Project Number: RLP 1001

Design of a Molding System to Recapitulate Skeletal Muscle Fiber

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

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Abstract

Systems for the development of skeletal muscle tissue *in vitro* can be used to study muscle tissue development and pathophysiology. Many systems for the development of myofibers involve the presence of a scaffold, which can be a physical barrier to cell proliferation and cause incongruities within the myofiber. We developed a system by which myogenic cells can be seeded within a mold to form a myofiber without the need for a scaffold. A "dog" bone shaped double negative mold made of acrylic was used to form a final agarose mold. Experiments were conducted to compare the seeding of only C2C12 mouse myoblast cells versus myoblast cells embedded in fibrin or collagen gels. Tissue formation in the no-gel system was observed one day post-seeding, while minor incongruities were seen in the fibrin gel system and major breaks in the tissue were found in the collagen gel system. C2C12 cells were characterized using BrdU staining to find the optimal time point after induction of differentiation for seeding and showed that differentiation occurs at Day 4 post-induction. Myosin staining of C2C12 cells seeded on plates confirmed maturation of the formed myofibers. Further studies in optimizing the molding system should focus on improved manufacture of the mold to enhance the long-term stability of the myofiber.

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Chapter 1: Introduction

In all individuals, skeletal muscle damage and repair occur continuously when physical stresses from daily activities are exerted on the muscle. For the muscle to repair itself, satellite cells, which are progenitor cells found within muscle fibers, are released from the basement membrane and are activated to proliferate, then differentiate and fuse into myotubes. Some of the satellite cells remain undifferentiated to maintain the sateliite cell pool. The new myotubes then integrate with the surrounding undamaged skeletal muscle and then mature into myofibers. Ideally, the newly formed skeletal muscle will proceed to become innervated and mechanically linked to bone via tendons in order to resume full functionality.

On a large scale, skeletal muscle damage can be caused by atrophy, disease, or injury. In these cases, the extent of the damage often makes it difficult for the muscle to naturally regenerate completely. During the repair process, fibroblasts deposit collagen and other extracellular matrix proteins, which contribute to the formation of scar-tissue. If fibrosis occurs, the repaired muscle will have significantly decreased functionality since the scar tissue affects the strength of the muscle contraction and the elasticity of the muscle. Transplantation of muscle fibers into the damaged region can allow the fibers to incorporate with the existing fibers and potentially reduce the amount of fibrosis that occurs during the natural regeneration process.

The current system of creating muscle fibers involves seeding of cells onto collagen or fibrin microthreads using a rotational seeding system. However, the effectiveness of this system is limited by the inability of the cells on the surface of the microthread to proliferate throughout the center of the microthread to accurately recapitulate the structure of a muscle

fiber. Thus, creating a system that involves a channel within a mold that will guide cells with myogenic potential to align in the appropriate formation to develop muscle fibers may be a more effective method of creating muscle fibers that are comparable to those found within the body. The molding system would also allow for precise control over physical characteristics of length and width of the myofiber and will provide optimal uniformity of mechanical properties through the length of the myofiber.

By enclosing the cells within a biological gel matrix and seeding this gel within the seeding channel, the cells will receive the benefits of the biological cues provided by the gel and will be able to degrade the gel over time as they form their own ECM which penetrates throughout the volume of the channel. Two anchor points, one on either end of the seeding channel, will provide support for the cells to grow around and pull on each other to maintain tension throughout the fiber. This tension will promote optimal alignment of the myofiber and will prevent collapse of the myofiber as the cells contract upon each other.

In the following chapters of the project report, a summary of current approaches used in skeletal muscle regeneration and potential future advancements is presented. The report also describes the project strategy by including a breakdown of the client statement presented to the design team and the approach that the design team took to develop a solution to the client statement. The report then describes alternative designs that were explored during the design process and the analytical tools that were used to evaluate and determine the design that best meets the criteria established by the client and the user. The design verification chapter of the report presents data and findings from the testing of the system. This section is followed by a discussion of the results and the final design validation. The next chapter relates

the group's results in context of the current approaches discussed in the literature review. Also, the report contains experimental methods for validation of the design that can be used by future project groups. The final section contains conclusions about the results and recommendations for future work.

2. Literature Review

2.1 Structure of Skeletal Muscle

The human body is characterized by 660 skeletal muscles, which are composed of hundreds to thousands of individual muscle fibers. The smallest muscle in the body, the tensor tympani, which is found in the ear, contains a few hundred muscle fibers, while the largest muscle of the body, the medial gastrocnemius muscle of the calf is comprised of over a million muscle fibers (MacIntosh, Gardiner, & McComas, 2006). The length of each muscle fiber depends on the number of sarcomeres in series that make up the fiber. The length of the longest muscle fiber found in humans is 12 centimeters, so any muscles that are longer than 12 centimeters are made of series of multiple fibers (MacIntosh, Gardiner, & McComas, 2006).

Figure 1: Human Skeletal Muscle System. Image from anatomyposter.com

2.1.1 Structural Hierarchy

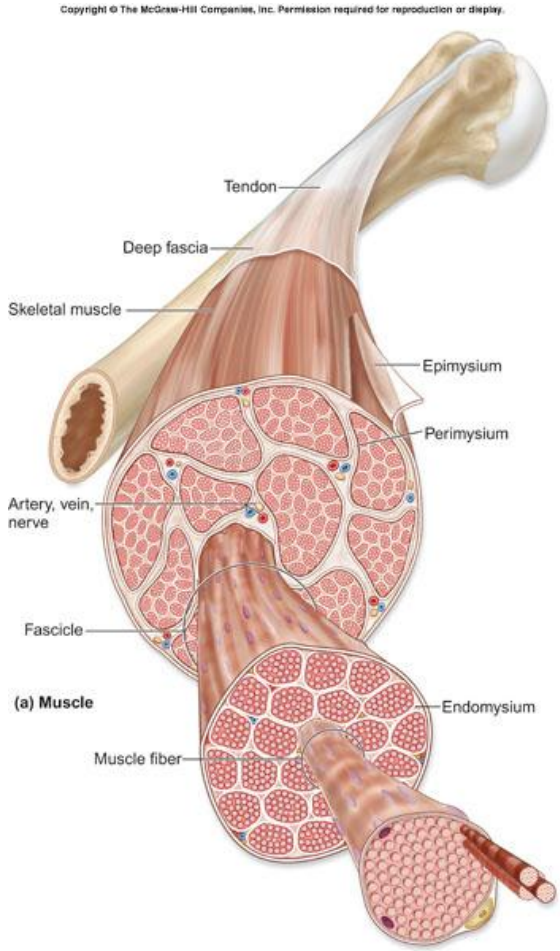


Figure 2: Structural Hierarchy in a Skeletal Muscle

Each muscle fiber, or myofiber, is composed of several nuclei, organelles, a sarcolemma, and hundreds of myofibrils. Surrounding the muscle fiber is the sarcolemma, which consists of the basement membrane and plasmalemma. The plasmalemma is the plasma membrane that immediately surrounds the muscle fiber and functions as both an anatomical barrier and a selective barrier between the cellular contents and the surrounding connective tissue. Anatomically, it separates the aqueous solution containing inorganic ions, sugars, amino acids, peptides, and proteins found within the muscle cell from the connective tissue. The

plasmalemma acts as a selective barrier as it contains protein channels and pumps to maintain the appropriate protein concentrations inside and outside of the muscle fiber.

Immediately surrounding the sarcolemma is the basement membrane, composed of the basal lamina and the reticular lamina (Sanes, 2003). The functions of the basement membrane involve providing mechanical support to the cells and providing a scaffold for cells during the development and regeneration processes. The basement membrane also has a significant role in helping the sarcomere to provide contractile abilities to muscle and in providing elasticity to the muscle through the extracellular matrix (Sanes, 2003). Between the basal lamina and the plasmalemma lie the stem cells responsible for muscle regeneration, the satellite cells.

Myofibrils found within individual myofibers are responsible for producing the motions that result in muscle contraction and force production. Myofibrils are thread-like structures aligned along the axial direction of the myofiber, which are mainly characterized by two contractile filaments: actin and myosin. These two proteins are the components of the myofibril that are responsible for the elastic and contractile properties of muscles. Actin and myosin filaments are assembled into functional units called sarcomeres, which appear as longitudinally repeating banding patterns.

2.1.2 Sarcomere Structure

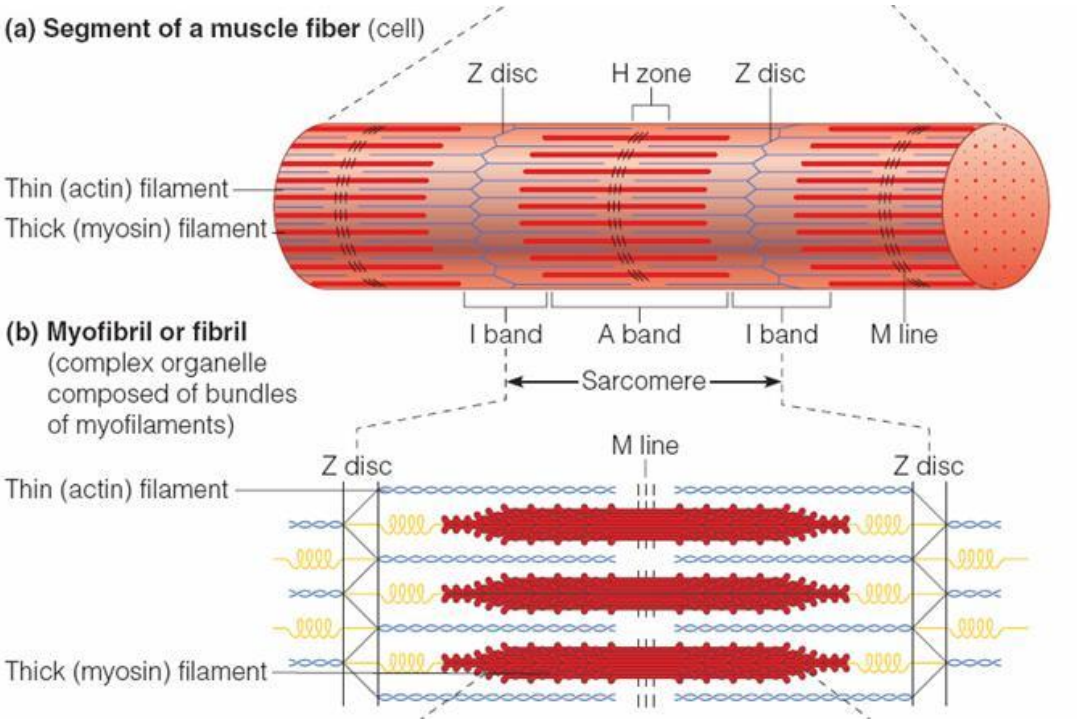


Figure 3: Structure of a Sarcomere. Image from shoppingtrolley.net

The sarcomere itself is characterized by different structures based on the arrangement of actin and myosin filaments. The Z-lines mark the boundaries of each sarcomere and anchor the actin filaments at each end. The A band represents the length of the myosin filaments and contains an H zone in its center, which represents the region where there are no actin filaments overlapping with myosin filaments. The I-band represents the region of the sarcomere where there are no myosin filaments overlapping with actin filaments.

This particular arrangement of bands is what gives muscle cells their striated appearance. Besides the aforementioned contractile units, the sarcomere has supporting protein structures; titin and nebulin. Titin is a filamentous protein which is anchored to the Z-disk at one end, and to the M-line on its other end. This structural assembly allows anchorage of the myosin filaments, which contributes to their structural stability. Nebulin is another

filamentous protein located on the I-band and which provides structural and mechanical support to actin filaments (Liu, 2007).

2.1.3 Extracellular Matrix

The extracellular matrix of muscle fibers includes both interstitial connective tissues and proteins. There are four main functions of the extracellular matrix: (Liu S. Q., 2007)

1. Support and organization of cells, tissues, and organs
2. Shape formation of tissues and organs
3. Mechanical strength and protection of tissues and organs from injury
4. Regulation of cell adhesion, proliferation, migration, and apoptosis

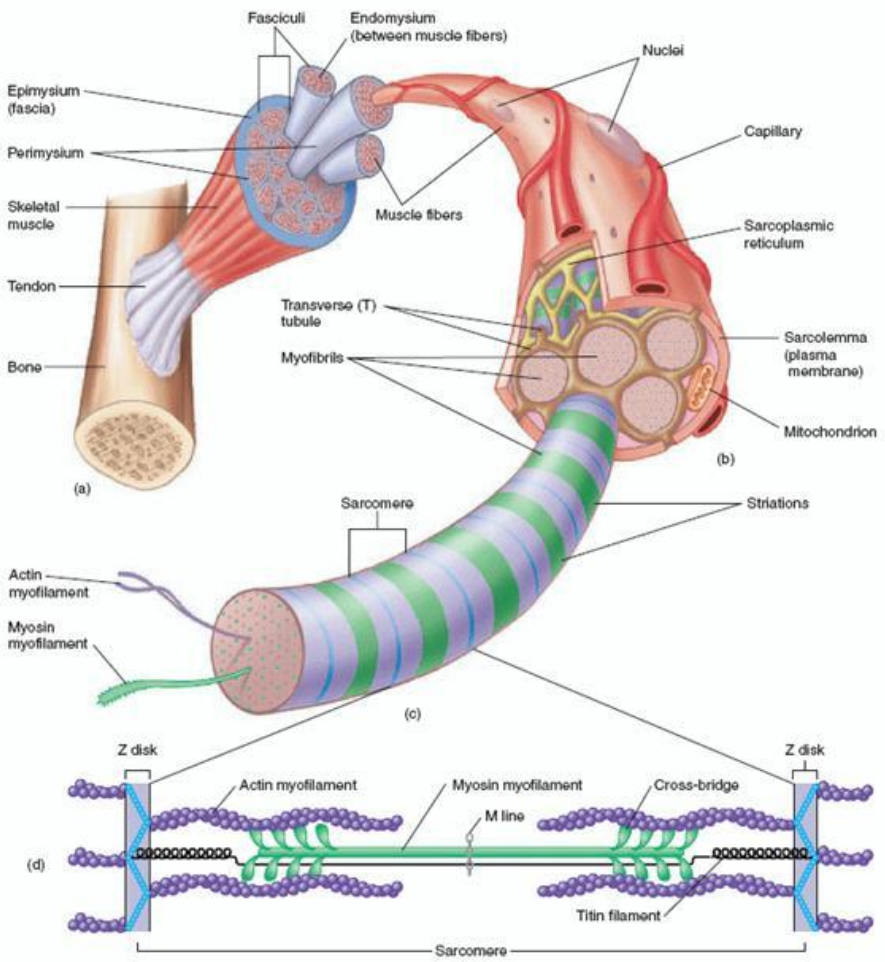


Figure 4: Muscle Structural Hierarchy Including Connective Tissue

2.1.3.1 Interstitial Connective Tissues

The interstitial connective tissues make up from 1% to 10% of muscle tissue and function to support surrounding blood vessels and nerves and to translate the force generated within the myofiber into a muscle movement (Schiaffino & Partridge, 2008). The types of connective tissue found in skeletal muscle are the epimysium, perimysium, and endomysium. The epismysium is a layer of connective tissue that surrounds the entire muscle, the perimysium surrounds a fascicle, or bundle of myofibers, and the endomysium surrounds individual myofibers.

Collagen fibrils, which are abundant in these connective tissues, provide mechanical strength to the tissues. Collagen is characterized by polypeptide chains organized into a triple helix structure. There have been twenty-seven types of collagen identified in vertebrates but the three main types of fibrillar collagen are types I, II, and III (MacIntosh, Gardiner, & McComas, 2006). Out of these, collagen type I makes up the majority of interstitial connective tissue and tendons. Collagen type IV is an integral component of the basement membrane and binds collagen types I and II in order to securely anchor the interstitial connective tissue to the basement membrane (MacIntosh, Gardiner, & McComas, 2006).

2.1.3.2 Proteins

Adhesion of cells to collagen is facilitated by glycoproteins that are found within the extracellular matrix. One glycoprotein that has a role in cell adhesion and migration is fibronectin when it is in its insoluble fibrillar form. On one side, fibronectins bind to cells via receptors on the cell membrane matrix known as integrins, and on the other side, fibronectins

bind to collagen fibrils. The binding of collagen and fibronectin to the muscle fiber is the first step in the processes of cell adhesion, cell proliferation, and cell migration (Liu S. Q., 2007).

Another class of glycoproteins is the tenacins, which have a role that counters the effects of fibronectin. Tenacin-C (one of the four members of the tenacin family) is an anti-adhesive for cells and is also found to increase dramatically in concentration when the muscle is bearing a large mechanical load or has been damaged. Tenacin-X is expressed in high concentrations in the extracellular matrix during muscle development, and Tenacin-W and Tenacin-R are both involved in re-innervation and neural function relating to skeletal muscle (Schiaffino & Partridge, 2008).

Integrins also bind to laminins, which are proteins that assemble with collagen type IV to form networks within the basement membrane (Schiaffino & Partridge, 2008). Laminins also bind to the dystrophin glycoprotein complex (DGC) which forms a link between the muscle fiber and extracellular matrix for transmission of the force produced by sarcomere contraction (Schiaffino & Partridge, 2008).

Other components of the extracellular matrix include glycosaminoglycans (GAGs). Those found in the extracellular matrix of skeletal muscle are heparan sulfates, chondroitin sulfates, and dermatan sulfates. Heparan sulfate is the main GAG found in the basement membrane of skeletal muscle and functions as a regulator of muscle development, normal function, and regeneration (Schiaffino & Partridge, 2008).

2.2 Causes for Skeletal Muscle Loss

2.2.1 Sarcoma

Skeletal muscle injury may occur as the result of disease, trauma, ischemia, exposure to myotoxic agents or to excessively hot or cold temperatures, and rapid, explosive muscle contractions. One of the major causes of skeletal muscle loss is the removal of rhabdomyosarcoma, or a sarcoma of skeletal muscle. The most common treatment is surgical removal of the sarcoma, during which the surgeon must ensure that no cancerous cells remain in the area to minimize the possibility of a recurrence. Since the procedure is highly invasive and requires the removal of a significant amount of tissue, patients often require reconstructive surgery to rebuild the lost skeletal muscle (National Cancer Institute website, 2007).

2.2.2 Compartment Syndrome

Another common cause of skeletal muscle loss is compartment syndrome in which swelling or bleeding occurs in a muscle, causing pressure within the muscle and surrounding nerves and capillaries to rise to an extreme level. This pressure buildup impairs the normally continuous blood flow to the muscle, so oxygen is unable to reach muscle cells and nerves. Acute compartment syndrome, which usually develops as the result of a serious injury such as a fracture or a bruise from a severe accident, can often result in permanent tissue death. Treatment of acute compartment syndrome usually requires a surgical procedure in which the surgeon makes an incision in the skin and fascia of the affected area and closes the skin incision once the swelling subsides.

Chronic compartment syndrome can be caused by overexertion of a muscle, usually by an athlete, and can often be relieved by discontinuing the exercise that is causing the swelling.

Treatment options include physical therapy and anti-inflammatory drugs. If the pain and swelling does not respond to the non-surgical treatment, surgical intervention is also an option. The surgical treatment of chronic compartment syndrome involves the same procedure used for acute compartment syndrome, but can often be treated with a smaller incision (American Academy of Orthopaedic Surgeons, 2009). In both forms of the disease, surgical treatment leaves an incision which must be repaired for the muscle to become fully functional again.

2.2.3 Traumatic Injury

Other major causes of skeletal muscle loss are traumatic injuries, like those seen in soldiers injured on the battlefield. When an injury is too severe for the muscle to fully repair itself but not severe enough to require amputation of the limb, the patient will have decreased functionality to a degree depending on the severity of the injury. In injuries involving the loss of a relatively small amount of muscle tissue in the elbow or forearm, tissue transfer from another area of the body can be performed (Merritt, 2009). However, this is not a viable solution for injuries that involve loss of a greater amount of skeletal muscle since the amount of tissue needed for the transplant cannot be removed from another part of the body. In these larger muscle injuries, neither natural repair mechanisms nor tissue transplants are sufficient methods for repairing the tissue, so other repair mechanisms must be introduced for functional recovery.

2.3 Process of Skeletal Muscle Regeneration

The degree of skeletal muscle regeneration is dependent upon the nature of the injury, but the process of myofiber repair and regeneration follow relatively the same pathway regardless of how the injury arose.

When a large portion of a myofiber's sarcomere is disrupted, the myofiber becomes necrotic. Two outcomes can follow this scenario. If blood flow is hindered, fibers will become a necrotic mass of non-contractile tissue. On the other hand, if the blood supply is not interrupted, the regeneration process follows the process outlined in three major stages:

1. Inflammatory response
2. Activation, proliferation, differentiation, and fusion of satellite cells
3. Development of myofibers

(Ciciliot & Schiaffino, 2010)

During the inflammatory reaction to necrosis, the plasma membrane breaks down and an influx of calcium into the myofiber causes breakdown of the myofibrils (Ciciliot & Schiaffino, 2010). This process attracts leukocytes, neutrophils, and macrophages of the immune system to the necrotic site (Ciciliot & Schiaffino, 2010). They remove the debris remaining from the breakdown of the myofibrils and are also responsible for secreting anti-inflammatory factors to signal the end of the inflammation phase. This usually occurs between two to four days after the initial injury to the muscle (Ciciliot & Schiaffino, 2010).

The second phase of the regeneration process begins on the second day after injury with the activation of satellite cells by various signaling factors. Satellite cells begin dividing mitotically, after which they differentiate into myoblasts and fuse with one another as well as with unimpaired segments of the fiber. It is very important that there is cell-to-cell contact between myoblasts since the fusion of myoblasts is dependent on physical proximity of the myoblasts. This fusion causes the differentiation of the myoblasts into myotubes, which are a

new source for myonuclei. Throughout this process, myoblasts also fuse and form multinucleated myotubes inside the basal lamina of damaged myofibers.

This is followed by the production of muscle-specific proteins and maturation of myotubes into mature myofibers. The proliferation and differentiation of satellite cell-derived myoblasts occurs rapidly, resulting in the formation of plenty of myotubes in as little as 3 to 4 days (Brooks and Faulkner, 2003). During this process, satellite cells are constrained between the myofiber and basal lamina. This helps to prevent migration of the satellite cells away from the area of damage and helps to ensure that, once formed, the myofibers produced are oriented properly with respect to the existing myofibers (Sanes, 2003).

There are several factors involved in this myofiber regeneration process, all of which together regulate the proliferation, fusion, and differentiation of myoblasts. The initial injury to the muscle promotes the release of growth factors, which bind to ECM proteins. Two of these factors are FGF-2 and HGF, both of which promote proliferation and inhibit differentiation of myogenic progenitor cells. After sufficient time has passed to allow for adequate proliferation of myogenic cells, IGF-1 is expressed. IGF-1 promotes differentiation of myogenic cells and also enhances protein synthesis of the differentiated myofiber by activating a translation factor and inhibiting protein degradation.

In the third and final phase of muscle regeneration, the myotubes develop further and may become revascularized and reinnervated by blood vessels and nerves, respectively (Ciciliot & Schiaffino, 2010). Ideally, the regenerated myofibers will be oriented in the same fashion as the natural myofibers but there is still the possibility that the regenerated tissue will be structurally different from the native skeletal muscle tissue since a significant amount of

remodeling occurs during the regeneration process (Ciciliot & Schiaffino, 2010). If the fibers do not fuse fully with each other, separate clusters of fibers or forked fibers may form (Ciciliot & Schiaffino, 2010). If satellite cells are able to migrate out of their location between the myofiber and basal lamina, individual muscle fibers may form within the interstitial tissue (Ciciliot & Schiaffino, 2010).

Highly severe injuries result in the complete deterioration of the myofiber, leaving only the basal lamina in place. Preserving the integrity of the basal lamina is vital for skeletal muscle regeneration as it functions as a scaffold for the formation of new myofibers while minimizing fibrosis. However, in injuries where the basal lamina is heavily damaged, muscle regeneration can still take place. Without a basal lamina to provide support during regeneration, the myotubes formed will not be oriented parallel to each other, so the net force produced by contraction of the muscle may be insignificant (Sanes, 2003).

For the muscle tissue to completely recover function as well as structure, it must be reinnervated by axons forming new neuromuscular junctions. Though the first steps of skeletal muscle regeneration can occur without nerve activity, reinnervation has been shown to have effects on protein turnover rate, gene expression, and the proliferation and differentiation of satellite cells (Schiaffino & Partridge, 2008). When damage to the skeletal muscle tissue is minimal, axons regenerate at the original synaptic site 95% of the time (Sanes, 2003). The basal lamina of the nerve is thought to cause axon regrowth along the same path through connective tissue as the original nerve (Sanes, 2003).

Reinnervation occurs more completely and successfully when the basal lamina of the muscle is intact since it provides structural support and plays a role in promoting regeneration

(Schiaffino & Partridge, 2008). The neuromuscular junction and basal lamina are thought to take part in promoting the differentiation of a region on the regenerating myofiber into a postsynaptic terminal since studies have shown that this process occurs more rapidly during regeneration than during normal development (Schiaffino & Partridge, 2008).

2.3.1 Properties of Native Skeletal Muscle Fiber

The properties of a native skeletal muscle fiber that allow for its contractile functionality in the body include its elastic modulus, tensile strength, and percent elongation until failure. There are mechanical forces provided by bone *in vivo* that mechanically stimulate the developing myofiber to improve these properties (Powell et al., 2002). When developing a system to regenerate skeletal muscle, mechanical stimulation can be applied to the myofiber as it is being formed to mimic the naturally occurring process by which the myofiber is mechanically stimulated by bone.

Studies have been performed to determine the changes in mechanical properties of a muscle fiber with and without mechanical conditioning. In avian and rodent skeletal muscle cells, mechanical conditioning involving 3 days of uniaxial stretching and 2-3 weeks of repetitive stretching has been shown to improve various properties of the muscle fiber (Powell et al., 2002). Mechanical conditioning was found to improve the lengthening, orientation, and organization of the myofiber, as well as the composition of the surrounding ECM (Powell et al., 2002). In addition to improving the physical properties of the myofiber, mechanical conditioning also improves gene regulation, endogenous protein expression, protein accumulation, protein localization, and metabolic activity to mimic the processes occurring *in vivo*.

Mechanical conditioning was applied to human skeletal cells with forces approximating those found in muscle development *in vivo.* Normally, engineered skeletal muscle does not reach the diameter of native skeletal muscle, but added mechanical conditioning was found to increase the myofiber diameter by 12% (Powell et al., 2002). Additionally, only 2-15% of engineered skeletal muscle is made up of myofibers, as compared to 90% of muscle *in vivo.* The remainder of the tissue is ECM. The use of mechanical conditioning has been shown to improve the percentage of myofibers in engineered skeletal muscle to 40%. These results could be due to the facilitation of enhanced diffusion of nutrients into the core of the tissue by the mechanical stimulation.

