuity in the mechanically stimulated tissue was due to the fact that it had not undergone enough maturation and was mechanically stimulated prematurely.

5.5 Process of Creating Dog Bone Gel Molds

The dog bone model was chosen because the characteristics of its shape promote easier cyclic uniaxial mechanical stimulation. The process of creating the dog bone mold consisted of several steps. Ultimately three parts were produced; two gel molds and one mold wall.

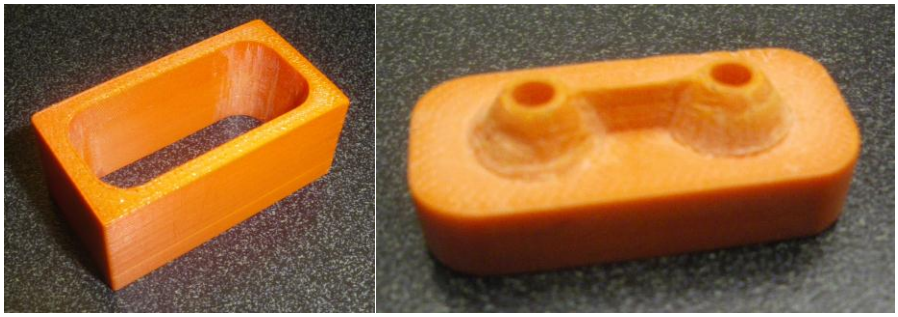


FIGURE 22: GEL WALL (LEFT) AND MOLD (RIGHT)

Firstly, the two mold negatives were created which required the use of computer aided design (CAD) to draw a three dimensional model of the gel seeding channel (Figure 22). The "V" shaped cell seeding channel was selected to limit the width of our tissue and concentrate the cells to bottom of the channel. The purpose of this shape was to isolate the smallest possible fiber in the seeding channel and promote cell self-assembly into tissue without the need for exogenous ECM. There are two models of the "V" shaped channel dog bone model created. One has an expanded well size to accommodate tissue ring implementation and the other does not (Figure 23).

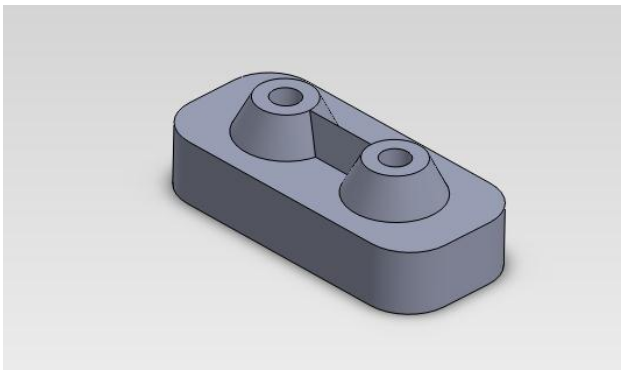
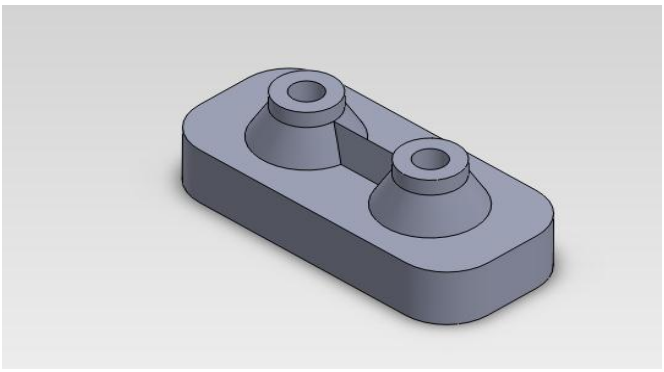



FIGURE 23: AGAROSE MOLD NEGATIVE WITHOUT (LEFT) AND WITH (RIGHT) TISSUE RINGS

The second half of the gel mold assembly is the mold walls. The mold walls fit around the mold negative to allow for molten agarose gel to be poured in. The height of the mold walls was determined by measuring the allowable vertical space inside of a 150ml diameter petri dish. The mold walls are interchangeable between the two gel molds.

The initial design the dog bone gel mold was going to be machined out of high density polyethylene (HDPE) blocks. The resolution of the machining process using the MiniMill limited the construction of the part. Additional alternatives to manufacturing included rapid prototyping. This process allowed a bottom up model of the mold design to be constructed with ABS plastic, a common acrylic. Although this manufacturing process created the parts, it was less than ideal. The resolution of the prototyping is approximately 1mm and the resolution of the design is approximately .1mm. This discrepancy was remedied using manual sanding and filing to reduce the seeding channel to a point. Sterilization can be accomplished using ethylene oxide gas, UV light treatment or soaking in 70% isopropanol. Autoclave sterilization is not an option because the temperature required exceeds the melting temperature of ABS. Despite the limitations of ABS and rapid prototyping, through experimentation the gel mold and mold walls have proven successful in creating agarose gel molds.

5.6 Mechanical Proof of Concept Testing

Upon completing the manufacturing of the mechanical stimulation plates and assembly, the team conducted proof of concept testing to confirm the original objectives. This was done to ensure that the design would mechanically stimulate a developing tissue at 10% strain through a sterile actuation process.

The first test that was conducted was to determine if the backstop that was created only allowed the mechanical stimulation plates to separate by 1.5mm. The articulating plate was pulled to the open position and rested flush against the backstop while the distance between the plates was measured. The total displacement of the articulating plate was 1.3mm. This means that when the stimulation plates are completely separate during actuation the device is providing the 15mm long developing tissue with 8.7% strain.

The next test was to determine if the plates could be actuated through the use of a magnetic connection to allow for a sterile stimulation process. The plates were placed in the petri dish with the backstop and locked into place on the mechanical stimulation assembly. After stimulation had begun, the team observed 10 complete actuations of the stimulation plates. This confirmed that the design could successfully separate the plates sterilely.

The team was concerned that the magnetic connection would not be strong enough to separate the plates sufficiently with the agarose gel connecting them. A proof of concept test was conducted in which the plates were separated using the magnetic connection while an agarose gel was connecting the plates. As expected, the plates did not separate when the motor arm magnet pulled on the articulating plate. This was due to the stiffness of the gel. It was determined that the gel would need to be cut in the center of both sides of the V shaped channel to allow for proper displacement. A final test was conducted in which the stimulation plates were separated through a magnetic connection with the

pre-cut gel on them. In this test the stimulation plates were able to be separated magnetically to a total displacement of 1.3mm.

To ensure that our design could endure an entire stimulation regime, a test was conducted in which the actuating device was observed over thirty minutes. Because the synchronous gear motor operated at 1 rpm the team expected that the plates would actuate thirty times in the continuous thirty minute test. After running the stimulation regime, it was determined that the device was fully prepared to mechanically stimulate a growing muscle tissue.

Overall the team has produced a device that provides mechanical stimulation to a developing muscle tissue through a sterile process. Although the team did not reach its specific goal of 10% strain, the mechanical stimulation objective has been met. The device provides a means of observing the effect mechanical stimulation has on 3D tissue development.

5.7 Electrical Stimulation Functionality Test

After the tissue has been cultured for the appropriate amount of time a test is done to determine if contractile function is achieved. This is accomplished by stimulating the tissue with an electrical current. (If muscle has been created then it should contract) A protocol adopted from Radisic et al. (2004) which includes stimulating with 5 volts in rectangular pulse duration every 2ms using standard electrical leads in the culture medium around the tissue. The results from this test will indicate if there are myoblast cells or fibroblast cells present. This step needs to occur after the tissue has matured enough to form actin and myosin protein structures in the cells or else there will not be any contraction.

CHAPTER 6: FINAL DESIGN AND VALIDATION

6.1 TISSUE CONTRACTILE MEASUREMENT

A contractile test will be performed on the two tissues, one mechanically stimulated and one self-assembled. The goal of this test is to determine if the uniaxial stimulated tissue performs better than the tissue that was not stimulated. In theory, the fibers in the stimulated tissue should be aligned in parallel increasing the contractile force in on direction. Without the mechanical stimulation the fibers may be aligned in multiple directions. Mechanical stimulation has been proven to increase cell proliferation, increase myofibril alignment, and produce extracellular matrix (Powell et al 2002). In order for proper contraction when an electrical stimulus is applied the necessary ion channels need to be present to transmit the signal through the tissue by the release of calcium ions.

Once the tissues are formed, positive and negative leads will be connected on opposite ends of the tissue. A BioPac system will be used to control the amount of voltage and time of charge. Once contracted the tissue will be measured and compared to the original length. The mechanically simulated tissue should contract uniformly in one direction while the non-stimulated tissue should not contract as well.

6.2 ELECTRICAL STIMULATION VERIFICATION

Two tissues were electrically stimulated after five days of maturation, one made from C2C12 cells and the other from primary human myoblasts. The static C2C12 tissue was electrically stimulated on day 6 of culture. Because the tissue was not continuous after contraction during maturation, only small portions of the tissue could be tested. When the charge was applied, there was not any visible contraction or movement. The primary myoblasts were electrically stimulated on day 5 of culture using

the method stated in the Electrical Stimulation Functionality Test section in chapter 5. This tissue was not continuous due to the strain applied during formation. When stimulated with 5 volts, the there was no visible movement or contraction. These results could indicate that there is a larger fibroblast population than myoblast; this would align with the results because the fibroblasts do not have the protein structures to contract.

6.3 Effects of Agarose Gel on Tissue

The agarose gel mold allowed was successful in encouraging tissue growth. The media was able to diffuse through the gel, ensuring that nutrients would always be available to the growing cells so they could continue to differentiate as they formed a tissue. Also, the cells did not adhere to the agarose gel, so that the tissue could easily be removed from the gel for analysis after it had formed. This was essential for the histology of the tissue. The structure of the gel was also highly beneficial to the tissue growth. The "v" shaped channel allowed for the diameter of the tissue to be controlled, so that the final tissue was approximately 16 cells in diameter, a good diameter for testing and not easily achieved. Finally, the dog-bone shape was successful in helping the tissue to anchor on the mold so that it did not contract off of the mold during mechanical stimulation, which has often been a limitation in the past.

6.4Immunocytochemistry

Immunocytochemistry is an assay to determine the presence of specific antigens in a tissue. It does this through the use of either fluorescent dyes or enzyme markers. Immunocytochemistry was completed on both the myosin stained cells and the BrdU stained cells.

6.4.1 Myosin

C2C12 cells are a cell line that is prone to develop fibroblastic characteristics if allowed to become too confluent during cell culture. Fibroblastic characteristics inhibit differentiation of the C2C12 cells and their ability to form myotubes. Myosin tests were completed to display both if the C2C12 cells were forming myotubes and at what time point in after exposure to differentiation media the cells began to display myotube formation. It was observed that myotube formation began on day 3 of culture in differentiation media and that few myotubes were forming in the cell culture.

