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4D Maturation of Bioartifical Muscle for High Content Drug Screening

A Major Qualifying Project Report submitted to the faculty of
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Abstract

Muscular dystrophies are a set of very serious muscular diseases that lack treatment as there are no animal models that accurately mimic the human 3D *in vivo* environment to provide accurate testing in clinical trials of various drugs and therapies. Therefore, our team designed and manufactured a customized cell culture lid for a 96-well plate that interfaces with a programmable Arduino stimulation system to promote muscle maturation. To test the electrical stimulation, bioartificial muscle (BAMs) were developed and stimulated under various regimens to measure the effect on tissue maturation and viability. Based on haemotoxylin and eosin histology stains, stimulated tissue constructs qualitatively showed improved myotube alignment and limited necrosis, compared to unstimulated tissue. In conclusion, our device elicited the importance of electrical stimulation for BAM development, which is an improvement for current models on the market.

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Chapter 1: Introduction

Muscular dystrophies are a group of fatal disorders that lead to progressive weakness and loss of muscle mass [1]. Duchenne Muscular Dystrophy is caused by a mutated dystrophin protein that is necessary for the formation of healthy muscle, as it connects the cytoskeleton of muscle fiber to the extracellular matrix [1]. Duchenne's is the most fatal type of muscular dystrophy life a typical life expectancy in the early twenties. Fascioscapulohumeral Muscular Dystrophy (FSHD) is a primate specific disorder that is caused by the deletion of the terminal repeat sequence on chromosome [2]. FSHD gets progressively worse throughout a patient's lifetime and becomes extremely debilitating. Both of these diseases have no cure and there is a growing drug market researching for treatments [3]. One of these challenges with muscular dystrophy drug development is the lack of testing methods because animal models used in the drug screening for muscular diseases are not accurate due to physiological and genetic differences between animals and humans. Therefore, animal models cannot viably be compared to human models. These limitations create a need for better bioartificial muscle models that can replace excessive use of animal models for representative preclinical treatments before reaching human testing stages.

Currently, bioartificial muscle (BAM) models are being developed through tissue engineering to study skeletal muscle disorders to find new and improved methods of treating these diseases. Skeletal muscle tissue engineering is a promising field for the development of *in vitro* skeletal muscle tissue constructs to better understand the disease process. This industry utilizes scaffolds and progenitor cells to develop a representative cellular physiological environment for future therapeutic applications. The environment requires external signals (electrical and mechanical) which allow muscle fibers to achieve full maturation. Therefore, there is a need to more closely mimic innervation found in skeletal muscles, and emulate neural function through the delivery of low-frequency electrical impulses to the muscle fibers to increase tissue maturation

[4]. In addition, electrical stimulation is highly essential for proper skeletal muscle maturation and function as native skeletal muscle depends on neuromuscular electrical stimulation to facilitate muscular contraction. Electrical stimulation is especially important due to its ability to control cellular morphology, gene expression, and cell migration [5]. Electrical stimulation is characterized by its electrical field, frequency, and pulse duration. However, no combination of these stimulation parameters have been discovered to completely mimic the electrical impulses found *in vivo* when paired with bench-top models [6].

The current electrical stimulation used in the Page Lab at Worcester Polytechnic Institute (WPI) is the Myomics system [7]. In the Myomics model, 3D skeletal muscle tissues are developed in 96-well plates between flexible polydimethylsiloxane (PDMS) microposts that act as anchor points for the muscle tissue. Electrical stimulation can be applied individually to the tissue constructs outside of the incubator, however, this is not ideal as BAMs are optimized in conditions that mimic the *in vivo* environment. The lack of versatility in the operating conditions of the Myomics system greatly limits muscle tissue growth and maturation. Similar devices, such as the IonOptix model, generates electrical stimulation that produces contractile forces in myofibers *in vivo* that measure to be only 2-8% of that found in native skeletal muscle [8]. Since human skeletal muscle cannot be studied directly *in vivo* there is the need for a robust model, which both resembles the skeletal muscle tissue in humans, and allows for repeatable experiments to test various therapies and treatments. Drug development and testing for muscular disorders can be a long and expensive process that is not always successful. An improved BAM model that more closely mimics human muscle tissues can ensure more successful preliminary testing of drugs and therapies to conserve resources.

Limitations in artificial muscle maturation are the driving force behind the development of

this project. Based on the need to enhance maturation of skeletal muscle tissue and the limitations in the current gold standards, the purpose of this project is to design a device that administers electrical stimulation to enhance tissue engineered skeletal muscle maturation that can be applied to human skeletal muscle constructs *in vitro*. The primary goals for accomplishing our project are to develop a tissue-stimulating device that conducts an electric field to each well of a well plate simultaneously, simulate *in vivo* conditions in order to maximize tissue construct maturity, change media concentrations or add new substrates to optimize cell culture protocols, and create a BAM model by the micropost attachment method which will be used in the tissue-stimulating device.

To accomplish these goals the team will apply the engineering design process. The team will identify the client's needs and conduct research on current models and techniques, specifically in BAM development and current electrical stimulation devices and regimens. This will give the team an understanding of the morphological and functional differences between native muscle and current BAMs in terms of fiber diameter, contractile strength or force and alignment. Additionally, it will help identify important operational factors of the incubator environment to prevent corrosion or failure of the device and cell death. The direction of the project will be based on the client's needs, feasibility within time and cost constraints, in addition to pertinent suggestions from published resources.

Chapter 2: Literature Review

Skeletal muscle is comprised of a complex system which profoundly affects the health and well-being of individuals. Muscle mass loss, along with decreased function can create conditions which greatly hinder an individual's ability to carry out activities of daily living successfully. Rehabilitation of dysfunctional or damaged tissue is an important task to create a solution to provide full patient recovery. The development and maturation of *in vitro* skeletal muscle tissue is a promising method to help understand the etiology of muscle dysfunction as well as a platform to test new therapeutic approaches that address muscular dysfunction. Properties of skeletal muscle *in vivo* are mimicked in the *in vitro* environment which incorporate the qualities of the native tissue. Thus, this chapter contains the background addressing current devices that electrically stimulate tissue along with their strengths and limitations.

2.1 Clinical Significance

Skeletal muscle comprises of about 40% of an individual's body mass and contributes to many important biological processes, such as breathing and locomotion. In the advent of a disorder or injury, the afflicted individual is unable to independently perform activities of daily living [9]. Diseases, such as Duchenne muscular dystrophy, leads to the progressive loss of muscle mass, causing the eventual complete loss of muscle functionality, resulting in death as early as ages 9 to 24 years old [10]. Current treatments do not treat the disease, but only delay the fatal outcome. Facioscapulohumeral Muscular Dystrophy (FSHD) also undergoes progressive muscle degeneration, which decreases an individual's functional autonomy and quality of life [2]. Effective therapies addressing FSHD are still unavailable due to limitations in understanding the physiopathological mechanism of the disease [2]. The genetic mutations found in both types of muscular dystrophies are only expressed in humans, therefore animal models are not representative of the problem. Furthermore, a mismatch between human and animal model

physiology causes a failure of clinical models, creating a gap between preclinical and clinical trials.

Tissue engineering is a promising approach to develop custom treatments to target the root of the muscular diseases by providing more accurate and quick high-throughput diagnostic screening. Additionally, tissue engineering has been appealing due to the potential for reducing the use of animal models, as they often exhibit significant physiological differences from humans, particularly in the area of therapeutic efficacy and toxicity, which also highlights the ethical concerns associated with the use of animals in the development of therapies to treat human disease. Therefore, the development of a platform to better understand the disease process as well as provide high throughput drug screening still remains a big challenge.

Moreover, surgeries and regenerative therapies are being studied to help form muscle tissue, yet these methods are not guaranteed to return full muscle functionality and could have additional side effects. Potential new therapeutics are being developed with engineered tissue in order to closely mimic disease and muscle repair processes. Yet, maturation of such bioartificial muscle models still remain a great challenge, especially recreating neural stimulation responsible for propagating muscle fiber contraction without any tissue innervation. Therefore, to address such challenges the team will be creating a device to permit an electrical stimulation regimen to bioartificial muscle models using an industry standard high content multiwall plate format that can be used to better test various treatments and therapies for tissue-engineered skeletal muscle and develop cures for intractable muscular disorders.

2.1.1 Muscular Dystrophy

Muscular dystrophies are a type of fatal diseases that are denoted by progressive weakening and loss of muscle [11]. Upon damage, satellite cells will continuously reproduce to make up for the lost tissue until there is an inability to reproduce. Without the satellite cells, muscle cells cannot regenerate due to the loss of muscle mass. Duchenne Muscular Dystrophy is characterized as a

X-linked recessive genetic mutation, where the mutated Duchenne Muscular Dystrophy gene interferes with the open reading frame and causes an incomplete translation of the dystrophin protein [1][11]. The dystrophin protein is commonly found in skeletal muscle, cardiac muscles and brain and is responsible for regulating membrane integrity when contraction occurs [11]. Dystrophin plays an important role in connecting the myocyte cytoskeleton to collagen IV, VI or laminin-211 in the extracellular matrix (ECM). If the protein is mutated, then the connections between the ECM and protein are compromised resulting in a continuous breakdown and remodeling of the muscle. This process ultimately results in a loss of muscle function and muscle tissue deterioration (Figure 1).

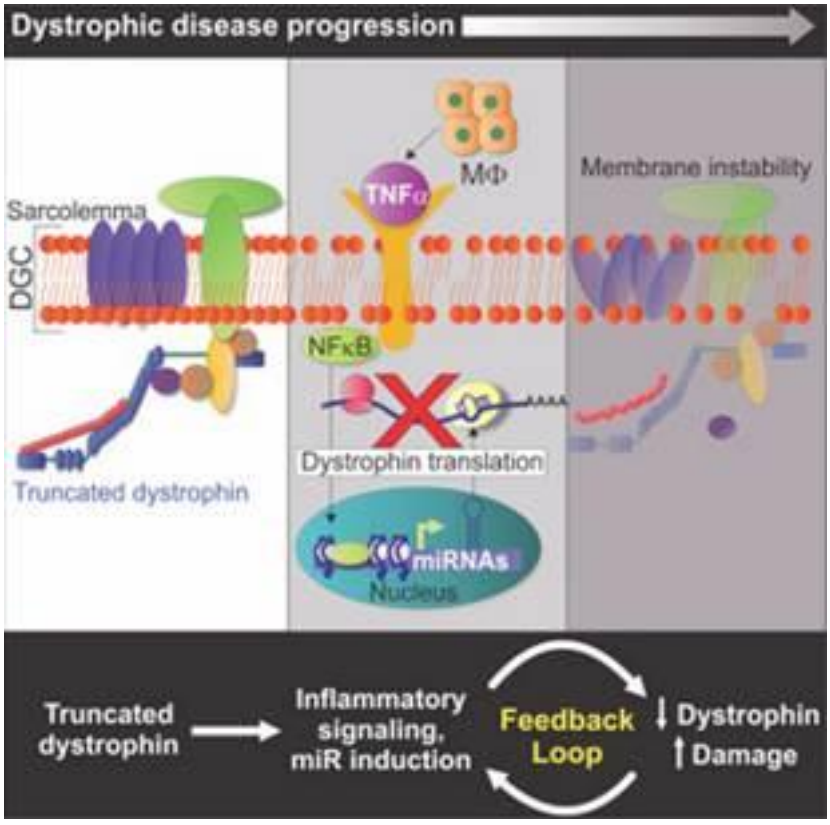

Figure 1: Dystrophin Disease Progression; Dystrophin feedback loop and dystrophin relationship intracellular interactions as muscle disease progresses [12]

The first symptoms of Duchenne's typically appear when the patient is between the ages of two to five. Some of these symptoms include muscle weakness, which can be seen in how the

patient walks, stands up, climbs stairs, speech delays or overall delayed motor development and lower limb muscle weakness [12]. A patient can be diagnosed with Duchenne's through muscle biopsy or molecular genetic testing. Duchenne's Muscular Dystrophy affects about one out of 3,500 males [11]. Most patients need a wheelchair by age ten and die in their twenties because of respiratory and cardiac complications, about 58% of patients will survive past the age of twenty-four, but with a very poor quality of life [11][13].