Mechanical conditioning has also been shown to maintain the elastic modulus of the myofiber over a period of 8 days of stretching, while non-mechanically conditioned myofibers show increased stiffness and elastic modulus over time (Powell et al., 2002). This stiffening is due to collagen cross-linking, which is inhibited by the repetitive mechanical stimulation (Powell et al., 2002). Overall, mechanical stimulation can be used to supplement fiber formation to enhance the biological and physical properties of muscle fibers. These enhancements can be used to develop muscle fibers with improved properties and stability than non-mechanically conditioned fibers.

2.4 Clinical Motivation

Though muscle is able to regenerate itself naturally, development of a skeletal muscle construct similar to natural skeletal muscle in structure and function can be transplanted into patients with functional deficits. Two pathways for achieving this goal are *in situ* tissue engineering, and *ex vivo* tissue engineering. *In situ* tissue engineering involves the targeted

introduction of satellite cells to the area of muscle damage. Ideally, these cells would fuse with the existing muscle and improve the functionality of the muscle. The disadvantage of this method is that there is often extensive cell death and typically, only less than 3% of the injected cells engraft at the target site (Mooney & Vandenburgh, 2008). Additionally, the size of the area that the transplanted cells can travel from the site of introduction is relatively small, so this method is not optimal for large scale regeneration (Mooney & Vandenburgh, 2008).

Ex vivo tissue engineering involves the development of a functional tissue construct using myogenic cells and the implantation of the construct into the site of injury *in vivo*. This often entails the use of a biological scaffold made of extracellular matrix material and the seeding of myogenic cells on the scaffold. The scaffold serves many purposes as a substrate for cells to adhere to, a means to control to the cell localization at the site of damage, and a template for the new tissue to form around (Mooney & Vandenburgh, 2008). The major requirements of a scaffold are:

1. Biodegradability at a rate that can be controlled
2. Biocompatibility throughout the degradation process
3. Mechanical properties that approximate those of native tissue
4. Biofunctionality: the ability to support cellular proliferation and differentiation, to secrete extracellular matrix , and finally, to form tissue

   (Liu, Ramanath, & Wang, 2008)

2.4.1 Current Solutions

2.4.1.1 Polymer Fibers

Electrospinning of polymer fibers has been shown to be a viable method for developing skeletal muscle constructs. The process of electrospinning allows for the creation of a complex scaffold structure that can accurately direct the migration of cells into an aligned myofiber (Aviss, Gough, & Downes, 2010). The material used by Aviss et al. was a poly(lactide-co-glycolide) (PGLA) polymer, a biodegradable, elastomeric polymer which degrades into products found naturally in the body.

The architecture of the electrospun fibers was found to be highly effective in the creation of aligned myofibers which contain sarcomeres with the banding patterns found in native skeletal muscle (Aviss, Gough, & Downes, 2010). The myofibers were found to appropriately support adhesion, proliferation, and differentiation of C2C12 myoblasts and when stimulated, were found to contract properly (Aviss, Gough, & Downes, 2010). The disadvantage to this method is the lack of cellular infiltration into the scaffolding material. The use of porous electrospun polymer foams could be more effective in creating a three-dimensional skeletal tissue construct that more closely resembles native myofibers.

2.4.1.2 Hydrogels

Hydrogels are highly hydrated hydrophilic polymer networks (greater than 30% water by weight) that are crosslinked by physical or chemical means. They can be made of either synthetic or biological polymers, but are usually formed from biological materials such as collagen or peptide-modified alginate for skeletal muscle regeneration applications (Drury & Mooney, 2003). Since hydrogels are three-dimensional structures, their architecture facilitates

cell adhesion, proliferation, and differentiation (Drury & Mooney, 2003). The major use of hydrogels is in the area of highly critical defects in localized areas since they do not have load-bearing capabilities to regenerate significant amounts of tissue.

2.4.1.3 Biological Gels

2.4.1.3.1 Fibrin

Fibrin gels have been investigated for use in the development of skeletal myofibers because of their three-dimensional architecture and degradability (Huang, Dennis, Larkin, & Baar, 2004). The structure of a fibrin gel allows cells to migrate and proliferate both on the surface of, as well as throughout, the fibrin gel matrix (Huang, Dennis, Larkin, & Baar, 2004). The fibrin gel matrix degrades within three to four weeks after implantation, by which time the cells have produced their own extracellular matrix proteins.

The experiments conducted by Huang et al. show that by using a fibrin gel matrix, tissue constructs formed in ten days, as compared to thirty-six days when cells are left to self-organize into fibers (Huang, Dennis, Larkin, & Baar, 2004). One limitation to this method is that the myotubes formed did not grow beyond a diameter of 10 μm, while the diameter of a native fiber is around 100 μm. Additionally, the force produced by the engineered constructs was a fraction of the force produced from natural skeletal muscle (36.3 kN/m^2 in the engineered tissue and 260 kN/m^2 in human skeletal muscle).

2.4.1.3.2 Collagen

Collagen is used in scaffolds and gels as since they are natural, biodegradable polymers that have roles in the formation of the muscle's framework. Since it is a protein that is found in the connective tissue of native ECM, it is involved in biological processes such as cell

attachment and tissue regeneration (Grant et al., 2001). These properties of collagen make it a potential candidate for a gel matrix with the capability to support and enhance skeletal muscle regeneration.

2.4.1.3.3 Matrigel™

Matrigel™ is an extract of extracellular matrix formed from Engelbreth-Holm-Swarm (EHS) tumor in mice that contains components of the extracellular matrix of the basement membrane. These components include type IV collagen, regularly arrayed sulfated macromolecules, and a high content of glycosylated molecules (Kleinman and Martin, 2005). Matrigel™ has been shown to promote cell morphogenesis and differentiation (Kleinman and Martin, 2005).

Studies of the mechanics of myogenic cell proliferation and differentiation have shown significant differences between the differentiation of C2C12 cells with and without use of a Matrigel™ coating to the plate. Results have shown that there is enhanced differentiation and reduced proliferation when Matrigel™ is used as a coating (Kleinman and Martin, 2005). Additionally, the use of a Matrigel™ coating has been shown to enhance the rate of myotube formation and the alignment of the myotubes into an organized axial pattern (Kleinman and Martin, 2005). Therefore, the addition of Matrigel™ to either a collagen or fibrin gel can provide the cells with the advantages of the gel, in addition to the capabilities of the Matrigel™ including differentiation, rapid myotube formation, and alignment.

2.4.1.4 Extracellular Matrix Threads

Braided collagen threads can be used as scaffolds since collagen is biodegradable and biocompatible, and since the architecture of the braided system provides a large surface area

for cell migration (Makridakis, Pins, Dominko, & Page, 2009). In experiments conducted by Makridakis et al., the collagen threads were chemically crosslinked and results after one day of culturing of muscle derived fibroblastic cells showed significant cell attachment. The use of braided collagen threads as scaffolds is a promising area of research but still poses some limitations. Since the cells are only able to migrate and proliferate on the surface of each individual thread, the system may not be able to produce a fiber that is structurally analogous to a native muscle fiber.

2.4.1.5 Current Molding Systems

There are some molding systems that are currently used to produce muscle fibers. Many of these systems rely on a substrate, usually made of ECM components, for the cells to proliferate and differentiate on top of. However, these extra components that act as substrates often add an extra level of complexity to the molding system that could be eliminated with the introduction of a molding system that relies mainly on the architecture of the mold for proper fiber formation and alignment.

In a study conducted by Lam, Huang, Birla, and Takayama, a laminin substrate was used to coat a wavy micropatterned PDMS mold (Lam et al., 2009). Myoblasts were seeded on top of the laminin layer and once the plate was 80-90% coated in myotubes, fibrin gel was added on top of the cell layer (Lam et al., 2009). Acting as a provisional matrix, the fibrin gel caused the myotube layer to detach from the laminin substrate and wrap around the gel (Lam et al., 2009). As differentiation proceeded, the cells degraded the fibrin and produced their own ECM components (Lam et al., 2009).

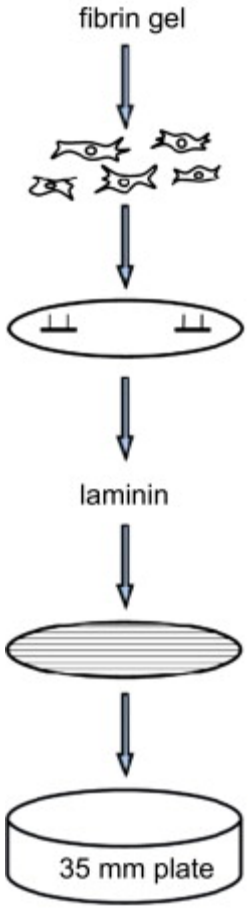


Figure 5: Representation of Fibrin Gel, Myoblasts, and Laminin Substrate Model (Lam et al., 2009)

In another study, a silicone on insulator (SOI) wafer was micropatterned with cantilevers and the culture surface of the silicone was coated with a diethylenetriamine (DETA) substrate (Das et al., 2006). DETA is a synthetic, non-biological material which has, in other studies, proved to be an appropriate surface for neuron and endothelial cell growth due to its cell attachment properties that result from its hydrophilicity and presence of amine groups (Das et al., 2006). The cells were then cultured onto the DETA substrate and myofibers conforming to the cantilever shape were formed.

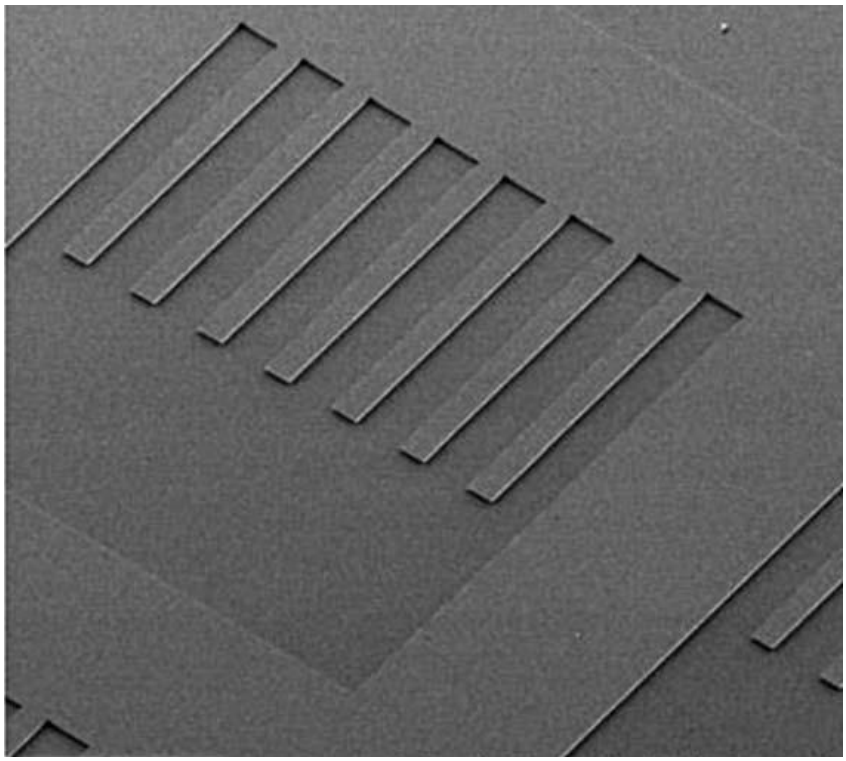


Figure 6: Cantilever Molding System (Das et al., 2006)

In each of the previous molding systems mentioned, the growth of the myofiber is dependent on the presence of the substrate, whether it is a biological material like laminin or a synthetic material like DETA. The development of a molding system that does not involve the coating of a substrate on the mold will represent a simplified system that requires only the presence of cells, a mold containing anchor points, and if necessary, a provisional matrix made of a biological material.

2.5 Cell Lines used for Study of Muscle Regeneration

The cell line that is often used to study muscle regeneration is the C2C12 mouse myoblast line. The C2C12 cell line differentiates rapidly and is able to form myotubes with contractile abilities and producing the proteins found in muscle tissue (Blau et al., 1985). (Product Description , 2010). They can be used in a variety of applications such as the

development of myofibers as *in vitro* models of skeletal muscle formation and function to represent the parallel processes in the body.

2.5.1 Medium Components for Myogenic Cells

There are several components that can be added to standard culture medium formulations to enhance specific functions of myogenic cells. These enhancement factors can increase cell proliferation, increase myoblast differentiation, or promote dedifferentiation into progenitor cells. Table 1 contains descriptions of some medium enhancing factors that can be used to promote certain types of cellular behavior.

Table 1: Medium Enhancing Factors

Enhancing Factor	Outcome
Insulin	Promote myoblast differentiation and fusion (Ewton and Florini., 1981).
TGF-β1	Triggers dedifferentiation of myoblasts/generates progenitor cells (Musgrave et al., 2001).
Sodium Ascorbate	Decreases the material stiffness and increases the tensile strength of tissue construct. Increases cell proliferation. (Camozzi et al., 2006)
Serum-free medium	Increases the extent of cell differentiation (Karger 2000).

Chapter 3: Project Strategy

The ultimate goal of this project was to design a molding system that recapitulates skeletal muscle fiber structure and into which myogenic cells can be seeded such that skeletal muscle tissue is formed. In order to attain this goal, it will be necessary to use a gel matrix to create a three dimensional structure and a scaffold-free molding system for the proper formation of a structurally-accurate skeletal muscle fiber. These two requirements of developing a 3-D structure and forming a muscle fiber of the proper structure must be met in order to achieve the final goal. The purpose of this chapter is to explain the step-by-step design process the group underwent to determine objectives for the project and to weigh the each of the project objectives. The chapter conclusion will provide an overview of the group's approach to solving the design problem and the methods by which to fulfill the objectives and meet the client statement.

3.1 Design

In general, the design process allows the designer to break a complex system down into specific parts and offers tools that will help determine the most cost-effective and optimized product that will satisfy the client. The design process is important in creating a product as it allows the design team to make logical, unbiased decisions in discussions and decisions about product details. This section describes the steps the group took in the design process to revise the initial client statement, decide on project objectives, functions, and constraints, and finally to prioritize each of the objectives. Ultimately, the design process provided the tools for the group to create a prototype of a molding device that can recapitulate human skeletal muscle fiber.

A precursor to the design process is the identification of the stakeholders of the product. The stakeholders for this project are the designers of the product, the users of the product, and most importantly, the client who initially expressed the need to create the product. For this project, the client is Professor Raymond Page. He provided the group with an initial client statement that described the current techniques of creating muscle fibers, why there is a need for the product we were tasked to develop, and what deliverables were expected from the designers at each project milestone. The potential users of this product will be the graduate students and researchers at WPI whose research involves muscle regeneration. The design team, made up of Sahil Bhagat, Carlos Donado, and Deepti Kalluri, aimed to understand the needs and wants of both the client and the users and to create a product that is feasible and will satisfy all parties.

3.2 Initial Client Statement

The next step is to identify the goals of the project and to clarify them in order to understand exactly what the stakeholders want the product to accomplish. Our initial client statement from Professor Page was:

> **"Biopolymer micro-threads produced from materials such as fibrin and collagen have proven useful as scaffolds to seed anchorage dependent cells to facilitate formation of tissue with linear structure. These materials are also useful for *in vivo* applications where the scaffold material must be degraded and replaced with new extracellular matrix produced from the transplanted cells. This approach is particularly useful for formation of skeletal muscle for both modeling skeletal muscle tissue formation *in vitro* and delivering cells to replace lost or damaged skeletal muscle *in vivo*. Currently, the micro-threads are produced first and then cells with myogenic potential are seeded onto the micro-threads 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 micro-threads. For cylindrical tissue such as skeletal muscle fibers to form, the cells must degrade the micro-thread 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 micro-thread production process involves a stretching and drying step to produce axially aligned fibers, which is not compatible with seeding the cells within the micro-threads at the time of formation. The goal of this project is to design a molding 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 cells over time. The size of fibers produced by the mold should approximate those of native skeletal muscle and must strong enough to permit handling for implantation (similar to micro-threads produced currently). The fibers produced must be able to be anchored to prevent tissue collapse and ideally will be made such that mechanical stretch can be applied to mechanically condition the newly formed muscle tissue."

As a design team, it is important to understand all parts of the client statement, but for simplicity, the client statement must be distilled down to a few sentences that express the objectives and constraints of the project. The group accomplished this task by asking the client, Professor Page, numerous questions during weekly meetings in order to clarify exactly what the final product is supposed to accomplish. After the client meeting, the design team had a group meeting where the new ideas were discussed and design ideas for the molding system which incorporated the information from the client meeting were brainstormed.

3.3 Objectives, Constraints, and Functions

Once the design goals are clarified, it is necessary to separate the goals into objectives and constraints. The next step is to list some possible functions that will allow a product to meet those objectives and constraints. The difference between objectives, constraints, and functions is that objectives are the tasks that the product should be able to perform in order to satisfy the client and the users and to be considered a successful product, constraints are

specific conditions the product design must meet in order to be functional, and functions are the means by which the objectives and constraints will be achieved.

Objectives:

1. Develop a molding system of defined dimensions suitable for *in vitro* analysis of mechanical properties which allows formation of skeletal muscle fibers *in vitro* using skeletal muscle myoblasts
2. Determine the composition of a provisional ECM to be used during seeding that will structurally support the overall tissue architecture until the myoblasts differentiate and fuse to form multinucleated myotubes and muscle fibers
3. Produce fibers that approximate the diameter of native skeletal muscle
4. Produce fibers that are strong enough to handle during *in vitro* testing of mechanical strength and contractile force, and ultimately, implantation into skeletal muscle wound models
5. Anchor fibers in place to prevent tissue collapse during formation or following differentiation
6. Allow for mechanical stretch that can be applied to mechanically condition newly formed muscle tissue
7. Allow for electrical stimulation to assess contractile function.

Constraints:

- Must fit within an incubator
- Sterilizable with on-site resources (Autoclave, ethylene oxide, alcohol bath)
- Materials must be commercially available or easily manufactured using campus or collaborator resources
- System must be easy to monitor and maintain (keep sterile while changing culture medium)
- Materials must not be cytotoxic
- Individual fiber diameter should close to that of native skeletal muscle (~100 µm)
- $368 Budget

Functions of the Molding System:

- Able to seed cells in a spatially controlled manner and at a specific cell density
- Able to perform viability assays
- Able to confirm effectiveness of provisional ECM on cell differentiation and maintenance of overall tissue integrity
- Able to secure tissue during formation to prevent collapse
- Able to mechanically condition the fiber

- Able to manipulate the formed tissue for testing for mechanical strength and electrically stimulated contractile force
- Able to control fiber properties (fiber diameter and length)

3.3.1 Pruned Objectives

After creating a general and comprehensive list of objectives, the design team created a pruned objective list based on meetings with the client and the design team's brainstorming sessions. Some objectives were deleted while others were combined. Each final objective was broken into sub-objectives that need to be fulfilled in order for the main objective to be completed successfully. The list below is the final pruned objective list following numerous revisions.

<u>Objectives:</u>

1. Find the optimal cell density to form muscle fiber
    A. Consistent Cell Seeding
    B. Composition of provisional ECM to support tissue structural integrity while myoblasts are differentiating to form myotubes
    C. Define differentiation conditions to ensure myoblasts are not proliferating while tissue is being formed
    D. Appropriate culture medium to support cell fusion and tissue formation

2. Produce strong fibers that approximate native skeletal muscle
    A. Structural fiber integrity and uniform alignment along the axis of tissue forming mold
    B. Uniform fiber dimensions
    C. Fiber stability/viability (over 10 – 14 days)
    D. Consistent fiber dimensions (batch to batch)

3. Anchor fibers in place
    A. Consistent initial position of anchors
    B. Consistent distance increase of the anchors at a determined frequency and magnitude
    C. Strong fiber attachment to anchor points
    D. Allow for electrical stimulation, mechanical stretch, and observation/measurement of contractile function

4. User –friendly
    A. Easy to use (Integrated system and minimum components)
    B. Easy to clean for reuse
    C. Safe

5. Time-efficient
    A. Takes less time than current standard using micro-threads

6. Cost-effective

The list above shows the design team's six main objectives:

1. Finding an optimal cell density to form human skeletal muscle fiber
2. Producing strong fibers that have similar cross sectional dimensions to those of native human skeletal muscle
3. Anchoring muscle fibers in place
4. Developing a product that is time-efficient
5. Developing a product that is cost-effective
6. Developing a product that is user-friendly

The optimal cell density needed to form skeletal muscle tissue is the one that allows adequate intercellular contact so the cells can align and fuse into multinucleate myotubes. Thus, the cells need to be previously amplified in proliferative culture conditions in the undifferentiated state to generate numbers required for seeding into the molding system using culture conditions to promote differentiation. Finally, the mold/seeding system should facilitate cell fusion and myotube alignment in the axial direction.

To complete the second objective of producing strong fibers that are similar to native human skeletal muscle, the design team would need to ensure that when the fiber is initially formed, it is strong enough to be moved and stretched. Fiber strength and integrity is important because the fiber should be strong enough to be moved during transplantation procedures and stretchable to allow for mechanical conditioning. Furthermore, the fiber should be uniform in diameter throughout its length and the molding device should produce

fibers of the same dimensions during each use of the molding system. Lastly, the muscle fiber should be able to maintain its mechanical properties long enough to be conditioned and be implanted into an animal model.

The design team had to determine a way to anchor the newly formed fiber in a way such that the fiber will be anchored in the same place every time in order to be consistent with every use of the molding system. In order to stretch the fiber for mechanical stimulation, the increase in distance between the anchor points for stretching should also be consistent each time to stimulate fiber formation in the same direction and in the same manner. The anchor points are also important for structural integrity since they keep the fiber taut and prevent its collapse. The molding system must also be produced in the most economical way possible while still fulfilling all the other objectives. Additionally, the device should be easy to use and should allow the user to develop muscle fibers in a timely fashion.

3.3.2 Quantitative Analysis of Objectives

The designing of a device involves the process of optimizing a device to perform specified functions safely and in a cost and time effective manner that will satisfy the client's needs. It is impossible to fulfill each objective with the best solution, as that solution might conflict with another objective. Thus, in order to produce the best possible product, it was necessary to weigh and prioritize the objectives.

This was done by creating pairwise comparison charts. Since the client, Professor Page, and the design team are the stakeholders, each of them has equal weight in their pairwise comparison of the objectives. In a pairwise comparison, the objectives in the same tier, such as

the main objectives and the sub objectives under each main objective, are compared against each other.

Each two objectives were compared at the same time and the objective determined to be more important receives a score of 1 while the objective determined to be less important receives a zero. If both the objectives are determined to be of the same importance, a score of 0.5 is given to each of the objectives. The scores need to be normalized by adding a 1 to the aggregated score in order to ensure a non-zero value for all objectives. Finally, a weight is created for each objective to allow a reader to quickly understand which objectives were found to be the most important and by what percentage. Table 2 shows the results of the pairwise comparison of the main objectives. For each objective, the client's score is on the left column while the design team's is on the right.

Table 2: Pairwise Comparison Table of Main Objectives

Main Objectives	1		2		3		4		5		6		Aggreg. Score	Normalized Score	Weight
1. Find optimal cell density			0.5	0.5	0.5	0.5	1.0	1.0	0.5	1.0	0.5	1.0	7.0	8.0	.229
2. Fiber similar to native muscle	0.5	0.5			0.5	0.5	1.0	1.0	0.5	1.0	0.5	1.0	7.0	8.0	.229
3. Anchor fiber in place	0.5	0.5	0.5	0.5			1.0	1.0	0.5	1.0	0.5	1.0	7.0	8.0	.229
4. Time efficient	0.0	0.0	0.0	0.0	0.0	0.0			0.0	0.0	0.5	0.0	0.5	1.5	.043
5. Cost effective	0.5	0.0	0.5	0.0	0.5	0.0	1.0	1.0			0.5	1.0	5.0	6.0	.171
6. User-friendly	0.5	0.0	0.5	0.0	0.5	0.0	0.5	1.0	0.5	0.0			3.5	3.5	.100

The pairwise comparison chart above shows that while all the main objectives are important, certain objectives are more important than others. The three most important objectives, according to both of the stakeholders, are finding the optimal cell density, producing a fiber that is similar to native human skeletal muscle fiber and being able to anchor the fiber in place. Following those three objectives in importance are having a device that is as cheap as possible to produce and use, a device that is user-friendly, and a device that is simple enough

that it does not take a long time to set up and use. Table 3 is a comparison of the sub-objectives of the first main objective.

Table 3: Pairwise Comparison Table of Sub-Objectives I

1. Find optimal cell density	**A**		**B**		**C**		**D**		**Aggregated Score**	**Normalized Score**	**Weight**
A. Consistent Cell Seeding			0.0	0.0	0.0	0.5	0.0	0.5	1.0	2.0	.125
B. Strong ECM	1.0	1.0			0.5	0.5	0.5	0.5	4.0	5.0	.313
C. Proliferate/Differentiate	1.0	0.5	0.5	0.5			0.5	0.5	3.5	4.5	.281
D. Cell Fusion/ Tissue formation	1.0	0.5	0.5	0.5	0.5	0.5			3.5	4.5	.281

Within the major objective of finding optimal cell density, it was established by client and the design team that having a strong ECM was the most important sub-objective. It was followed closely by ensuring that proliferation and differentiation are occurring at the appropriate times, and having the proper culture medium for cell fusion and tissue formation. Ensuring that there is consistent cell seeding during each use of the molding system was considered to be the least important sub-objective.

Table 4: Pairwise Comparison Table of Sub-Objectives II

2.Fiber similar to native muscle	**A**		**B**		**C**		**D**		**Aggregated Score**	**Normalized Score**	**Weight**
A. Fiber Integrity			0.5	1.0	0.5	0.5	1.0	1.0	4.5	5.5	.344
B. Uniform Fiber	0.5	0.0			0.5	0.0	1.0	1.0	3.0	4.0	.250
C. Fiber Stability	0.5	0.5	0.5	1.0			1.0	1.0	4.5	5.5	.344
D. Consistent Fiber (Batch to Batch)	0.0	0.0	0.0	0.0	0.0	0.0			0.0	1.0	.063

The stakeholders decided that, with respect to the main objective: having a fiber similar to native muscle, developing a fiber with continuous integrity, and developing a fiber with long-term stability were tied for the most important sub-objectives. The development of a uniform fiber was the next most important sub-objective, while producing consistent fibers from batch-to-batch was considered the least important sub-objective.

Table 5: Pairwise Comparison Table of Sub-Objectives III

3. Anchor fibers in place	**A**		**B**		**C**		**D**		**Aggregated Score**	**Normalized Score**	**Weight**
A. Consistent initial position			1.0	0.5	0.5	0.0	1.0	0.0	2.0	3.0	.200
B. Consistent distance increase	0.0	0.5			0.0	0.0	0.0	0.0	0.5	1.5	.100
C. Prevent fiber collapse	0.5	1.0	1.0	1.0			1.0	1.0	5.5	6.5	.433
D. Allow for Elec. Stimulation	0.0	1.0	1.0	1.0	0.0	0.0			3.0	4.0	.267

The most important sub-objectives under objective for anchoring fibers in place were that the device should prevent the fiber from collapsing and allow for electrical stimulation. Furthermore, the device should facilitate the placement of a consistent initial position of the fiber's anchors. Consistent distance increase of the anchors in order to stretch the fiber would be desirable but it was found to be a fairly low-priority sub-objective.