As the cells in this experiment were accidentally shocked for 24 hours through experiencing a dramatic shift from a 10% FBS to no serum media environment, it is hypothesized that the myoblastic population of cells was accidentally removed. If this hypothesis were to be confirmed in the lab through plating cells in suspension after allowing the cells to sit in no serum media and plating the cells left in suspension, cells adhered to the tissue culture surface, and a control population of C2C12 cells without exposure to no serum media, a method for isolating myogenic C2C12 cells from the total cell population might be obtained. Another explanation for the lack of myotube formation is that the C2C12 cells had become too confluent during cell culture and were losing their myogenic potential. However, even if the C2C12 cells used to produce tissues had lost their myogenic capacity, the ability of our device to provide a skeletal muscle tissue was proven through the formation of a continuous tissue using primary human myoblasts.

6.4.2 BrdU

BrdU testing with media concentrations of 0%, 1%, and 2% horse serum indicated that C2C12 cells still have approximately 10% of the cell population proliferating at day 3 with culture medium containing 1% horse serum. Additionally, approximately 25% of the cell population is still proliferating.

Cell proliferation at this time point when myotube formation is beginning could indicate the presence of fibroblastic cells in the C2C12 population as the myogenic cells should be differentiating at this time point. If the fibroblastic population continues to proliferate, the tissue will decrease its myogenic capacity in terms of the ratio of fibroblastic to myogenic cells within the tissue. As almost no cellular proliferation was occurring in serum free media conditions, switching the differentiation media form 2% horse serum to no serum could prevent the continual proliferation. Obviously cellular viability of the tissue would need to be determined for the tissues under these culture conditions. Use of primary human myoblasts would eliminate the need for this step in culture. However, if the myoblasts were co-cultured with fibroblasts, evaluation of the replication of the fibroblasts via BrdU would need to be assessed and the culture conditions suggested above might prove useful to follow.

6.5 MANUFACTURABILITY

The stimulation plates and mechanical stimulation assembly could be reproduced in any properly equipped machine shop. Using the technical drawings that can be found in Appendix K, the parts could be recreated using solid modeling and a CNC mini mill. HDPE was the primary material used due to its biocompatibility, low cost, availability, and ease of manufacturability. While the mechanical stimulation plate and gel mold design is easy to manufacture at its present scale, smaller plates and molds would aid in reducing the size of the tissue. A smaller muscle fiber will allow for a better understanding of developing 3D muscle tissue and decrease the number of cells needed to create a 3D tissue. The scale of the design was limited by the manufacturing capabilities available to the team. In particular, the gel mold scale could not be reduced due to the resolution capabilities of the 3D polymer printer in Higgins Laboratories.

6.6 SUSTAINABILITY

In order to reduce the impact a product has on the earth, engineers must evaluate their design's sustainability. It is the job of all scientists and engineers to minimize the resources needed to reach the desired goal. The proposed design is highly sustainable and uses easily obtainable resources needed for cell culture. As expected, the manufacturing process of the stimulation assembly and stimulation motor used electricity throughout the course of the project. As stated previously, the design is made from HDPE but utilizes less than one square foot for the entire design. The team believes that the device could be made smaller to reduce the resources needed to sustain a developing muscle tissue. Currently the bottom of a large petridish is filled with media to provide the tissue with nutrients. By creating smaller stimulation plates, gels, and tissues, a smaller petridish could be used to house the entire assembly. Ultimately this would reduce the amount of media needed. In addition, creating a smaller stimulation assembly will reduce raw materials needed and thusly reduce the designs environmental impact.

6.7 ECONOMIC IMPACT

Today the National Institute of Health (NIH) spends over 97 million dollars on research toward finding treatments and cures to Muscular Dystrophy (MD) (NIH Data Resource Sheet). This is a prevalent disease that affects many people across the world. The application of a reproducible engineered muscular tissue model could be widespread for institutional research and private research for MD. Many researchers use live animal testing in drug and treatment protocols. An *in vitro* tissue model could replace some of these tests and therefore decrease project spending. This would allow the grant money to be allocated in smaller amounts decreasing spending. There are many other disease and traumatic

injury patients that could be affected by the research that a 3D tissue model could provide. Some of them are listed below in Table 6.

TABLE 6: YEARLY NIH SPENDING ON MUSCULOSKELETAL DISEASES

Disease	**NIH Spending in 2011 ($)**
Facioscapulohumeral Muscular Dystrophy	5,536,294
Duchenne/ Becker Muscular Dystrophy	31,662,032
Muscular Dystrophy	75,083,359
Sarcopenia	37,035
Rhabdomyosarcoma	880,311
Total	**112,318,720**

6.8 Societal Impact

This device, if sold commercially, would have a lasting impact on society. The almost 90 thousand people who are affected by MD and traumatic muscle loss in the United States (Hoffman, 1987) could finally have therapeutic treatments to help with their diseases, which currently have no cure (Emery, 1993). People everywhere would no longer have to fear giving birth to a child that will suffer from muscular dystrophy their entire life. Also, the average individual would benefit from this device because it could assist in small scale drug testing for different muscle disorders that could be applicable for drug testing in the future. Politically, this device would also have an effect in terms of animal testing. This device, if sold commercially, would decrease the need for animal testing. If tissues could be easily grown in an *in vitro* setting, then research on new drugs could be conducted first on the tissues, limiting the need for animal testing. This would appease animal conservationists all over the world.

6.9 Ethical Considerations

In terms of ethics, designing an *in vitro* skeletal muscle tissue model will provide a means through which treatments for muscular dystrophy and other degenerative muscular diseases can be studied on the benchtop. Through verifying treatments first *in vitro* for their effectiveness, the number of animals used for in vivo studies can be minimized. As there are a number of ethical concerns with the use of animals for scientific purposes, limiting the need for multiple animal studies is both cost effective as well as ethically conservative.

6.10 Health and Safety Considerations

In terms of health and safety, *in vitro* testing helps determine whether a particular treatment will be detrimental to the health of a patient. Thus, it is an important aspect of maintaining health and safety concerns for novel drug development. Additionally, in vivo animal testing is costly and time consuming process. With an *in vitro* skeletal muscle model, the functionality of novel drugs on target tissues can be determined quickly on a pass fail basis leading to faster drug discovery through eliminating the study of drugs with poor functionality early on. With quicker turn around for novel drug treatments for musculoskeletal diseases, the health of the population can be improved.

CHAPTER 7: DISCUSSION

This chapter discusses the specific aims that were met throughout the project, including all of the manufacturing aims and limitations, the gel mold and mechanical stimulation aims, and how the process of seeding a gel was actually conducted.

7.1 MECHANICAL STIMULATION PLATE MANUFACTURING

The first step of manufacturing the mechanical stimulation plates was to finalize our solid model made in SolidWorks 2011. Dimensions, tolerances, and materials all had to be reviewed so that once built, the stimulation plates functioned properly. From here CAM program files were generated from the solid model by creating tools, tool paths, and cutting operations in ESPRIT software. Once the program had been refined, the HAAS MiniMill was loaded with the program and began cutting our stock material. The mechanical stimulation plates were cut from a 12”X16”X1/8” sheet of HDPE and did not contain posts after machining. This can be seen in Figure 24. The posts were added afterwards by cutting ½” sections of HDPE welding rod and gluing them, with silicone medical adhesive available in Professor Billiar’s Lab, into holes that were previously drilled. Magnets were also glued into their appropriate spots after the plates had been machined.

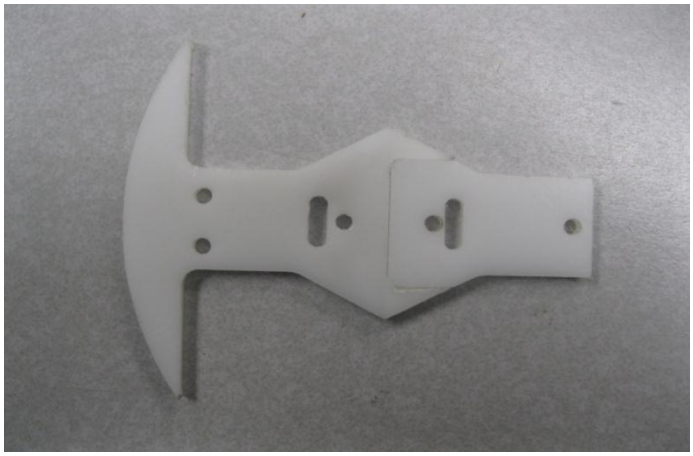


FIGURE 24: MECHANICAL STIMULATION PLATES WITHOUT POSTS

It was later determined that the silicone medical adhesive increased the friction of the mechanical stimulation plates and inhibited their movement within the petri dish. To solve this problem, microscope cover slides were glued to the bottom of the plates and sanded smooth. This drastically reduced the friction and allowed the articulating plate to move smoothly against the petri dish.

7.1.1 Limitations of Mechanical Design

While the team accomplished its three main objectives there are a number of limitations with the proposed design. Among these limitations are the design's low throughput, scale, and inability to provide varying tension to developing tissue.

The first limitation is the low throughput of the mechanical stimulation plates. Currently only one tissue can be mechanically stimulated at a time. To improve the designs throughput a future iteration could be made to allow for multiple tissues to be seeded and stimulated on one stimulation plate. This would increase the number of results that could be obtained and save resources needed for tissue culture.

Another limitation the team identified was the size of the design. Ideally the designs scale would be decreased to allow for a smaller tissue to be developed. This would also minimize the resources necessary to culture and mature the tissue while providing a better model to study the development of skeletal muscle tissue. The team was limited to the current design scale due to the manufacturing capabilities available.

A final design limitation includes the rigidity of the stimulation plates. During muscle tissue development the tissue contracts slightly pulling on its anchor points. Because the posts of the design allowed for no give, the team believes that many tissues pulled themselves apart during development. To remedy this limitation the team proposes a stimulation design that accounts for the varying tension the tissue needs during development. The post-to-post distance could be decreased after a certain

number of days to ensure that the developing tissue doesn't pull itself apart. This would allow the tissue to fully mature before it was mechanically stimulated.

7.2 CAM AND DISH HOLDER MANUFACTURING

To move the stimulation plate within the Petri dish while maintaining sterility it was determined that a magnetic connection point was needed. After the files were finalized on SolidWorks 2011 a CAM file was created using ESPRIT. The dish holder and CAM apparatus consisted of five custom designed parts and one previously purchased motor (Appendix X).

The stock material used to manufacture the base, dish holder, and magnet arm was a 12"X 4.5" X 6" block of HDPE. HDPE was used because its ease of manufacturing both with prototyping and the MiniMill. Both the dish holder and magnet arm were cut using the Haas MiniMill in Higgins Laboratory. The dish holder was fixed to the base part by four 1/4 -20 bolts and rests flush with the end of the base part.