Fascioscapulohumeral Muscular Dystrophy (FSHD) is a digenic disease which causes progressive asymmetrical muscle weakness, and is the most common myopathy in adults [2]. Currently, the incidence of FSHD affects 1:10,000 people and is characterized by necrosis of the muscle fiber generated by a cascade of events that induce degeneration [2]. This form of muscle weakness generally spreads from the face to the limbs causing muscle atrophy and impairment. FSHD is classified into types 1, and 2 which have similar symptoms. FSHD type 1 patients (95%) have a deletion of chromosome 4q35, while type 2 patients have a genetic mutation in genes [14]. Clinical findings of FSHD include asymmetry and weakness in the muscles of the face, shoulder, abdomen, and lower extremities; starting at distal extremities and moving to the proximal lower extremities [14].

The cause of FSHD is still not well understood and remains incurable, with therapeutic treatments only targeting symptom management. Furthermore, pharmacological strategies designed to help slow down or halt the progression of FSHD remain inconclusive [14]. Treatment of symptoms include surgery to decrease scapular pain and allow patients to regain range of motion in the shoulder to potentially improve its function. However, surgery comes at a risk and may have drawbacks such as further potential complications with brachial plexus injuries and loss of functional gain [14].

The mismatch between human and animal model physiology cause clinical models used for drug testing of muscular dystrophies to fail. Therefore, a gap exists between preclinical and clinical trials resulting in no cure for the disease. Combined with an estimated growth in the global market from $8.2 million in 2014 to $990 million in 2019, there is a significant need for a platform to study the disease etiology on the molecular level and conduct drug screening [3]. This need can be addressed by creating representative 3D muscle models for accurate and rapid testing of new treatment strategies targeting an increase in muscle strength and growth.

2.2 Skeletal Muscle Development

Skeletal muscle development and maturation is essential to understand each step of myogenesis to be replicated in an *in vitro* environment. Therefore, it is important to create conditions that closely resemble the native *in vivo* conditions to optimize the development and maturation of the 3D muscle models.

2.2.1 Myogenesis

Myogenesis is the process of permanent change of proliferation-competent myoblasts to fused, multinucleated secondary myotubes called myofibers [15]. During the embryonic development, muscle fibers are generated by mesoderm-derived structures due to the fusion of myoblasts into multinucleated myofibers [15]. An important component of myogenesis is the ability to produce and control positive and negative signaling, which comprises the body's electrical stimulatory system. Molecular signals from tissues nearby stimulate the beginning of the muscle formation process, therefore inducing myogenesis [16]. Myogenesis undergoes three stages for the generation of new muscle tissue, (1) determination, (2) differentiation, and (3) maturation. These phases are shown in Figure 2.

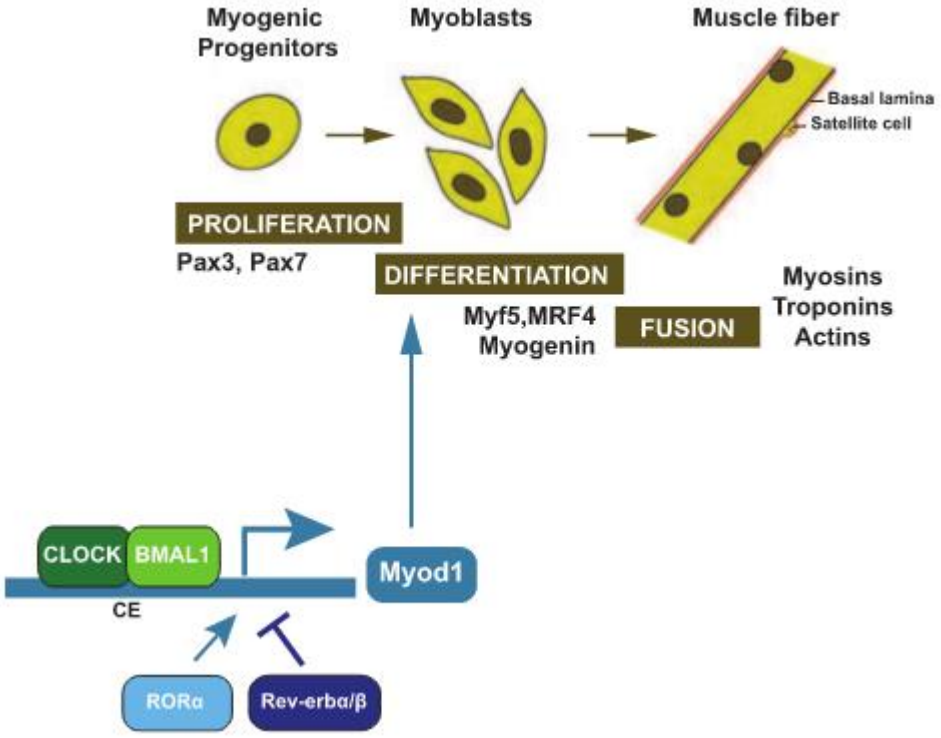

Figure 2: Skeletal muscle cell differentiation process [9]

The determination phase in skeletal muscle development is the activation of the genes in the Myo-D family, which regulates muscle formation as well as myotome induction [16]. A myotome is the coupling of muscle innervated by one spinal nerve root. Determination and eventual differentiation of myoblasts is composed of an essential cross-regulatory transcriptional network regulated by myogenic regulatory factor 5 (MYF5) and myoblast determination protein (MYO-D) [5]. The spatial and temporal onset of myogenesis is regulated by secreted paracrine factors, which directly stimulate the signal transduction mechanism leading to the activation of transcription factors responsible for myoblast determination. The paired box (PAX) gene family is also activated due to external stimuli, which subsequently starts stimulating myoblast determination protein to prepare the myoblast for differentiation. Once gene expression is activated, the progenitor cells become myoblasts.

The second phase of myogenesis is the differentiation of myoblasts. Myoblast proliferation begins upon stimulation of the cell with growth factors. Cells cannot differentiate while they are proliferating, therefore, a feedback mechanism signals for phosphorylation of the retinoblastoma protein (Rb) to block cell growth and cell cycle progression, while impeding stimulation due to growth factors. As the cell cycle ceases, cells begin to align and anchor on the extracellular matrix (ECM). The ECM has an essential function in muscle fiber force transmission, maintenance and repair, and can vary greatly for both injured and diseased states of the cell [17]. The ECM directly bears most of the passive load of the muscle, which relates to the motion and stiffness of the ECM properties [17]. Aligned myoblasts begin to fuse and form elongated, multinucleated, cylindrical structures called myotubes, which are approximately 10-100 μm in diameter [16]. During this differentiation process, myotubes begin expressing acetylcholine receptors, which contribute for direct stimulation of the myotubes for cellular maturation and develop muscle fiber's contractile function. Additionally, during this phase the cells are receiving their nutrient supply through vascularization of the tissue.

The third phase of myogenesis is the process of myotube maturation into muscle fibers, primarily initiated by the mechanical and electrical stimulation of the fibers. Electrical stimulation is achieved *in vivo* through cellular innervation and development of neuromuscular junctions between the muscle fiber and nerve. Innervation is important to provide continuous communication between the nerve and muscle fibers in order to apply mechanical, electrical, and chemotropic stimulation for optimal maturation [5]. For example, the organization of actin and myosin (contractile proteins) into sarcomeres create an organized and structured contraction to skeletal muscle myotubes [18]. Acetylcholine signaling transmits an electrical stimulus which depolarizes the muscle cell muscle maturation essential for contraction to occur. Essentially,

electrical stimulation of myofibers contributes to the control of cellular morphology by providing alignment and anchorage, gene control, and cell migration [5]. For the proper maturation of myotubes include innervation for neural stimulation, vascularization for nutrient supply, tissue anchorage for axial alignment, and a load applied for mechanical stimulation.

2.3 Bioartificial Muscle (BAM) Models in Research

Bioartificial muscle models (BAMs) are tissue engineered three-dimensional structures of cells created in an *in vitro* environment that serve as a functional unit of muscle tissue. It represents a promising method for drug screening, because it allows researchers to recreate the diseased muscle and understand the disease in order to enhance drug testing performance. Current methods of fabricating BAM models include three primary tissue engineering techniques including assembly scaffolds with the use of synthetic polymers, natural extracellular matrix (ECM) proteins - collagen or fibrin- and self-assembly systems. Rossi et al compared the use of satellite cells and muscle progenitor cells to maintain cell-like properties once placed into scaffold form. However, the results showed that the muscle progenitor cells were not able to have inadequate regeneration [19]. Another type of method for BAM was completed by Fuoco et al. This research group delivered mesoangioblasts with injectable polyethylene glycol-fibrinogen hydrogel for the enhancement of cell engraftment and limit chronic muscle degeneration. Unfortunately, this system lacked differentiation potential of the skeletal muscle lineage. The use of BAMs is a promising method to study skeletal muscle physiopathology to identify therapeutic treatments to halt the progression of atrophy and re-enable proper muscle function. Since human skeletal muscle cannot be studied directly *in vivo*, there is the need for a robust model which both resembles closely the skeletal muscle tissue in humans, and allows for repeatable experiments to test various therapies and treatments.

In the development of BAM models, it is important to address all the components that will allow it to mature and grow, therefore creating a representative functional unit of the muscle fibers for future testing. An improved BAM model that more closely mimics human muscle cells and tissue will ensure more successful preliminary testing of drugs and therapies to conserve resources.

2.4 BAM Maturation

Creating conditions for optimal BAM maturation are key components in the development of viable tissues for drug-screening. As previously mentioned in section 2.2.1, the main components needed for maturation *in vivo* are mimicking the extracellular matrix, providing tissue anchorage, delivering nutrient supply, applying a load for mechanical stimulation, and transmitting signals to mimic neural stimulation.

2.4.1 Scaffold Development

For mimicking native muscle's ability to form dense, parallel-aligned muscle, it is important as this incorporates well-organized extracellular matrix [20]. Cell-to-extracellular matrix interaction provides the structure for muscle's growth and development, such as myogenesis and muscle regeneration. Other purposes of the extracellular matrix are its contributions to muscular attachment, myotubular alignment, and differentiation precursor.

Currently, 2D studies have shown that using extracellular matrix substrates are able to increase myogenic differentiation markers as well as promote myoblast fusion for multinucleated myotubes. These studies have shown that extracellular matrix function greatly impact proper muscle development, and therefore highlighting importance of extracellular matrix in the transitions to 3D models. Typically, research being conducted for tissue-engineered skeletal muscle consists of using naturally derived hydrogels, such as collagen, fibrin, or Matrigel. These grow and differentiate skeletal myoblasts due to high cell density and migration abilities, unidirectional cell alignment, and ability to have measurable contractile forces. For instance, Hinds

et al used a silicon hydrogel matrix and after two weeks of growing tissue constructs, they found that the average bundle diameter ranged from 1.1 ±0.08mm to 2.7 ±0.18mm [20]. Additionally, Hinds et al found that stimulation rates increasing up to 40Hz caused cytoplasmic calcium concentration to increase at a constant rate, thus supporting tetanic force generation [20].

The muscle ECM *in vivo* gives muscle fibers the structure to determine fiber growth and function. Therefore, tissue engineered muscles need a scaffold that can replicate the ECM to encourage proliferation and differentiation of progenitor cells. The differentiation process of myofibers is challenging to initiate and regulate *in vitro*, which increases the need for an improved bioartificial muscle model [21].