Table 6: Pairwise Comparison Table of Sub-objectives IV

4. User-friendly	**A**		**B**		**C**		**Aggregated Score**	**Normalized Score**	**Weight**
A. Ease of use			0.5	0.0	1.0	1.0	2.5	3.5	.389
B. Safe	0.5	1.0			1.0	1.0	3.5	4.5	.500
C. Easy to clean	0.0	0.0	0.0	0.0			0	1.0	.111

Last but not least, under the main objective of User Friendly, user safety was the highest weighted sub-objective. The second highest-ranked sub-objective in this comparison was ease of use. Easy to clean was ranked the least important of the three sub-objectives.

After completing the pairwise comparison tables, each of the sub-objectives were weighted together. This exercise allowed the design team to understand which sub-objectives required more focus for the development of a successful device. To summarize the results, a weighted objectives tree, as seen in Figure 7, was created. Within the weighted objective tree, the number on the left is the weight of the objective within its branch. The number on the right

is the weight of the objective when compared to all the objectives in the whole tree. A more simplified version of the weighted objective tree is shown in Figure 8 as a bar graph.

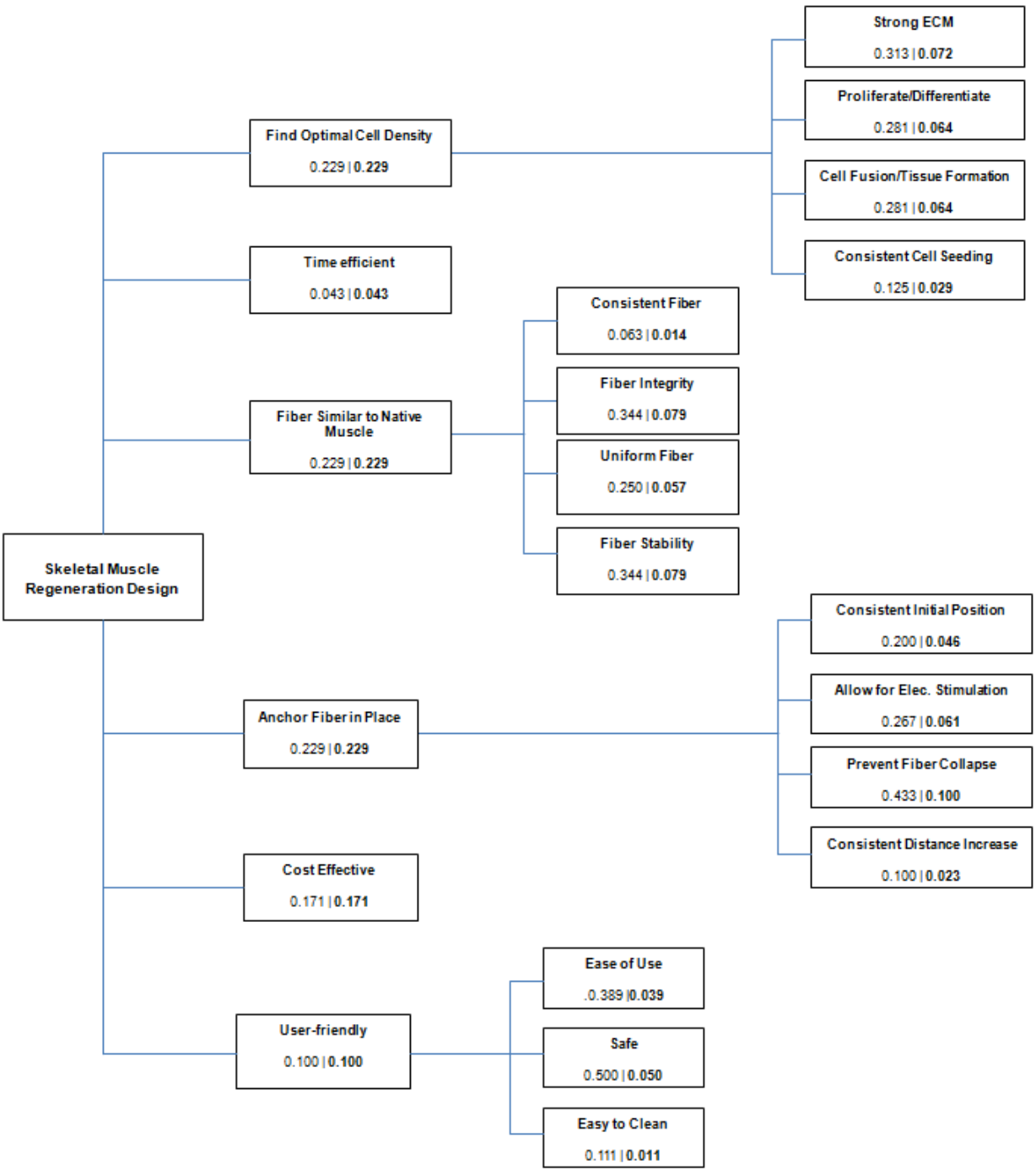


Figure 7: Weighted Objective Tree

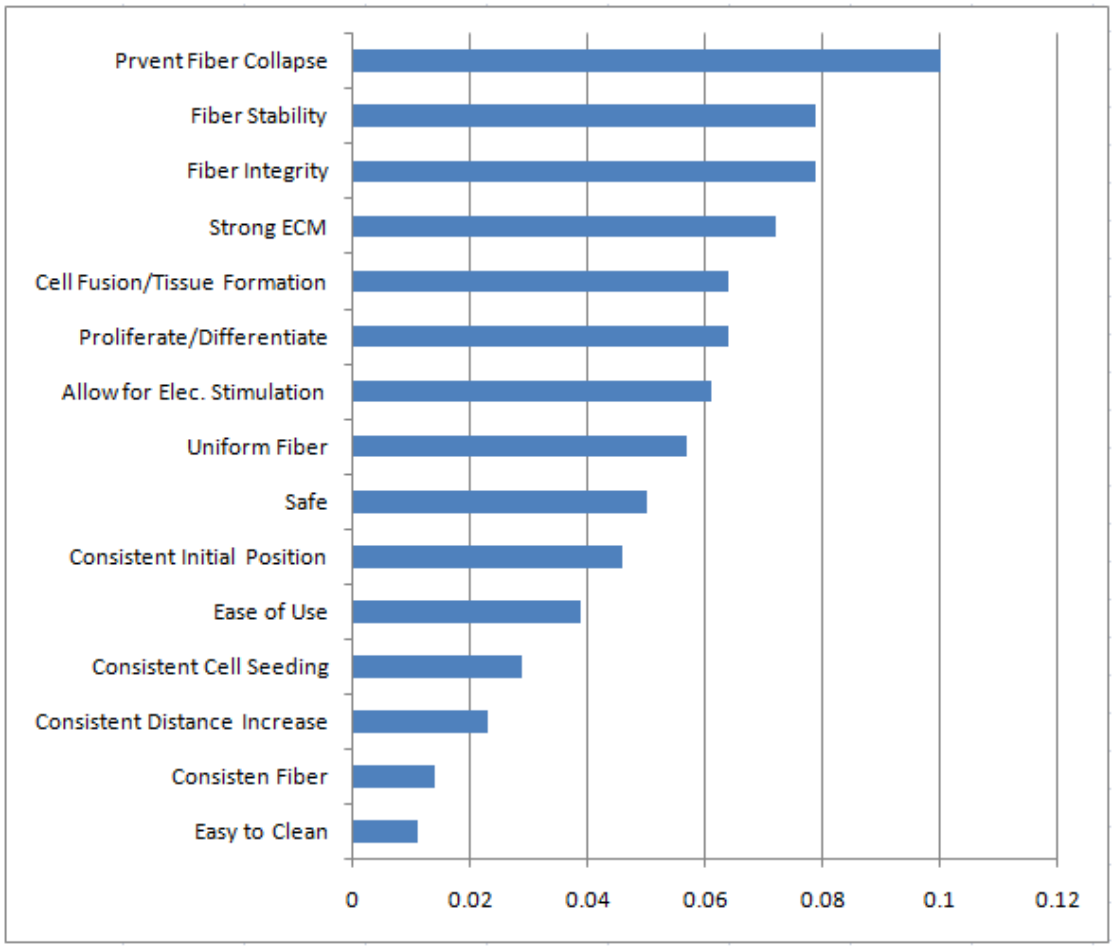


Figure 8: Summary of Weighted Objective Tree

3.4 Revised Client Statement

After meeting with the client and user, brainstorming several ideas, and conducting analysis of the objectives using pairwise comparison tables, the original client statement was revised. The revised client statement is:

Design a sterilizable molding device where myogenic cells can be seeded to recapitulate native skeletal muscle fiber which can be anchored to prevent tissue collapse and can be mechanically conditioned.

3.5 Project Approach

The group developed a two-step approach to represent the process by which myogenic cells are seeded into a gel, the cell-gel solution is placed within a mold, and differentiation is promoted by a switch to differentiation medium. This process is represented in Figure 9.

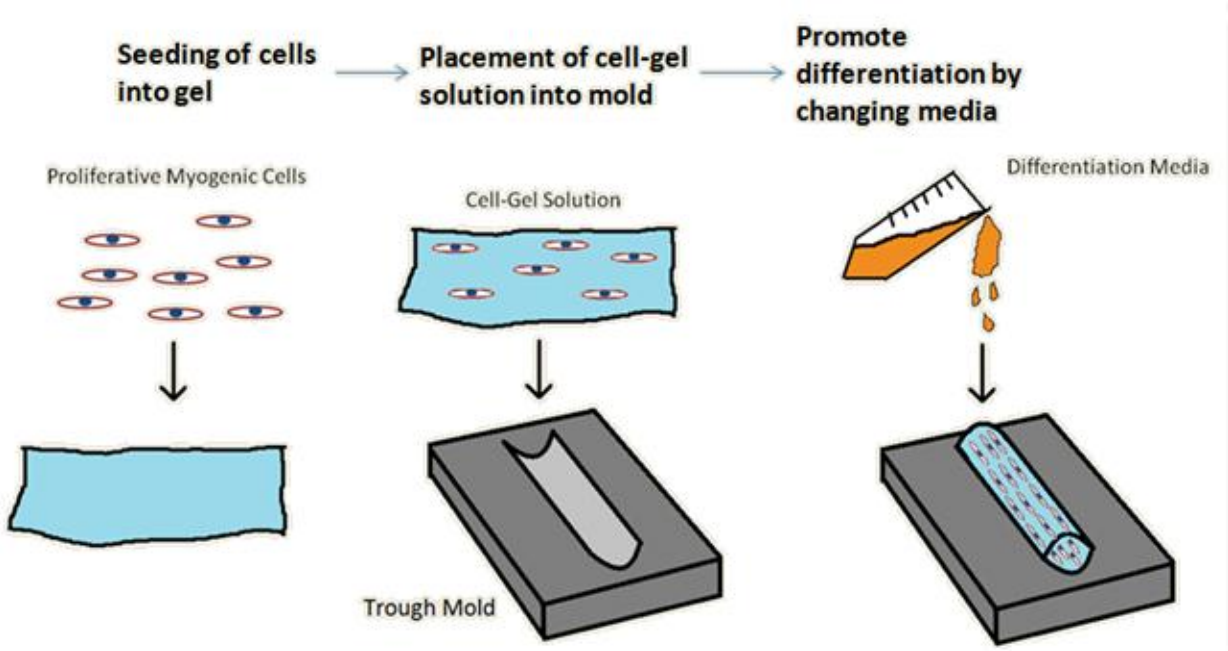


Figure 9: Schematic of Project Approach for the Seeding of a Cell-Gel Solution into a Mold

The main aims that must be achieved for this approach to be effective are to:

1) **Provide a gel matrix for the cells to proliferate within**

There are several advantages to using a gel as a provisional matrix for the cells. The use of a gel matrix allows for a 3-D structural geometry that represents that of a native muscle fiber. Though the gel is initially in a liquid form, its polymerization causes it to transition into a more solid state. The polymerized gel forms a 3-D structure, while conforming to the space that it is placed in. When the cells are within this gel matrix, they will have the advantage of taking the shape provided by the gel. Additionally, since

the gel is often made of biological materials such as collagen or fibrin, the biological cues provided by the gel to the cells stimulate their proliferation and differentiation into myofibers. Over time, the biological gel will be degraded by the cells and the cells will form their own ECM, while maintaining their initial structure.

2) **Provide a constrained seeding channel for the cells to conform to**

The use of a seeding channel within which to seed the cell-gel solution allows for the development of a muscle fiber into a desired shape. For example, a seeding channel with a rounded bottom will lead to the creation of a cylindrical cell-matrix structure. A long seeding channel with a length approximating that of a native skeletal muscle fiber can be used to create muscle fiber of the same length.

3) **Provide anchor points for the cells to pull on and align themselves**

Anchor points within the molding system will act as points around which cells can attach. Cell congruity at these anchor points will lead to a cohesive network of cells that will pull on each other from each anchor point, promoting uniaxial alignment of the cells. The pulling from each anchor point will also prevent tissue collapse, as there will be tension throughout the length of the fiber.

Chapter 4: Alternative Designs

The team held a brainstorming session to develop alternatives designs. Five design alternatives for the molding system were generated. Each of these five alternatives was evaluated and an illustration and pros and cons of each design are outlined below.

4.1 Needs Analysis

After several brainstorming sessions and client meetings, it was determined that the molding system to be designed must form a skeletal muscle fiber that is similar in morphology, contractile properties, and mechanical properties to a native human skeletal muscle fiber. This requirement is important because a muscle fiber without the proper morphology will not integrate properly with native muscle in the tissue recipient. Also, a muscle fiber without similar contractile and mechanical properties will not react properly to neural signals or contract with the magnitude necessary for functionality. The molding system also needs to produce a skeletal fiber that is at least 1 centimeter long. There is a minimum fiber length requirement because any mechanical testing on any fiber that is shorter than 1 centimeter would be difficult.

If possible, it would be desirable for the molding system to allow for mechanical stimulation of the skeletal muscle fiber. Previous studies have shown that mechanically-stimulated muscle fibers have better mechanical properties than non-stimulated muscle fibers. In the future, if the muscle fibers are properly formed, they would be used for *in vivo* testing where they would be implanted into mice. Thus, it would also be desirable to design the molding system with a method of easy removal of the fiber. Also, due to economical and manufacturing constraints, the molding system currently being designed will not be able to

produce a muscle fiber that is close to the size of a native muscle fiber. However, it is desirable, when the affordable manufacturing capabilities exist, to machine a molding system that can produce a human skeletal muscle fiber with a diameter of 100 µm.

4.2 Conceptual Designs

4.2.1 Circumferential Molding System:

The use of a molding system to grow tissue rings was adapted from the Rolle Lab at WPI. An acrylic mold is machined with annular wells containing inner posts with a diameter of 1 millimeter. The annular wells are positioned both vertically and horizontally next to each other, separated by the same distance. A mixture of Polydimethylsiloxane (PDMS) and curing agent (10:1 ratio of PDMS to curing agent) is poured onto the acrylic mold to create a negative template. A solution of agarose mixed with Dulbecco's Modified Eagle Medium (DMEM) is then poured onto the PDMS negative to cast annular agarose wells, which would be removed from the negative and placed in 6-well plates.

C2C12 cells are seeded into each well at a concentration of 1.5 million cells per well kept at 60 percent confluency. The cells, at this stage, are cultured in proliferative media made of DMEM, F12, FBS, glutamine, penicillin/streptomycin, and insulin (Lluri and Jaworski, 2005). After proliferating for two days and reaching 100 percent confluency, the proliferation media will be replaced with differentiation media made of DMEM and F12, and supplemented by Insulin- Transferrin- Selenium (ITS), glutamine, and horse serum (Lluri and Jaworski, 2005). Once the rings form, each ring is cleaved at one specific point, resulting in one myofiber per well, each with dimensions approaching those of skeletal muscle (100 microns in diameter, and

4 millimeters in length). At this point, they are they are prepared for histological analysis and mechanical testing.

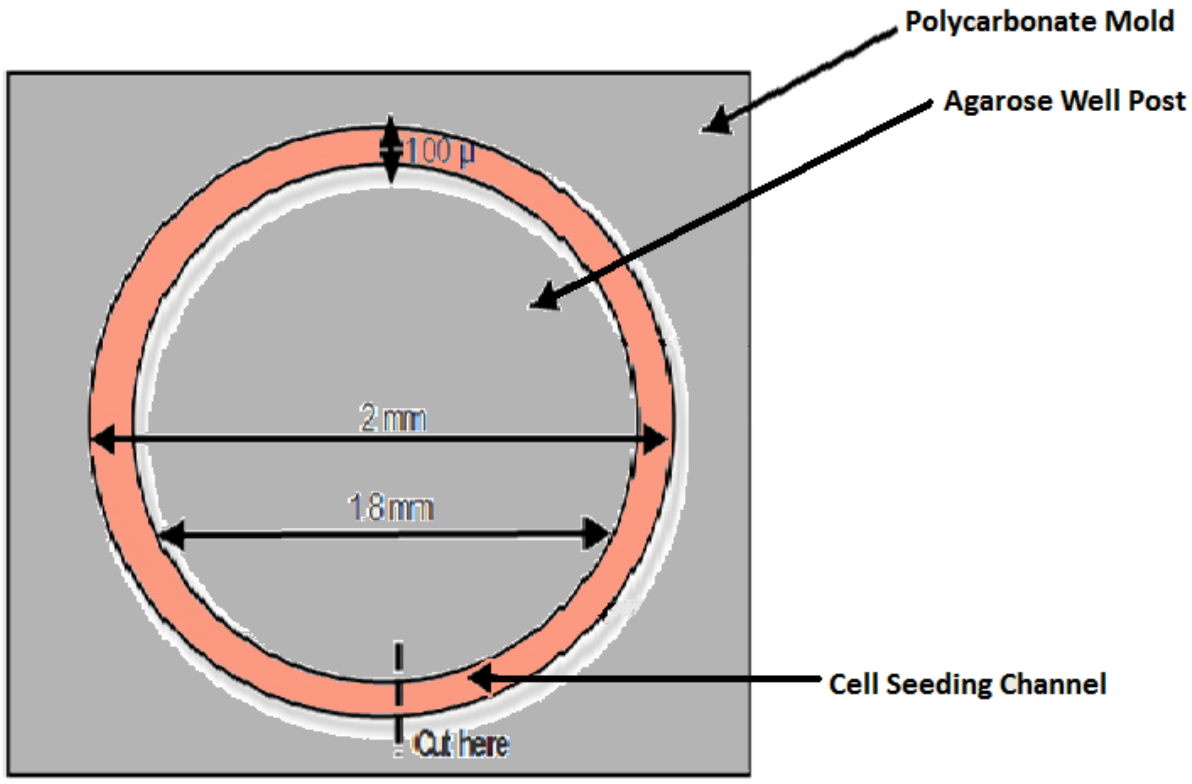


Figure 10: Individual well in the Circumferential Molding System

Pros:

- Batch uniformity
- Control over fiber dimensions

Cons:

- Difficult to stretch for axial alignment
- Non-uniformity of tissue construct due to circular shape

4.2.2 Dog Bone System:

An acrylic mold is created by machining two cylindrical posts, each with a diameter of 5 mm on a base made of acrylic. Each post is separated horizontally from the other by a distance of 12 mm. A mixture of Polydimethylsiloxane (PDMS) and curing agent (10:1 ratio of PDMS to curing agent) is poured onto the acrylic mold to create a template, after which a solution of agarose mixed with DMEM is poured onto the PDMS negative to cast a mold of the same structure as the original acrylic mold. The mold itself is in a "dog bone" shape with the two posts located in the circular portions of the mold. The edges of the mold are raised to create a well within the structure and the two posts are raised above the level of the surface to serve as anchor points for the cells seeded within the well.

C2C12 cells are seeded into the well at a concentration of 1.5 million cells per well kept at 70 percent confluency. Cells are cultured in proliferative media made of DMEM, F12, FBS, glutamine, and penicillin/streptomycin are incubated at a temperature of 37° C for two days (Lee et al., 2007). After that, the cells reach 100 percent confluency and the proliferation media will be replaced with differentiation media made of DMEM, F12, ITS, glutamine, and horse serum. Once the tissue forms around the posts, it will go through histological analysis and mechanical testing.

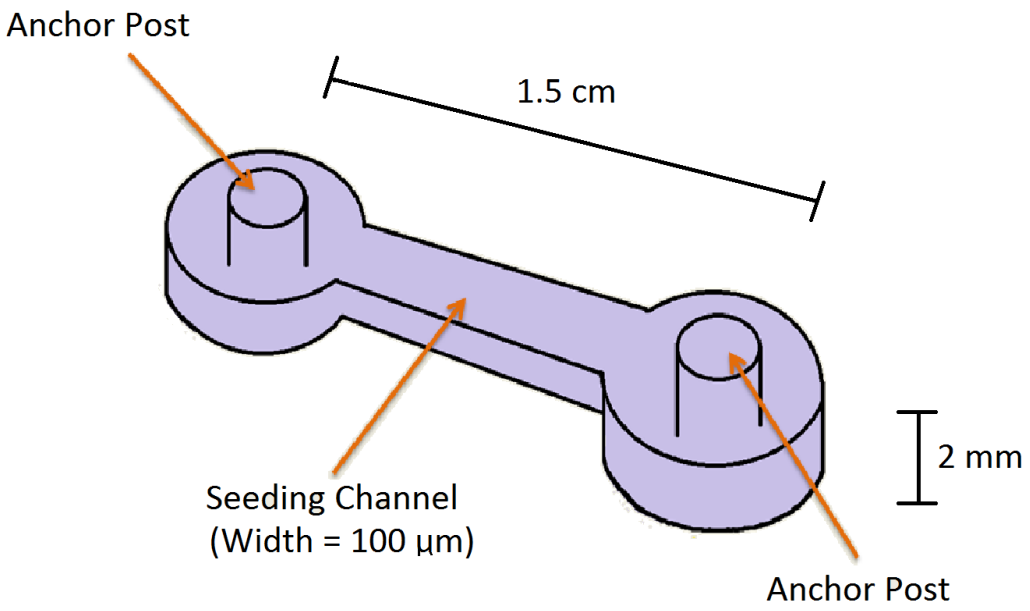


Figure 11: Individual Cell Seeding Channel in the "Dog Bone" Molding System

Pros:

- Ability to anchor cells
- Batch uniformity
- Control over fiber dimensions
- Uniformity of tissue construct

Cons:

- Mechanical properties are not completely uniform throughout fibers

4.2.3 Micro-thread Extrusion System:

4.2.3.1 Fibrin micro-threads

A fibrin micro-thread co-extrusion system was adapted from the Pins Lab. Fibrinogen and thrombin from bovine plasma are placed in separate syringes, and are coextruded using a stabilized crosshead on a threaded rod through a blending connector in which both solutions are mixed and extruded through polyethylene tubing. The resulting fibrin micro-threads are released into a buffered bath (HEPES, pH 7.4 at room temperature) and after 15 minutes, they

are removed from the bath to be air-dried overnight, and finally stored in a desiccator at room temperature.

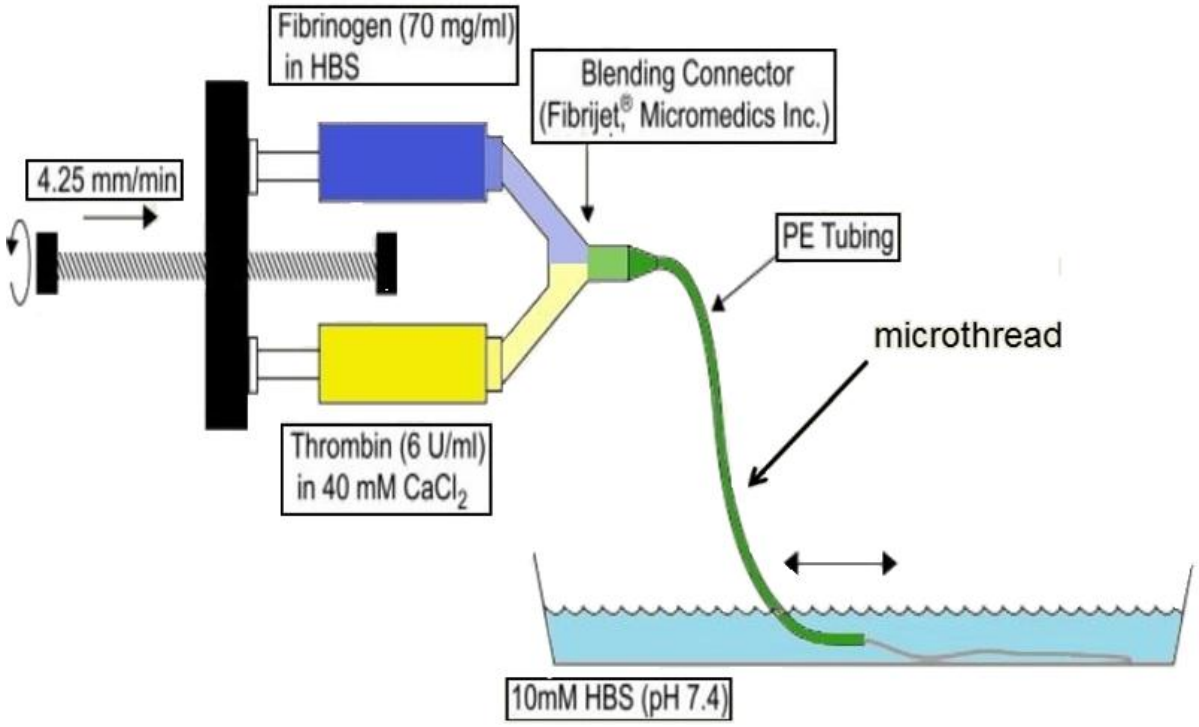


Figure 12: Co-extrusion System for Fibrin Micro-threads (Cornwell and Pins, 2006)

4.2.3.2 Collagen micro-threads:

As a first step, acid soluble type I collagen is extracted from rat tails. Collagen threads are extruded either from solutions of insoluble or soluble type I collagen. Collagen solution is stored in a syringe and is extruded through polyethylene tubing by using a syringe pump set at a certain flow rate. Threads are released into a bath and maintained initially in fiber formation buffer overnight, followed by fiber incubation buffer for 24 hours and lastly by distilled water for another 24 hours. Finally, the threads are removed from the water bath and stored in a desicator at room temperature.