The motor arm was cut using the laser cutter available to MQP students in Washburn labs. A .dwg file was prepared and loaded into the machine and while the acrylic stock material was mounted inside the laser cutter. Acrylic is the material of preference when using the laser cutter because of its low melting point but stiff mechanical properties. After preparing the machine it took less than a minute to cut the motor arm out of the stock material.

The motor mount was created by bending and cutting a stock aluminum sheet to the appropriate dimensions (Appendix K). A total of four holes were drilled in the motor mount to facilitate the mounting of the motor to the base of the dish holder apparatus. The fabrication of this part was done in the Washburn machine shop.

The final piece fabricated was the motor cog. The main purpose of this part is to turn the rotational motion of the motor into linear motion. A one inch diameter aluminum rod was cut to size and drilled to the appropriate dimensions. The part consisted of a hole for the shaft of the motor to be inserted into and a set screw to tighten this connection. A second hole was drilled on the opposite face of the motor cog exactly one quarter of an inch from the center to allow for a total linear displacement of one half inch. The lathe and drill press were used in the Higgins machine.

7.3 Assembly of Gel Mold and Mechanical Stimulation Platform

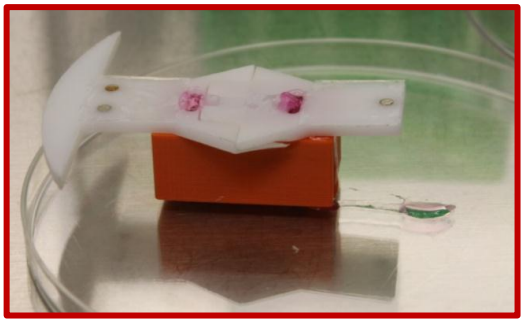
The assembly of the gel mold and mechanical stimulation platform in the petri dish in preparation of cell seeding is a multistep process. This process is simplified into three distinct steps including assembly, gel formation, and wall removal.

Step 1: Assembly *(takes place in the cell culture hood after all pieces of the device have been sterilized)*

- Using autoclaved forceps place the mold negative into the inside lid of a sterile 150mm petri dish
- Place the mold walls around the mold negative
- Insert the inverted mechanical stimulation platform into the holes in the complete gel mold

Step 2: Gel Formation

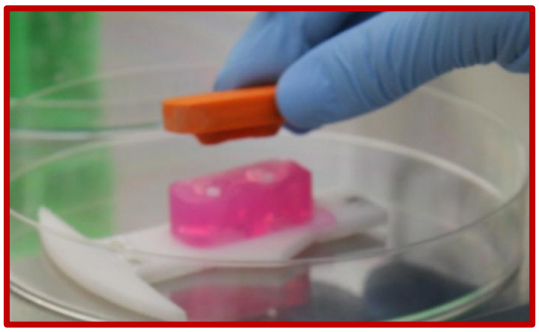
- Prepare the autoclaved 2% agarose gel with DMEM as specified in the gel preparation section (5.4).
- Pipette 6ml of molten agarose through the slots in the mechanical stimulation platform into the gel mold.
- Allow the gel to cure for 10 minutes in the cell culture



hood.

Step 3: Wall Removal

- Once cured, flip the gel mold, walls, and mechanical stimulation platform into the bottom of the petri dish such that the mechanical stimulation platform in resting in the petri dish.
- Using forceps to slowly remove the gel mold walls before removing the gel mold.



- Replace the lid on the petri dish and place the dish into the dish holder.

The outlined procedure stated above is the process necessary to prepare the agarose mold for seeding the C2C12 cells into. It is important for contamination issues that all instruments and devices remain sterile.

7.4 Cell Seeding

Once the agarose mold is prepared, the cells can be seeded in the dog-bone shape. After completing a series of tests to define the ideal cell seeding number, the team determined that approximately 3 million C2C12 cells in 100µl of differentiation media is the appropriate number for seeding into the mold. The cells need to be spread evenly throughout the dog-bone shape, so that the channel and the circles around both posts have an adequate number of cells. This will help ensure that the tissue will grow uniformly. The best way to accomplish this is to use a 25µl micropipette, so that the cells can be seeded in four different cycles- one for each of the sections around the posts and two for the v-shaped channel down the middle. Upon seeding all 3 million cells, the lid is placed back on the petri dish and the whole system is placed in the incubator for growth.

CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS

Our design consists of two primary components, the agarose gel mold and the mechanical stimulation apparatus. The conclusions for each of these devices is given below following a short section on conclusions made based on ICC data obtained from C2C12 cell analysis.

8.1 C2C12 IMMUNOCYTOCHEMISTRY CONCLUSIONS

After myosin staining it was determined that C2C12 cells did not undergo any myofibril formation until day 3 of maturation in both serum free and 2% horse serum media. Myofibril formation observed was also low for the C2C12 population. After BrdU analysis, it was concluded that the C2C12 cells were still replicating during the process of cell differentiation, at day 3 of culture, even in 1% horse serum containing medium. No DNA synthesis was detected with serum free medium; however, at day 3 with 2% horse serum medium at least a quarter of the cell population was still proliferating. This indicates that a quarter of the cell population was not undergoing differentiation and that the presence of a fibroblastic population of myoblasts within the C2C12 cells may have developed during cell culture.

8.2 MECHANICAL STIMULATION DEVICE

Magnetic actuation can be used to maintain sterility within the petri dish. Posts on the mechanical stimulation plates allow for anchorage of the muscle tissue during maturation and mechanical stimulation. A single synchronized gear motor and CAM system can be used to eliminate the need for programming and decrease in the cost of production of the device. The strain magnitude can be controlled via distance the mechanical stimulation plates travel during actuation and the duration of the mechanical stimulus can be controlled via the number of cycles the tissue experiences. Through

creating various backstops that allow the mechanical stimulation plates to travel various distances, the magnitude of the strain applied to the tissue can be both regulated and altered to fit multiple mechanical stimulation regiments.

As mentioned earlier, the duration of the strain applied to the tissue can be controlled via the amount of time the muscle tissue is exposed to cyclic strain cycles. The current single synchronous gear motor and backstop combination applies a full cycle of 8.7% strain every minute. However, purchase of a single synchronous gear motor which cycles at faster time intervals, such as one strain cycle every 30 seconds, allows for adjustment of both the number of strain cycles as well as the duration of the strain applied to the tissue.

8.3 Use of Polyethelene (PE) for the Mechanical Stimulation Device

The PE material used to create the mechanical stimulation device was easy to manufacture and withstood incubation under humidified conditions well. However, in terms of the mechanical stimulation plates, the PE had to be ethylene oxide sterilized as it encourages fungal and bacterial growth under exposure. These issues with contamination were prevented through use of fungicide and penn-strep in the culture medium. However, for future work a different material for mechanical stimulation plate manufacture that could be autoclaved and would decrease fungal and bacterial growth would be recommended. It is also recommended that the mechanical stimulation plates be manufactured to contour a smaller petri dish to decrease the amount of media needed for tissue culture.

8.4 Magnet Coatings

In terms of coating the magnets to prevent corrosion and nickel poisoning, it was found that silicone glue can be used to seal device. However multiple coats will be necessary as it was observed that after three to four weeks in culture media as well as storage in 70% isopropanol, the silicone adhesive loosens to expose the magnets to corrosion. Nail lacquer was also used to seal the magnets and given two full coatings provided a barrier to corrosion. Again multiple coats are needed to prevent corrosion. Coating the magnets in PDMS was attempted earlier on in the process. However, the coating was found to easily peel off of the polyethylene surface when applied. The coating was also much bulkier than the medical adhesive or nail lacquer.

8.5 Electrical Stimulation During Tissue Maturation

In the next generation of our mechanical stimulation apparatus, it would be recommended to incorporate a conducting metal (ie a copper alloy) into the posts for tissue anchorage. Through doing so, electrodes can be attached to the posts and an electric current can be passed through the tissue. Electrical stimulation of the tissue will allow for improved maturation. In addition, it will provide a simple means through which the contraction of the tissue at different stages of mechanical stimulation can be assessed. Electrical stimulation could also be integrated into the device design through electrodes placed on the top of the petri dish which would be engineered to the appropriate length and distance apart where the tissue could be electrically stimulated via a top vs. bottom approach.

8.6 Agarose Gel Mold Conclusions

As cells will not adhere to the surface of agarose gel, a 2% agarose gel mold can be used to allow for self-assembly of C2C12 and human primary myoblasts into a continuous 3D tissue. The formed 3D

tissue maintained a dog bone-like structure. Thicker tissue formation occurred around the PE posts for increased anchorage of the tissue, while a thin fiber of tissue, approximately 16 cells in diameter, formed within the V-shaped channel. This thin tissue fiber provides an ideal tissue size with which to complete in vitro skeletal muscle tissue testing. The diameter of the tissue within the V-shaped channel can also be controlled through the number of cells seeded into the channel, with increased cell populations forming tissues of slightly larger fiber diameter. It was also suggested that the gel be designed to allow for the seeding of multiple tissues at once in order to give the design a higher throughput for in vitro skeletal muscle tissue testing.

In addition to providing shape for the tissue, the agarose gel mold also provides a means for nutrient diffusion through the uptake of media from the petri dish through the gel and to the cells within the developing tissue. It was observed that a cell seeding density of for the agarose mold should be at least 2 million for C2C12 and 1.5 million for primary cells within 100ul of differentiation media. Moreover, formed skeletal muscle tissues within the agarose gel should be allowed to mature for at least a week prior to mechanical stimulation, as tissue continuity was lost after 4 days of tissue maturation.

8.7 Muscle Tissue Contraction During Maturation

As the C2C12 cells or human primary myoblasts assemble and mature into myofibrils within the differentiation medium, the tissue formed begins to contract. The mechanical stimulation posts anchoring the tissue do not allow for any variable tension. Thus, at this point in tissue maturation, approximately 5 days after cell seeding, the tissue breaks due to contractile forces. As a result, in the future it is recommended that variable tension be integrated into the device design. Otherwise the tissues formed within the agarose mold will break before they are able to be mechanically stimulated in

vitro. Variable tension could be added to the design through designing a mechanical stimulation plate that allows the anchorage posts to angle themselves inward during tissue contraction. Obviously the strain distance applied to the tissue would have to be adjusted so that no more than 10% strain would be applied to the tissue during mechanical stimulation. An electro-active shape memory polymer could be used for this application as well.

8.8 Co-culture with fibroblasts

It is suggested in future work that primary human myoblasts or C2C12 cells be co-cultured with fibroblasts to improve the integrity of the tissue during the maturation process. Fibroblast play a significant role in the formation of muscle tissue *in vivo*, and it is hypothesized that they will strengthen the muscle tissue *in vitro* through preventing breakage of the tissue during contraction within the myofibril maturation process.