2.4.2 Anchorage

Anchorage is an important factor for skeletal muscle maturation *in vivo.* In the human body, myoblasts are able to grow and attach to bone [22]. For *in vitro* environments it is important to create a method to mimic the hard-soft tissue, tendon to bone, interactions that are needed to mature muscle.

The current techniques used for manipulating anchorage in 3D tissue cultures are the ring formation and the micropost attachment methods [23]. In the ring method, cells are grown around circular posts with circumferential alignment to apply positive and negative strain. This method is commonly used on cardiovascular research, such as the Rolle Lab at Worcester Polytechnic Institute (WPI) who focuses on development of tissue-engineered blood vessels. Figure 3 demonstrates the ring formation method used in the Rolle Lab [24].

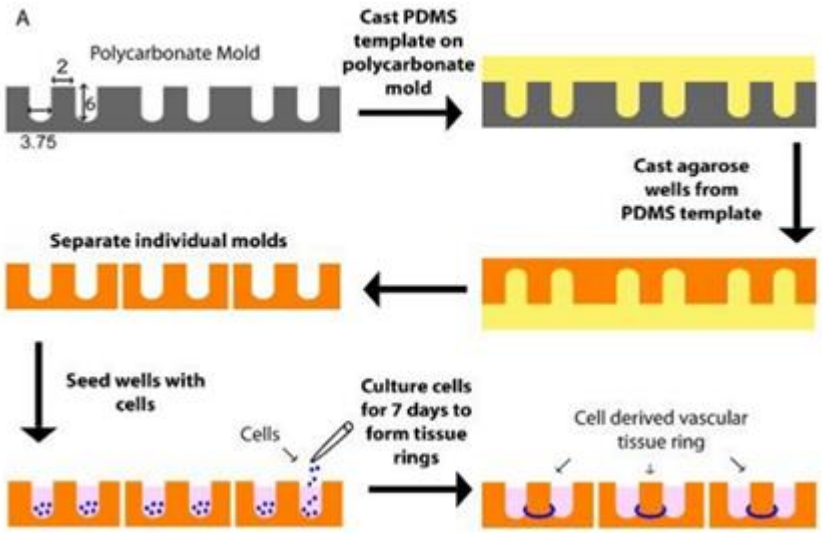

Figure 3: Shows the Rolle method of creating the tissue constructs using the ring shaped technique [24]

The micropost attachment method is when cells are attached around two flexible posts to provide uniaxial alignment to create aligned skeletal muscle tissue constructs. Stimulation is applied back and forth which provides this uniaxial alignment, allowing for the muscle to mature. Micropost anchorage helps promote self-assembly, which would enable the cells to wrap themselves around polydimethylsiloxane (PDMS) posts without using ECM or other proteins as seen in Figure 4 done by the Vandenburgh group [7]. The posts are made out of PDMS because this material is bioinert, and sturdy enough to provide anchorage but still allow enough malleability for muscle contraction and force generation. Self-assembly could be used in the micropost attachment method by seeding the constructs with a fibrin and collagen gel, yet there is an unfavorable distribution of native ECM to synthetic ECM. Excess synthetic ECM could disrupt proper self-assembly. This technique is able to mimic skeletal muscle attachment, as cells surround the posts and can simulate simplified *in vivo* movement and directly mimic tissue attachment to tendons and bones. Additionally, the muscle fibers are able to form parallel fibers between the posts with similar contractile forces.

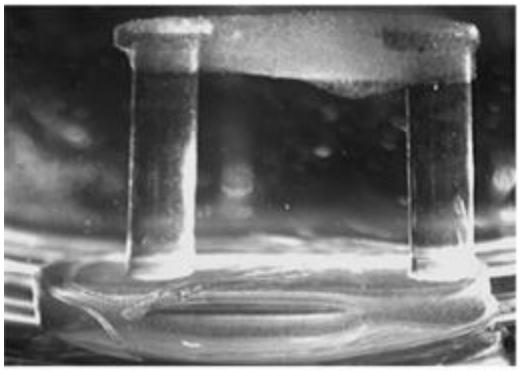

Figure 4: Represents Vanderburgh method of creating the tissue constructs using the micropost attachment method [7]

2.4.3 Nutrient Supply *In Vitro*

Nutrient supply *in vivo* is achieved by vascularization of the muscle tissue. Therefore, to create functioning BAMs, a constant supply of nutrients is needed for the fibers to maintain integrity and growth. The length and diameter of the BAM constructs are of essential in creating a minimal functional unit which does not need blood supply and can retrieve needed nutrients solely by exposure to cell culture media. Therefore, to be able to forgo the blood supply needed to deliver necessary nutrients the ideal BAM dimensions fall between having the diameter of 200-250µm and the length of 3-4mm [7]. In this case, the tissue is small enough to retrieve needed nutrients without vascularization. However, it will be large enough to withstand its own contractile force, and prevent rupture. Furthermore, its size allows for the tissue to fit within a 6.35mm well of a 96-well plate, required for high content drug screening.

2.4.4 Mechanical Stimulation of BAMs

Mechanical stimulation on its own represents the application of load and frequent use of muscle tissue. In an *in vivo* system, mechanical and electrical stimulation are applied at the same time to improve muscle maturation. For instance, to pick up an object, electrical impulses *in vivo* in the form of action potential are delivered to the skeletal muscle tissue used in the motion, leading to the performance of mechanical work in picking up of the object. This represents the simultaneous nature of both mechanical and electrical stimulation *in vivo*, which must also be

present *in vitro* for the maturation and increase in BAM development to be closer to native skeletal muscle [25]. Currently there are approaches to providing mechanical stimulation to skeletal muscle constructs which are primarily focused on strain regimens. One standard is the Vandenburgh method, where a computerized mechanical cell stimulator is used to stimulate skeletal muscle constructs [26]. The strain regimen involved passive sample stretching for one cycle, with positive 10% to 20% strain for thirty minutes [26].

The second standard involves determining optimal strain rates for cell fusion in stem cell and skeletal myoblast co-cultures. In this method, 2D tissue cultures (density of 5000 cells/cm^2) are placed through pulses of 15% strain at 0.5Hz [27]. This regimen applied to 3D tissues has been found to be optimal for stem cell and myoblast alignment and maturation. Additionally, these stimulation parameters have shown to produce the least rupture [27]. This regimen also appeared to accelerate the differentiation process in C2C12 cells, which formed a greater number of myotubes compared to controls.

2.4.5 Electrical Stimulation of BAMs Remains a Challenge

Innervation of the skeletal muscle refers to the motor neurons that form networks within the muscle tissue. It provides nerve-derived trophic factors with growth factors as well as electrical impulses that regulate skeletal muscle maturation. In the absence of innervation, myotubes cannot fully mature, facing limited regeneration, muscular atrophy, and loss of functional properties [28]. As described on Table 1, the fiber diameter and specific force of immature myofibers are smaller than for mature myofibers, hence highlighting the need for electrical activity to promote maturation. Replication of neural stimulation in an *in vitro* system remains a challenge due to lack of innervation of the BAMs. To develop representative skeletal muscle *in vitro*, a method must be incorporated to mimic the effect of neural stimulation [25].

Table 1: Tissue properties of immature vs. mature myofibers

Tissue Properties	Immature Myofiber	Mature Myofiber
Fiber diameter	≤ 10 µm	approx. 100 µm
Specific force	5-20 $kN \cdot m^{-2}$	25,000 $kN \cdot m^{-2}$
Alignment	SIMILAR, but low density for BAM	

Multiple electrical stimulation regimens for the development of BAMs exist and the current gold standards mainly differ by their electrical stimulation parameters. The electrical stimulus applied to the muscle fibers is measured by its electrical field. This electrical field magnitude serves to replicate nerve stimulation found in our bodies by mimicking the electrical field caused by action potentials that ultimately lead to muscle fiber membrane depolarization and contraction upon sarcoplasmic release of Ca^{2+}. Since 1997 to 2014 the range of applied electrical field is between 3 to 8.3V/mm.

Regimen duration and frequency generally deal with the duration of the whole electrical stimulation and how long single impulses are applied. It takes time and repeated stimulations to induce skeletal muscle maturation, and thus the duration of stimulation is adjusted depending on the magnitude of other parameters, such as electrical field and the frequency.

2.5 Current Methods for Electrical Stimulation

The current gold standard for benchtop models targeting electrical stimulation of BAMs includes two devices: (1) IonOptix C-Pace EP Culture Stimulation System (CEPSYS), and the (2) Myomics' Myoforce Analysis System (MFAS™). This section highlights the benefits and limitations of each device in its current use.

2.5.1 IonOptix C-Pace EP Culture Stimulation System

The IonOptix CEPSYS device is mainly a customized circuit board used to create electric fields to mature tissue engineered skeletal muscle [8]. Nedachi and colleagues used this model to study how skeletal muscle responds to exercise [29]. Progenitor cells become differentiated in 2D culture plates and then are moved to the electrical stimulation dish to mature [29]. Then contractile force is applied to the newly created myofibers [29].

This device was used by Dennis et. al to apply an electrical stimulation regimen to BAMs over a 10 day period to monitor the effects of different electrical fields on the specific forces and alignment of BAMs [30]. This device is beneficial over the Myomics device in the sense that it can provide an electrical stimulation to BAMs in an incubator. However, this study used a co-culture of immortalized mouse myoblasts (C2C12) and rat fibroblasts, which are not ideal representatives of BAMs used for drug screening. Additionally, the device electrically stimulated a 6-well culture plate, which is not appl)icable to the standard 96-well format needed for high content drug screening [28]. It was observed that the length of the BAMs were approximately 12mm, lengths in large sizes cause BAMs to have inadequate nutrient supply due to the absence of vascularization, leading to necrosis. Ideally, BAMs with lengths of 3mm should be developed [28]. Dennis et al. showed that the measured contractile forces due to electrical stimulation from this device was about 2-8% of the forces produced by native skeletal muscle [30].

2.5.2 Myomics Myoforce Analysis System (MFAS™)

The Myomics MFAS™ system focused on producing BAM tetanic force production in a 96-well plate format. The Myomics model utilized 3D myotubes in the plate and did not transfer cultured tissue after it was differentiated [7]. This model produces artificial skeletal muscle to mimic native muscle properties. To maximize nutrient supply to the tissue, this system used a temperature responsive polymer, N-isopropylacrylamide (NIPAAm), to suspend BAMs in the

middle of the well preventing the tissue constructs to develop on the bottom of the plate. As the myoblast differentiate, the coalesce around the flexible microposts [7]. Upon NIPAAm removal, media was added to the tissue providing the essential nutrients, hormones and antibiotics needed for its development and maturation [7].

The electrical stimulation provided by this device was designed for a 96-well plate. It utilized an automated system that provided electrical stimulation to one well at a time, taking approximately twenty minutes for one round of stimulation [7]. One limitation with this system was that stimulation occurred outside of the incubator, therefore depriving the tissue from its optimal environment to survive. Since actual electrical stimulation regimens require multiple periods of electrical stimulations and rests, the Myomics device cannot be used to electrically stimulate BAMs to improve maturity. To mimic the *in vivo* tissue, the device would need to deliver 2700 stimulation repetitions, translating to 54,000 minutes of work (approximately one month), for a regimen needed to be done in one day. This translates to taking about ten months to complete which would normally take ten days [28]. Therefore, limitations with the current systems must be addressed in order to create functional BAMs for high content drug screening.

Chapter 3: Project Strategy

In this section the team states their initial client statement and the steps taken to achieve the final revised client statement. The team also describes the objectives, design specifications and constraints that were determined through literature reviews and client interviews. These objectives, specifications and constraints ultimately shaped the design of the project. The team also formulated a plan for their project approach to maximize time and efficiency. Lastly the team found International Organization for Standardization (ISO) International Standards that were relevant to their project and necessary to consider in future developments.