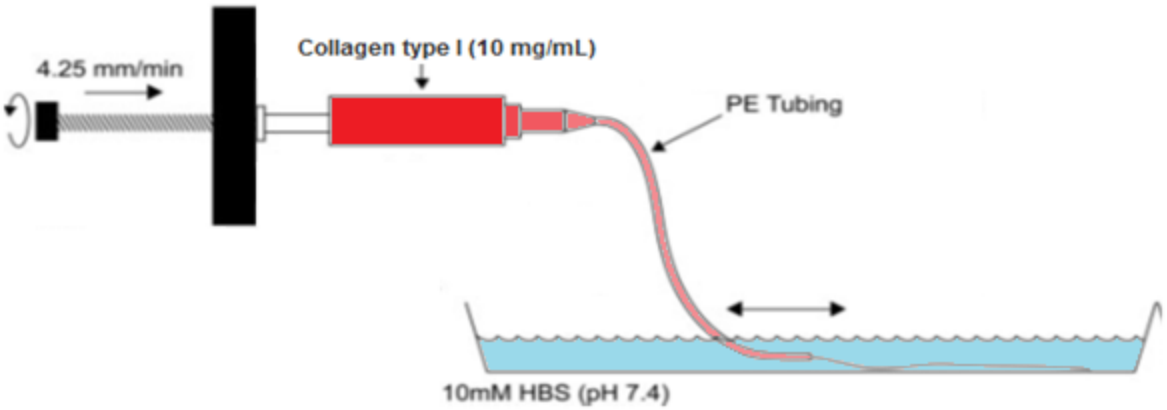


Figure 13: Extrusion System for Collagen Micro-threads

In the next step, micro-threads are placed on a reflective aluminum foil surface and cross-linked by exposing the micro-threads to UV radiation emitted from an ultraviolet crosslinker for a certain period of time. This is done with the aim of increasing the strength and stiffness of the threads.

Bundles of 10 micro-threads are then placed on 35 mm culture dishes by gluing them onto coverslips supplemented by silicone adhesive. Each thread is seeded with 100 µl of cells in medium and incubated with 10% FBS for 30 minutes. After the incubation period, thread bundles are released from coverslips and cell proliferation and attachment, and mechanical properties are analyzed.

Pros:

- Enhanced rate of cell migration
- Provides some structural alignment for cells

Cons:

- Inconsistencies in scaffold preparation
- Possibility of insufficient cell migration into the thread's core

4.2.4 Series Molding System

An acrylic mold is created by machining several elongated posts on an acrylic base. Each post is separated longitudinally from neighboring posts by 100 µm. A mixture of Polydimethylsiloxane (PDMS) and curing agent (10:1 ratio of PDMS to curing agent) is poured onto the acrylic mold to create a template, after which a solution of agarose mixed with DMEM is poured onto the PDMS negative to cast a mold of the same structure as the original acrylic mold. The mold has a series of elongated posts which are separated longitudinally from the neighboring elongated posts by 100 µm. Above and below each elongated post would be an oval shaped post, to serve as anchor points for the fibers forming around the elongated posts.

C2C12 cells are seeded into the molding system at a concentration of 6 million cells per molding system, kept at 70 percent confluency. Cells are cultured in proliferative media, and are incubated at a temperature of 37 °C for two to three days, after which the proliferation medium is removed and replaced with differentiation medium. Once the tissue forms around the posts, it will go through histological analysis and mechanical testing.

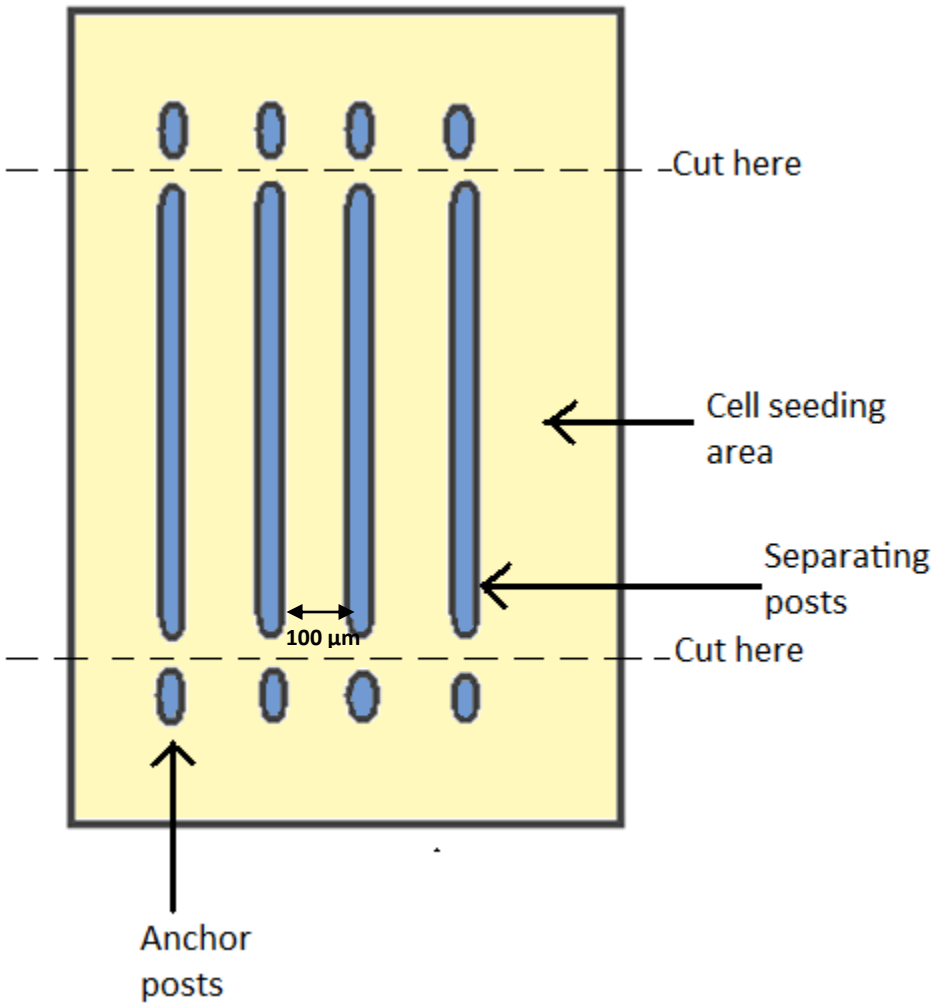


Figure 14: Cell Seeding System for the Series Molding System

Pros:

- Control over fiber dimensions
- Ability to anchor cells

Cons:

- Mechanical properties are not completely uniform throughout fibers

4.2.5 Pin and Mesh Molding System

In the pin and mesh molding system, a base made of acrylic is machined such that there are semi-circular channels running parallel to each other. The anchoring system within the model consists of a silk thread, either end of which is tied around each of two minutien pins. The silk thread is tied at the midpoint of the minutien pin so that it is suspended at about the middle of the tubular structure. The final component is a mesh-like structure made of a biocompatible metal such as titanium, which is placed on top of the system.

C2C12 cells, suspended within a gel matrix, are seeded into the tubular structures. The minutien pins, connected by the silk thread, are then placed along the length of the tube, and the metal mesh is placed on top of the PDMS mold. The metal mesh provides structural support to the gel, so that it forms a cylindrical structure, while still allowing oxygen diffusion to the cells through the holes within the mesh.

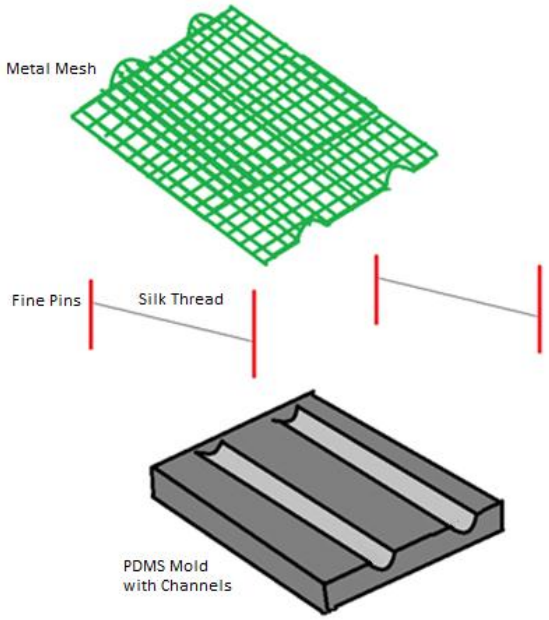


Figure 15: Cell Seeding in a Pin & Mesh Model

Pros:

- Control over fiber dimensions
- Provides structural alignment to cells
- Ability to anchor cells

Cons:

- Mechanical properties are not completely uniform throughout fibers
- Large number of components and complexity of device

The team performed a qualitative analysis on the design alternatives to determine the final design. The final design chosen by the design team was the dog-bone molding system. The reason was because out of the five total designs, the dog-bone molding system fulfilled all constraints and reached the largest number of the specified goals and objectives.

After the design team met with the client, the client mentioned that it would be preferable for the recapitulation of the muscle fiber to occur without the aid of a scaffold. Hence, the Microthreads system was removed for further consideration.

The Circumferential molding design was not chosen because producing a skeletal muscle fiber, which is usually linear, in a circular shape could potentially cause fiber formation and integrity issues. Slight non-uniform seeding could cause the cells in the highest density to start pulling on the cells adjacent to them. This cell aggregation could prevent many cells from receiving oxygen and nutrients and could cause the cells to die. Even if uniform cell seeding occurred and a fiber formed, there would be the possibility of the contracting force of the fiber causing it to break. Additionally, due to its circular shape and lack of points from which to perform mechanical conditioning of the tissue, the circumferential molding system is not amenable to mechanical conditioning.

The Series molding system could potentially produce muscle fibers at sizes very close to native muscle fiber, however the problem with the molding system is that it must be manufactured by photolithography, which is cost prohibitive with the current budget.

Through discussions with the client, the group was informed that because of the large number of parts involved in the tubular gel molding system, the manufacture would be difficult. Additionally, it would be difficult for the user to seed the cells within the device properly and consistently during each use.

After performing the brief qualitative analysis on the design alternatives, the team performed a quantitative analysis by generating a Best of Class chart to compare the relative ability of each of the five design alternatives to meet the weighted objectives. Each of the design alternatives was ranked from 1 to 5, according to the ability of each alternative to fulfill the project's objectives. A score of 1 was given to the alternative that best addresses each of the project's objectives. If two design alternatives were determined to address a particular objective equally well, the score for both alternatives is the average of 1 and 2 (1.5). The team used this approach as a way to compare each of the design alternatives. Weighted scores for each of the design alternatives were calculated by multiplying the scores determined through the Best-of-Class chart by the weights previously assigned for each of the objectives. Finally, the alternatives with the lowest total weighted scores were proposed to be the best design choices.

Table 7: Best-of-Class Chart

		DESIGNS									
		Circumferential molding system		Dog Bone molding system		Fibrin/collagen micro-threads		Series Molding System		Tubular Gel Molding System	
OBJECTIVES	**Weight**	**Score**	**WS**	**Score**	**WS**	**Score**	**WS**	**Score**	**WS**	**Score**	**WS**
Consistent cell seeding	0.029	1.5	0.044	1.5	0.044	4	0.116	5	0.145	3	0.087
Composition of provisional ECM	0.072	3	0.216	4	0.288	2	0.144	5	0.720	1	0.072
Define differentiation conditions	0.064	1.5	0.096	1.5	0.096	5	0.320	3	0.192	4	0.256
Appropriate culture medium	0.064	1.5	0.096	1.5	0.096	3	0.192	4	0.256	5	0.320
Fiber integrity, uniform alignment	0.079	3	0.237	1	0.079	5	0.395	2	0.158	4	0.316
Uniform fiber dimensions	0.057	2	0.114	3	0.171	5	0.285	4	0.228	1	0.057
Fiber stability/viability over time	0.079	5	0.395	4	0.316	2	0.158	3	0.237	1	0.079
Batch to batch fiber consistency	0.014	1	0.014	2	0.028	5	0.070	3	0.042	4	0.056
Consistent initial anchor position	0.046	5	0.230	1	0.046	3	0.138	4	0.184	2	0.092
Consistent anchor-fiber elongation	0.023	5	0.115	1	0.023	3	0.069	4	0.092	2	0.046
Prevent fiber collapse	0.100	3	0.300	1.5	0.150	5	0.500	1.5	0.150	4	0.400
Allow for electrical stimulation	0.061	5	0.305	1.5	0.092	1.5	0.092	4	0.244	3	0.183
Easy to use	0.039	1.5	0.059	1.5	0.059	4	0.156	3	0.117	5	0.195
Easy to clean	0.011	2	0.022	3	0.033	1	0.011	5	0.055	4	0.044
Time efficient	0.002	1.5	0.003	1.5	0.003	4	0.008	3	0.006	5	0.010
Cost effective	0.029	1.5	0.044	1.5	0.044	4	0.116	3	0.087	5	0.145
TOTAL			2.290		1.568		2.770		2.913		2.358
RANK			2		1		4		5		3

According to the Best-of-Class chart, given our objectives, constraints, and expected functions, the top two systems were found to be the Dog Bone Molding System and the Circumferential Molding System. These molding systems will allow for the creation of skeletal muscle fibers that are close to the dimensions of a native muscle fiber, based on the seeding channel width and height. The molding systems are also very simplistic in design so they will not be difficult or time-consuming to manufacture.

The quantitative results from the Best-of-Class chart supported our qualitative judgment based on the background and pros and cons of each device. In particular, we anticipated that these methods would create uniform and mechanically stable fibers with a high degree of cell viability. The advantages of a scaffold-free system for cell-based applications have been described in the Background section. It was determined that important features of a scaffold-

free system and a gel matrix allow for the requirements of three-dimensional structure, proper cell differentiation, and proper axial alignment.

4.3 Feasibility Study/Experiments

The list of materials needed for the manufacturing process of the device is shown below:

- Acrylic
- PDMS
- Agarose

It was determined that the use of these three materials is feasible since they are all readily available and their costs lie within the budget. In terms of manufacturing the device to the intended specifications, however, it was found that using the techniques available through the machine shop would not be sufficient to develop a device with such small specifications (100 µm, 250 µm, and 500 µm). It was found that laser cutting, which is available at the WPI machine shop, can be used to create a device that contains channels at approximately 10x the specified dimensions.

Other methods of manufacture such as photolithography and 3-D printing can be used to accurately create a device of the desired dimensions. However, these methods are not available through the machine shop and are more expensive than using a traditional CNC machine for development of the device. It was determined that it would not be feasible to outsource the machining at a high cost before performing preliminary testing on an affordable and more easily manufacturable device. Therefore, the design specifications were revised to include channels that were ten times their original widths so that the device could be manufactured at WPI.

4.4 Mold Material Choice

Each of the proposed materials (Acrylic, PDMS, and agarose) was evaluated against a list of attributes that were determined to be desirable for a molding system. For the template, the desired attributes were determined to be manufacturability and reusability. For the final mold, the desired attributes were determined to be autoclavable, non-cytotoxic, moldable, optically transparent, diffusible for media, and non-adherent to cells.

Table 8: Comparison of Mold Materials for Template

Attributes	Acrylic	PDMS	Agarose
Manufacturable	✓	✖	✖
Reusable	✓	✓	✖

The desired characteristics for the final mold are listed in Table 8. Though the final mold should be moldable into a specified shape, the template should be sturdy to allow for the development of final molds from the template. This template itself should be manufacturable so that it can take the exact shape and dimensions of the CAD drawing. Of the three materials analyzed, only acrylic is manufacturable, so it must be used as the template.

Reusability is another important characteristic of the template because the ability to reuse the template as many times as necessary will allow for the development of several final molds from the template. Reusability will eliminate any need to manufacture more than one mold using the machine shop and will allow the group to make all further molds in the lab. Both acrylic and PDMS are reusable, but since PDMS is not manufacturable, it is not suitable for use in the template.

Table 9: Comparison of Mold Materials for Final Mold

Attributes	Acrylic	PDMS	Agarose
Autoclavable	✖	✓	✖
Non-cytotoxic	✖	✓	✓
Moldable	✖	✓	✓
Optically transparent	✓	✓	✓
Media Diffusion	✖	✖	✓
Non-adherent to cells	✖	✖	✓

The desired attributes for the final mold (autoclavable, non-cytotoxic, moldable, optically transparent, diffusible for media, and non-adherent to cells) are shown Table 2in Table 9. The first desired attribute, autoclavable, was chosen because the proper sterilization of the mold is necessary to ensure that the cells that are seeded into the mold are in a sterile environment necessary for survival. Of the three materials, only PDMS is autoclavable in its molded form. Acrylic is not autoclavable, and therefore, cannot be used for the material on which to directly seed the cells. Agarose, in its molded form, is not autoclavable, but when it is in a liquid form prior to being molded, it can be autoclaved. Therefore, either PDMS or agarose can be suitable for use as a final mold, in terms of autoclavability. Since the cells will be seeded directly onto the mold and will be in contact with the mold, the material used for the final mold must also be non-cytotoxic. PDMS and agarose are non-cytotoxic, while acrylic is cytotoxic. This indicates that acrylic is not suitable for use as the material of which the final mold is made.

Another desirable quality for the final mold is for it to be moldable. This will allow for it to be molded to fit the shape of the template and will allow for multiple final molds to be

obtained from one template. Both PDMS and agarose are moldable, while acrylic is not, indicating that either PDMS or agarose can be used as the final mold and that acrylic cannot.

The final mold should also be optically transparent to allow for optimal visualization of cells and the resulting fiber. All three materials are optically transparent, indicating that any material's optical properties are suitable for the final mold.

One very important requirement of the final mold is that it should allow for media diffusion. When the cells are seeded into a channel within the mold, they will still need nutrients from the media. The media that is covering the mold from the top will not be able to penetrate throughout the forming muscle fiber through the sides and bottom of the fiber. Therefore, the media must be able to diffuse through the mold and into the sides and bottom of the fiber. The only material of the three that allows for this diffusion is agarose, indicating that it is the optimal choice for the final mold material.

The other critical attribute for the final mold material is that it should be non-adherent to cells. This quality is important because a material that cells adhere to will not allow for the formation of a congruent muscle fiber. This is because the cells may adhere to the material with greater strength than the strength with which the cells interconnect. This will cause disconnects within the fiber. Additionally, a material that is non-adherent to cells will allow for relatively easy removal of the muscle fiber from the mold once it has formed. The only material of the three that is non-adherent to cells is agarose, which supports the use of agarose as the final mold material.

Once the materials for the template and the final mold were chosen, it was determined that PDMS would be the optimal material choice for the negative mold. Since it is sturdy and

reusable, one PDMS mold can be used for the development of several final agarose molds. Additionally, since PDMS is autoclavable, it can be sterilized and then be used as the negative for the final mold to ensure sterility of the final agarose mold.

4.5 Manufacture of the Primary Mold

Once it was determined that the two possible molding shapes would be a dog bone mold and circumferential mold, the group decided to test each of the molds with three different channel widths that could form fibers of varying diameters. To minimize the number of components to each device, the device was conceptualized with three dog bone molds on one block and three circumferential molds on another block. By placing three molds on one block, the seeding process can be expedited.

In designing our device, the material proposed for the double negative mold was acrylic. Laser cutting can be used to accurately etch the design into one of two materials: acrylic poly (methyl methacrylate) or Delrin (polyoxymethylene). Since acrylic was readily available in the WPI machine shop, it was chosen as the material for the template.

By developing a double negative mold using a material that is sturdy and will remain usable for the length of the project, negative molds can be developed any time that they are needed. The negative mold would then be formed by pouring PDMS into the double negative. Using a negative mold made of a stable and durable material like PDMS, several of the final molds can be made in a timely manner, without the need to machine a new mold. Each final mold will be made by pouring agarose into the negative mold made of PDMS. Each agarose mold will be used for a seeding experiment and then discarded once the fiber has formed and has been removed from the mold.

4.6 Device Specifications

The mold was initially developed according to the set of initial specifications. Preliminary tested indicated, however, that the specifications would need to be revised for optimal fiber formation. Therefore, a revised mold was developed according to a new set of specifications.

4.6.1 First Iteration of Molding Device

In order to manufacture the device, a set of specifications that includes the dimensions of the device and the materials that make up the device must be determined. For the dog bone molding system, it was determined that three different dog bone molds would be made onto the same block in order to facilitate simultaneous testing of three molds of varying dimensions. Initially, the channel widths that were chosen were 100 μm, 250 μm, and 500 μm. The 100 μm channel width was chosen because this is the diameter of a native skeletal muscle fiber and ideally, the device would be able to produce a muscle fiber as close to native dimensions as possible. The 250 μm and 500 μm channels were chosen in case the 100 μm channel was too difficult to seed with cells because of its very small size. However, from conversations with staff at the WPI machine shop, the group was informed that channels of such small widths could not be machined using the CNC machines available at the machine shop. The dimensions would have to be enlarged to 10x their current size for the device to be manufactured at all and would need to be enlarged to 40x their current size for the device to be manufactured with accuracy.

The group decided that accuracy of the mold's outline was not as large of a concern as the objective of creating a muscle fiber that approximates native skeletal muscle as much as

possible. Therefore, it was determined that the channel widths would be increased to 10x their original values for channel widths of 1 mm, 1.5 mm, and 2 mm. The post diameters on each end of the dog bone were chosen to be 2 mm so that the posts would be sturdy enough to withstand the contraction of the muscle fiber once it forms. A post with too small of a diameter might break once the muscle fiber becomes continuous throughout the mold and begins to contract against the post. The heights of the posts and depths of the channels were chosen to be 2 mm, the same value of the largest channel diameter. Assuming that the muscle fiber that forms will be perfectly cylindrical, the post height and channel depth would have to be as least as large as the channel width. All post heights were chosen to be 2 mm, even for the smaller channel widths, since a post that is higher than the channel is wide will still allow for a cylindrical fiber to form.

For determining the specifications of the circumferential molding system, which contains three circular molds on one block, the same channel widths of 1 mm, 1.5 mm, and 2 mm were chosen for the reasons stated previously. Since the dog bone molds were based on of the concept of the circumferential molds used in Professor Rolle's lab, the depths of the channels were found using the ratio found in Professor Rolle's molds. The ratio of channel width to channel diameter was 1:1.75 in Professor Rolle's molds, so by using the same ratio for the circumferential device, a channel diameter of 1 mm would correlate with a channel depth of 1.75 mm, a channel diameter of 1.5 mm would correlate with a channel depth of 2.63 mm, and a channel diameter of 2 mm would correlate with a channel depth of 3.5 mm. The CAD

representation of the mold is shown in Figure 16.

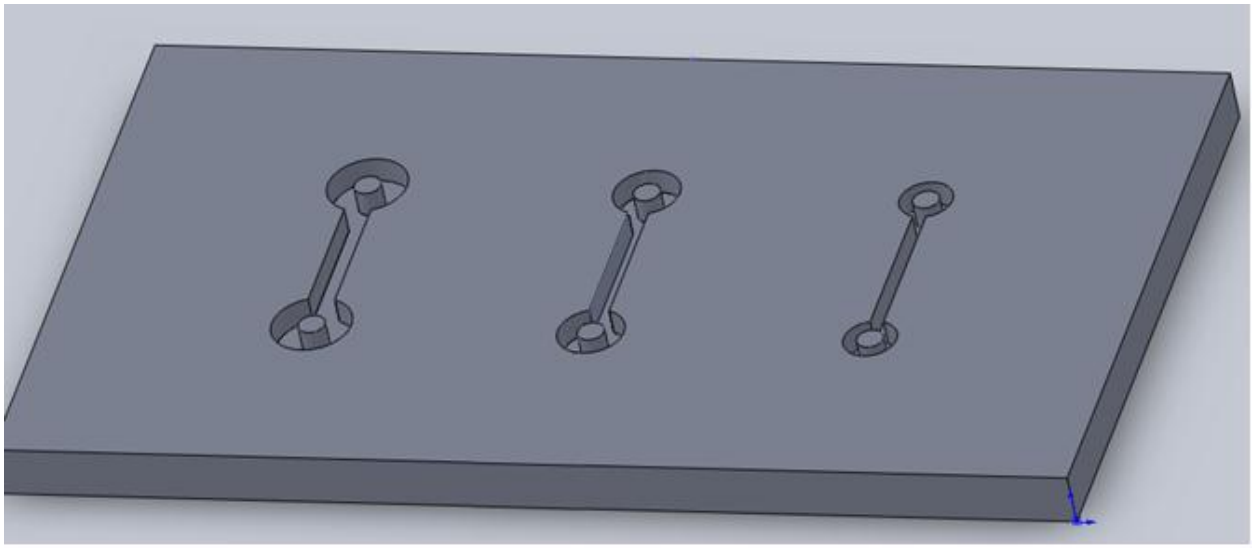


Figure 16: CAD Representation of Initial Dog Bone Mold

4.6.2 Second and Final Iteration of Molding Device

Preliminary testing of the initial mold suggested that the specifications were not optimal for proper fiber formation. It was found that the channel width that was most effective in forming myofibers was the smallest channel width of 1 mm. Therefore, the new mold was manufactured with all three dog bones with channels 1 mm in width. Additionally, it was determined that an increase in channel depth and post height would be beneficial since it would ensure longer anchoring of the fiber around the post. Therefore, the channel depth was changed from 2 mm to 3.5 mm. The CAD representation of the final iteration of the molding device is shown in Figure 17.

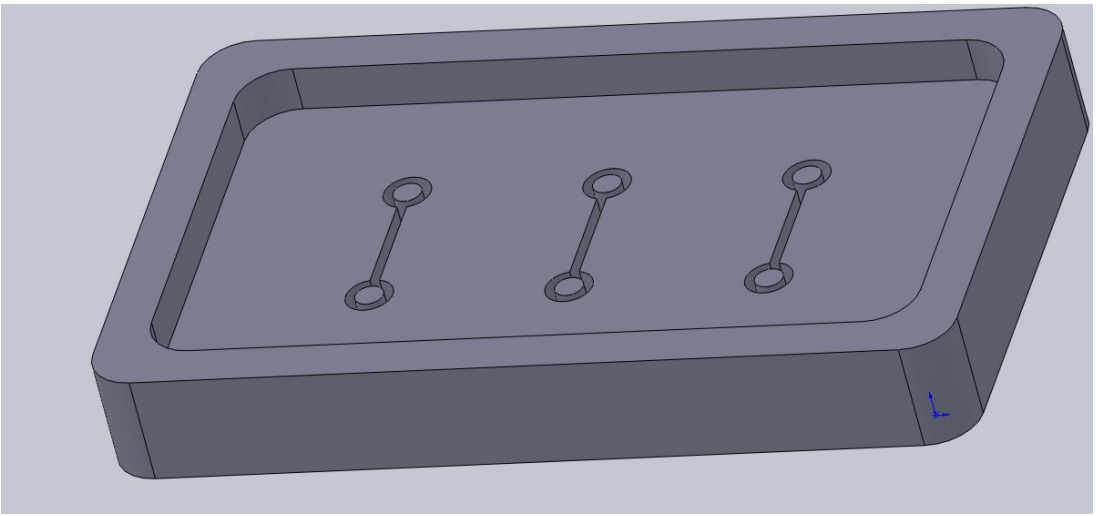


Figure 17: CAD Representation of Final Dog Bone Mold

Chapter 5: Design Verification

From the design process, it was determined that the dog bone mold would be machined of acrylic and would be seeded cells within a matrix of collagen or fibrin gel. The primary acrylic molds were developed through the WPI Machine Shop and subsequently, the PDMS and then the agarose molds were made. Gels were then prepared, seeded with cells, and placed into the seeding channels within the dog bone molds. The cells and any resulting fiber were then subjected to BrdU staining to ensure proper proliferation of cells, myosin staining to ensure differentiation into a muscle fiber, and live/dead staining to ensure cell viability. The final fiber was also subjected to electrical stimulation to demonstrate the functionality of the tissue.

5.1 Development of Final Molds

Once the double negative mold was made from acrylic in the machine shop, the negative mold and the final mold had to be developed. As shown in Figure 18 below, the acrylic double negative mold would lead to a PDMS negative mold, which would, in turn, lead to the final agarose mold.