8.9 Global Conclusions

Overall, a basic in vitro device for mechanically stimulating a single skeletal muscle tissue has been designed. The design allows for a small fiber of muscle tissue to be formed through seeding C2C12 cells or primary human myoblasts into a 2% agarose gel mold. The V-shaped channel within the mold design allows for the diameter of this muscle fiber to be controlled through the number of cells seeded for culture. Additionally, the mechanical stimulation apparatus allows for sterile actuation of formed muscle tissue through a magnetic connection between the motor arm and mechanical stimulation plates. Though the device is a first stage prototype, it shows promise as a base structure through which

other modifications can be made to increase the efficiency of the device and provide a means for in vitro skeletal muscle testing in the future.

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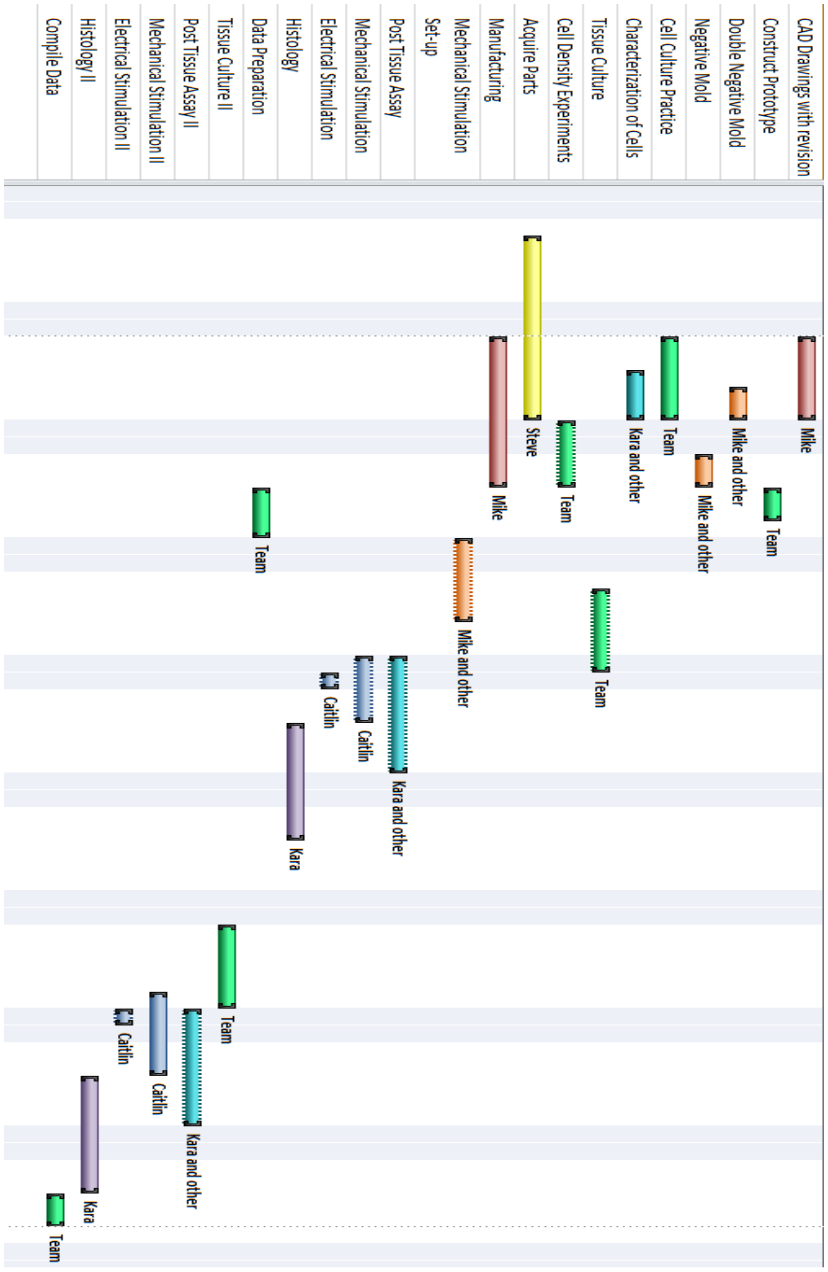
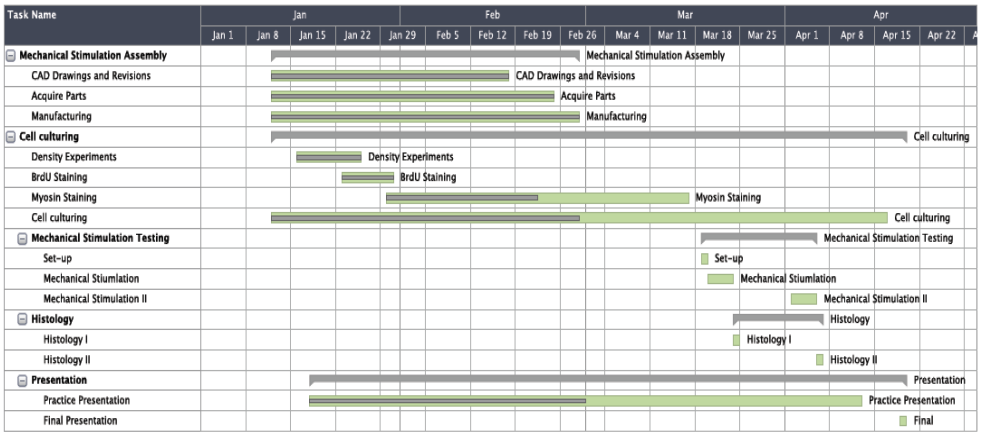
APPENDICES

APPENDIX A: GANTT CHART

ORIGINAL GANTT CHART



Revised Gantt Chart


Task Name
Jan
Feb
Mar
Apr
Jan 1
Jan 8
Jan 15
Jan 22
Jan 29
Feb 5
Feb 12
Feb 19
Feb 26
Mar 4
Mar 11
Mar 18
Mar 25
Apr 1
Apr 8
Apr 15
Apr 22
Mechanical Stimulation Assembly
CAD Drawings and Revisions
Acquire Parts
Manufacturing
Cell culturing
Density Experiments
BrdU Staining
Myosin Staining
Cell culturing
Mechanical Stimulation Testing
Set-up
Mechanical Stiumlation
Mechanical Stimulation II
Histology
Histology I
Histology II
Presentation
Practice Presentation
Final Presentation
Mechanical Stimulation Assembly
CAD Drawings and Revisions
Acquire Parts
Manufacturing
Cell culturing
Density Experiments
BrdU Staining
Myosin Staining
Cell culturing
Mechanical Stimulation Testing
Set-up
Mechanical Stiumlation
Mechanical Stimulation II
Histology
Histology I
Histology II
Presentation
Practice Presentation
Final

APPENDIX B: BUDGET

Before implementation of the project plan began, it was necessary for the project group to outline all of the expected spending to ensure the budget would not be exceeded and all supplies could be purchased. Below is that outline:

		Total ($)
Initial Money	$156pp	624
Lab Fee	$100	524
Lab notebook	$23	501
PE sheet	$15	486
PE rod	$8	478
Plaster of Paris	$7	471
Motor x2	$80	391
PE blocks	$23	368
Calipers	$10	358
Rapid prototyping	$2	356
Total left		356

APPENDIX C: WORK BREAK DOWN STRUCTURE

APPENDIX D: THAWING CELLS

1. Place an empty 15mL conical tube in the hood, then obtain a cryotubeof the desired cells from the -80 ° C freezer. Place the cryotube in the 37° C water bath to thaw quickly. While thawing label the conical tube with your initials, and transfer your warmed media to the hood along with other necessary supplies. Cells must not be left in the water bath any longer than needed for thawing (1-2 min).
2. When thawed, rinse the outside of the vial with ethanol and wipe dry, then place the tube in a rack in the hood. Make sure all ethanol has evaporated before opening the tube as ethanol is toxic to the cells!
3. Using a 1 mL pipet gently re-suspend the contents of your cryovial by pipetting up and down, without creating bubbles, then gently transfer entire suspension to the 15 mL conical tube. Very slowly add 10 mL of warm medium down the inside to the tube: one drop, then 2-3, then 4-6 drops, etc. with gentle hand mixing (tap tube on side) between each addition, for at least the first 5 mL. It should take 2-3 minutes to add all of the media.
4. Cap tightly, gently mix the cell suspension by slowly inverting the tube 2-3 times, then centrifuge for 5 minutes at around 1200 rmp. Re-wipe the tube with 70% EtOH (taking care not to disturb the cell pellet) and return to the hood.
5. Aseptically attach a sterile, glass, Pasteur pipet to the end of the vacuum line. Turn on the pump, uncap the tube and aspirate off as much medium as possible without disturbing the pellet.
6. Re-suspend the pellet in 2 mL of medium. Perform a cell and viability count. Determine the total live cells/mL, which will be used in the next step. Note: if cell number is already known proceed to step 8 and do not perform a cell and viability count.
7. Determine the volume of cell suspension ('V') that contains the appropriate # of live cells for the experiment. For subculturing 200,000 cells are needed for a T75 flask.
8. Plate your cells in a T75 flask with a final total volume of 10 mL:
    a. Place a T75 flask in the hood and add 10 - V mL ('V' from step 7) of media down the back of the flask. This is the large surface that will be on the bottom, when you set the flask down so that the canted neck is pointing upward; example: If you will be adding 0.5 mL of cells, you must put 10.0 - 0.5 = 9.5 mL in the flask. *Note: if V is less than 0.1 mL use a full 10 mL of media.*
    b. Gently re-suspend the cells, then remove (use an appropriate size pipet) the required volume of cell suspension determined in step 7 and place it in the T75 flask by gently dispensing the liquid down the back of the flask. If you do not have enough cell suspension, add all that you have.
    c. Cap the flask tightly (vented flasks only), mix and distribute the cell/media mixture by gently rocking the flask to coat the entire bottom with cell suspension (hold horizontally, with the canted neck slanting up). When finished, place the flask in the incubator. Aspirate any excess cell suspension, then clean the vacuum line by rinsing with 70% EtOH, and discard cryovial in biohazard can.

("Thawing cells", 2010)

APPENDIX E: CRYOPRESERVATION

1. Use freezing media with 70% DMEM, 20% DMSO, and 10% FBS.
2. Follow trypsin protocol for C2C12 cells through step 4.
3. Do a cell count and viability determination for this flask.
4. With the information from your cell count, calculate the volume of cell suspension which contains 2.5 x 10^5**LIVE** cells and remove it to a 15 mL conical, sterile tube. If you do not have enough cells, use the entire contents of your flask and determine the number of live cells actually used. Spin for 5 min. at about 1200 rpms. While the cells are pelleting, put a cryovial in the hood and label it with your initials, the date, the cell line, the total number of live cells, and the media it will contain.
5. Aspirate medium from pellet and resuspend in 1 mL of freezing medium. Working quickly, transfer the resuspended cells to the labeled cryovial and immediately place in a cold Nunc cooler, which will then be placed in the -80°C freezer until next lab period. Normally, for long term storage they would subsequently be moved to the liquid nitrogen dewar for storage.
6. Aspirate all remaining media from all containers then discard in biohazard waste.
7. Remove and discard the glass pipet in the aspirator line (sharps container on floor), then rinse the line with enough 70% EtOH to wash all media into the trap flask.
8. With the vacuum tube open turn off the pump.