3.1 Initial Client Statement

The team originally met with the client to learn about the problem statement and to identify objectives, functions, and constraints that would guide the project. The following consists of the initial client statement presented to the team by Professor Raymond Page:

"Engineered bioartificial muscle in a 96 well format has the potential to provide high content screening for therapeutic effects on muscle function, fatigue and toxicity. The current system enables electrical stimulation only one well at a time and muscle tissue development and maturation requires frequent simulation over time as the tissue develops. This simulates intermittent motor neuron stimulation of muscle twitches. Most tissue engineering strategies address this using small numbers of individual muscle constructs cultured in the same vessel.

Design and build a system that enables programmed electrical stimulation of bioartificial muscle constructs formed in 96-well plates. The stimulation regime must be able to be applied while the plates are in an incubator. Determine the optimal method of applying the electrical stimulation to the engineered tissues as well as optimal stimulation parameters. Address material selection issues with respect to electrode corrosion and placement of electrodes with respect to the muscle constructs. Design and implement methods to measure contractile force due to

electrical stimulation and fiber strength. Determine the effect of repeated electrical pulses on muscle fiber alignment, degree of maturation and contractile strength. Address issues associated with sterility and multiple uses of the device".

Based on this client statement, the team conducted literature review and background research to have an in depth understanding about the gap that currently exists in the field and certain limitations that would affect the project. Based on this combined information, the team identified objectives and constraints of the design.

3.2 Design Requirements: Technical

In this section, the design objectives, constraints, functions and specifications are described. These technical requirements are essential in establishing the foundation for the device and ensuring that the device encompasses the appropriate design features to guarantee functionality.

3.2.1 Objectives

An objective can be identified as a characteristic or behavior that the design should display [31]. The team conducted multiple client interviews, with both the client, Professor Page, and the graduate student, Jason Forte, predominately working to determine the features that they wanted to see in the device. The team also executed a vast literature review to gain more insight on qualities that should be incorporated within the device. The following list contains the project objectives:

- Adjustable Electrical Stimulation Regimen
- Reliable Electrical Stimulation Regimen
- Sustainable in Incubator Environment
- User Friendly Operation

These objectives were described in bi-weekly presentations to the client, Professor Page, for his review.

A Pairwise Comparison Chart (PCC) was completed by the team to rank the importance of the design objectives [31]. A PCC works by comparing two objectives against each other, and assigning a point value based on which objective is understood to be a higher priority to the design. A point value of 1 indicates the objective in review is more important the other, 0.5 indicates both objectives are of equal rank, and a score of 0 indicates that the objective in review is of lesser rank than its pair.

The team used feedback provided by Professor Page and Jason, and what they considered the most important features for the project in order to create a PCC for the project. The team also incorporated the information they gathered from the literature review while prioritizing these objectives to make sure they were feasible. Table 2 shows the project PCC.

Table 2: Objectives Pairwise Comparison Chart for the team's project

	Adjustable	Sustainable in Incubator Environment	Reliable	User Friendly	Total
Adjustable	X	1	0	1	2
Sustainable in Incubator Environment	0	X	0	1	1
Reliable	1	1	X	1	3
User Friendly	0	0	0	X	0

The ranked objectives as result of the PCC are shown below. The specific reasoning behind these rankings in relationship to the team's project will be described later in this section.

1. Reliable Electrical Stimulation Regimen
2. Adjustable Electrical Stimulation Regimen
3. Sustainable in Incubator Environment

4. User Friendly Operation

These five objectives serve as the team's primary objectives for the project. A primary objective is the main, overarching goal for a specific feature of the device. These primary objectives were also divided into secondary objectives. Secondary objectives serve to provide a more detailed explanation of the expectations of the primary objective. Figure 5 below is an objective tree that represents the relationship between the ranked primary and secondary objectives. In this objective tree, the primary objectives are shown as green circles and the secondary objectives are shown as yellow squares. Both the primary and secondary objectives are displayed in this diagram in order of highest to lowest importance (read from left to right).

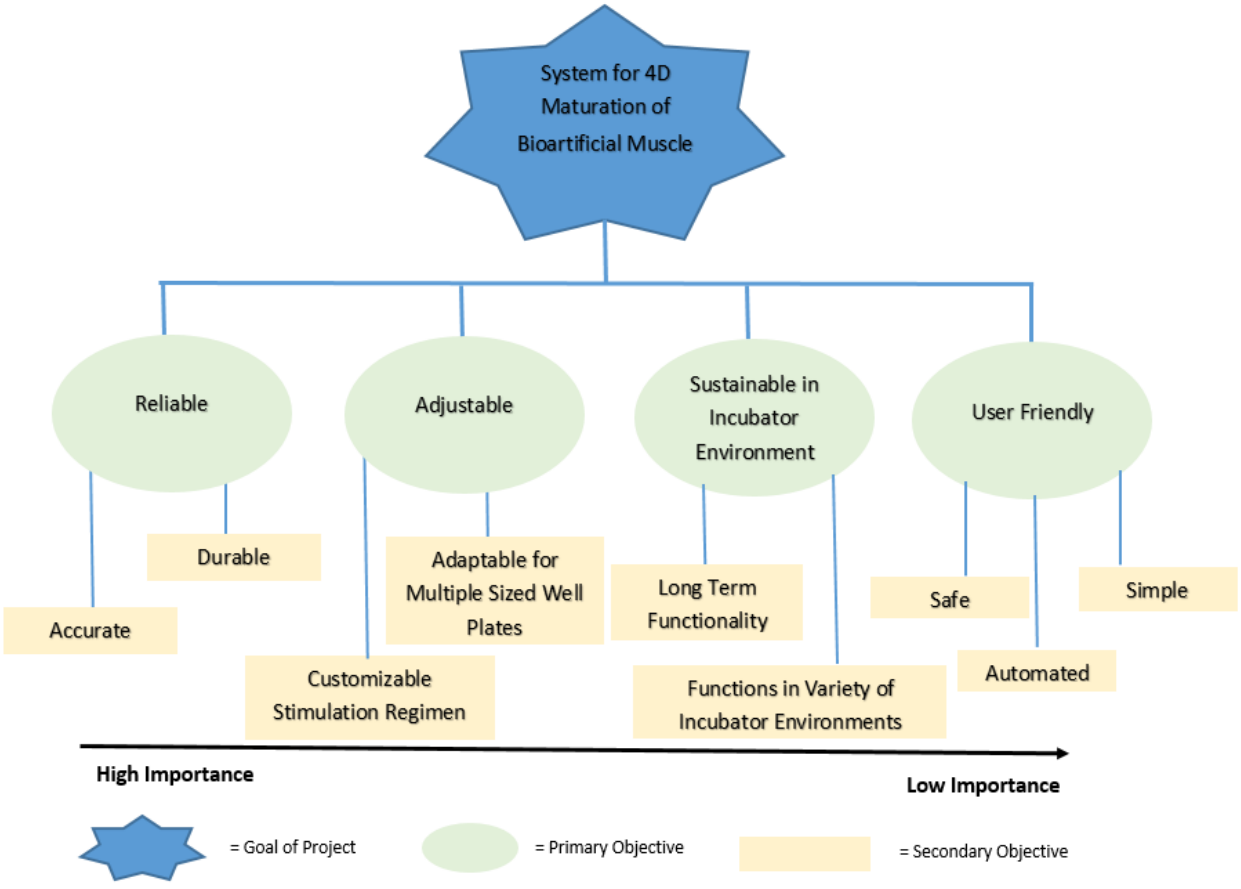

Figure 5: Objective Tree for the Improved Bioartificial Muscle Model; This chart organizes and ranks primary and secondary objectives from most to least important

Based on the PCC and the team's discussions with Professor Page, a reliable electrical stimulation regimen is the highest ranked objectives. The ultimate goal of this device is to be able to replicate neural signaling *in vivo* into electrical stimulation *in vitro*, to better mimic native human muscle properties. By creating an effective electrical stimulation regimen, the team can optimize the maturation of the BAMS, making it available for high content drug screening of muscle disorders. Reliability of the electrical stimulation regimen refers to the device being able to run consistently without error. The electrical stimulation applied from the system, should be trusted that it is the same stimulation that is actually received by the tissues. The device should be dependable to provide consistent stimulation for as many cycles as needed. This objective is very important when conducting experimental tests using the device to ensure validity of results. Reliability can be further classified into the secondary objectives of accuracy and durability. Based off of requirements for the functionality of the device from meeting with Professor Page, the secondary objectives are ranked as follows:

1. Accurate
2. Durable

As the client mentioned, the device needs to be accurate to guarantee that the voltage input being programmed to stimulate the cells matches the output being administered to the cells. Also if we set a particular stimulation regimen, the device should be accurate and reliable in delivering that regimen, and the user can expect consistent results with multiple trials of the device. Therefore, accuracy is the most important secondary objective in order to maintain device constraints, because incorrect voltage or frequency could harm the tissue and ruin the test. Durability is ranked second as the device needs to be able to withstand a variety of testing parameters, such as frequency, voltage, duration, and environmental conditions. The wider range of conditions that it can function

in the better and will allow for the most possible applications. In addition, the device should be durable enough to be used multiple times without failure. These secondary objectives come together to ultimately ensure that the device will function reliably.

Adjustable electrical stimulation regimen is the next ranked objective. The following are the team's secondary objectives: The secondary objectives for this section are as follows:

1. Customizable Regimen
2. Adaptable Size

This device could be used for a variety of different applications and therefore should be a system that allows for customized electrical stimulation parameters based off the needs and conditions of the respective test being performed. In addition, the device should allow for multiple, stimulation regimens within the same well plate with the ability to adjust the parameters as needed. Current models only allow one stimulation regimen throughout the whole well plate, therefore, the team aimed to create a system that permits multiple conditions to test multiple stimulation regimens. The next highest objective is sustainability in an incubator environment. This objective was ranked as the third most important because according to Professor Page the tissue used with the device must remain in an incubator. Therefore, the device should be able to continue working in physiological conditions that replicate the *in vivo* environment. Research obtained during the literature review states that the human native tissue functions in the environmental conditions of 37^0 C, 5% CO2, 95% humidity [32].This means in terms of a secondary objective that the device can either function in an incubator that is set at conditions that model the *in vivo* environment or can provide homeostatic incubation. Homeostatic incubation involves the maintenance of constant temperature, CO_2 levels, and humidity for the electrical stimulation of tissue. The ranked secondary objectives are:

1. Long Term Functionality
2. Functions in a Variety of Incubator Environments

Long term functionality is the highest secondary objective because ideally the device will last for many years and be able to program many different stimulation regimens for long periods of time (days, weeks, months, etc.). This would increase the quality of the device and widen its applications as it could be used more than once. The device is designed to function in standard incubator conditions without harming its components or the tissues themselves.

The lowest ranked objective was user friendly operation. Although it received zero points on the PCC, it is still necessary to consider ease of use in the product design. This will ensure that the device can meet the needs and abilities of a wide range of users. The Myomics Myoforce Analysis System (MFAS™) currently utilized in the Page lab is complex and requires its own technician to service and troubleshoot problems. Therefore, the secondary objectives are to create an automated and simple to use device to decrease human intervention as well as maintain the engineered tissue in ideal environmental conditions as much as possible. In addition the device should incorporate the safety of both the user and the tissue being tested. The secondary ranked objectives are as follows:

1. Safe
2. Automated
3. Simple

The device should be safe so that it will not harm either the user or the tissue during stimulation. There should also be some level of biocompatibility in the sense that the device should withstand treatments and therapies that are made for human cells. Safety is the highest ranked secondary objective as it encompasses the compatibility and sterility of the device with the

stimulated tissue. The device should not cause any form of corrosion, and it must maintain the BAMs sterile. In addition, the device needs to be safe for the user so that it does not hurt the electrical current does not electrocute the user.

Based off of conversations with the client, there is a stronger need for the device to be programmable and automated, over being simple. Therefore the device can run on its own without a technician being present in order to accommodate stimulation regimen and the user's time. If the process is a bit more complicated, users can be taught how to use it, so simplicity is an additional feature that could increase the market value of the device.