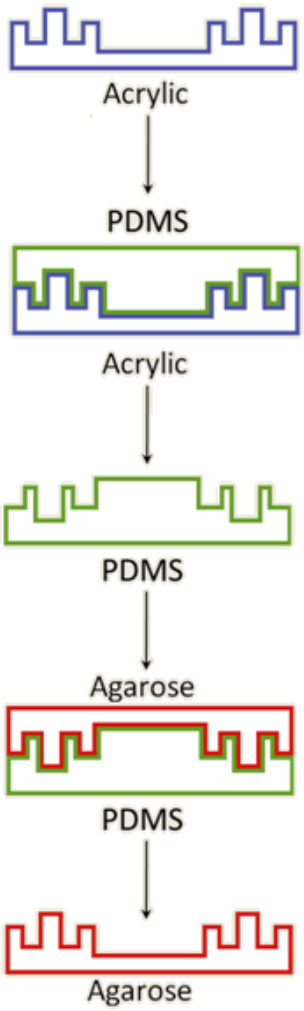


Figure 18: Schematic of Double Negative Mold to Final Mold

The primary mold made of acrylic that was machined through the WPI machine shop is shown in Figure 19.

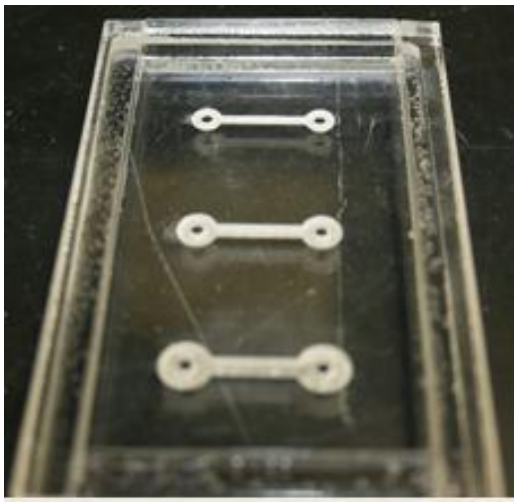


Figure 19: Primary Mold of Acrylic

A mixture of PDMS and curing agent (10:1 ratio of PDMS to curing agent) was poured onto the acrylic mold. The PDMS/acrylic complex was placed in the de-gasser for 1 hour to eliminate any bubbles followed by 4 hours in the oven at 60° C to allow for polymerization of the PDMS. Once the de-gassing and polymerization was complete, the PDMS negative mold was removed from the acrylic mold. The PDMS mold is shown in Figure 20.

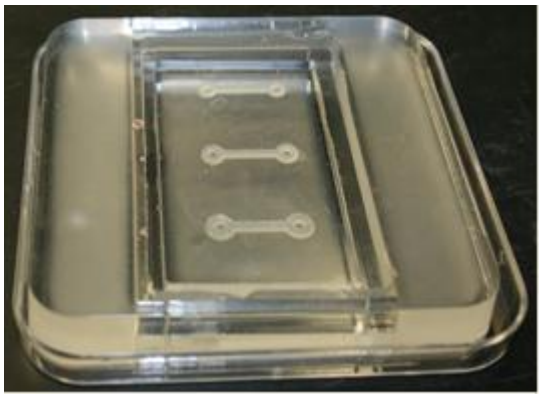


Figure 20: PDMS Mold Developed from Acrylic Mold

In order to form the agarose mold, 2.5% agarose was dissolved in DMEM within a glass container and autoclaved using the liquid cycle. Initially, 2% agarose was used to make the final molds but it was determined that in order to increase the rigidity of the posts and prevent

further tissue collapse, a higher concentration of agarose should be used. The PDMS templates made previously were autoclaved using the gravity cycle. Autoclaved tongs were used to transfer the PDMS template into the hood and the autoclaved agarose was then filled into the mold using a micropipetter. The pipetting was done slowly to avoid bubble formation.

After being left in the hood for 10 minutes, the PDMS/agarose complex was transferred within a plate into the refrigerator for storage until use. The mold was then transferred back into the hood and using scalpel blades, the agarose mold was carefully removed from the PDMS template. The agarose mold, shown in Figure 21, was then placed within a 100 mm petri dish and incubated in DMEM for two days prior to seeding.

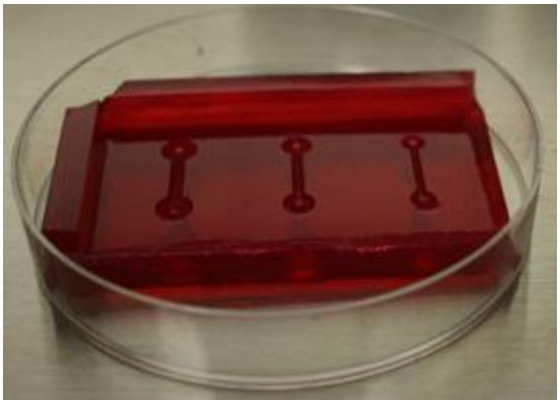


Figure 21: Agarose Mold Made from PDMS Mold

5.2 Culturing of C2C12 Cells

Proliferation and differentiation media was used in experiments with C2C12 cells to sustain the cells during each phase of fiber formation. The base of the proliferation media was 50%/50% DMEM/F12, and it was supplemented with 10% Fetal Bovine Serum (FBS), 1% Glutamine, and 1% Penicillin/Streptomycin. For differentiation media, the base was similarly 50%/50% DMEM/F12, and was supplemented by 1% ITS, 1% Glutamine, and 2% Horse Serum.

The cell culturing procedure for C2C12 cells begins by breaking down the extracellular matrix holding the cells to the surface of the plate by removing the media within the flask and adding 3.5 mL of trypsin to the flask. Trypsin is a protease, which means that it breaks down protein, and therefore, is able to detach the cells from the plate's surface. After being placed in the incubator for 7 minutes, the flask was viewed under the microscope and if the cells were found freely floating across the surface of the plate, they were centrifuged. If most of the cells were found to be not detached after seven minutes in trypsin, they were placed back into the incubator for 3 more minutes. The cells must not be left in trypsin for greater than 15 minutes because the trypsin will begin to break down the cells themselves. 5 mL of medium was then added to the cells in trypsin since the FBS within the medium inactivates the trypsin.

This solution was pipetted into a 15 mL conical tube and at this point, 100 µL of the cell suspension was removed from the conical tube and placed in an Eppendorf tube. 100 µL of trypan blue was added to the same Eppendorf tube and then 20 µL of the solution was placed into the hemocytometer to determine the cell concentration. The cell count was performed according to the procedure in Appendix B.

The cell suspension remaining in the conical tube was centrifuged for 5 minutes at an RCF of 700 xg. At the end of the five minutes, a cell pellet was seen at the bottom of the conical tube, which was otherwise filled with the trypsin/media solution. The solution was aspirated from the conical tube and the cells were resuspended in the appropriate amount of medium for re-plating or experiments.

5.3 Preparation of Gels

Two types of gels, collagen gel and fibrin gel, were investigated as potential provisional matrices within which to resuspend cells prior to seeding within the channel of the mold.

5.3.1 Collagen Gel

The collagen gel was prepared with the addition of several components to form a solution, which is then added to bovine Type 1 Collagen. First, culture medium was added to FBS, which was then added to 5X DMEM, which was then added to sodium hydroxide (NaOH), which was then added to acetic acid. Next, the solution was added to cell solution of the correct density for the mold that it was being seeded into. Finally, the cell suspension was added to the 2 mg/mL collagen. This process is represented in Figure 22.

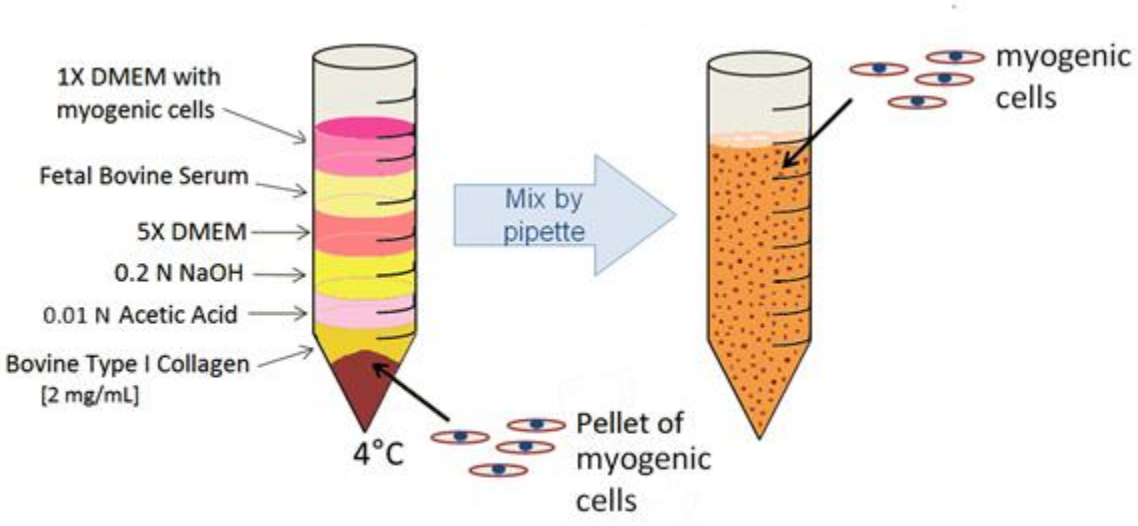


Figure 22: Representation of Collagen Gel Components and Cell Suspension

5.3.2 Fibrin Gel

The components of fibrin gel are fibrinogen and thrombin. The fibrin gel was made according to the protocol described in Appendix C3. Briefly, calcium chloride ($CaCl_2$) solution

was mixed with thrombin, which was then mixed with fibrinogen. In the formation of fibrin from its constituents, thrombin initially catalyzes the conversion of fibrinogen into fibrin monomers, and then the fibrin monomers polymerize into fibrin strands. The role of calcium in forming fibrin is that it is a co-factor in the process, which causes cross-linking of the fibrin strands, thereby providing cohesiveness to the gel. The time required for polymerization of the fibrin gel was approximately 10-15 minutes. Within this time frame, the cells were resuspended within the appropriate amount of gel to seed the mold. This process is represented in Figure 23.

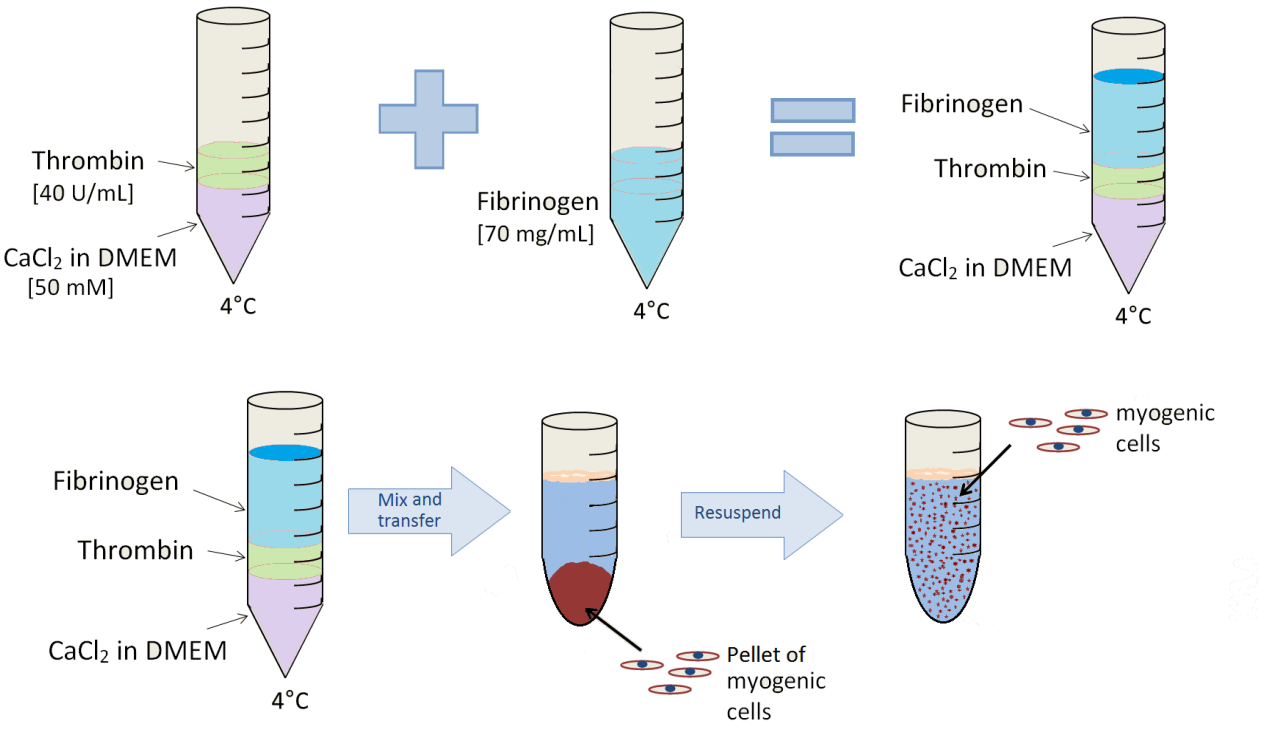


Figure 23: Representation of Fibrin Gel Components and the Process of Cell Suspension

5.4 Seeding of Cell-Gel Solution

The seeding of the cell-gel solution was dependent on the volume available within the seeding channel. In order to measure this volume, a measured amount of water was slowly pipetted into the seeding channel. The three dog bone molds created on the same base had seeding channels with diameters of 1 mm, 2.5 mm, and 5 mm, and the volumes of water that could fit within the seeding channel were measured to be 60 µL, 80 µL, and 100 µL, respectively. Additionally, the number of cells to be seeded within each well was calculated based on the surface area of the seeding channel. It was determined that for the 1 mm diameter seeding channel, the number of cells would be 2 million cells. For the 5 mm diameter seeding channel, the number of cells seeded into the mold would be 3 million cells.

5.4.1 Collagen Gel

After the collagen gel containing cells was prepared, it was pipetted into the molds and placed in the incubator for 1-2 hours. Within 1-2 hours, the polymerization of the collagen gel was complete, so the mold was removed from the incubator, filled with media for adequate nutrition and hydration, and placed back into the incubator for cell growth. This step-by-step process is depicted in Figure 24.

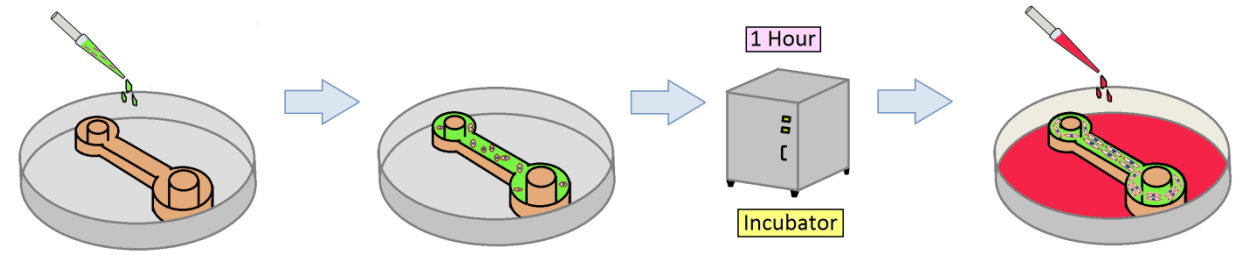


Figure 24: Representation of Seeding of Collagen Cell-Gel Solution into Dog Bone Mold

5.4.2. Fibrin Gel

The fibrin gel, once prepared, and mixed with cells, was then seeded into the molds using a pipettor. After 15 minutes of being placed in the incubator for polymerization of the fibrin gel, the mold was removed from the incubator, filled with media for adequate hydration, and then placed back into the incubator for cell growth. This process is shown in Figure 25.

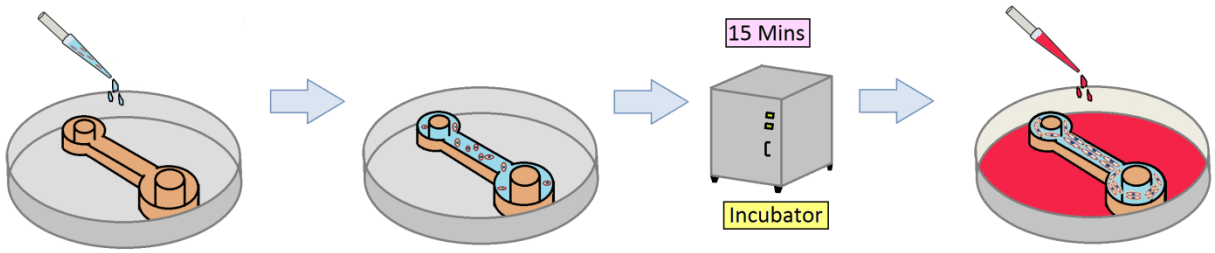


Figure 25: Representation of Seeding of Fibrin Cell-Gel Solution into Dog Bone Mold

5.5 Immunocytochemistry

Several immunocytochemistry assays were performed in order to characterize the behavior of C2C12 cells. A study of the progression through proliferation and differentiation of C2C12 cells was performed to understand the time point within the cell cycle at which the cells begin differentiation. This time point is important because when in their proliferation phase, cells do not produce ECM components, but while in the differentiation phase, the cells are producing ECM components, which contribute to structural integrity. Fiber formation would, therefore, be enhanced if the mold was seeded with cells entering differentiation since the cells would produce ECM components immediately and form a strong tissue reinforced with ECM.

Myosin staining was performed in order to determine whether C2C12 cells cultured on a plate are capable of forming mature myofibers. This study was performed for the characterization of C2C12 cells in determining whether myofibers would form if the cells were

left to proliferate without the addition of differentiation media and to perform a comparison between myofiber formation on fibrin and collagen gels.

5.5.1 Bromodeoxyuridine (BrdU) Assay for Cell Proliferation

A BrdU incorporation assay is a common assay to detect proliferating cells in culture. BrdU is a synthetic nucleoside that is an analog to thymidine. The mechanism of BrdU staining is that during the S phase of the cell cycle, BrdU incorporates into the replicating DNA by substituting for thymidine. Once the BrdU is taken up by the cells, specific antibodies are used to detect BrdU using immuno-fluorescence. However, the antibodies can only bind BrdU once the DNA is denatured. The denaturation process is usually done by exposing the cells to heat or acidic conditions.

An experiment was set up for the determination of the time point at which differentiation occurs. The information gained from this study could be used for more accurate cell seeding into the molds since the optimal time point at seeding is at the beginning of the cells' differentiation phase. While the cells are in their proliferative phase, the number of cells increase but the cells do not produce extracellular matrix components. Once the cells enter the differentiation phase, they begin to form extracellular matrix, which provides integrity and structure between the cells. Therefore, the seeding of cells at the appropriate cell density into the molds at the time of differentiation would enhance the structural integrity of the fiber since the extracellular matrix proteins would be formed immediately.

The experiment was set up with a total of five 24-well plates (Plate #1 – Plate #5), each with four different cell densities (5,000 cells/well, 10,000 cells/well, 15,000 cells/well, and 20,000 cells/well). After seeding the cells into the 24 well plates, the cells were allowed to

proliferate until the cells at the highest density reached a confluency of 80-90%. At this point, the media within the wells was switched to differentiation media. This time point was recorded as Day 0.

On Day 1, the cells in Plate #1 were fixed according to the protocol in Appendix B2. On Day 2, the cells in Plate #2 were fixed, on Day 3, the cells in Plate #3 were fixed, on Day 4, the cells in Plate #4 were fixed, and on Day 5, the cells in Plate #5 were fixed. After being fixed, all plates were stained at once for BrdU and were visualized using fluorescence microscopy.

The Hoescht stain used in the protocol would stain for all of the nuclei present in the sample, while the BrdU stain would stain for all of the nuclei in the proliferative phase of the cell cycle. If the majority of cells stained blue were also stained green, this would indicate that most cells within the sample are within their proliferative phase. If most of the cells stained blue were not also stained green, this would indicate that most cells within the sample had exited the proliferative phase and begun differentiation.

5.5.2 Myogenic Differentiation Study

A myosin staining assay can be used to detect myosin protein within the sample of interest. Presence of myosin within the aligned fibers indicates the presence of contractile units and maturation of the cells into contractable fibers. A myosin stain was performed on plates with cells of varying densities, which were allowed to proliferate for 10 days. The study was performed to characterize C2C12 cells and determine the natural differentiation potential and maturation of the cells without the addition of any differentiation media. The proliferation study was conducted in a 24 well plate with cell densities of 5,000 cells to 20,000 cells at 1,000 cell intervals. Cells were seeded at the appropriate densities into each well and 1 mL of

proliferation media was added on top of the cells. The proliferation media was changed every 3 days during the course of the study.

5.6 Cytotoxicity Study

A cytotoxicity study was performed in order to investigate the effects of the collagen and fibrin gels used in the cell seeding process on C2C12 cell maturation and to observe any differences in cell differentiation based on the type of gel used. For this study, cells of varying densities were seeded into wells coated with collagen and fibrin and images were taken daily in order to follow the behavior of the cells. The gel study was conducted in a 24 well plate with cell densities of 5,000 cells/well, 10,000 cells/well, 15,000 cells/well, and 20,000 cells/well for each the collagen and fibrin gel.

Initially, four wells of the plate were coated with 150 µL of collagen gel each and four wells of the plate were coated with 150 µL of fibrin gel each. The cells were then added at the appropriate densities so that four different cell densities were added to each gel-coated well. Then, 1 mL of proliferation media was added to each well. After reaching 80-90% confluency, the media was switched to differentiation media, which was replaced every 3 days through the course of the study.

5.7 Live/Dead Assay

Once the tissue had formed within the mold, it was necessary to determine cell viability of the formed fiber. A live/dead staining was performed to visualize the parts of the tissue with live cells and the parts of the tissue with dead cells. Hoechst staining was used to visualize all nuclei within the fiber and propidium iodide at a concentration of 500 nM was used to visualize only the dead myocytes. It would then be inferred that the cells stained by the Hoechst stain

but not stained by propidium iodide were live cells. The following protocol was used to perform the live/dead stain:

1. Aspirate PBS from the wells containing the tissue fibers
2. Add PBS (1.0 ml/well for 24-well or 0.5 ml/well for 48-well plate) and add 1 μg/ml Hoechst 33342 (stock is 1 mg/ml) and incubate for 10 min at RT
3. Aspirate the PBS and wash 2X with PBS
4. Dilute propidium iodide 1:3000 in PBS+0.05% Tween-20
5. Add propidium iodide solution (300 μl/well for 24-well plate or 900 μl/well for 48-well plate) and incubate at RT for 10 min.
6. Aspirate antibody solution and wash 2X with PBS and add PBS (1.0 ml/well for 24-well or 0.5 ml/well for 48-well plate).
7. Cells are ready for observation by fluorescence microscopy. Plates can be stored at 4C wrapped in foil to protect from light.

5.8 Contractile Testing/ Electrical Stimulation Testing

Some of the tissues that had formed were also tested for contractile ability since a fully formed fiber should contain the appropriate contractile units for contractility upon electrical stimulation. Depolarization of the membrane potential can allow for the manipulation of intracellular calcium currents through the use of electrical pulses, and is known to accelerate sarcomere assembly in myofibers (Fujita et al., 2007; Nedachi et al., 2008). The team used a pair of electrodes interfaced with a BIOPAC system to supply a series of electrical pulses to both ends of the formed tissue in each agarose dog bone mold. Initially, the stimulator was set up to provide 5 volts for a 4 millisecond pulse length at 500 millisecond intervals. A number of attempts to test for contraction were carried out at 60 second intervals. Jumper wires were attached to the stimulator's positive and negative electrode leads and these were placed on either side of the tissue immersed in medium within the agarose dog bone mold channel. After testing for contraction at 5 volts, contraction was also tested using 10 volts at the same pulse length and time intervals. The set-up for contractility testing is shown in Figure 26.

Figure 26: Jumper Wires Connected to Both Ends of Tissue Construct

Chapter 6: Final Design & Validation

6.1 Cell Seeding Experiments

The cell seeding experiments performed allowed the team to determine the parameters by which the most contiguous tissue would form and remain anchored around the posts. Initially, the first version of the dog bone mold was tested, followed by a modified dog bone mold which was expected to allow for the development of more congruent tissue constructs. The team seeded different cell densities within the molds and compared the resulting tissue when seeding the cells without a gel, versus seeding them in a collagen or fibrin gel matrix. It was determined that the optimal cell density at which to seed the cells in the agarose dog bone molds is 3.5 million cells per mold, and the most contiguous tissue developed after seeding this density using no gel. The following are the results obtained using no gel, versus using collagen or fibrin as matrices.

6.1.1 Initial Dog Bone Mold

The team analyzed the results of the initial dog bone mold and determined that its dimensions were not the optimal ones. The depth of the seeding channel, which was 2 mm, was found to be insufficient to allow the seeding of a cell-gel solution since it provided inadequate space for media retention. As a result, the group was required to flood the mold with media, which often caused detachment of the tissue from within the seeding channel. None of the experiments performed using this first iteration of the mold developed contiguous, full tissues, and none of the tissue fragments were found to be successfully anchored around the posts.

6.1.1.1 No-gel Cell Seeding

After different attempts at forming a contiguous, full tissue, the team was unable to attain the desired results throughout the entire seeding channel. As seen in Figure 27, the tissue had adopted the post's circular shape at one of its ends but had come off the post one day after cell seeding at a density of 3.5 million cells per mold. Although not shown in Figure 27, none of the experiments performed using no-gel seeding systems caused the development of a full, contiguous tissue. The tissue was found ruptured at one or two different spots, as soon as one day post-seeding.

Figure 27: Tissue formation at 1 day post-seeding of cells seeded without a gel in initial mold at 3.5 million cells/mold (5X)

6.1.1.2 Collagen Gel Seeding

The results obtained after seeding a density of 3.5 million cells into a collagen matrix also resulted in fragmented tissue constructs. One day post-seeding, tissue fragments were found throughout the seeding channel. As shown in Figure 28, some of the fragments appeared as if they had begun contracting into a circular shape, instead of maintaining their

linear orientation. None of the tissue fragments formed when using collagen as a matrix were found to be anchored one day after seeding.