("Cryopreservation," A2010)

APPENDIX F: TRYPSINIZING, COUNTING AND PASSAGING ADHERENT CELLS

1. Place a sterile, glass pipet on the end of the aspirator tube, turn on the vacuum pump and using the aspirator, remove all of the medium from the T75 flask. Add 5 mL of sterile PBS (Phosphate Buffered Saline), swirl to wash the surface, and aspirate this as well. This will remove dead cells and debris, as well as any components in the DMEM or serum, which might inhibit trypsin.
2. Add 1 mL of 0.25% trypsin, hold horizontally (cell layer down) and gently rock the flask to cover the entire layer of cells with trypsin. Let sit for 30 seconds, gently rock flask, then start looking at the cells by eye. Alternate gently rocking and observing until all of the monolayer has released and most of it has broken into small piece. At this point tap the side of the flask sharply with the heel of your hand 2-3 times. Small pieces should now separate into single cells. As soon as most of the monolayer completely breaks apart go to step 3.
3. Add 5 mL of proliferation media (DMEM+10%FBS) and vigorously wash the bottom surface of the flask, pipetting up and down. Squirt the suspension several times against the bottom of the flask, to break up all cell clumps. When you have a uniform suspension transfer it to a 15 mL conical tube and spin at about 1200 rpms for 5 minutes.
4. Aspirate off the medium, re-suspend the cells in 5 mL of proliferation media. Again, pipet up and down until no clumps are visible
5. Remove a 120-150 mL aliquot and do a cell and viability count (follow cell count procedure).
6. Determine the volume of cell suspension which contains 2 x 10^5 live cells. (Determine the volume of DMEM + 10% FBS needed to bring the final volume up to 10mL. Add the required volume of media to a clean T75 flask.
7. Re-suspend the cells in the tube from step 4 by gently pipetting up and down several times, then remove and add the required volume of cell suspension to the new flask. Tighten the cap, then holding the flask horizontally with the slanted neck pointing up, rock gently to cover the bottom with cell suspension.
8. Label flask with today's date, your initials, the cell line and media, then place in the incubator.
9. Aspirate any leftover culture. Discard the aspirator pipet and clean the aspirator line with 70% EtOH. Open aspirator line and turn off pump.

(Trypsonizing and plating of adherent cells", 2010)

APPENDIX G: MAKING MEDIA

Note: All media should be warmed prior to adding to cells. Cold media, from the refrigerator may kill your cells.

Proliferation Media
("Cell culture manual-Detailed", 2011)

Component	**Volume for 100ml**	**Final Concentration**
Penn Strep	1ml	1%
Glutamax	1ml	1%
FBS	10ml	10%
50%/50% DMEM/F12	88ml	88%
Final volume	100 ml	100%

Differentiation Media
("C2C12 differentiation," 2011)

Component	**Volume for 10ml**	**Final Concentration**
Horse Serum	200ul	2%
ITS	100 ul	1%
Glutamax	100 ul	1X (2mM)
Penn Strep	100 ul	1X (1%)
50%/50% DMEM/F12	9.5 ml	95%
Total volume	10 ml	100%

Freezing Media
("Cell culture manual-Detailed", B2011)

Component	**Volume for 10 ml**	**Final Concentration**
DMSO	2 ml	20%
FBS	1 ml	10%
DMEM	7 ml	70%
Total volume	10 ml	100%

1. Determine the total volume of media needed, then calculate the volume of each component which needs to be added to reach this total volume of media.
2. Pipet the required amount of media and components into a 50ml, 150ml, or 500 ml filter unit.
3. Filter sterilize the media into a clean, sterile container. Cap the containers and leave in the hood until you are finished.

4. Attach vacuum line to the Steriflip side-arm, turn on vacuum and continue pulling until all media has mostly filtered through. Turn off pump. Remove vacuum line when all media has been pulled into the lower tube (ignore bubbles).
5. Remove Steriflip filter and top tube; Throw away filter and top tube (biohazard trash). Cap the bottom (media containing) tube with the sterile cap provided in the Steriflip package.

("Making media", 2010)

APPENDIX H: CELL COUNTING

COUNTING CELLS VIA HEMOCYTOMETER

1. Prepare the hemocytometer: If dirty, clean the surface of the hemocytometer with 70% alcohol taking care not to scratch the semi-silvered surface. Clean and dry the coverslip, then place it over the grooves and semi-silvered counting area. Make sure both pieces are completely free of ethanol before placing the coverslip on the hemocytometer.
2. Use a 1 mL pipet to remove 0.12 - 0.15mL of cell suspensionand transfer it to an Eppendorf tube
3. Make a 1:2 dilution of cell suspension withtrypan blue as follows:
   a. Re-suspend the cells in your non-sterile tube by gently tapping the tube, and then pipeting the cell suspension up and down.
   b. Immediately remove 100 µL of the cell suspension and place in another non-sterile tube.
   c. Add 100 µL of trypan blue. Note, the dilution factor is now 2 since you diluted 1 volume of cells (100µL) in a total of 2 volumes final solution (100µL of cells + 100µL of trypan blue).
   d. Mix gently by pipetting up and down. The trypan blue will pass through damaged cell walls. So, blue cells are dead or dying and uncolored cells are living.
4. Load both chambers (sides) of your hemocytometer, with a single sample of 100 µL, and count a total of at least 100 cells or a minimum of one $1mm^2$ area ("large square") in each chamber (see fig. 1 and detailed instructions on the next page). Count total cells and dead cells for each area.

Note, if you cannot see any living cells adjust the light source diaphragm to decrease the amount of light passing through the slide (increases contrast).

("Cell counting", 2010; Freshney, 1994)

CELL COUNT CALCULATIONS

a. Calculate the average cell number in a 1-mm^2 area: add all counts on both halves of the hemocytometer and divide by the total number of 1-mm^2 squares counted. Do this for both total cells and dead cells.
b. Determine the number of live cells counted = $\frac{\text{ave. \# of total cells- ave. \# of dead cells}}{}$
c. Determine the concentration of cells c = (n/v) x d

c = concentration (amount of cells in a specific volume, the value you are calculating)
n = average number of cells
v = volume counted ($0.1mm^3$, or 1×10^{-4} mL)
d = dilution factor for the sample you counted
Thus, for a normal dilution factor of 2 (1 part cells + 1 part trypan blue): **$c = 2n \times 10^4$ cells/mL**
Notes:
a. Trypan blue is toxic, thus if you leave living cells in this mixture the cells will begin to die. This can lead to an underestimation of the % viability in your samples.
b. Partially because of the need to count cells quickly, some sick, non-viable cells may not have time to take up enough dye to turn blue. Thus, dye exclusion viability, when done correctly, tends to overestimate the percentage of cells that are truly viable.

("Cell counting", 2010; Freshney, 1994)

APPENDIX I: CELL PROLIFERATION ASSAY USING BROMO-DEOXYURIDINE (BRDU)

1. Add 1.0 µl of BrdU stock solution per ml of culture medium to cells being assayed and incubate for 4 hours or the time required by the experimental protocol.
2. Aspirate culture medium and wash cells in 2X in DPBS+.
3. Aspirate DPBS+ and add ice cold (-20C) methanol (1.0 ml/well for 24-well plate). Incubate for 10 min at -20C
4. Aspirate methanol and wash with 1.0 ml PBS for 10 min (plates can be stored at 4C with PBS in wells if analysis is not to be done right away.
5. Aspirate PBS and add 1.5 N HCl (0.5 ml/well for 24-well or 0.25 ml/well for 48-well plate) and incubate at RT for 20 min.
6. Wash 3x with PBS, 5 min each
7. If cells were cultured with serum, blocking is not necessary. If cultured in serum-free system, block at RT for at least 15 min with 5% FBS in PBS+0.05% Tween-20.
8. Dilute anti-BrdU antibody 1:100 in PBS +0.05% Tween-20.
9. Add antibody solution at 150 µl/well for 24-well plate or 75 µl/well for 48-well plate) and incubate at RT for 30 min.
10. Aspirate antibody solution and wash 3X with PBS for 5 min each.
11. Add fluorescent dye conjugated secondary antibody diluted 1:500 in PBS+0.05% Tween-20 (150 µl/well for 24-well plate or 75 µl/well for 48-well plate) and incubate at RT for 30 min.
12. Wash 3X with PBS (without Tween).
13. Add 0.5 µg/ml Hoechst 33342 to last wash (stock is 1 mg/ml) and incubate for 10 min at RT
14. Aspirate Hoechst solution, wash with PBS and add PBS (1.0 ml/well for 24-well or 0.5 ml/well for 48-well plate).
15. Cells are ready for observation by fluorescence microscopy. Plates can be stored at 4C wrapped in foil to protect from light.

(BrdU-Cell Proliferation Assay, 2011)

APPENDIX J: MYOSIN HEAVY CHAIN IMMUNOCYTOCHEMISTRY PROTOCOL

MATERIALS

- PBS (MediaTech Cat # 21-040-CM, VWR Cat # 45000-448)
- Tween-20
- DPBS+ Dulbeco's phosphate buffered saline with Ca/Mg, MediaTech Cat # 21-030-CV , VWR, Cat. # 45000-430)
- FBS (use blocking FBS, stored at -20C), or any bovine serum, or serum derived from species of the secondary antibody
- Alexafluor-488 conjugated anti-mouse IgG (Invitrogen), or Alexafluor-568 conjugated anti-mouse IgG (Invitrogen)
- Antimyosin antibody (mouse IgG), MF20 from Developmental Studies Hybridoma Bank, stored at 4 C
- Ice cold methanol (JT Baker, Cat 9070-13, VWR Cat # JT9070-13, 250 ml aliquot stored at -20C)
    - PBS with 0.05% Tween-20 (PBS/Tween)
- Cells grown in 24-well plates
- Glycerol DNase, RNase, protease free (Shelton Scientific, Cat # IB15672, VWR Cat # IB15762)
- Hoechst 33342 (EMD Cat # 382065-100MG, VWR Cat # 80056-706)

PROCEDURE

1. Aspirate culture medium and wash cells with DPBS for 1 min.
2. Aspirate DPBS and add 1 ml/well ice cold (-20C) methanol and let stand for 10 min.
3. Aspirate methanol and add 1.0 ml/well PBS and store plates at 4 C until ready for staining

4. Aspirate PBS and add blocking solution (PBS with 3% FBS or appropriate serum) at 150 µl per well and incubate at RT for 30 min.

5. Aspirate blocking solution and add primary antibody (1:500 dilution in PBS/Tween) at 150 µl per well and incubate at RT for 30 min. Secondary antibody-only controls will skip this step.