3.2.2 Constraints

Constraints are strict limitations to a design so that solutions to the problem are met, thus making the design acceptable [31]. Constraints are used to help narrow the design space in order to optimize the design based on numerical ranges, conditions and materials. After interviewing the client, our team determined that the following are the constraints needed to achieve our objectives:

- Device must withstand physiological conditions (internal incubator environment)
    - Must mimic or operate in physiological conditions (37°C, 5% CO_2, and 95% humidity)
    - Must be biocompatible to allow tissue constructs to develop and mature over time
- Device and materials must be sterile and maintain sterility during operation
    - Must prevent contaminations to allow repeated use of the device
    - Must have a sterile environment for cell culture
    - Safe from foreign body (bacteria, fungal) effects on BAMs
- Device must not over-stimulate the cells
    - Maximum electrical field and frequency of about 2V/mm and 3Hz, respectively to allow for muscle tissue maturation *in vitro* [33]

    - Stimulation pulse width must be less than 0.25ms to allow for correct triggering of action potential [28]
- Device must not cause corrosion
    - Must not evaporate media contents
    - Must not cause toxicity or damage cells and tissues
    - Possible electroplating must not negatively affect BAMs
    - Must not cause evaporation of media contents due to heat production
- Time limitation
    - Must be completed in only 28 weeks to create preliminary, conceptual, alternative and final designs
- Budget
    - Must meet $524 budget

The constraints created for this design are important to take into consideration as the team is aiming to properly develop tissue engineered skeletal muscle by applying electrical stimulation. Sterilization is essential for this design as cells in contact with contaminants can disrupt the maturation process and inhibit growth. The proof of concept for this design will primarily be working with a co-culture of C2C12 cells (mouse myoblasts) and human fibroblasts under a regulated regimen that optimizes the myogenic potential.

Another constraint needed for the design to function is electrical stimulation. The MFAS™ system currently used in the Page Lab performs electrical stimulation outside of an incubator, solely in a heated sterile environment, without the CO_2 level and humidity needed for muscle growth. This exposes the skeletal muscle to environmental changes as well as contamination. The team needs to create a device that is capable of withstanding the incubation environment.

Additionally, electrical stimulation needs to have certain amount of stimulations to occur within a day. Throughout literature, the team identified that the electrical field and frequency applied to the tissue engineered skeletal muscle need to be less than 2.5V/mm and 3Hz as maturation can be inhibited [33]. Since the device will be exposed to high humidity, one of the main constraints for this design is preventing corrosion. Additionally, the potential use of electrodes to deliver a current across a well with media could cause electrode corrosion and deliver harmful components to the cell. When corrosion occurs in the system, cellular toxicity occurs, causing immediate damages to the tissue constructs.

The purpose of these constraints are focused on maintaining homeostasis for the tissue construct growth and maturation. With these constraints, the device will be designed to satisfy the objectives in order to involve the constraints.

3.2.3 Functions

The following functions are necessary for the device to be able to create a system that enables the creation of improved bioartificial muscle models.

1. Provide programmable, controlled and accurate electrical stimulation regimen
2. Encourage muscle tissue maturation through customizable electrical stimulation
3. Interface with industry standards for BAM development and high content drug screening

These functions were established to satisfy the design objectives and constraints in the development of the team's project. It is important for the device to have the ability to apply a customizable electrical stimulation regimen and that it can be programmed, or automated, so that there is very little user involvement. This will allow for more realistic tests as the system can function on its own at all hours of the delay without relying on someone to constantly program the system, in addition the parameters applied by the stimulation system should be accurate as to what is delivered to the BAMs. The device should have features as well as a stimulation regimen that

encourage myofiber anchorage, attachment and alignment in order to promote muscle maturation which is ultimately needed to create a bioartificial muscle model. Lastly, should be able to function according to industry standards for BAM development and high content drug screening by interfacing with a standard 96-well plate format. The goal is that this project can meet industry regulations for high content drug screening for muscular dystrophy.

3.2.4 Specifications

This sections describes the design specifications for each portion of the team's project. These published guidelines for bioartificial muscle development, culture plate parameters, and electrical stimulation systems were important for the team to consider in the creation of their project strategy.

Bioartificial Muscle Development

It is essential to the team's project to create a functional BAM. This BAM must be able to maintain the functions and properties of engineered muscle tissue as described in section 2.4. The team must be aware of potential sources of corrosion that would induce cytotoxic chemicals and harm tissue development, alignment, assembly and anchorage.

As mentioned in 2.3.1 the cell density of the myotubes for *in vivo* tissue is between 30-57 nuclei-mm^2 and greater than 90% Myosin (+) and therefore the BAM should attempt to replicate this cell density [7]. The cell density of the BAM is important as it plays a role in tissue alignment and is a large factor in reducing seed cell quantity. Furthermore, the ideal BAM size will have the diameter of 200-250μm and length of 3.5mm [7]. These dimensions make the BAM large enough to resist its contractile force and small enough to forego blood supply when using this minimal functional unit as mentioned in section 2.4.3.

Culture Plate Parameters

After discussing the wants and the needs of the project with the client, the culture plate parameters should be based on a 96-well plate metrics, which is the standard for drug screening. For example, Corning makes clear polystyrene plates with dimensions consisting of 128mm in length, 85mm in width and 17mm in depth [34]. The size of the plate is able to stack within an incubator as well as being able to fit in the MFAS™ device. This specification is important to take into account as this will influence the space and sizing for the circuitry.

Other culture plate parameters that were mentioned by the client's needs and wants is defining the features of the microposts in the culture dish. In the Page Lab, microposts are inserted into each of the wells, which is essential for engineered skeletal muscle as this provides anchorage to the tissue constructs. These microposts have dimensions of 0.8mm in diameter, 4mm pitch with 3.5mm from center-to-center. For *in vitro* 3D tissue constructs, anchorage to the microposts mimics the *in vivo* attachment points to the direct the axial alignment of myofibers. This allows tissues to generate uniaxial tension, a contributor to muscle maturation. Current methods of developing *in vitro* anchorage include micropost attachment, ring formation, and self-assembly.

Electrical Stimulation System

An integral component of this design is to transmit electrical stimulation to promote maturation of tissue-engineered skeletal muscle. Therefore, a regimen is needed to prevent any hindrance of the BAM myogenic potential due to a high frequency of electrical stimulus. Electric stimulation is achieved by passing an electric current through electrodes in the media. Electrode placement in the culture media will allow current to be conducted through the tissue, which in turn depolarize the cells causing muscle tissue contraction. For example, the MFAS™ device provides electrical stimulation with electrodes placed perpendicular to tissue constructs as described in section 2.5.2. This allows for better cellular alignment and maturation. After given this design

specification from our client, the team studied current parameters from previous research in order to compare and determine the best method for the electrical stimulation regimen better visualized in Table 3.

Table 3: Various skeletal muscle tissue parameters used from 1997 to 2014

Study	Electrical Field (V/mm)	Frequency (Hz)	Timing (ms)	Duration
Thelen, et al 1997 [35]	3	2	6	2 days
Radisic, et al 2004 [36]	5	1	2	5 days
Pedrotty, et al 2005 [37]	6	0.5 - 10	0.5 – 250	1 - 14 days
Fujita, et al 2007 [38]	6.7	0.1 - 10	24	1 - 9 hours
Ito, et al 2014 [39]	3	1	4	10 days
Yamasaki, et al 2009 [40]	8.3	0.5-10	80	10 days
Donnelly, et al 2010 [28]	2.5	10	400	10 days

These parameters are important factors in the development of the device as they have been used for comparison in the development of a regimen to optimally achieve maturation of muscle tissue constructs.

3.3 Design Requirements: Standards

The International Organization for Standardization (ISO) creates and publish International Standards to provide specifications that guarantee that products and services are reliable, safe, efficient and of high quality [41]. In terms of business, ISO International Standards are beneficial

in ensuring that costs are minimized as much as possible by reducing mistakes and waste, and also raising productivity. These standards enable companies to enter different markets, enable 162 countries to be on the same playing field with the same standards, and initiate free and just global trade [41]. There are over 19,500 International Standards ranging from almost all fields; such as food safety, healthcare, agriculture and more [41]. The team's MQP project is intended to be used more for research purposes, yet if the team decided to move forward with the device on a clinical level, ISO Standards would be need to be followed. The ensuing ISO Standards provide an overview as to which standards would be necessary for further developing the team's device. Table 4 below shows some of the ISO standards that must be considered in the design of the team's device.

Table 4: ISO Standards necessary for the development of the team's designs

Sterility	Safety	Well Plates	Corrosion	Medical Products Testing	Management
ISO 17664:2004 - Sterilization of medical devices	**ISO 13688:2013 -** Protective clothing	**ANSI SLAS 1-2004 - ANSI SLAS 4-2012 -** 96-Well Plate Standards	**ISO 11845:1995 -** Corrosion of metals and alloys -- corrosion testing	**ISO 13022:2012 -** Medical products containing viable human cells- requirements for processing practices	**ISO 9001:2015 -** Quality management systems - Requirements
	ISO/IEC 63:2012 - Guide to the development and inclusion of safety aspects in International Standards for medical devices			**ISO 10993-5:2009 -** Biological evaluation for medical devices -- Part 5: Tests for in vitro cytotoxicity	

3.3.1 ISO 13022:2012

ISO 13022:2012 (en) is the ISO Standard of Medical products containing viable human cells - Application of risk management and requirements for processing practices. From meetings with the client, the team was able to identify that the ultimate goal for this device is to test the effects of various treatments and drugs on patient's specific cells. Therefore this standard would be necessary to make sure that the team's medical device is accounting for any potential risks and requirements for using human cells.

Some aspects this standard accounts for includes contamination, complications from materials causing unexpected immunogenic reactions, product degradation, toxicity and more. This standard is used particularly in regenerating a patient's own tissues, so this is relevant for clinical applications for the team's project such as finding treatments to muscular dystrophy and muscle injury, where there is a need to create muscle cells [42].

3.3.2 ISO 10993-5:2009

ISO 10993-5:2009 is titled Biological evaluation of medical devices -- Part 5: Tests for in vitro cytotoxicity**.** This standard is details information regarding testing procedures to evaluate *in vitro* cytotoxicity of medical devices. Some of the specific methods addressed is the process of incubating cultured cells that come in contact with a medical device and/or elements of a device either directly or by diffusion [43]. Ultimately, this standard would help to predict the biological response of mammalian cells *in vitro* while using relevant biological guidelines.

As previously mentioned, the device has to function in an incubator in order to simulate the *in vivo* environmental conditions for BAM development. Therefore this standard is useful as the team would need to make sure that none of the device materials or cells used would be toxic to the user, especially after incubation. In addition, this standard takes into account biological parameters, or the conditions of the body. Also this standard would be appropriate to use in

preclinical testing using specific human (mammalian) cells to test the effects of various drugs and treatments and ensure that there is no cytotoxicity. If corrosion of the device occurred *in vitro*, this standard would help determine if there are any toxic effects on the cells.

3.3.3 ISO 17664:2004

ISO 17664:2004 is called Sterilization of medical devices -- Information to be provided by the manufacturer for the processing of resterilizable medical devices. Within this standard are guidelines for how to process medical devices so that they are resterilizable and can safely continue to maintain performance standards. Some of the elements of this standard are preparation, cleaning, disinfection, drying, inspection, maintenance, testing, packaging, sterilization, and storage [44]**.**

Sterility of the device is an important component to maintain viability of the BAMs. The device cannot cause contamination to ensure accuracy of test results and maintain integrity of the tissue being tested. The team will need to be wary of the sterilization method chosen as sometimes, over-sterilization can harm the cells or distort the environmental conditions that they would not accurately simulate an *in vivo* environment. There are a variety of ISO standards on sterilization, so once the team finalizes their method of sterilization they sure reference the ISO standards again to ensure that appropriate measures are being followed for specific techniques.