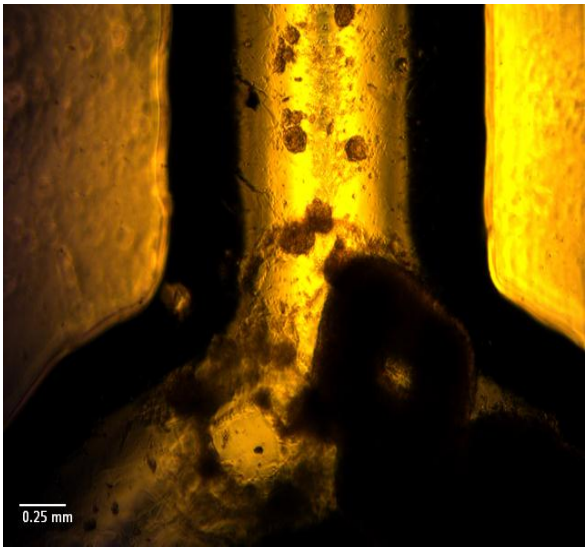


Figure 28: Tissue formation at 1 day post-seeding of cells seeded in collagen gel in initial mold at 3.5 million cells/mold (5X)

6.1.1.3 Fibrin Gel Seeding

The results obtained after seeding the molds with a density of 3.5 million cells in a fibrin matrix resulted in fragmented tissue, and then contracted tissue fragments. Seeding the cell-matrix solution within the small dimensions of the seeding channel was particularly difficult when using fibrin as its components are more viscous than those of collagen. As seen in Figure 27, the cell distribution was inconsistent throughout the channel, as the tissue had ruptured and there were no additional tissue fragments in close proximity to the one shown in the picture. The tissue fragments were found to not be anchored at the posts.

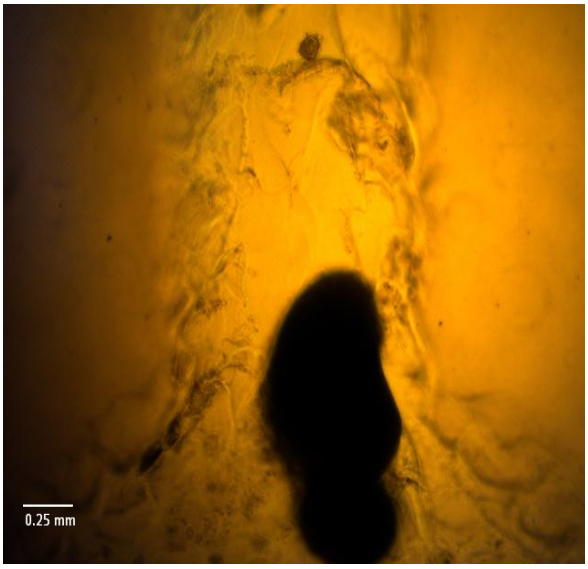


Figure 29: Tissue formation at 1 day post-seeding of cells seeded in fibrin gel in initial mold at 3.5 million cells/mold (5X)

6.1.2 Revised Dog Bone Mold

After a thorough analysis of the results of the initial dog bone mold, the group decided to modify the dimensions of the design and machine a new mold. The increase in channel depth and post height, to 3.5 mm, was found to improve the overall tissue formation within the seeding channel. The best results were obtained when the cells were seeded into the mold without a gel matrix.

6.1.2.1 No-gel Cell Seeding

The results observed after seeding 3.5 million cells in the molds without using any gel showed optimal tissue formation. At day 1, represented in the rightmost section of Figure 30, the tissue was fully anchored around the posts, and there were no ruptured areas within the tissue construct. The cell distribution appeared to be very consistent throughout the tissue. As seen in the rightmost image in Figure 31, the tissue formed contiguously throughout the entire channel, and had remained anchored around both posts.

6.1.2.2 Collagen Gel Seeding

Seeding cells embedded within a type I collagen matrix in the seeding channels within the molds resulted in the least contiguous tissue formation. At day 1, as shown by the first picture in Figure 30, the tissue was somewhat anchored around one of the posts, but was fully ruptured below the post. Also, as shown in Figure 31, the entire tissue was fragmented at various locations throughout the channel and at the posts. Loss of anchorage occurred at the bottom post as the tissue fragment on the bottom of the seeding channel had adopted the circular post shape but was no longer connected to the remaining tissue.

6.1.2.3 Fibrin Gel Seeding

Using fibrin as a matrix gave significantly better results as compared to collagen, but were not comparable to the results obtained from seeding the cells without a gel matrix. At day 1, the resulting fiber was anchored on one of the posts, as seen in the middle picture of Figure 30, but the cell distribution close to the post seemed to be slightly less than ideal, indicating that it could be prone to rupturing. As shown in Figure 31, significantly better tissue formation was found throughout the entire channel, as compared to the collagen gel seeding experiment, but the fibrin tissue was still ruptured and lost its anchorage at the bottom post.

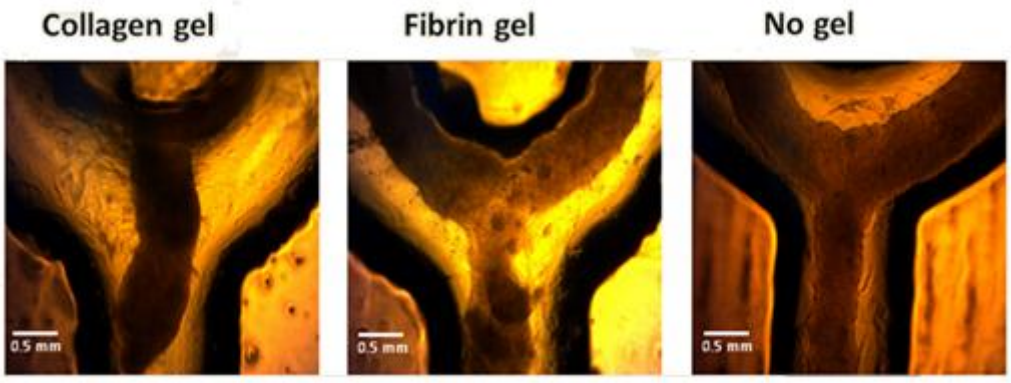


Figure 30: Tissue Formation 1 day Post-Seeding at 3.5 million cells/mold using Collagen, Fibrin, and No Gel

Figure 31: Complete Tissue Formation 1 day Post-Seeding at 3.5 million cells/mold using Collagen, Fibrin, and No Gel

6.2 Immunocytochemistry

The results obtained from the BrdU staining and myosin staining allowed the group to characterize the behavior of C2C12 cells under various conditions. The BrdU differentiation study indicated the time point at which the cells began differentiation and the myosin study confirmed the presence of mature myofibers within the sample.

6.2.1 BrdU Staining

Results from the BrdU study showed consistent levels of proliferation and differentiation across all four cell densities that were tested, indicating that cell density does not affect the time point at which cells begin differentiation. At all densities, proliferation was found to decrease sharply by Day 5, indicating that Day 5 is the time point when cells begin differentiation.

In the following images, the green staining is the BrdU stain, which represents proliferative cells, while the blue staining is the Hoechst stain, which represents all cells, regardless of their position in the cell cycle. The side-by-side images of each BrdU stain and

Hoechst stain were taken from within the same frame of view. Images taken from a well of seeding density of 10,000 cells/well are shown below, and images from all other seeding densities tested can be found in Appendix A.

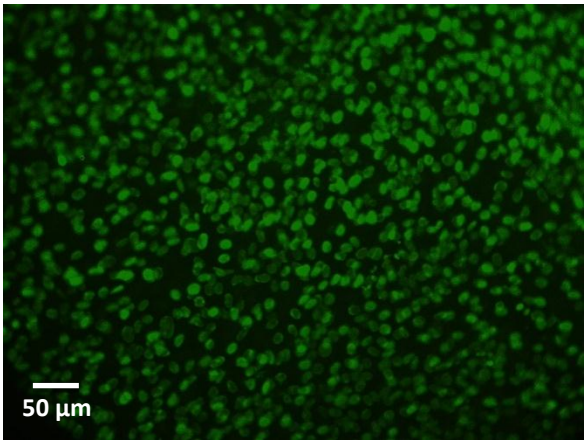
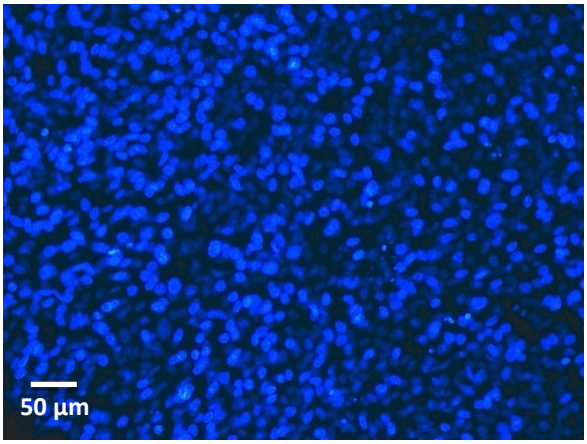


Figure 32: Day 1 BrdU Stain (10,000 cells/well



Day 1 Hoechst Stain (10,000 cells/well)

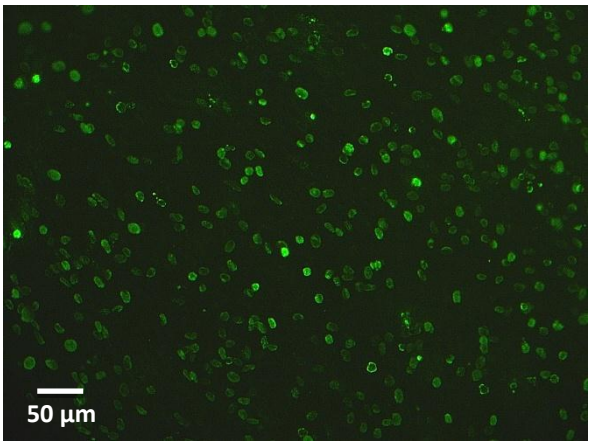
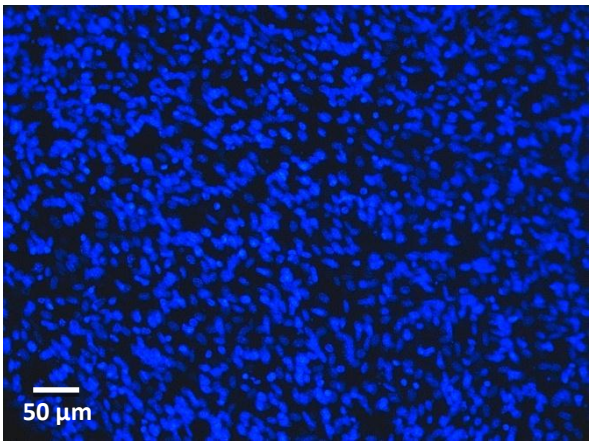


Figure 33: Day 2 BrdU Stain (10,000 cells/well)



Day 2 Hoechst Stain (10,000 cells/well)

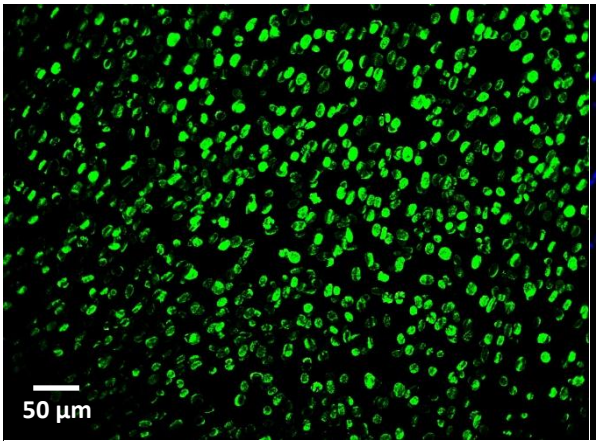
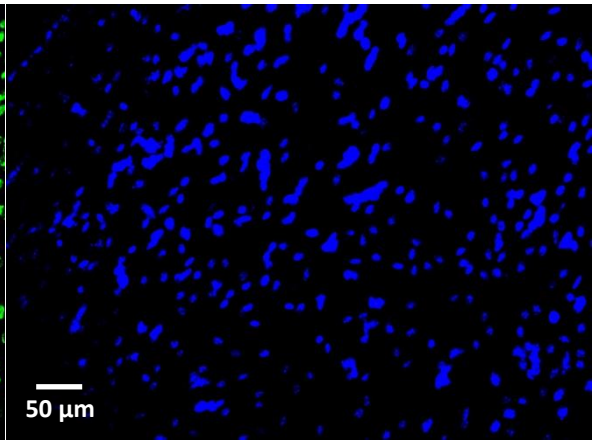


Figure 34: Day 3 BrdU Stain (10,000 cells/well)



Day 3 Hoechst Stain (10,000 cells/well)

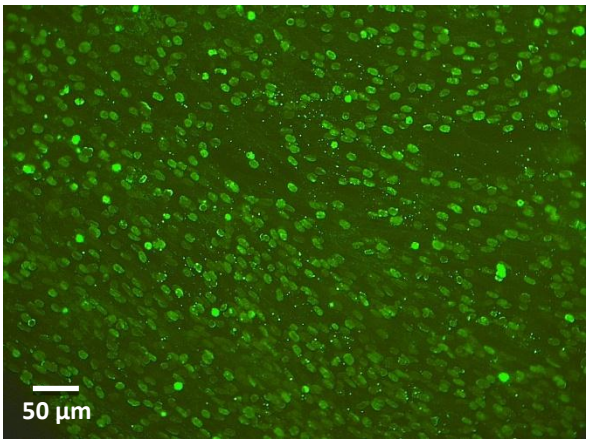
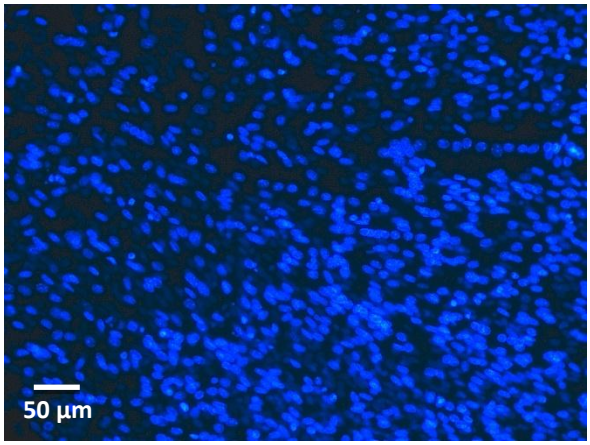


Figure 35: Day 4 BrdU Stain (10,000 cells/well)



Day 4 Hoechst Stain (10,000 cells/well)

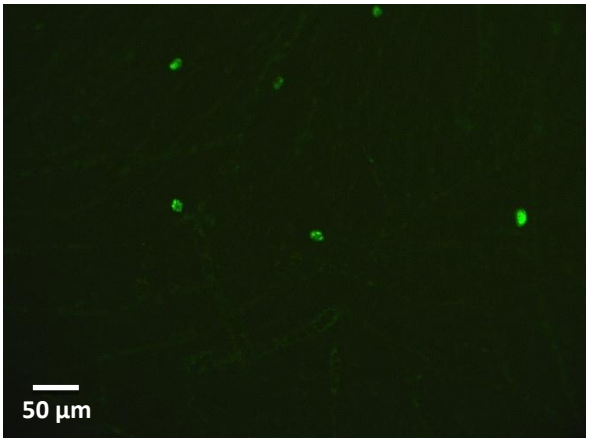
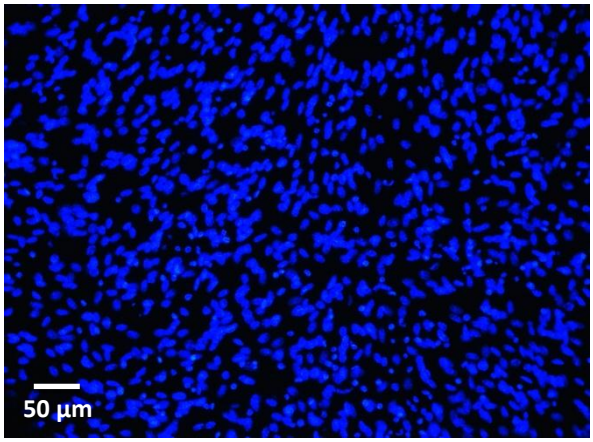


Figure 36: Day 5 BrdU Stain (10,000 cells/well)



Day 5 Hoechst Stain (10,000 cells/well)

As shown by the images, the number of proliferating cells remained relatively constant from Days 1 through 4. At Day 5, however, there was a sharp decrease in the number of cells undergoing proliferation.

The graph in Figure 37 presents quantitative data of number of cells undergoing proliferation and total number of cells at time points throughout the study. The data was obtained by overlaying a grid onto the images, counting 10 boxes from the grid, finding the average number of cells per box, and multiplying by the total number of boxes in the image to determine the total cell count.

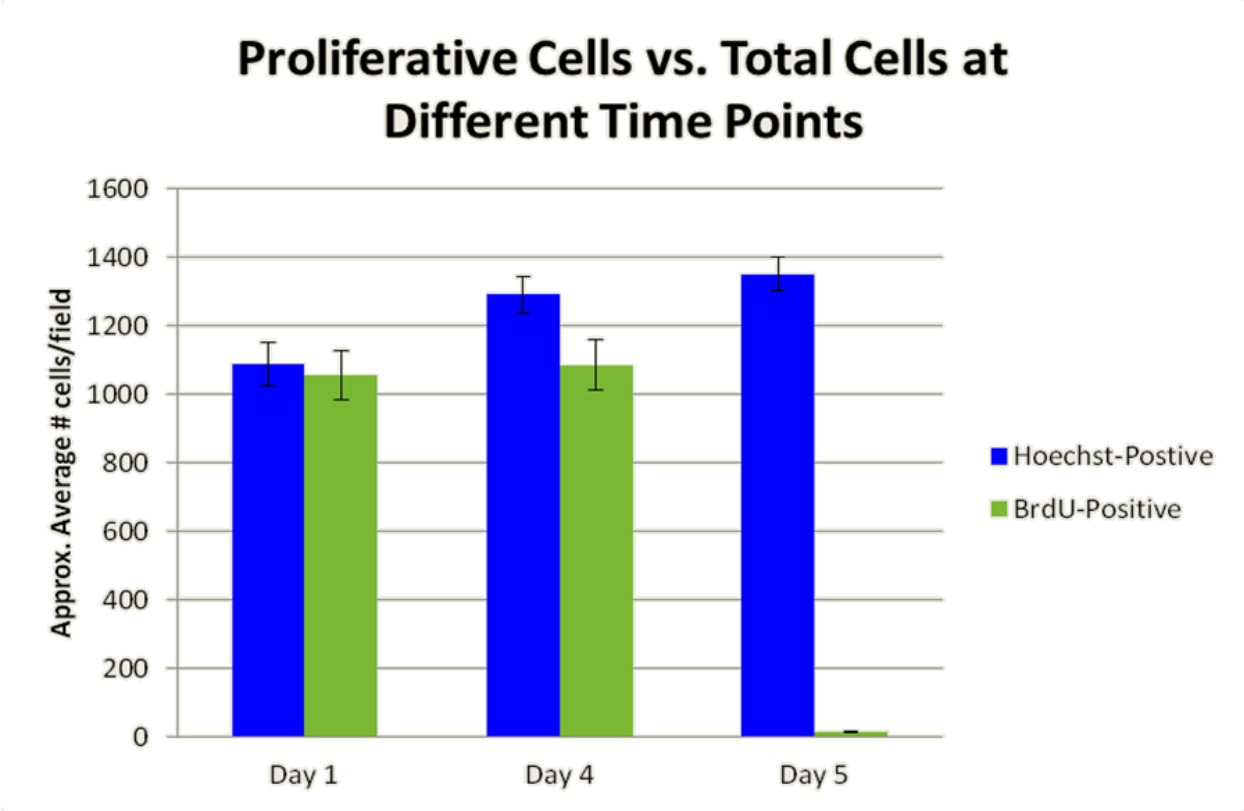


Figure 37: Number of Cells Undergoing Proliferation and Total Number of Cells at Various Time Points

The quantitative measurements from the graph support the qualitative assessments from the fluorescence images. The graph shows relatively stable levels of proliferative cells from Days 1 through 4, and a sharp decline in proliferative cells at Day 5. Only cell numbers from Days 1, 4, and 5 were counted because qualitative findings from the images indicated that the most significant discrepancy in cell number occurred between Days 4 and 5.

6.2.2 Myogenic Differentiation Study

Images obtained from the proliferation study showed formation of mature myofibers in all wells, as evidenced by the myosin staining that was found to be present along the fused nuclei of the myofiber. The staining was performed such that the myosin staining would stain for the myosin protein found in mature myofibers and the Hoescht staining would stain for all

cells found within the well. Figure 38 shows the myofibers that formed within a well, with the myosin represented by the green staining, and the cells represented by the blue staining.

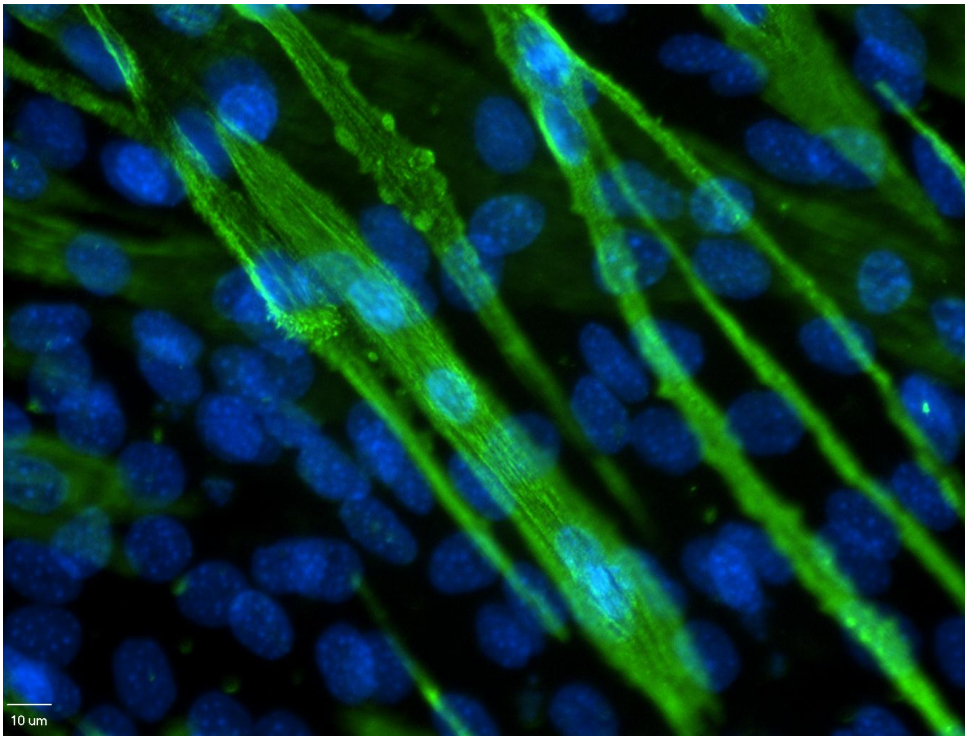


Figure 38: Nuclei Fused to form Myofibers

In this image, the presence of a myofiber is indicated by the three fused nuclei that make up the myofiber containing myosin protein.

6.3 Cytotoxicity Study

Images taken at several time points from the cytotoxicity study were used to determine the behavior of C2C12 cells when placed on collagen and fibrin gels. Shown below in Figure 39 and Figure 40 are images of the cells at Day 4 of proliferation, which were seeded at 10,000 cells/well onto collagen and fibrin gels, respectively.

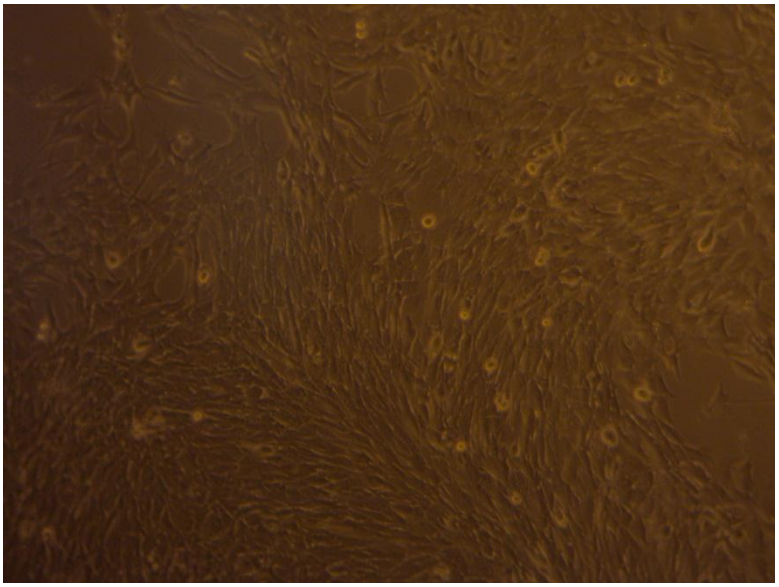


Figure 39: Cells on Collagen Gel at Day 4 of Proliferation

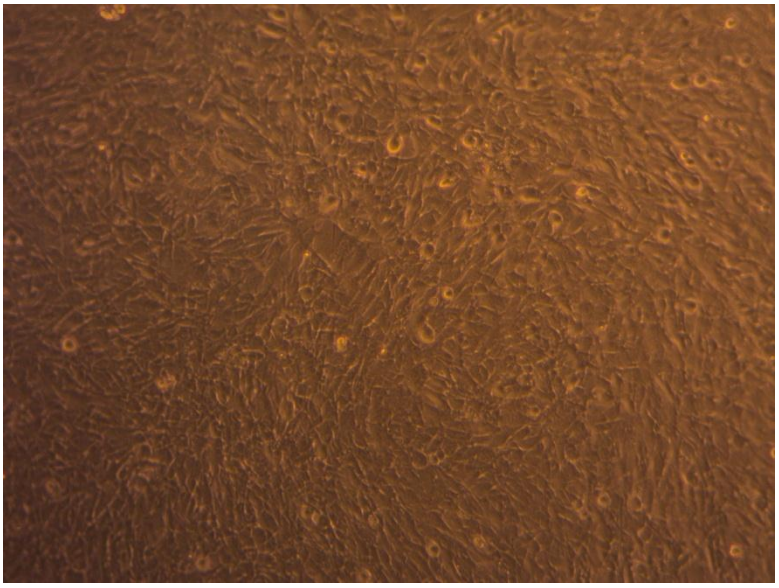


Figure 40: Cells on Fibrin Gel at Day 4 of Proliferation

These two images indicate that the cells were proliferating appropriately when seeded onto collagen and fibrin gel substrates.

Shown below in Figure 41 and Figure 42 are images of the cells at Day 1 post-differentiation, which were seeded at 10,000 cells/well onto collagen and fibrin gels,

respectively. These images can be used to understand the degree of differentiation that cells seeded onto each type of gel undergo once switched to differentiation media.

Figure 41: Cells on Collagen Gel at Day 1 Post-Differentiation

Figure 42: Cells on Fibrin Gel at Day 1 Post-Differentiation

These images show that cells seeded onto the fibrin gel differentiated to a greater degree than the cells seeded onto the collagen gel. This can be seen by the amount of

unidirectional alignment shown by the cells seeded on the fibrin gel, which is not present in the cells seeded onto the collagen gel.

6.4 Live/Dead Staining

Results from the live/dead staining performed on a tissue sample allowed the team to identify the regions of the tissue containing live cells and the regions of the tissue containing dead cells. The staining was performed with a Hoechst stain to visualize all of the cells present in the tissue and propidium iodide to visualize dead cells. In Figure 43, the cells stained blue represent all cells within the tissue sample, while the cells stained red indicate all dead cells. The white dashed line represents the approximate fiber boundary.