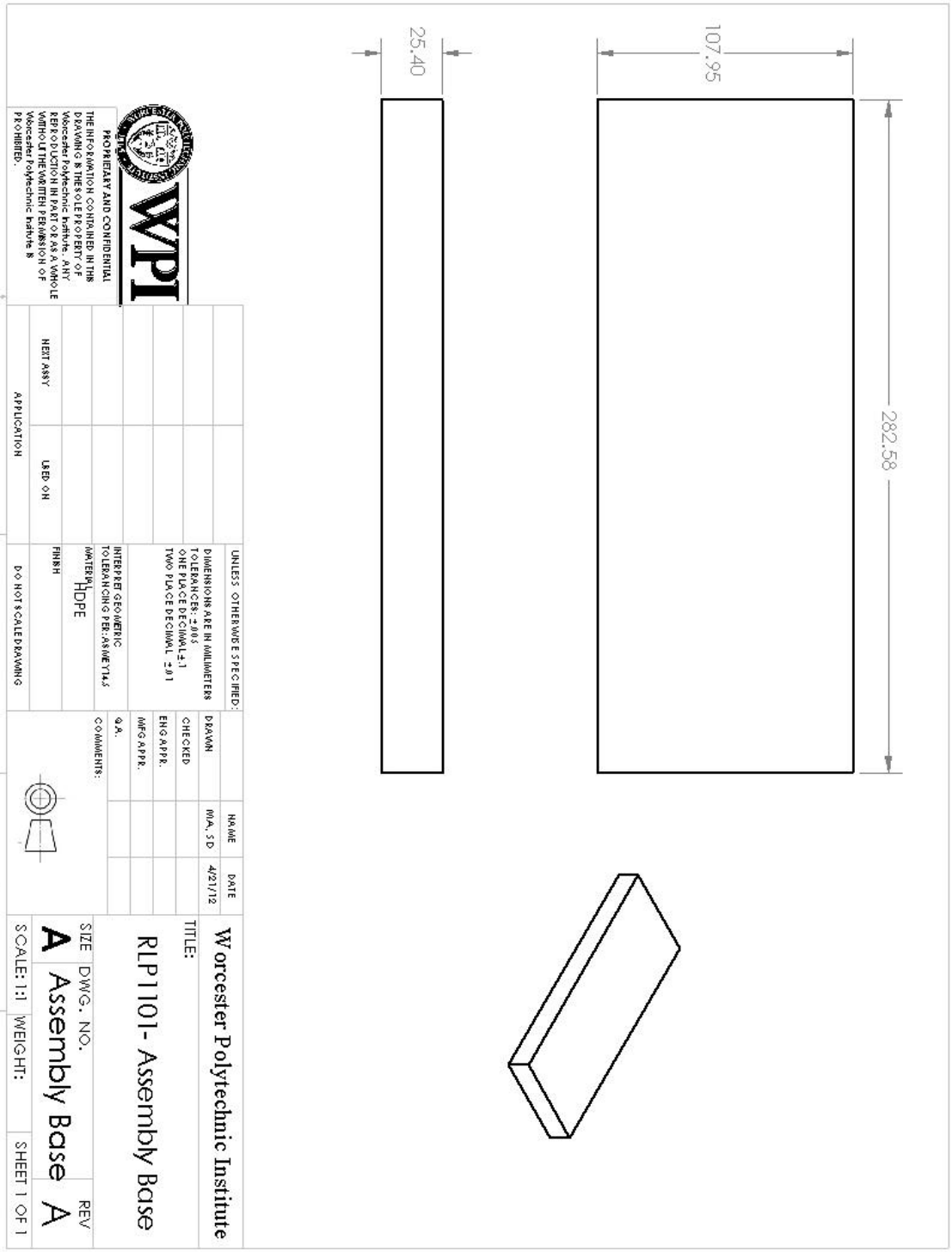
6. Aspirate primary antibody and wash 4X with PBS for 3 min each wash.

7. Aspirate PBS and add secondary antibody (diluted 1:500 in PBS/Tween) at 150 µl/well and incubate for 30 min.

8. Aspirate secondary antibody solution and wash 4X with PBS. If nuclear counterstain is desired, add Hoechst 33342 at 0.5 µg/ml in PBS to last wash and incubate for 10 min. Aspirate Hoechst and add 1 ml/well PBS.

9. Cells are ready for observation by fluorescence microscopy. Plates can be stored at 4C wrapped in foil to protect from light. If long term storage is desired replace PBS with PBS containing 0.1% Na-Azide to prevent bacterial growth.

APPENDIX H: TECHNICAL DRAWINGS OF MECHANICAL STIMULATION ASSEMBLY



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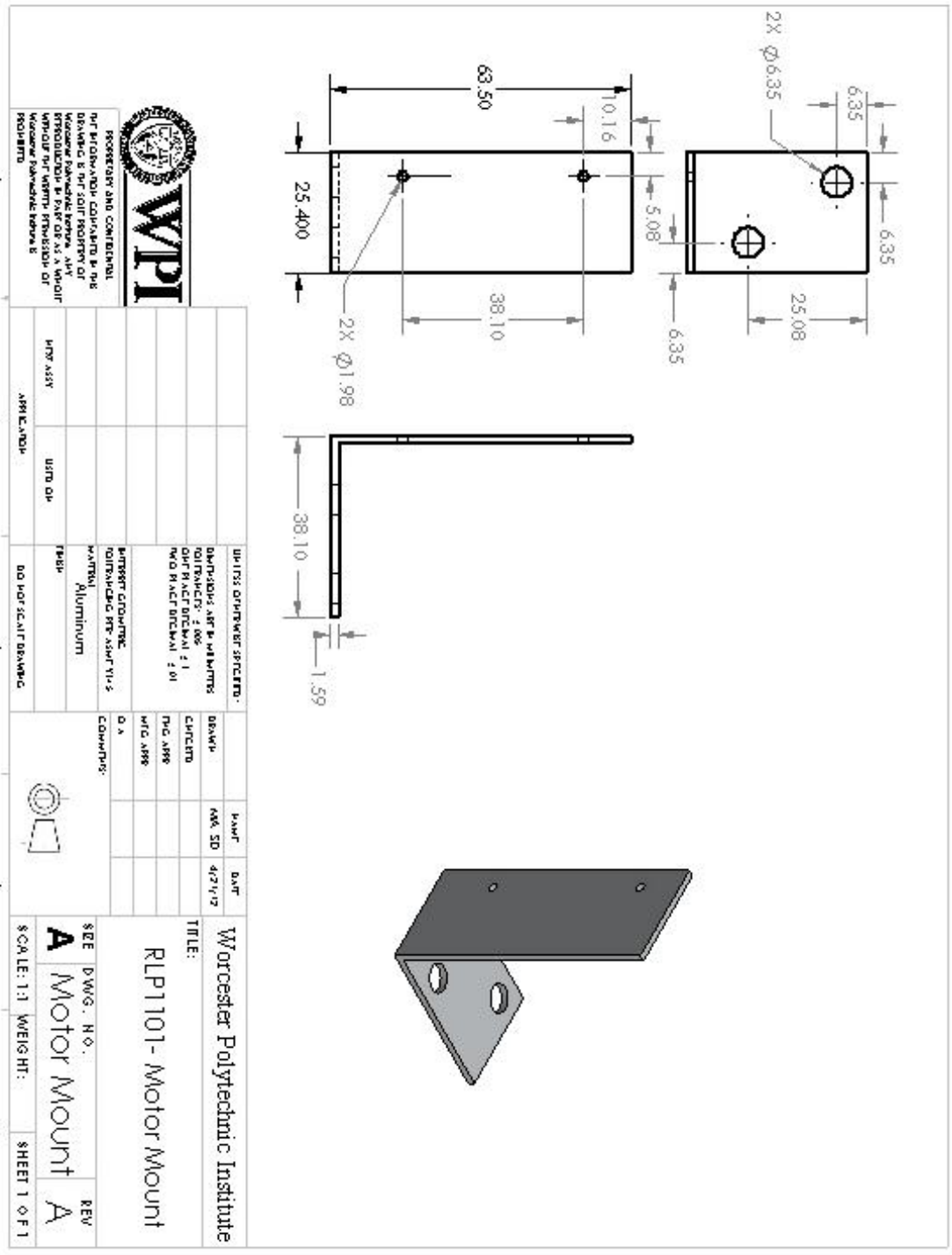

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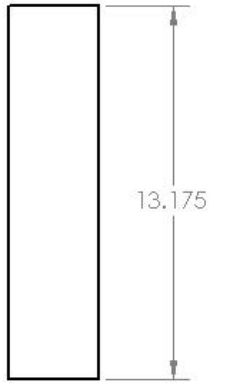

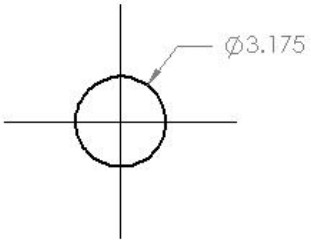

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Worcester Polytechnic Institute