3.3.4 ISO 11845:1995

The ISO standard 11845:1995 is called Corrosion of metals and alloys -- General principles for corrosion testing. This standard outlines a basic overview of the standards for executing tests for corrosion [45]. Corrosion must be prevented to assure tissue viability during testing. The metal that the team selects as an electrode could potential be corrosive in combination with the media used in the device as well as under extreme environmental conditions meant to replicate the *in vivo* environment.

3.3.5 ANSI SLAS 1-2004 – ANSI SLAS 4-2012

This series of standards all pertain to a microplate and include regulations regarding footprint dimensions, height dimensions, bottom outside flange dimensions, well positions and well bottom elevation [46]. Microplates are a crucial element of the team's project, especially as we are following the standards for high content drug screening. The well plate would contain diseased tissue that would be stimulated to mature and then test various drugs and therapies in order to help find cures for the disorders. The device would be able to stimulate multiple wells at once. Therefore, it is extremely important that the team follows these standards as a microplate is a requirement for the project**.**

3.3.6 ISO 13688:2013

ISO 13688:2012 is titled Protective clothing -- General requirements. Throughout the MQP process the team will need to be in the laboratory testing its product, culturing cells etc. It is imperative that the team takes safety seriously and is wearing the necessary protective clothing in respect to the tests being done. This standard gives a general overview of the specifications of protecting clothing, but needs to be used in conjunction with an additional standard that refers to the protective clothing that is required for the specific type of experiment being performed [47].

3.3.7 ISO/IEC 63:2012

ISO/IEC 63:2012 is titled Guide to the development and inclusion of safety aspects in International Standards for medical devices. This standard provides a framework on how to follow standards regarding medical device safety, but also integrate those standards with those of the risk management standard, ISO 14971 [48]. In addition this standard will include safety-related performance and usability regulations. Since the team's project is to create a medical device, and one that could potentially work with human tissue, it is very important that the team follows these safety precautions.

3.3.8 ISO 9001:2015

ISO Standard 9001:2015 is a process standard called Quality management systems - Requirements**.** This standard provides requirements as to what an organization needs to do in order to regulate processes that impact the quality of products and services [49]. In addition, this standard details what is need to consistently meet and also improve customer expectations and satisfaction of the organization's goods and services.

The team's project currently is geared towards a producing a medical device for research purposes. However, if the product was successful the team could market their product to various organizations on a commercial level. If the device was to be made on a large scale then it would be necessary for the team to follow this standard.

3.4 Revised Client Statement

After meeting with the client and thoroughly discussing the client statement, the team adjusted the client statement to capture the salient features of the problem. The team also met with Professor Page and Jason Forte to further discuss the client's problem. This extra communication was important to provide an additional perspective of the limitations to the current standard used for experiments in the client's lab.

From the interviews conducted, the team identified the needs and wants for the project should be aimed toward designing a device to fit a 96-well plate. The long term goals for this project was to create a model to test drugs and therapies for a variety of muscular disorders. In addition, Professor Page and Jason mentioned that they wanted the team to improve upon previous MQPs. The previous MQPs that dealt with the topic of electrical stimulation were not able to develop a working system that solves the problem, though they developed some very innovative ideas.

The team used the limitations of the current standard, the past MQPs and discussions with the client and his graduate student to set the scope of the project in developing the revised client statement, project objectives and constraints in this chapter.

The revised client statement is as follows:

In the science of drug screening and diagnostic testing in vitro, engineered bioartificial muscles (BAMs) are developed as biological samples in a 96 well format with the potential of providing high content screening for therapeutic effects on muscle function, fatigue and toxicity.

In the science of drug screening and diagnostic testing in vitro, engineered bioartificial muscles (BAMs) are developed as biological samples in a 96 well format with the potential of providing high content screening for therapeutic effects on muscle function, fatigue and toxicity.

There is a need to improve the efficiency and accuracy of in vitro bioartificial muscle models (BAMs) for high content screening for therapeutic effects on muscle function, fatigue and toxicity. The current systems have many limitations that prevent the models from accurately replicating in vivo skeletal muscle maturation and properties. These models are also expensive and inefficient. The goal of this project is to develop a system that provides an electrical stimulation to each well of a 96-well plate that will enhance muscle maturation in order to improve the utility of bioartificial muscle for evaluating therapies for muscle disease. The following characteristics and parameters are important to be incorporated within the team's design:

1. Mimicking the muscle-myotendinous junction attachment to provide tissue anchorage.
2. Electrical stimulation by electric field, to replicate in vivo motor neural stimulation for improvement of myofiber alignment and maximum differentiation.
3. Programmable and controllable electrical stimulation regimen.

4. Maintaining sterile tissue constructs and a sterile device that is as corrosion resistant as possible.

3.5 Management Approach

This section describes how the team plans to approach the project in terms of timelines and dividing up tasks among team members. Below is a general representation of the project approach, but this could be adjusted based off of the needs of the team, advisor and project progress.

The team's project is organized to last throughout the entire school year, from September to April. Therefore it is very important for the team to manage tasks and deadlines in order to ensure that the team stays on track for successful completion of the project by the final due date. The team created a Gantt Chart in order to organize the project timeline. This chart includes a list of tasks, individual due dates, and up to date statuses on the completion of each task. Figure 6 below represents the team's Gantt Chart for the year.

ACTIVITY	START	DURATION	COMPLETE
Project Research	1	7	100%
Client Interviews	1	3	100%
Client Lab Observations	3	5	100%
Project Brainstorm	1	7	100%
Chapter 1 - Introduction	2	5	100%
Chapter 2 - Literature Review	3	4	100%
Chapter 3 - Project Strategy	3	4	100%
Alternative Designs	6	2	100%
Lab Experiments	9	9	100%
CAD Drawings	9	2	100%
Order Materials	9	3	100%
Construct Prototypes	11	5	100%
Test Prototypes	12	5	100%
Modify Prototypes	14	4	100%
Finalize Prototypes	16	3	100%
Chapter 4 - Design Process	8	10	100%
Chapter 5 - Final Design Verification	11	7	100%
Tissue Analysis	18	4	50%
Construct Device	17	4	100%
Test Device	20	2	50%
Modify Device	20	2	50%
Chapter 6 - Final Design Validation	20	4	50%
Finalize Device	21	3	50%
Chapter 7 - Discussion	21	3	75%
Chapter 8 - Conclusions and Recommendations	22	4	50%
Finalize Report	23	3	0%
Presentation	28	1	0%

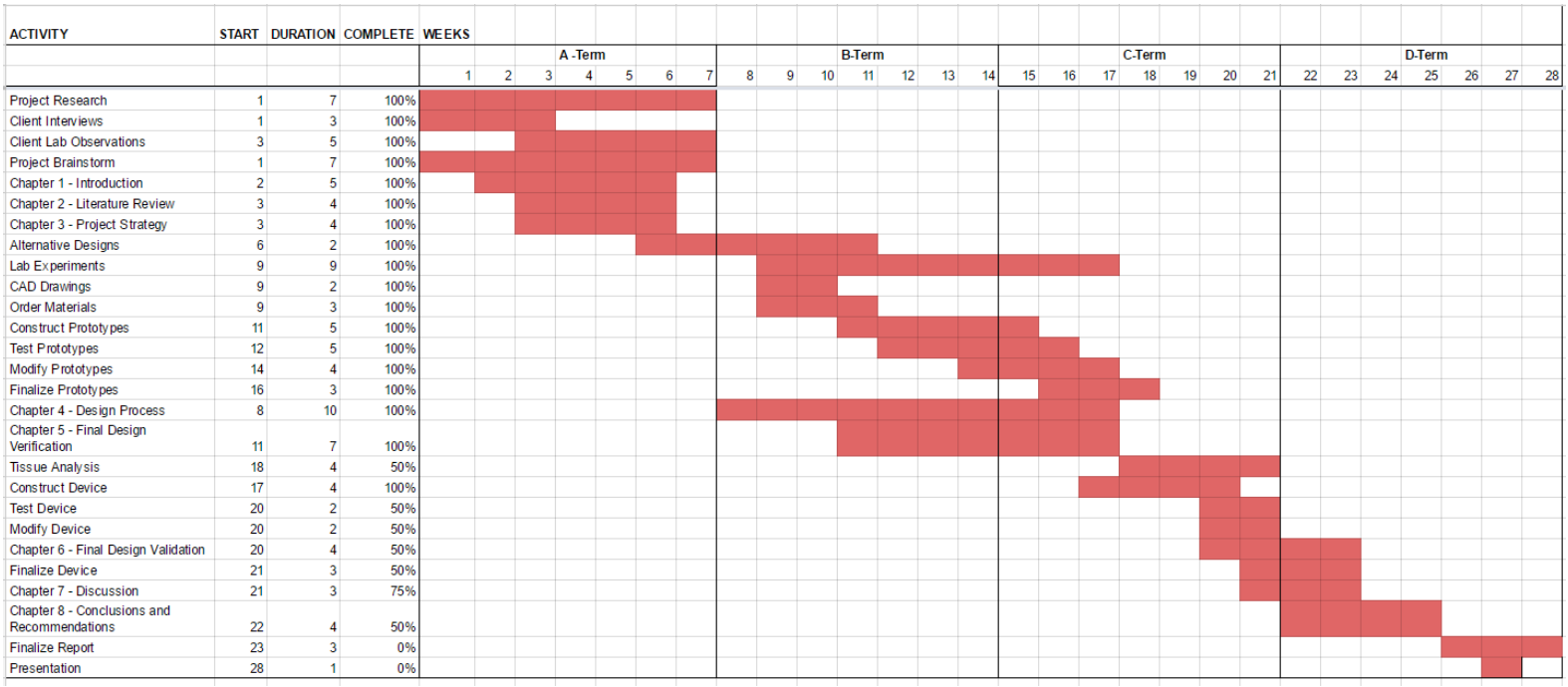

Figure 6: Gantt Chart; This chart is to monitor the progress of each task necessary to ensure the completion of the team's project

Based on this Gantt Chart the team further divided these general tasks into more specific steps so that each member could have a clear role and be responsible for ensuring the completion

of the project elements. Figure 7 below shows the team work breakdown structure. This structure helped to make the project even more efficient as each member was assigned tasks that suit their personal strengths and would allow the team to use their time effectively by making the most of each team member's skills.