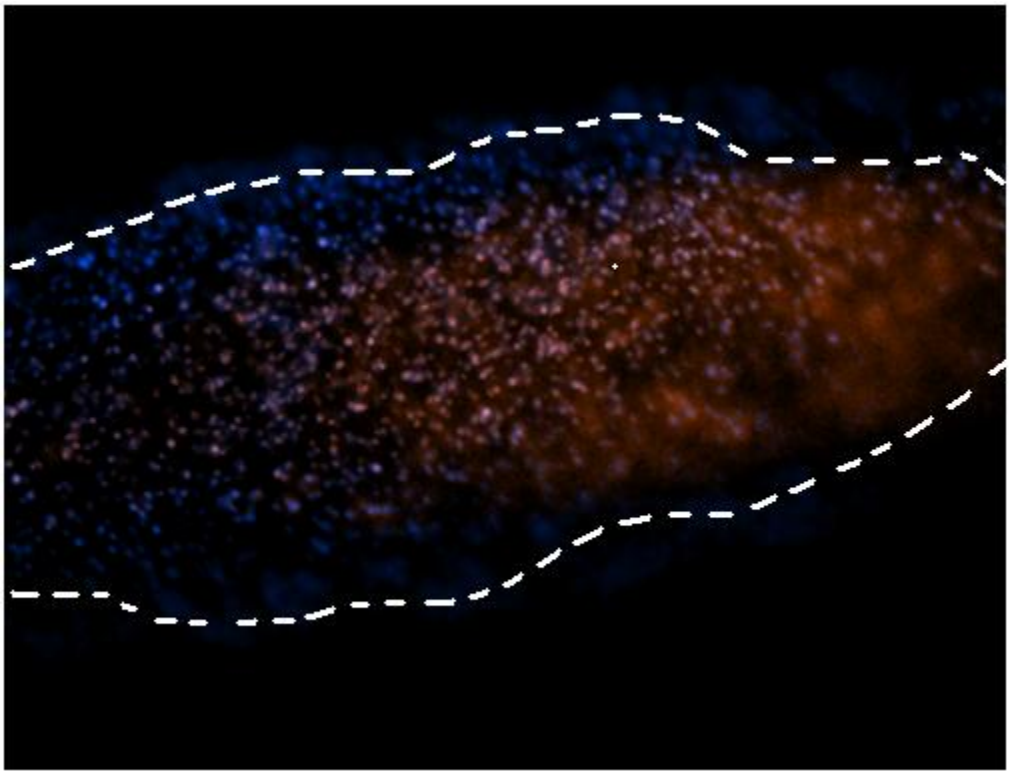


Figure 43: Live/Dead Stain of Tissue Sample

The tissue staining shown in Figure 31 shows staining of cells is apparent throughout the fiber, while the dead cells are concentrated towards the center of the tissue sample.

6.5 Contractility Testing

The tissues formed from cell seeding into the mold were subjected to electrical pulses ranging from 5 to 10 volts for a 4 millisecond pulse length at 500 millisecond intervals. After testing various tissue samples, no contraction was observed in any of the tissues. Bubbles formed around the jumper wires providing the electrical pulses, indicating that the electrical stimulus was being transferred to the agarose mold, but none of the fibers showed contractile function after the pulses were applied for 60 seconds.

Chapter 7: Discussion

7.1 Project Discussion

The main focus of this project was to design, develop, and test a scaffold-free system that can be used for the development of skeletal muscle fibers in vivo. Once the system was manufactured, it was tested with the seeding of myogenic cells at various seeding densities into the mold in no-gel and gel-embedded systems. C2C12 cells were characterized by fluorescently labeling cells seeded onto plates to determine the time point at which the cells exit the proliferation stage of the cell cycle and enter differentiation, as well as to determine whether the development of mature myofibers is possible in vitro.

Cell seeding experiments indicated that 3.5 million cells/mold is the optimal seeding density for formation of congruent tissue. Densities lower than 3.5 million cells/mold resulted in formation of fragmented tissue due to inadequate cell-to-cell contact throughout the mold. Densities higher than 3.5 million cells/mold resulted in clumps of cells in addition to the formed tissue, indicating that the cells were not incorporated into the tissue, and therefore, not necessary for fiber formation.

Comparison of seeding cells alone into the mold versus seeding cells embedded in a collagen or fibrin gel into the mold indicated that seeding cells alone is a more effective means of forming congruent tissue. Cells seeded into the mold at the appropriate density of 3.5 million cells/mold, without a gel matrix, were able to form completely congruent tissue throughout the dog bone structure by Day 1 post-seeding. The multiple sites of fragmentation that occurred in the tissue when cells were seeded in the collagen gel and the occasional fragmentation that occurred in the tissue when cells were seeded in the fibrin gel indicate that

the presence of the gel surrounding the cells inhibited proper tissue formation. This inhibition could be due to the physical presence of the provisional gel matrix preventing the complete proliferation of cells throughout the mold.

The BrdU study performed on the C2C12 cells cultured into a 24-well plate allowed for the determination of the time point of differentiation. Since the images taken of each time point indicate that the number of proliferative nuclei, relative to total nuclei, decreased drastically from Day 4 to Day 5, Day 5 was determined to be the point at which the cells switch to the differentiation phase. The quantitative results displayed in Figure 29 also show that there were similar levels of proliferations at Days 1 and 4, but significantly less proliferation levels at Day 5.

Due to time constraints in the project, the results of the BrdU study were not incorporated into the mold seeding to optimize tissue formation. In the future, however, the study suggests that the optimal time point at which to seed cells into the mold would be at Day 4. This is because cells begin to form ECM components while in the differentiation phase and these ECM components would contribute to increased cohesiveness and structural integrity of the myofiber. Therefore, seeding cells at the time point at which differentiation begins, rather than during the proliferative stages, as was done in the seeding experiments, could facilitate more congruent tissue formation. This study also indicates that if the cells are seeded while in proliferation, as was done in the seeding experiments, the tissue must retain its conformation for at least 4 to 5 days, in order to have enough integrity to be manipulated for immunocytochemistry assays and contractile testing.

Myosin staining performed on cells seeded within 24-well plates and allowed to proliferate within 10 days showed that there was myosin present in the conformation of a myofiber within the tissue. The fused nuclei in Figure 38 indicate myofiber formation and the myosin staining indicates maturation of the myofiber. These results suggest that in vitro development of skeletal muscle tissue can lead to the formation of mature myofibers. The findings from the myosin staining indicate that if the skeletal muscle tissue formed from within the dog bone mold remains viable for at least 10 days, it should contain myosin protein and appropriately stain for myosin, indicating its maturation.

The cytotoxicity study performed indicated that neither of the gels have negative effects on cell behavior. Cells seeded onto both collagen and fibrin gels showed proper proliferation, indicating that the gels are not cytotoxic. After addition of differentiation media to the cells, the cells seeded onto the fibrin gel were found to have better differentiation and alignment than those seeded onto the collagen gel. The limited alignment found in the cells seeded onto the collagen gel correlate with the fragmentation found in the seeding experiments with cells seeded within a collagen gel. The inadequate differentiation of cells, when embedded within a collagen gel, could have caused the tissue fragmentation.

The live/dead stain performed on the myofibers indicated that the majority of cells in the center of the tissue construct were dead. This result could be due to inadequate media diffusion into the center of the tissue, due to its thickness, which is greater than that of native myofibers. Additionally, the loss of tissue anchoring that occurred prior to the assay being performed could have caused cell death. Complete anchoring of skeletal muscle tissue is required for proper formation of skeletal myofibers, cell viability, and formation of contractile

units. Lack of anchoring could, therefore, be a contributing factor to the cell death observed at the center of the tissue construct.

Contractility testing was performed on the tissues that formed from cell seeding experiments by stimulating the tissue with electrodes that delivered 5 volts at 4 ms pulses. No contractions were observed in the tissue. The reason for the lack of response to electrical stimulation could be cell death within the tissue, due to either a loss of anchoring or insufficient media diffusion throughout the tissue. After Day 1 post-seeding, most formed tissues were found to have a loss of anchoring due to the tissue surrounding the post moving off of the post and settling within the channel.

This could be due to the contractile force produced by the cells, which may have caused the tissue to contract up the post and lose its anchoring around the post. Tissue death could also be due to insufficient media diffusion into the center of the tissue due to the large diameter of the tissue. Native skeletal muscle fibers are 100 µm in diameter, however, the molding system has a diameter of 1 mm. This discrepancy was caused by manufacturing limitations from the WPI machine shop, since the manufacturing of a channel less than 1 mm in diameter requires. Since the diameter of the formed tissue is approximately 10X that of native tissue, the extra tissue may be hindering the diffusion of media, which provides nutrients, to the cells at the center of the tissue structure. Cell death in the center of the tissue due to lack of nutrients from media could be contributing to the lack of contractile properties in the tissue.

Modifications to the design on the molding system could be incorporated into the system in order to solve the problems encountered with the loss of anchoring. By constructing a mold with taller anchor posts, the mold will provide a larger distance for the fiber to contract

upwards on, minimizing the chance that it will completely move off the post. Additionally, manufacture of the mold using more advanced technologies such as photolithography can allow for the development of a mold with channels of smaller diameters. The fiber resulting from seeding of these molds would be thinner than the fiber produced from the current molding system and would allow increased media diffusion into the center of the fiber.

Limitations to the molding system include the post height and channel width mentioned previously. Other limitations include the lack of an incorporated system for mechanical conditioning of the fiber as it forms. Previous studies have described the improvements in mechanical properties of myofibers due to dynamic mechanical conditioning. The dog bone molding system is, however, amenable to mechanical conditioning due to its linear shape. Future groups can expand upon the current system to incorporate a system to cyclically provide mechanical conditioning to the tissue as it forms a myofiber in order to enhance its mechanical properties.

Though the model that was developed was successful in allowing the team to meet the goal of achieving tissue formation, the proposed modifications could lead to its optimization. A thinner channel diameter, taller posts, and the addition of a mechanical conditioning system could be used for the development of skeletal muscle fibers with improved properties and greater potential for use in research applications.

7.2 Impact Analysis

The group conducted an impact analysis that shows how our project relates to global concerns in various ways. The topics of this analysis are: economics, environmental impact,

society influence, political ramifications, ethical concerns, health and safety issues, manufacturability, and sustainability.

7.2.1 Economics

The success of our product depends on how much of a need our users have for our product. As a design team, we must determine how much they are willing to spend on it. If our product makes it very easy for the user to produce human skeletal muscle fibers, then our product will sell. Furthermore, patients suffering from muscle loss may not mind paying for a state-of-the-art device that will help them regain some muscle mass even if is an expensive alternative.

7.2.2 Environmental Impact

The molding device will not have a significant impact on the environment. Any potential effects on the environment due to our device are discussed in the sustainability section later in the report. An indirect environmental impact is that if our device is successful it can improve and possibly extend a patient's life. If humans live longer, they will contribute more waste and use up resources at a faster rate. This can add further stress on the economy and the non-renewable energy sources.

7.2.3 Societal Influence

The molding system can potentially have a drastic effect on a patient. Since this system will allow for the regeneration of lost muscle mass, it will greatly improve the quality of life of the patient and the patient's caretakers. If the molding system goes to market, it could have a beneficial psychological and societal effect on certain groups such as injured soldiers and cancer patients.

7.2.4 Political Ramifications

Currently, the molding device does not have any political ramifications. If, in the future, the device can be manufactured quickly and cost-effectively, it can have a big impact for soldiers and cancer patients around the world. Not only will this device make an impact in European countries, but it will especially be useful in poor Asian countries that have a variety of muscle diseases.

7.2.5 Ethical Concerns

The molding device does not have many associated ethical concerns. Since this device uses progenitor cell lines, people who are not fully informed about the cell types used in the device might think that stem cells might be used. Stem cells represent a polarizing subject in biological research and thus proper presentation of the device to users and patients is necessary. Another possible ethical concern is the practice of taking a patient's cells and using them in a biomedical capacity. This practice can conflict with certain individuals' religious beliefs.

7.2.6 Health and Safety Issues

The molding device was designed and built with the intention of improving a patient's quality of life. Theoretically, the device should improve a patient's quality of life by aiding in repairing and coercing the patient's body to regenerate lost muscle mass. The safety of the device can be tested by the FDA through in-depth animal and clinical testing. This will help the device earn an HCPCS code and confirm that the device is safe for patients. An HCPCS code will help the device to be covered by insurance companies.

7.2.7 Manufacturability

The manufacturing process of the molding system involves a one-time manufacture of an acrylic mold template, which takes about 2 hours. The PDMS mold, which is then developed from the acrylic template, is then created in a process that takes about 4 hours for the PDMS to be de-gassed. Finally, the agarose mold is developed in a 3 hour long process, using the PDMS mold as a template. The use of a double negative template ensures that there is always a means to develop single negative and final molds through processes that do not involve the use of the machine shop and the skills of a machinist, but rather components found in the lab. Once one PDMS mold is made, it can be used as the template for all future agarose molds. Therefore, the only mold that must be continually made for each experiment is the agarose mold. Additionally, the use of three dog bone molds on one block allows for the manufacture of three molds at once.

Compared to the use of scaffold systems, such as seeding cells onto microthreads, the manufacturability of the dog bone molding system is high because of the decreased time that it takes to manufacture each scaffold. For example, the development of standard collagen microthreads is a three day process and each thread must be extruded by hand. The relative ease of manufacture of the molding system makes it a feasible alternative to the current standard device.

7.2.8 Sustainability

Sustainability can be defined as using materials, processes, and systems that will not deplete resources or affect natural cycles. Using this definition, the design team analyzed the complete process of producing a muscle fiber using a gel matrix and a dog bone molding system

for its conformance to sustainability. Collagen, fibrin, agarose, and myogenic cells are naturally-occurring. They can be derived from animals or human patients and thus, they are renewable resources and will not be depleted. The myogenic cells can regenerate while collagen, fibrin, and agarose can be collected in several environmentally-friendly means. However, the master mold is made of acrylic, also known as PMMA. PMMA is synthesized using petroleum, which is a non-renewable resource. Hence, it is highly recommended to find an alternate material that has similar properties as PMMA but comes from a sustainable resource. Finally, most of the culturing equipment was made of various plastics that are discarded properly and can be recycled at a proper facility. All these factors make this device a very eco-friendly one and its sustainability is one of the added benefits of using this product.

Chapter 8: Conclusions and Recommendation

The main objective of this project was to achieve skeletal muscle tissue formation using a scaffold-free system. The team developed a dog-bone shaped molding system with a channel for the seeding of myogenic cells and anchor posts incorporated within the mold itself. Experimentation with seeding densities and the use of biological gels allowed the team to determine the optimal conditions for fiber formation using the dog bone mold. Assays were performed to characterize C2C12 cells to validate the seeding experiments and to determine ways to optimize the seeding process in future studies. Overall, the team developed an effective way to induce skeletal muscle tissue formation *in vitro* and provided a platform for the development of skeletal muscle fibers with optimized properties in the future.

While the goal of this project was to design a molding device that will recapitulate skeletal muscle fiber, this project can be expanded in various directions in the future. One way to optimize the current molding system would involve the manufacture of the mold using a process such as photolithography. The laser cutting process that was used to develop the dog bone molds in this project is not precise enough to create a smooth surface for the cell seeding channel. Photolithography is unavailable using the resources at WPI, but if used, could be used for the development of a dog-bone mold with a smoother surface area for the cells. The smoother surface area could facilitate increased cell-to-cell contact since the cells would be seeded onto a smooth surface, rather than one with grooves for cells to fill into.

Additionally, the bottom of the channel of the current dog bone mold has sharp edges, as shown in the schematic of the cross-section of the mold shown in Figure 44. The ideal seeding channel, also shown in Figure 31 has more rounded edges. In the current system, the sharp

edges inhibit full cell-to-cell contact at the bottom of the seeding channel because the tissue that forms is rounded. The shape of the ideal channel would more easily facilitate cell-to-cell contact and reduce the possibility of excluding cells at the edges of the channel from integrating into the tissue.

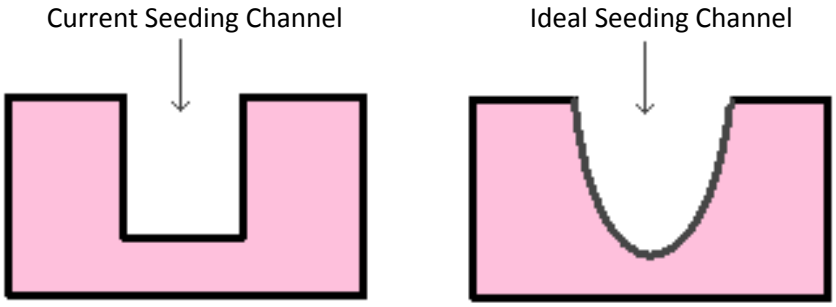


Figure 44: Schematic of Current Seeding Channel and Ideal Seeding Channel

Many studies have shown that mechanical conditioning of skeletal muscle fibers can provide the fiber with improved mechanical properties such as higher tensile strength, maintenance of desired elastic modulus, and a high percent elongation until failure.

There are many ways to mechanically condition the muscle fiber developed from the dog-bone molding system. One example involves the use of magnetic particles, which can be loaded into agarose posts. After the muscle fiber forms, a magnet attached to an actuator can be used to attract the magnetic particles, and can cause the muscle fiber to stretch in a cyclic manner. Another possible method is to carefully cut the agarose mold in half while keeping the muscle fiber intact and attached on either end to an agarose posts. One side of the agarose mold can be attached to an actuator and the actuator can be used to mechanically condition the muscle fiber.

In this project, the muscle fiber was electrically stimulated in order to observe whether the muscle contracted. More quantifiable experiments can be conducted where a force transducer can be used to measure any contractile forces. Furthermore, a mechanical testing device, such as the Instron can be used to measure mechanical properties such as tensile strength and elastic modulus. All of these mechanical properties of the engineered fiber can be compared to those of a native skeletal muscle fiber.

Reinnervation is an important process for full integration of skeletal muscle grafts and thus, it is important to show that the engineered muscle fiber allows for nerves to form neuromuscular junctions. An experiment can be conducted *in vitro* where PC12 cells, a widely used cell line derived from rat pheochromocytoma and which serves as a standard model system for neurons, can be co-cultured with muscle fibers formed from C2C12 cells. Neuroscreen-1, a subclone of PC12, does not aggregate with itself but is still able to develop neurite outgrowth. After two days, the Neuroscreen-1 cells should have enough neurite outgrowths to begin to form neuromuscular junctions.

Electrical stimulation tests can be conducted to electrically stimulated neural axons to determine whether an action potential is generated and, consequentially, whether the muscle contracts. If the engineered skeletal muscle fiber has the appropriate mechanical properties and allows for reinnervation, *in vivo* studies in rats can be conducted.

The ultimate end goal of this project is to regenerate human skeletal muscle that can be grafted into patients with muscle loss from injuries or disease. However, *in vivo* studies in small animals and large animals must be conducted prior to human clinical trials. The in vivo studies will demonstrate the degree of integration of the engineered skeletal muscle fiber into the

surrounding native muscle tissue. The results of the *in vivo* studies can also be used to measure the effectiveness of the engineered muscle in recapitulating native skeletal muscle. The *in vivo* test subjects with a muscle graft can be compared to a control group containing subjects with no muscle injury to determine the effectiveness of the engineered muscle in providing the animals with natural mobility.

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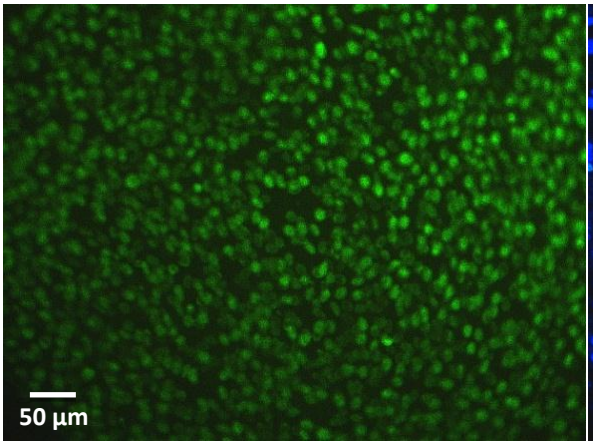
Appendix A: C2C12 Proliferation and Differentiation Studies

DAY 1



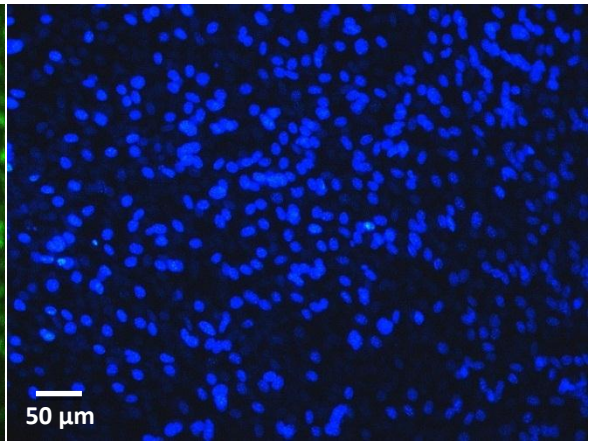
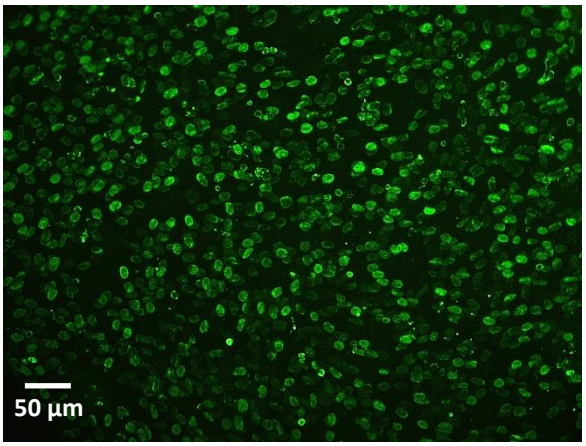


Figure 45: Day 1 BrdU Stain (5,000 cells/well) **Day 1 Hoechst Stain (5,000 cells/well)**



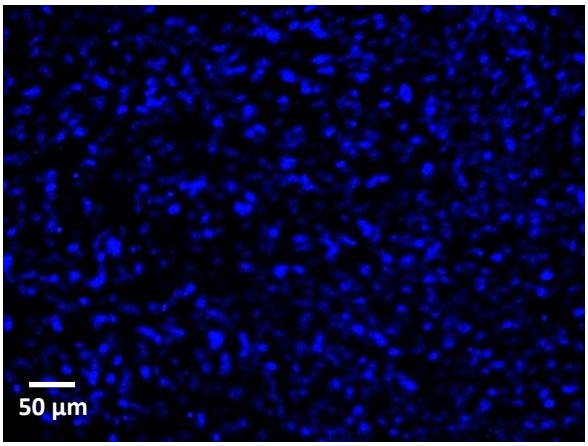
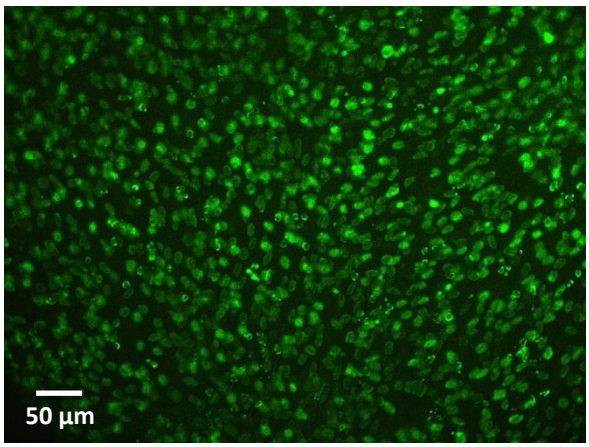


Figure 46: Day 1 BrdU Stain (15,000 cells/well) **Day 1 Hoechst Stain (15,000 cells/well)**



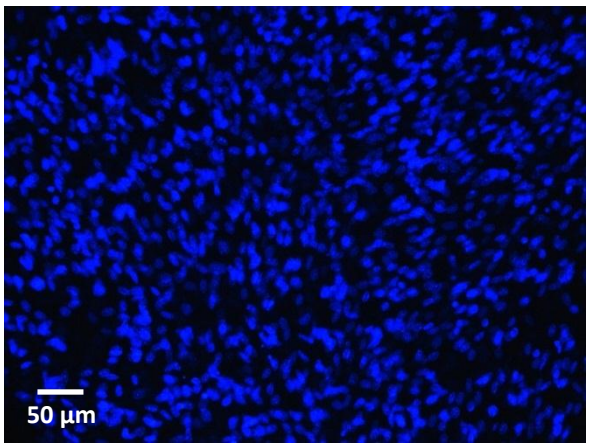


Figure 47: Day 1 BrdU Stain (20,000 cells/well) **Day 1 Hoechst Stain (20,000 cells/well)**

DAY 2

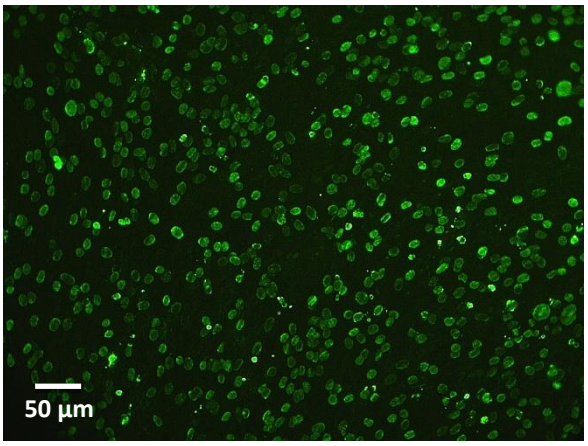
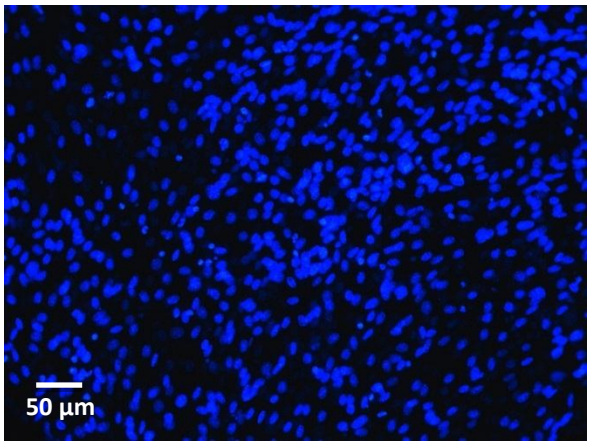
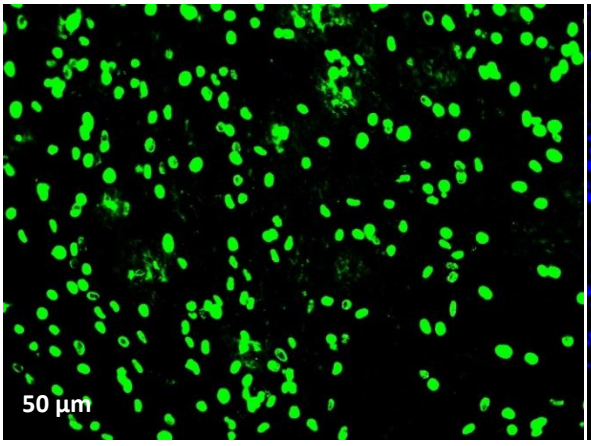