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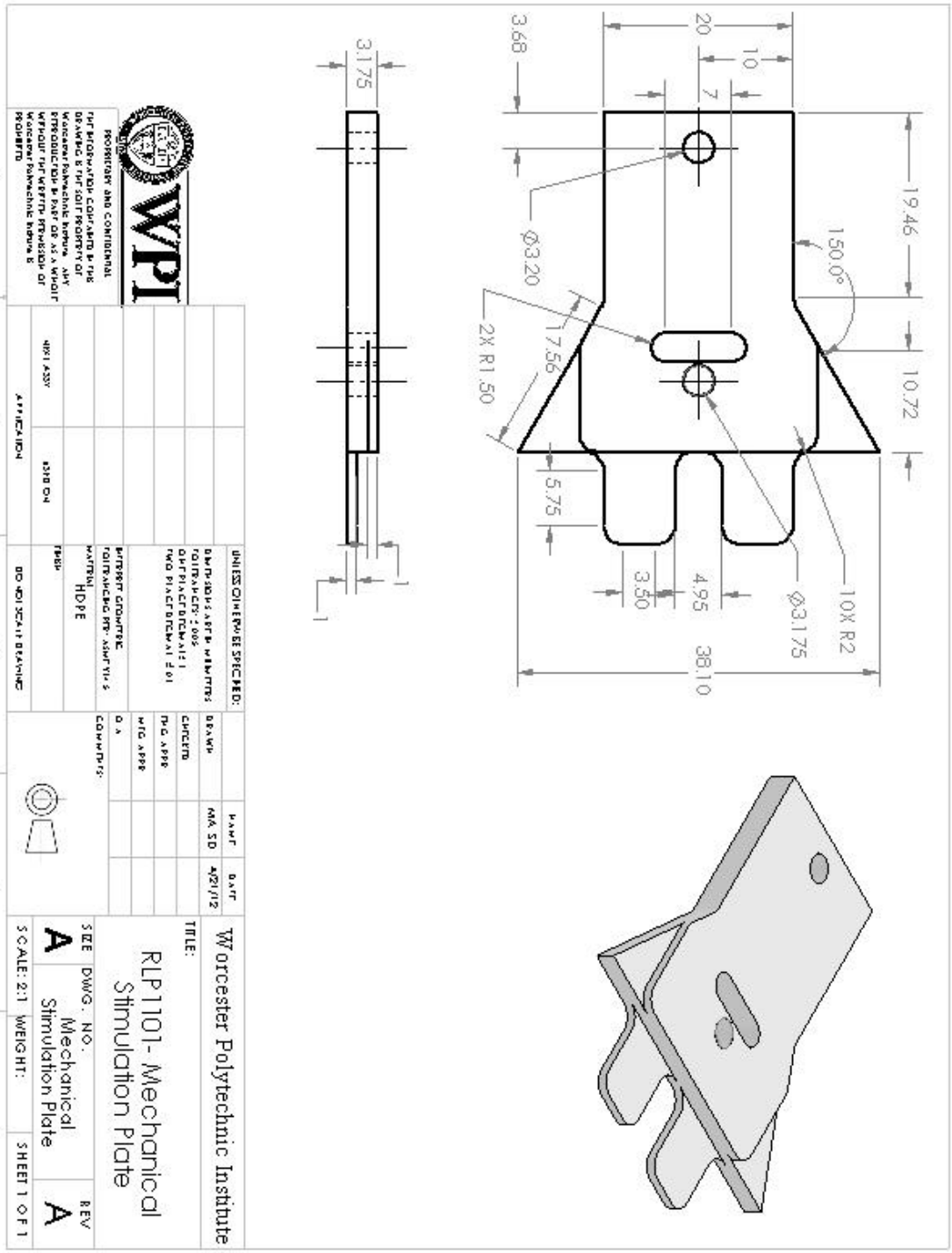

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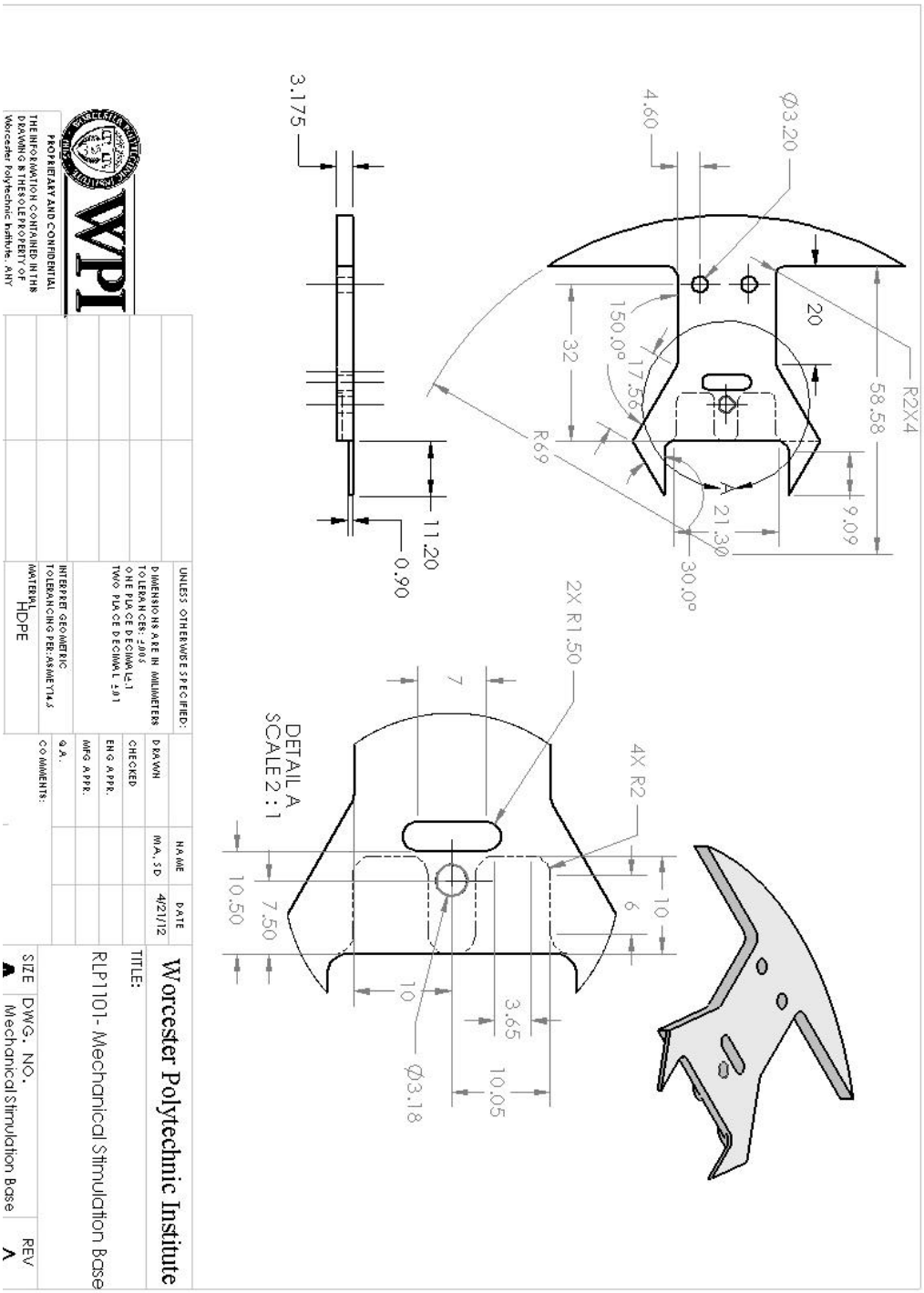

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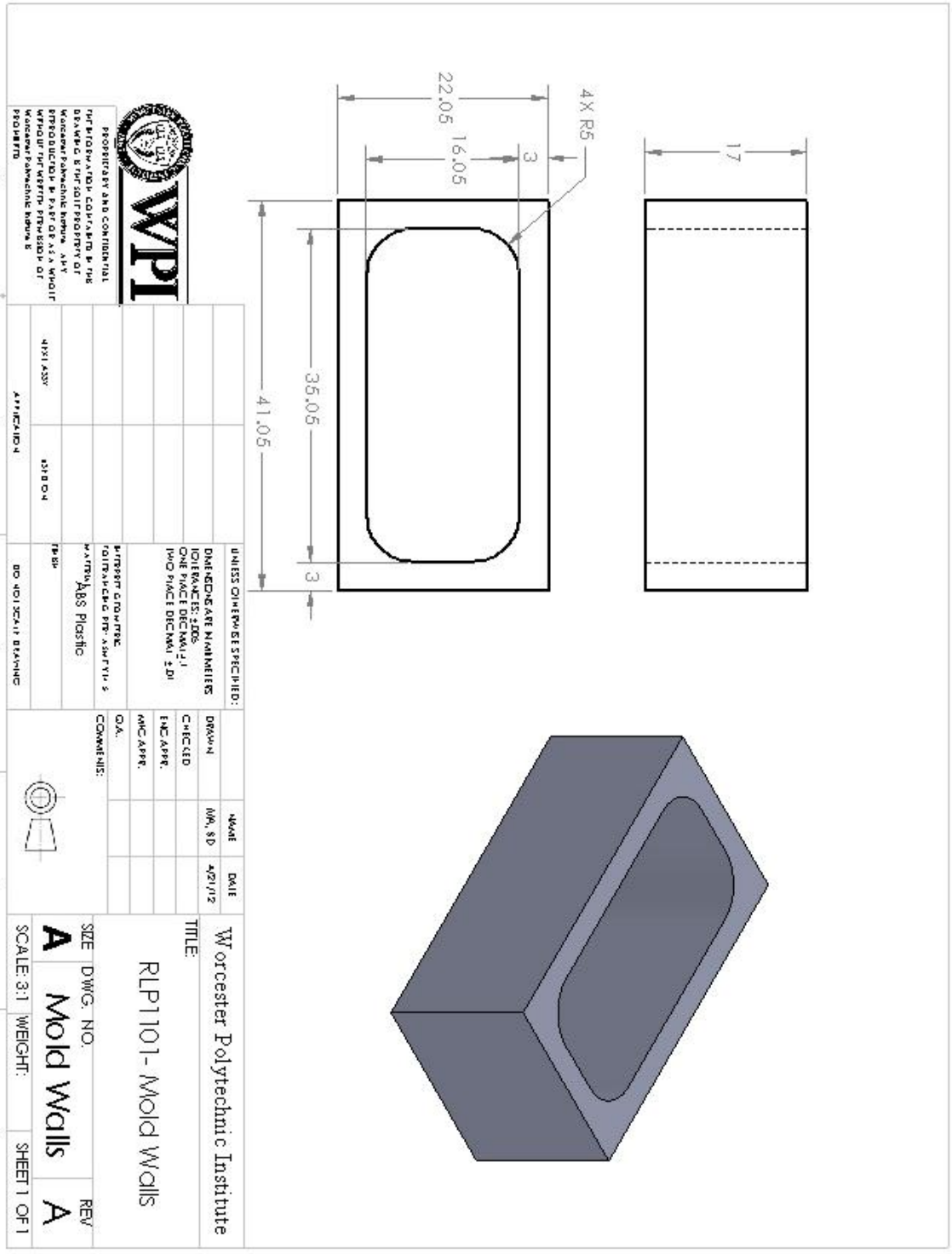