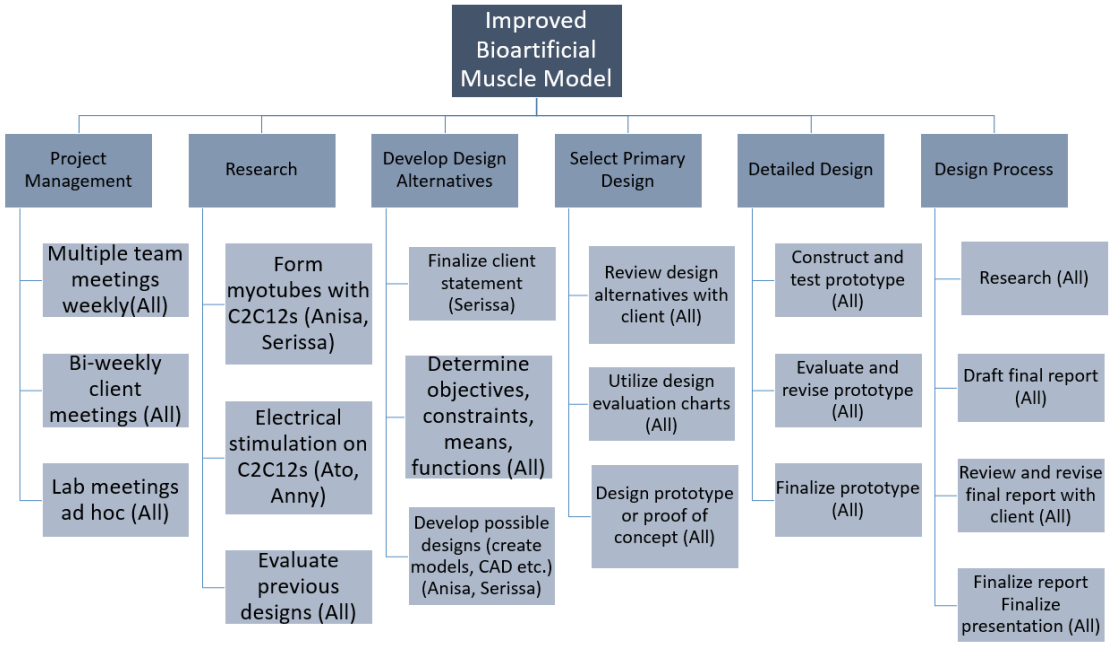

Figure 7: Work Breakdown Structure; this chart further classifies each step of the project and assigns responsibility to team members for completion

The whole team worked together to come up with the design for the well plate and electrodes. After those designs were developed, the team split the project into two categories; the electrical stimulation component and the tissue engineering/material components. Team members distributed themselves to work within those two categories based off of their interests and experience. Anisa focused on the tissue engineering and materials, while Ato focused on the electrical stimulation component. Anny and Serissa switched off between categories based off of which aspect of the project needed more attention or additional help. They also helped with manufacturability of the device.

Chapter 4: Design Process

After numerous meetings with the clients, the team revised the client statement to best encompass the objectives needed to have a properly functioned device. In this chapter, the team discusses the "needs" and "wants" of the device. Additionally, this section describes the team's conceptual and alternative designs that were determined. Lastly, the team focused on the potential advantages and limitations of each design, leading to the decision of the final method of operation of the electrical stimulation system.

4.1 Needs' Analysis

An analysis of the client's needs was created from the revised client statement found in section 3.4. This analysis consists of categorizing the primary objectives as project needs, and the secondary objectives as project wants. Therefore, the needs are the team's highest priority, while the wants are preferred, but not necessary for the completion of the device. Constraints, including time and budget, were important factors considered in creating the needs analysis. The following needs and wants were used when further creating alternative designs.

Needs:

1. Sterile tissue constructs (BAMs)
2. Adjustable and reliable electrical stimulation regimen
    a. Can set user-defined electrical field (current), number of pulse cycles, pulse width and pulse rest
    b. Can ensure that voltage delivered to tissues is consistent with the voltage that is being received by the tissues
3. Sustainable in incubator environment
    a. 5% CO2, 95% humidity and 37°C temperature

Wants:

1. Simple to operate, automated
2. Compatible with a wide variety of environmental conditions
3. Provide mechanical stimulation or be compatible with devices that deliver mechanical stimulation
4. Custom printed circuit board to replace the circuitry wiring and provide an optimized electric stimulation

4.2 Conceptual Designs

The team combined the design functions and means described in section 3.2.3 to create Table 5. This table categorizes the functions and means into conceptual designs. The conceptual designs in the table below were greatly used in the generation of alternative designs for (1) electrical stimulation, (2) tissue formation and (3) lid design.

Table 5: Function-means table for the device

Function	Means						
Provides Electrical Stimulation	Posts are electrodes	Posts are piezioelectric material	Line electrodes in base plate	Magnetic fields in top and bottom plates	Detachable, suspended electrodes in top plate (don't touch wells or media)	Two electrodes contact in the well	Contactless electrode array, with a glass coverslip
Provide Automation for Electrical Stimulation	Arduino board	Raspberry Pi					
Electrical Stimulation Feedback	Sensor	Filters	Potentiometer				
Tissue Anchorage	Ring formation	Micro-post Attachment	Self-assembly				
Attachment for Electrode	Grooved channels	Soldering	Buss Bar				
Corrosion Resistant Electrodes	316L Stainless Steel	Conductive polymer	Gold	Platinum	Platinum-Iridium Alloy	Silver	

The function-means table was used to generate unrestricted ideas of how to develop the device. By using this method of analysis, the team was able to have a comprehensive understanding of the components needed and identify the best approaches to successfully develop the device for the desired application. Each of the means were identified as materials/ methods commonly used in literature. Based on thorough literature review, the team was able to identify what components fit best in the development of the electrical stimulation device.

4.3 Alternative Designs

In this section, the alternative designs were broken down into three components to determine the device design: (1) electrical stimulation, (2) tissue constructs development, and (3) lid design.

4.3.1 Electrical Stimulation Component

After the determining the needs, objectives, constraints, functions and means for the conceptual designs, the team used Table 5 to create alternative designs for the electrical stimulation.

Contactless Stimulation: Electric Field

One method of providing electrical stimulation is by creating an electric field. This tissue culture plate would be placed in the incubator with a magnetic plate placed on top. An electric field would be generated when a voltage is applied to the device, causing current to flow through the plate. The electric field would be generated perpendicular of the tissue construct providing the current needed for enhanced tissue development. As the field is applied, the tissue would contract. The purpose of this approach is to avoid the use of electrodes, which would eliminate potential contamination.

Another method to provide electrical stimulation is using a static magnetic field. Similar to the method done by Coletti et al., Neodymium magnetic plaques were used to create an axial field with a magnetic pole in the North direction [50]. The magnitude and homogeneity of the magnetic field for the plaque in this system was 80 ±5mT [50]. The benefit of using this method is to drastically reduce the amount of heat that could affect culture media and the cells in culture themselves. However, using a method like this requires very advanced analysis and tests.

Contact Stimulation: Electrodes

Electrodes would be used to provide electrical stimulation in direct contact with the culture media. Typically, metallic electrodes are used because of their ability to modify their shape, connect to a power source, and provide direct contact when inserted into culture media.

Another electrode method to provide electrical stimulation is the use of conductive polymers. The benefit of using polymers is their ability to facilitate mechanical compliance, electrical conductance, and biological affinity. The following polymers have shown to enhance cell growth, differentiation, environmental stability and biocompatibility: polypyrrole (PPy), polyaniline (PANi), and polythiophene (PT) [51]. Therefore, these polymers provide stability and can conduct electrical stimulus without risk of corrosion, which is cytotoxic. Another option is the use of piezoelectric materials, which can provide simultaneous electrical and mechanical stimulation because these materials undergo physical change when voltage is applied.

The orientation and placement of the electrodes are perpendicular to the tissue constructs because there is a higher concentration of myoblasts in the center of the tissue and perpendicular electrodes apply the most electrical field to the middle of the construct. In addition a single well in a 96-well plate is very small and therefore the length of the tissue constructs are very small. The electrodes would likely touch if they were parallel to the tissue constructs, which would cause them to short-circuit and damage the tissue. This would allow for better maturation and fiber alignment than the current being applied parallel to the constructs. Additionally, the electrode attachment is another consideration for this design. One option for this is the creation of a "comb", in which a long wire would have soldered shorter wires attached. The same method could be created by utilizing a specific-metal epoxy, therefore attaching the smaller wires with a cold-solder.

Programming Electrical Stimulation

For the programming of the electrical stimulation, the team considered two different types of platforms to control the voltage frequency, voltage duration, number of stimulation cycles, and number of wells to stimulate: (1) Arduino and (2) Raspberry Pi.

An Arduino is a microcontroller used as an open source prototyping platform which is based on easy-to-use software and hardware [52]. Varying models of Arduinos contain different characteristics and capacities. Such boards are able to read inputs such as a finger on a button, a light on a sensor, even a twitter message and are used for embedded applications [52]. Such input is subsequently turned into an output which could activate a motor, publish something online, or turn on an LED light [52]. By utilizing an Arduino programming language the user can communicate with the board and give it instructions on what to do next. A benefit of using Arduinos is that it is a cross-platform microcontroller that can be utilized and run on Windows, Macintosh OSX, and Linux [52]. Additionally this microcontroller is inexpensive, has a simple programming environment, and has open source hardware and software.

Raspberry Pi is a microprocessor commonly used for graphic intensive control. It mostly functions as a computer because it has a dedicated graphics driver, processor, and memory. Additionally it has an output for HDMI and WIFI [53]. This microprocessor runs on the Linux operating system. A major limitation is that it does not possess internal storage and requires the use of SD cards for flash memory. This system also requires a constant 5V power to keep it turned on [53].

4.3.2 Tissue Constructs Component

Possible tissue formation methods included the use of either ring or micropost attachments for the development and anchorage of the BAMs. Both techniques promote anchorage and alignment of C2C12s but would have different effects on the integrity and development of the

constructs. The team also considered co-culturing with fibroblasts to test their effects on improving the quality of the engineered skeletal muscle constructs. C2C12 immortalized cell lines were used during testing due to their high rate of proliferation, cost-efficient, and present significant myogenic potential, compared to primary cells.

Ring Shaped Tissue Formation Methods

This method creates stable tissue constructs and is simple to execute and is previously described in section 2.4.2. Professor Page's previous MQP team working on this project created ring tissue constructs in 2% agarose molds that were made from reverse positive polydimethylsiloxane (PDMS) mold. These PDMS molds were filled with hot liquid agarose to make 4 annular wells that had a 2mm diameter post in the center. Once the PDMS solidified, the previous team withdrew the agarose from the mold and C2C12s were seeded into the wells to have a seeding density of 400,000/55μL. This method did allow for tissue self-assembly but in comparison to micropost attachment formation, it did not replicate the tissue's myotendinous junctions as closely. The team considered the results of last year's MQP ring shaped applications and results, as well as research, to create a comparison to micropost tissue. This comparison is important for determining the relevance of both tissue formation methods to the project.

Micropost Attachment Tissue Formation Method

Micropost attachment formation was also considered by the team as a tissue anchorage method, which is previously described in section 2.4.2. The process for micropost attachment anchorage is more complicated than ring anchorage as the cells need to merge around two posts to create tissue instead of only one post, while there is also a connecting section between the posts. Mechanical stimulation promotes uniaxial alignment for tissue constructs, which is important for encouraging tissue maturation. Micropost formation has been used by the Vandenburgh group to

create skeletal muscle tissue constructs [26]. The Vandenburgh group made modifications to the standard approach and created clamps on each end of the construct as an alternative to shaping the tissue around the two posts [26].

The molds in the micropost constructs are made from N-Isopropylacrylamide (NIPAAm) or agarose. NIPAAm is known for its thermoresponsive properties, while agarose undergoes polymer change. Agarose is solid at 45^{o}C and lower temperatures, and a liquid at temperatures higher than 45^{o}C. NIPAAm is solid at 32^{o}C and over and liquid at temperatures lower than 32^{o}C [54]. Therefore, agarose remains solidified at room temperatures while NIPAAm melts. NIPAAm has the advantage because it can be cooled from its gel state causing it to be liquefied and removed from the construct. Agarose has a much higher melting point than NIPAAm and would not be liquefied without boiling and killing the cells. These thermal properties of NIPAAm were much more suited to the project's parameters than agarose. Figure 8 provides a visual representation for creating tissue constructs using the Vandenburgh method [26].