Figure 48: Day 2 BrdU Stain (5,000 cells/well)



Day 2 Hoechst Stain (5,000 cells/well)



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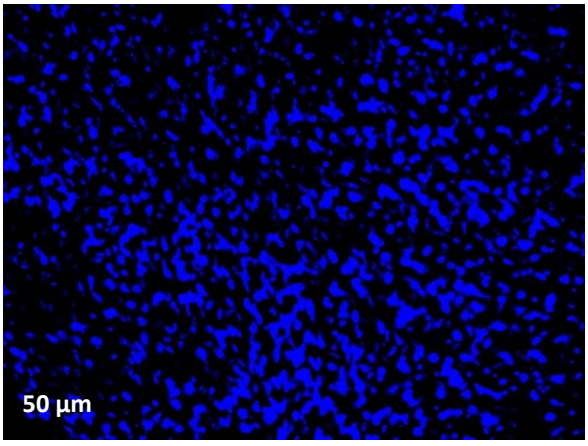

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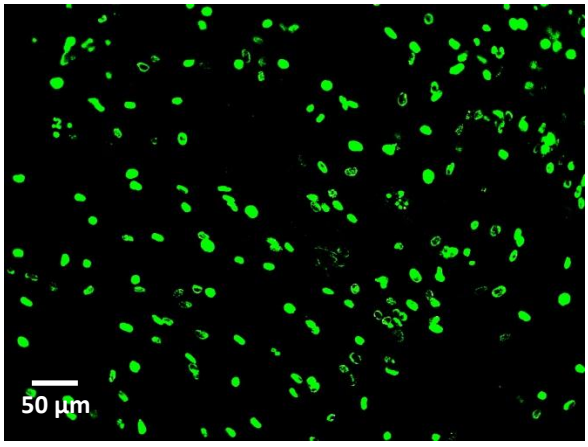

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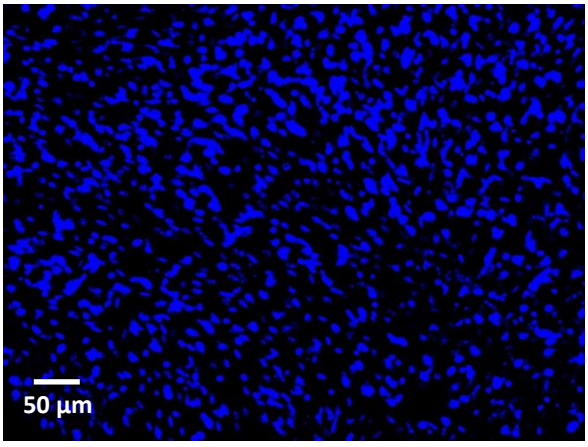
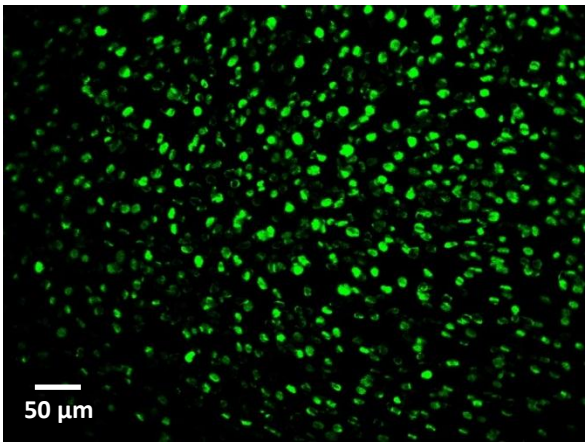


Day 2 Hoechst Stain (20,000 cells/well)

DAY 3



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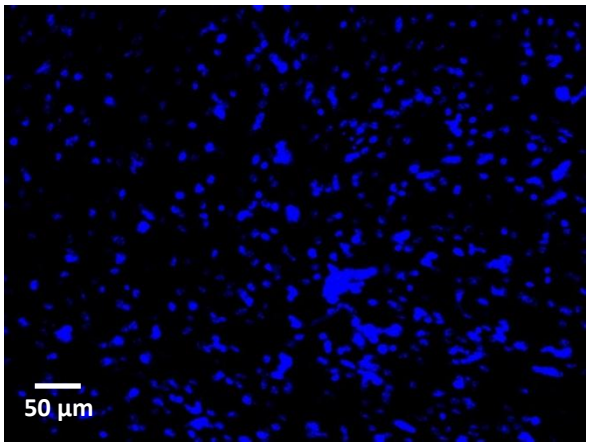

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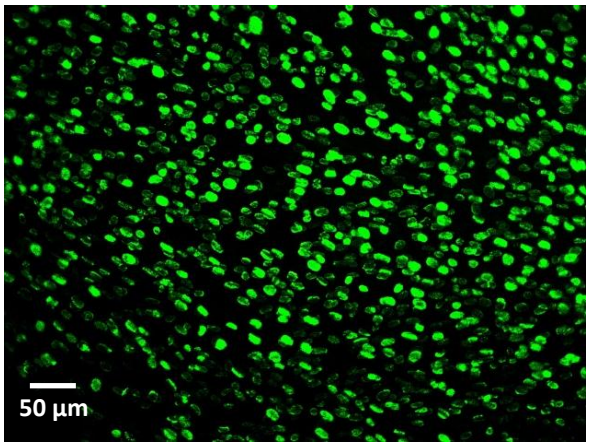

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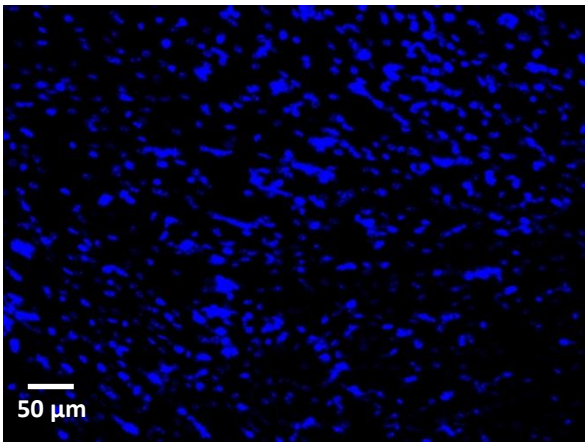

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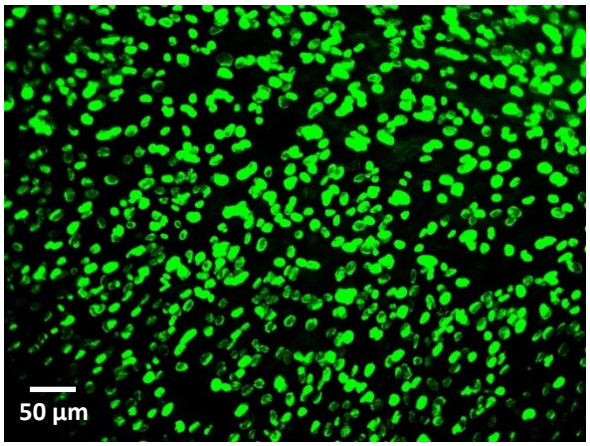
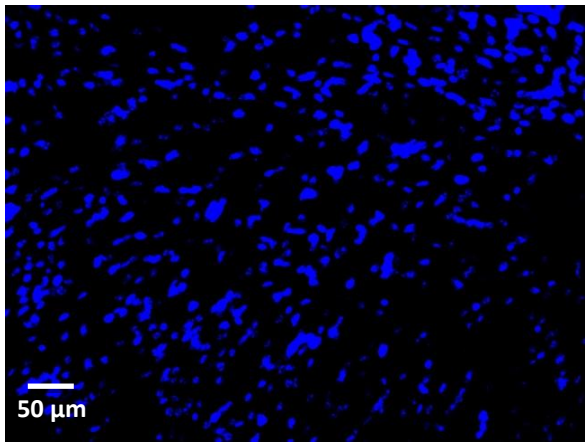


Figure 53: Day 3 BrdU Stain (20,000 cells/well)



Day 3 Hoechst Stain (20,000 cells/well)

DAY 4

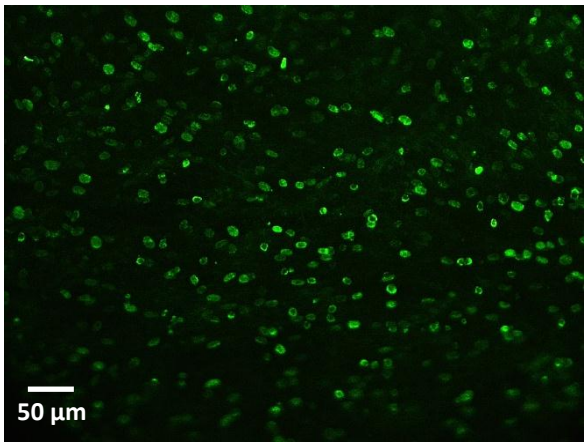
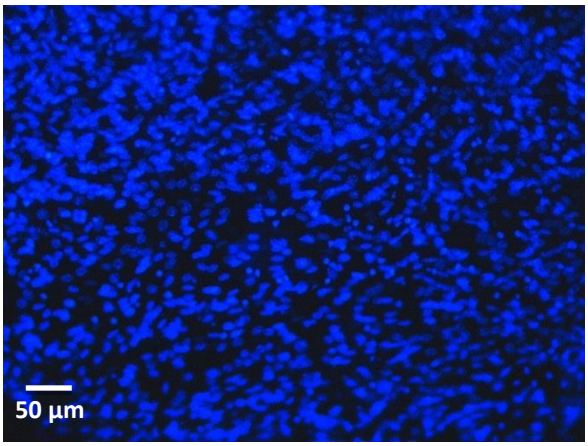


Figure 54: Day 4 BrdU Stain (5,000 cells/well)



Day 4 Hoechst Stain (5,000 cells/well)

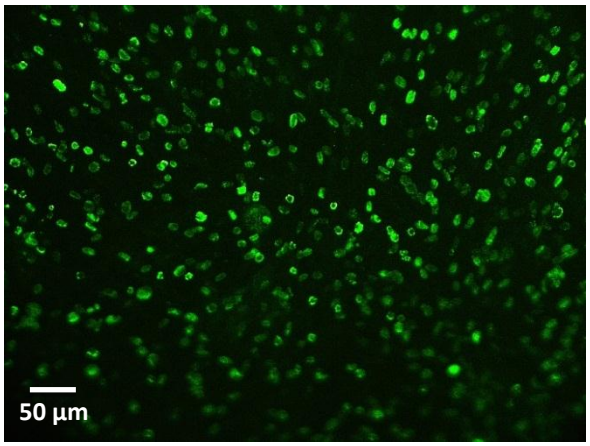
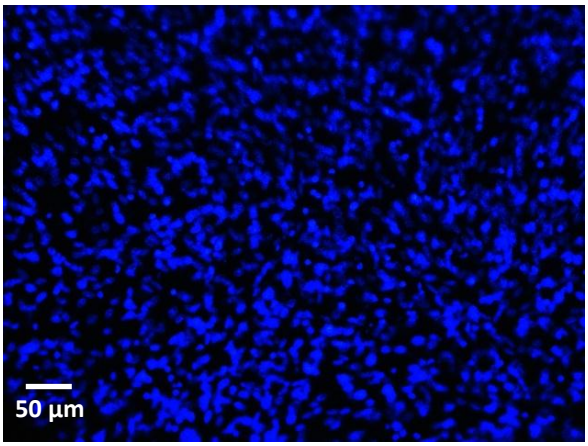


Figure 55: Day 4 BrdU Stain (15,000 cells/well)



Day 4 Hoechst Stain (15,000 cells/well)

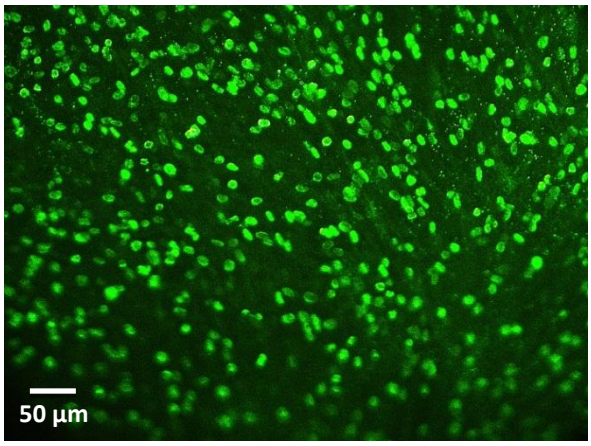
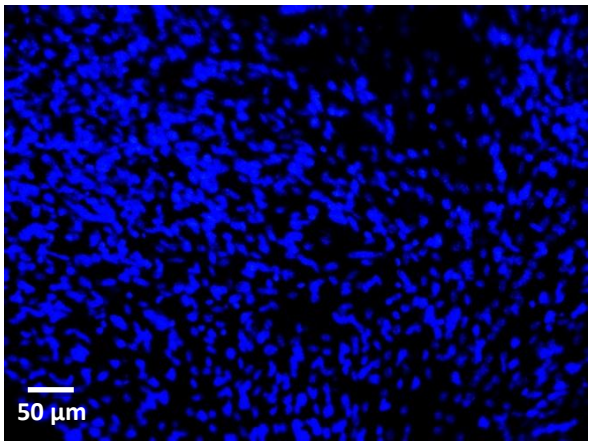


Figure 56: Day 4 BrdU Stain (20,000 cells/well)



Day 4 Hoechst Stain (20,000 cells/well)

DAY 5

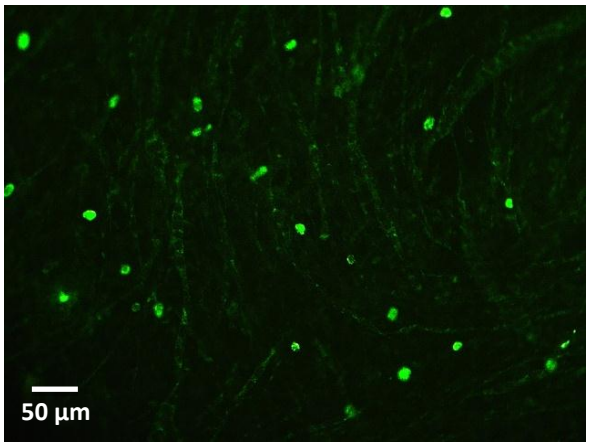
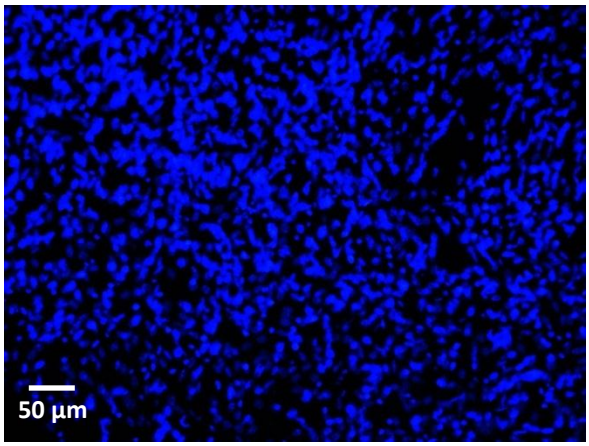


Figure 57: Day 5 BrdU Stain (5,000 cells/well)



Day 5 Hoechst Stain (5,000 cells/well)

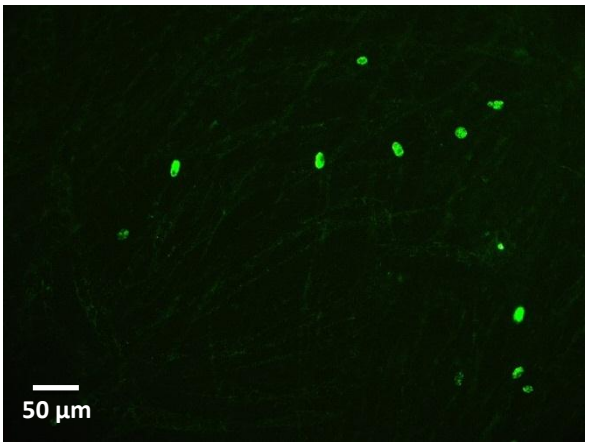
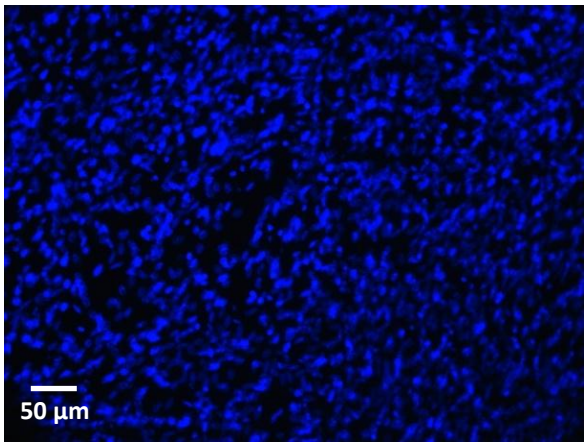


Figure 58: Day 5 BrdU Stain (15,000 cells/well)



Day 5 Hoechst Stain (15,000 cells/well)

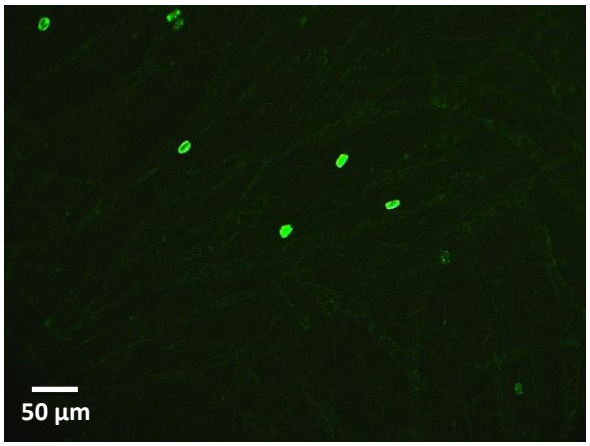
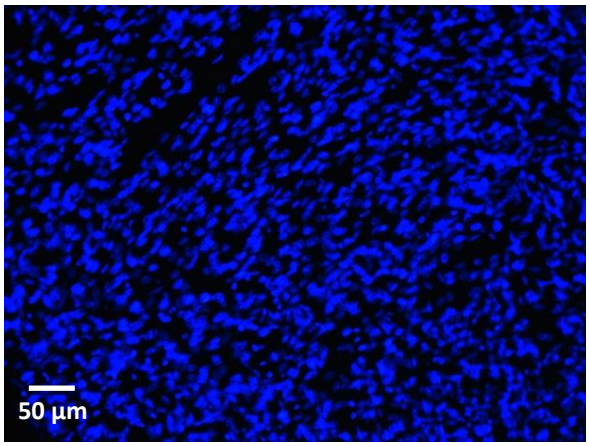


Figure 59: Day 5 BrdU Stain (20,000 cells/well)



Day 5 Hoechst Stain (20,000 cells/well)

Appendix B: Protocols

B1. Plating C2C12 Cells:

1) Wash hands, clean hood, clean aspirating tube with 70% ethanol
2) Aspirate media out of flask using glass pipette tip
3) Add 2mL of PBS to rinse flask
4) Swirl until PBS covers the surface of the plate
5) Use a glass pipette tip to aspirate all of the PBS
6) Add 2mL of trypsin to the flask
7) Wait 5 minutes for the cells to detach from the plate surface and observe under microscope to ensure cell detachment
8) Put 3 mL of media in the flask to neutralize the trypsin
9) Pipette the whole solution into a 10mL conical tube
10) Spin down the 10mL conical tube for 5 minutes at 800-1200 rpm
11) Aspirate out the supernatant, leaving the pellet in the conical tube
12) Add 3mL of media to resuspend the cells
13) Pipette up and down to break the pellet and for uniform cell distribution
14) Take a 50µL sample of media and add to Eppendorf tube
15) Add Trypan Blue (50µL) to the Eppendorf tube containing the media and cells
16) Mix the contents of the Eppendorf tube and remove 20µL of the solution and put in a hemocytometer
17) Using the light microscope, count the number of cells and calculate the current cell density
18) Based on the cell density, calculate how much media must be added to bring the cells to the right seeding density
19) Based on calculations, add the calculated amount of media into a T-25 flask
20) Seed the T-25 flask with the necessary amount of cells
21) Lightly swirl plate to have media covering the whole surface and ensure that there are no bubbles
22) Put flask in incubator at the right conditions

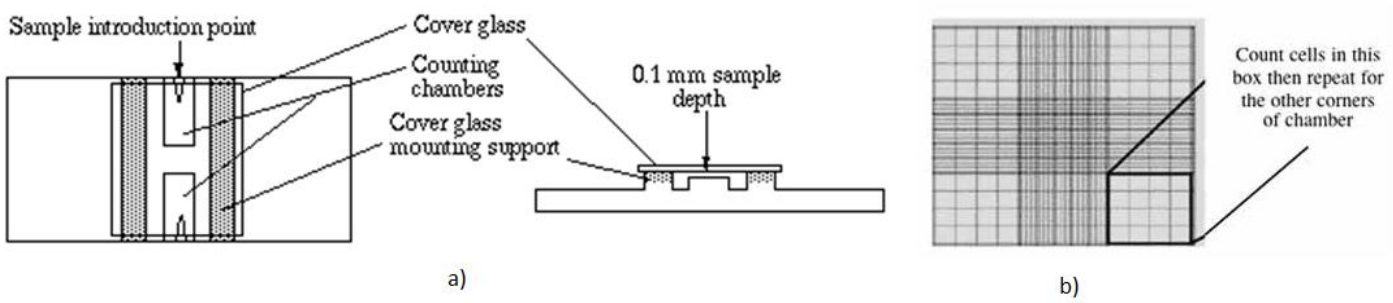


Figure 60: a) Hemocytometer b) One Grid within the Hemocytometer

- Count the number of cells in the regions of the hemocytometer, as indicated in Figure 59.

- For increased accuracy, count the cells in two hemocytometer blocks and take the average from the two blocks

Calculations for steps 17-20

-Divide the total number of cells counted by 2 to obtain an average

- Divide the number of cells by 4 to account for the four boxes of the grid counted

- Divide the number obtained by 2 to account for the dilution in Trypan Blue

- Multiply by 10,000 to obtain the value for the total number of cells (a)

- The optimal amount of media in a T-25 flask is 3mL, so for our protocol we are using 2.5 mL of media and 0.5 mL of cells

- If (b) is the number of cells that we want to seed in our T-25 flask, and we want (b) in 0.5 mL of media, then our desired cell density will be $(b) \times 2$

$$\frac{b}{0.5\ mL} \times 2 = \frac{2\ b}{1\ mL}$$

- Divide a/2b to obtain the value for the amount of media to add to the tube containing the cells

- Pipette up and down to ensure proper mixing

- Add 2.5 mL of media to the T-25 flask and 0.5 mL of cells to the same T-25 flask

- Continue from Step 21 of the protocol

B2. BrdU Staining Protocol

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 for10 min (plates can be stored at 4°C 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 (150 ul/well for 24-well plate or 75 ul/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 ul/well for 24-well plate or 75 ul/well for 48-well plate) and incubate at RT for 30 min.
12. Wash 3X with PBS **(without Tween)**
13. Add 0.5 ug/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/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.

B3. Myosin Staining Protocol

8. Aspirate culture medium and wash cells in 2X in DPBS+
9. Aspirate DPBS+ and add ice cold (-20C) methanol (1.0 ml/well for 24-well plate). Incubate for 10 min at -20C
10. Aspirate methanol and wash with 1.0 ml PBS for10 min (plates can be stored at 4°C with PBS in wells if analysis is not to be done right away.
11. 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.
12. Wash 3X with PBS, 5 min each

13. 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.
14. Dilute anti-myosin antibody 1:1000 in PBS+0.05% Tween-20
15. Add antibody solution (150 ul/well for 24-well plate or 75 ul/well for 48-well plate) and incubate at RT for 30 min.
16. Aspirate antibody solution and wash 3X with PBS for 5 min each.
17. Add fluorescent dye conjugated secondary-antibody diluted 1:500 in PBS+0.05% Tween-20 (150 ul/well for 24-well plate or 75 ul/well for 48-well plate) and incubate at RT for 30 min.
18. Wash 3X with PBS **(without Tween)**
19. Add 0.5 μg/ml Hoechst 33342 to last wash (stock is 1 mg/ml) and incubate for 10 min at RT
20. Aspirate Hoechst solution, wash with PBS and add PBS (1.0 ml/well for 24-well or 0.5/well for 48-well plate)
21. Cells are ready for observation by fluorescence microscopy. Plates can be stored at 4C wrapped in foil to protect from light.

B4. Fibrin Gel Protocol

Materials:

Fibrin 70 mg/ml
Thrombin 40 U/ml
$CaCl_2$ 40 mM
Thaw all materials on ice and keep on ice until ready for mixing

For 2 ml of total gel, use:

-0.25 ml Fibrinogen stock (2X)

- 212.5 μL $CaCl_2$ (at 40 mM in DMEM) mixed with 37.5 μL Thrombin stock for 0.25 ml of a 2X working thrombin solution

Mix the fibrinogen with the $CaCl_2$/thrombin solution to obtain a 40 mg/mL fibrin gel. This is 4X higher than the concentration used in most fibrin gel protocols (10 mg/mL).

Rapidly dilute the 0.25 mL of fibrin gel in 1.5 mL of media to obtain a 10 mg/mL fibrin gel

The gel will take approximately 10-15 mL to polymerize, so apply the gel to the mold within this time period.

B5. Collagen Gel Protocol

Materials:

Type I collagen stock solution 2 mg/mL
Culture Medium
FBS
NaOH

Mix each of the following components in order

a. 420 µL Culture medium
b. 300 µL FBS
c. 480 µL 5X DMEM
d. 300 µL 0.2N NaOH
e. 1500 µL Type 1 Collagen (in 0.02N Acetic Acid)
f. Cell solution: Density for each mold

This protocol can be used to make 3 mL of collagen gel and can be scaled down appropriately for smaller volumes of gel.

B6. PDMS Protocol

1. Place a weigh boat on top of a scale, and tare it.
2. Determine the total weight of PDMS you wish to make and carefully pour a 10:1 ratio of elastomer base to curing agent in the weigh boat. For example, if you wish to make 10 grams of PDMS, pour 18 grams of elastomer base followed by 2 grams of curing agent.
3. Remove the weigh boat from the scale and mix well with a stirrer. The mixing creates bubbles which indicate good mixing. Make sure you get a lot of bubbles while mixing.
4. Place the weigh boat containing the PDMS mixture in a plastic vacuum chamber (degasser), seal the chamber, and turn the vacuum valve on to connect vacuum to the chamber. Keep the PDMS mixture in there for one hour to eliminate the bubbles.
5. Shut off the vacuum valve, and remove the PDMS containing weigh boat from it.
6. Carefully pour the degased PDMS into your mold.
7. Set the oven at 60 degrees Celsius, and place the mold in it for 4 hours to allow it to cure.
8. After the 4 hours have passed, remove the mold from the oven, and carefully remove the PDMS from the mold using a spatula to gently separate it and slowly pry the newly shaped PDMS out.