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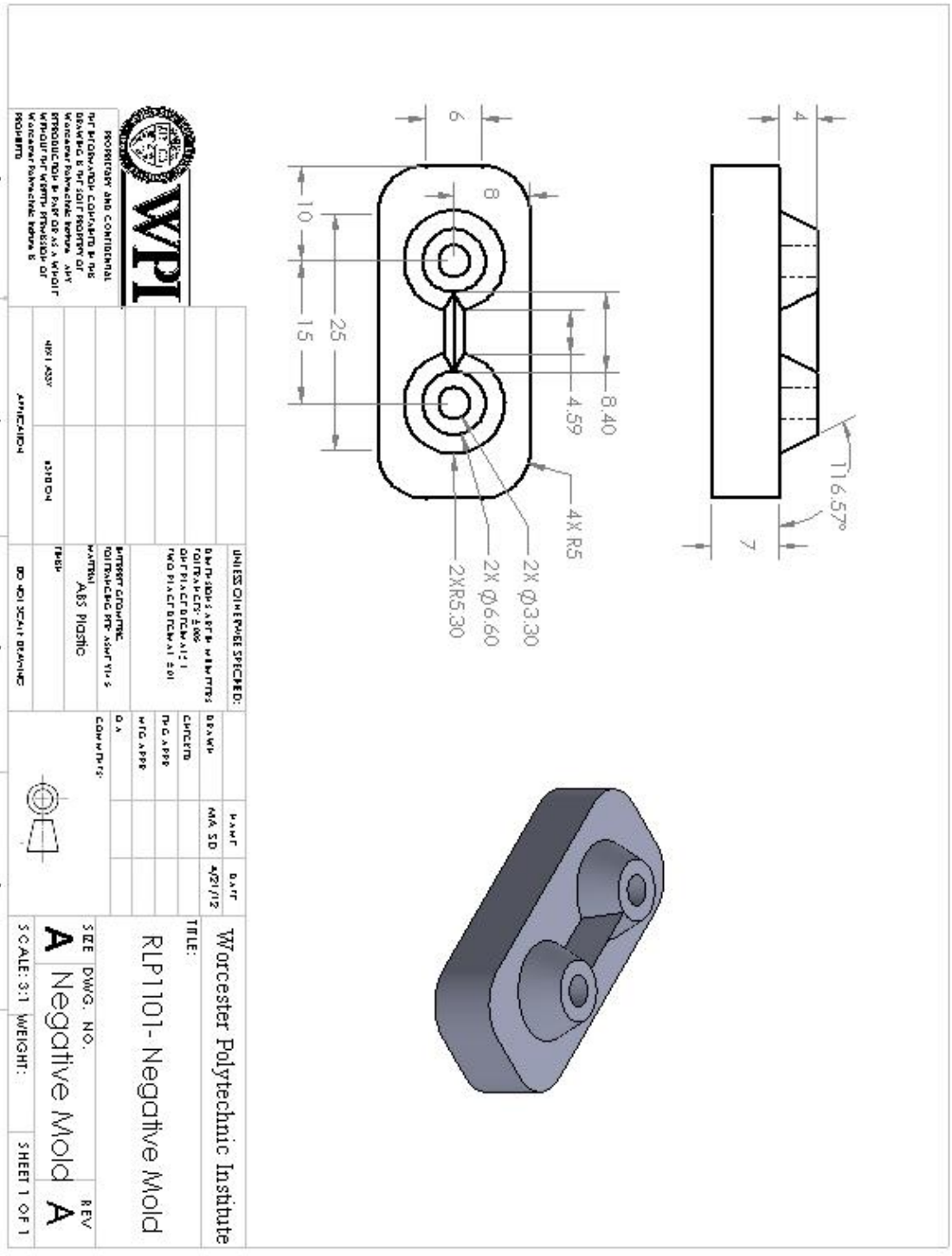

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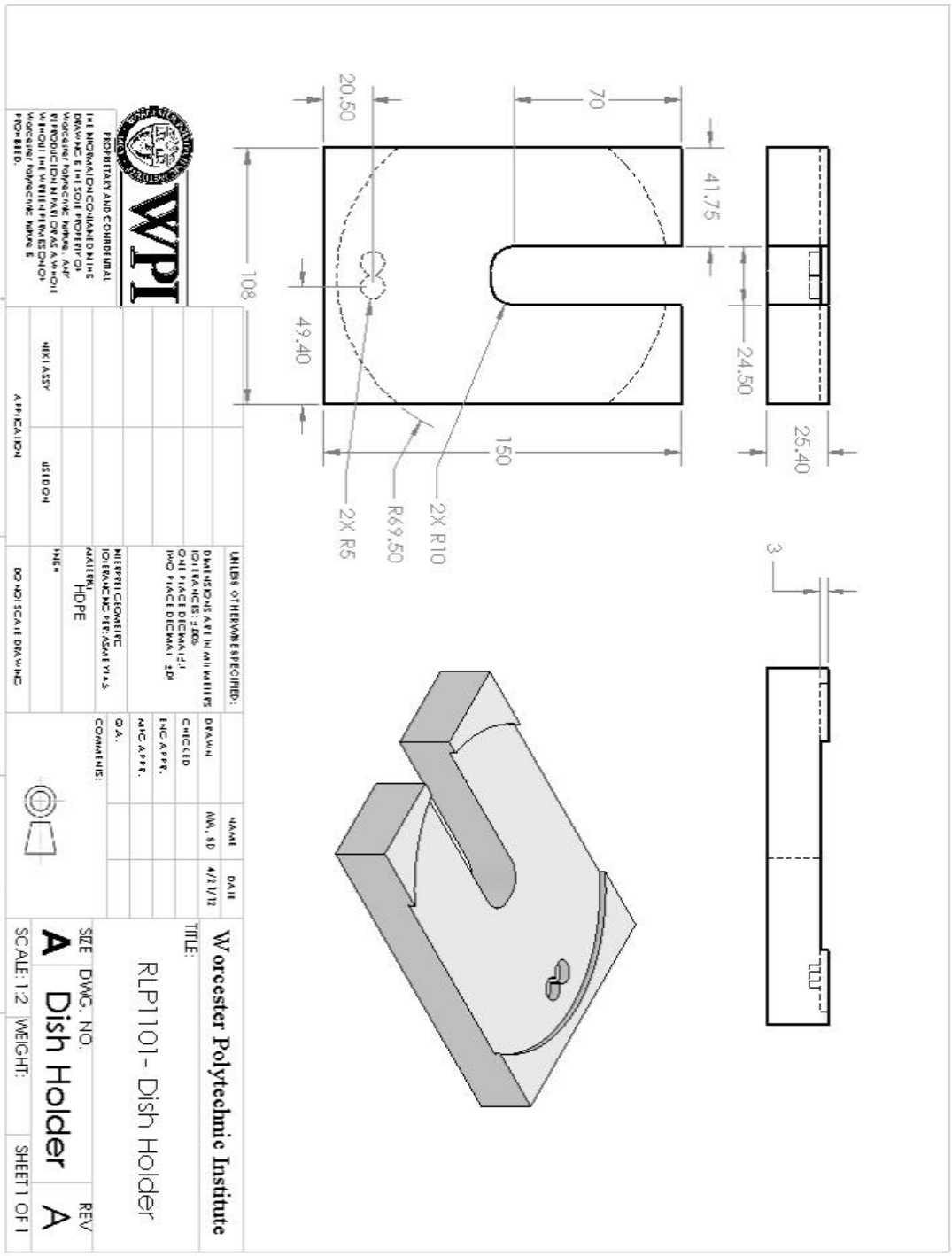

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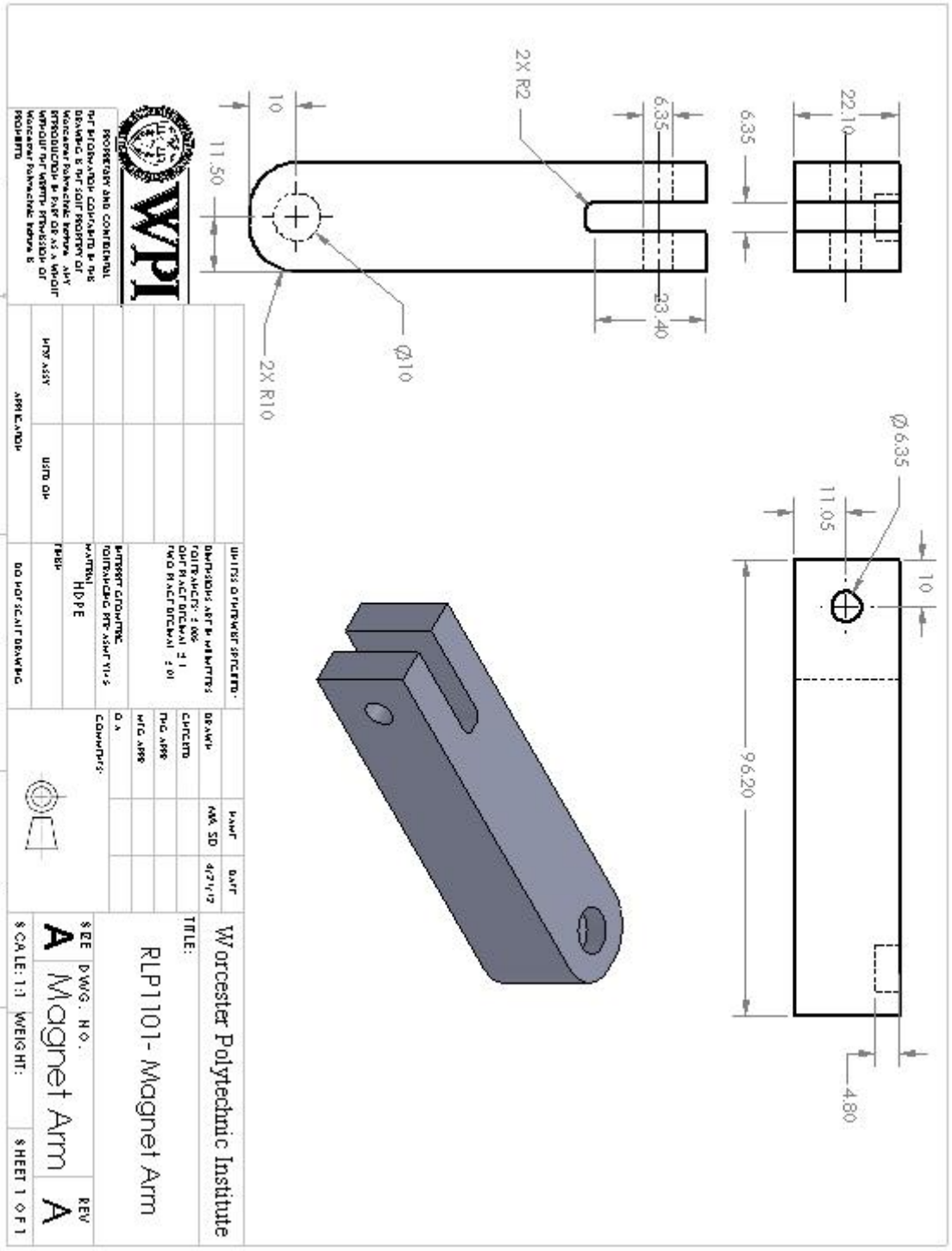

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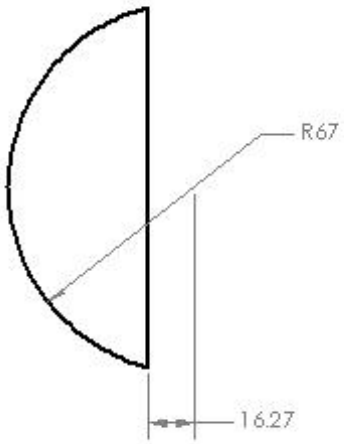

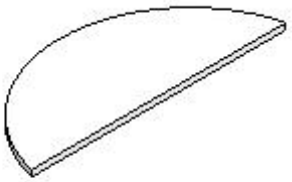

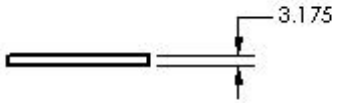

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Worcester Polytechnic Institute

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