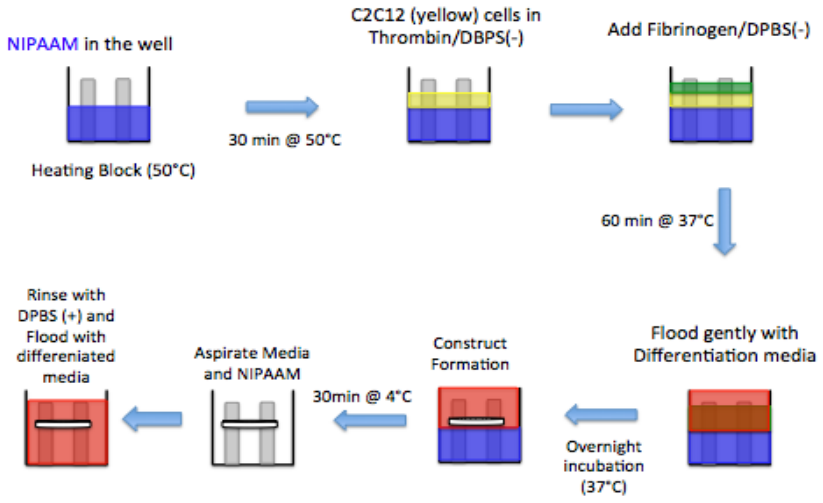

Figure 8: Vandenburgh method for creating tissue constructs [26]

4.3.3 Lid Component

For designing the cell culture lid that provides electrical stimulation, the characteristics that were needed are outlined in section 4.1 in the “needs” and “wants”. Overall this device needs to withstand incubator conditions, be easily sterilized, provide stimulation simultaneously to each well in a 96-well plate, and provide electrical stimulation without corroding or harming the tissue constructs.

More specifically, incubation and sterilization of the device are key in protecting the device and keeping the tissue constructs viable. Corrosion or damage to the electrical components of the device due to incubator conditions must be addressed. Therefore the team derived a few alternative designs which could either allow the device to function directly in the incubator with direct tissue stimulation or operate outside of the incubator while indirectly stimulating tissue inside the incubator. Additionally, these lid designs would be capable of providing electrical stimulation simultaneously to each well in a 96-well plate, increasing efficiency in conducting multiple experiments at a time.

Soldering Method

The soldering method consisted of drilling a 1mm hole, 3.5mm apart in each well of a standard 96-well cell culture plate, made out of polystyrene (Corning), with two 1mm drilled. Small copper electrodes (25mm) were secured and soldered onto a larger (~150mm) piece of copper wire. The electrodes were placed in the holes of the polystyrene cell culture plate and secured by using copper epoxy. Sterilization of this alternative lid design would be performed with ethylene oxide (EtO). Figure 9 shows the Solidworks version of the plate and Appendix A shows the drawing of the lid design.

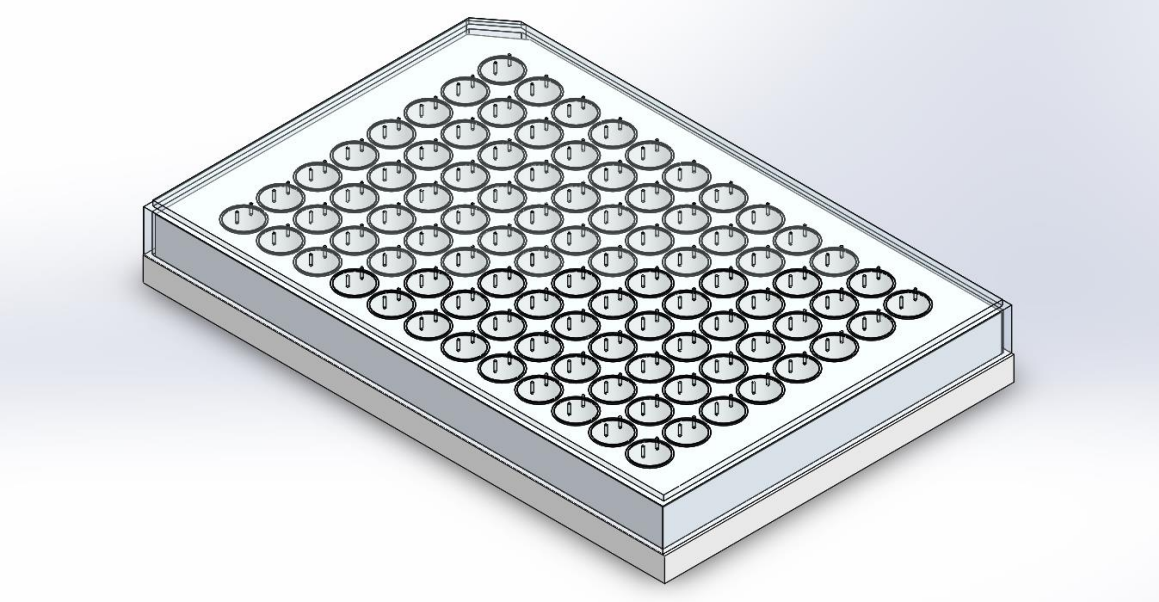

Figure 9: Shows the isometric view of the soldering lid design

Grooved Channel Method

The lid was designed in SolidWorks, where Figure 10 shows the overall design in exploded, isometric view and Appendix B shows the drawing of the grooved channel. The material chosen for the lid was acrylic because of its transparency record provides visual representation of the tissue contractions upon electrical stimulation creating easy to record measurements. Additionally, acrylic can be sterilized, capable of withstanding incubator conditions, and easily manufactured. Because the lid was designed to fit a standard 96-well plate, its dimensions were based on the width of 85mm and length of 127mm. The lid was composed as an assembly, as described below.

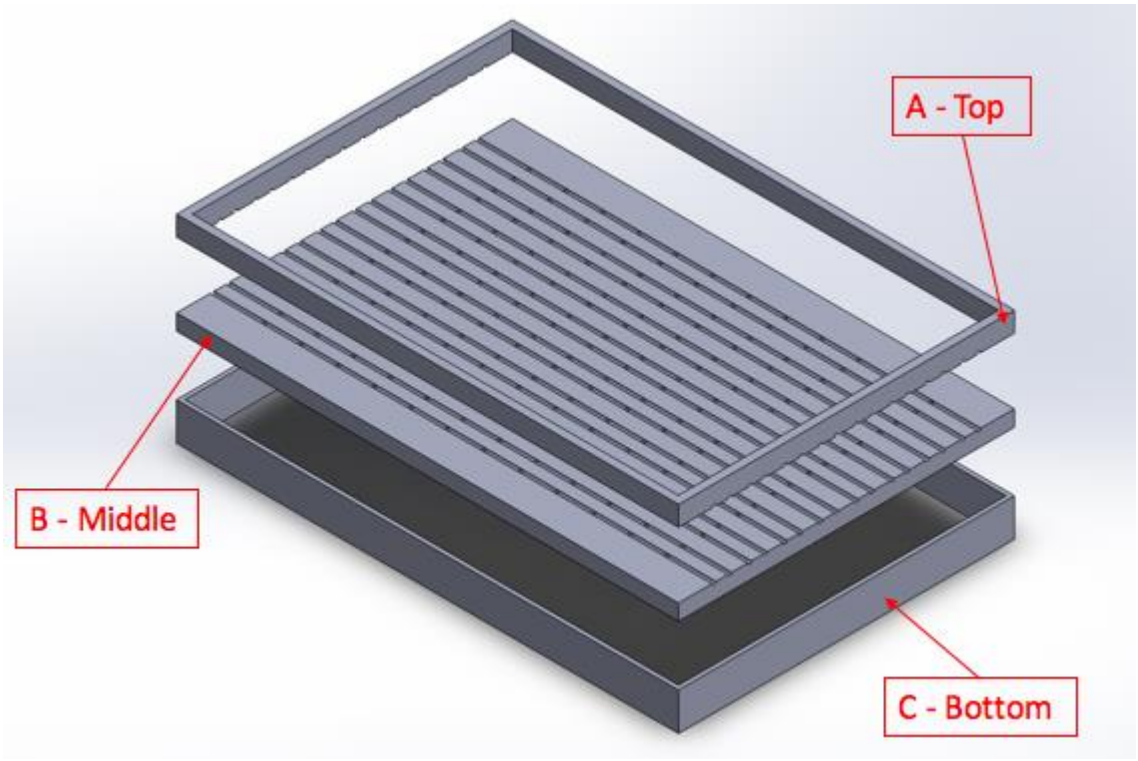

Figure 10: Shows the exploded, isometric view of the grooved channel design. Labeled (A) represents the top of the assembly, (B) represents the middle of the assembly, and (C) represents the bottom of the assembly

Top part (labeled as A): For this part, semicircles would be drilled into the side of the acrylic, with a diameter of 1mm. The height would be 4mm with a thickness of 3mm, as this would provide the placement for the soldered wires containing electrodes.

The middle part (labeled as B): This component contained 0.8mm diameter holes for the electrodes to be positioned. Each well has two holes located perpendicular to the PDMS posts. Each of the holes were separated 3mm apart from center-to-center. To create the grooves, holes would be drilled into the side of the lid, which would act as a channel for the placement of the soldered wires. In addition, when part A goes onto part B, this would act as enclosure for the ends of the wire, as this would provide the electrical connection. One groove would provide the positive current while the other groove would provide negative current. For each row of the cell culture plate, two grooves would be engraved into the acrylic.

The bottom part (labeled as C): This component has a height of 9.5mm and thickness of 3mm, which acts as the support for holding parts labeled as A and B. This helps provide closure and being loose in order for oxygen exchange to occur in the plate.

Buss Bar Method

The lid was designed in SolidWorks, where Figure 11 shows the overall design in isometric view and Appendix C shows the drawing version of this design. The material chosen for the lid was acrylic because of its transparency as previously described above in the grooved channel design. Additionally, in this lid design, the bottom of the plate would follow the industry standard of a 96-well plate, width of 85mm and length of 127mm. The lid was composed as an assembly, as described below.

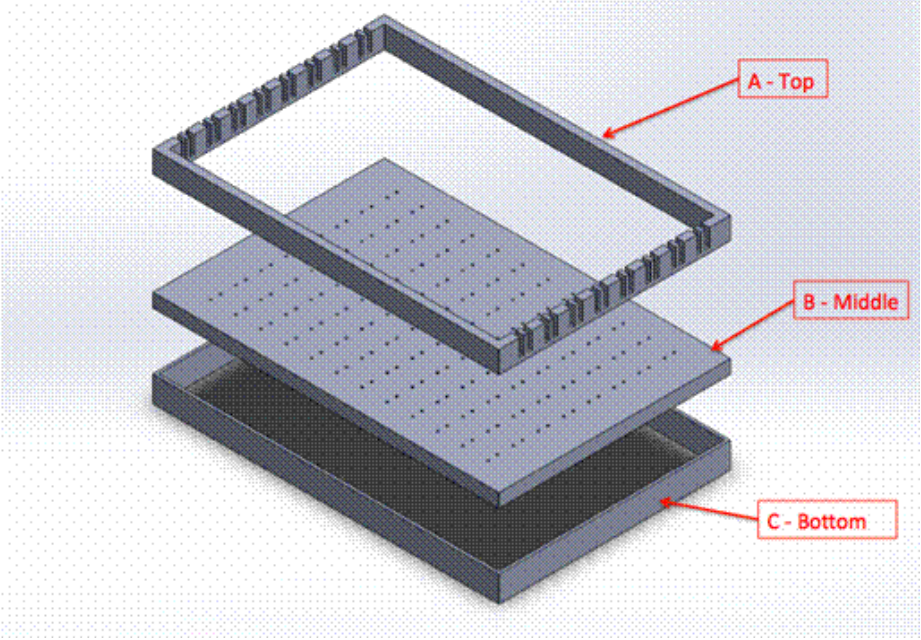

Figure 11: Shows the exploded, isometric view of the SolidWorks final design of the buss bar lid. Labeled (A) represents the top of the assembly, (B) represents the middle of the assembly, and (C) represents the bottom of the assembly

Top part (labeled as A): This part contains slits for the buss bars. Each row of the culture 1/16” x ¼” inch slits to insert the brass bars. One side of the plate provided a positive connection and the other provided a negative connection. The center-to-center distance of the slits were

6.25mm. These brass bars contained slots where 316L Stainless Steel electrodes were screwed into place.

The middle part (labeled as B): For this part there were 0.8mm diameter holes for the electrodes to be inserted into. Each well had two holes perpendicular to the PDMS posts. Each of the holes were separated 3mm apart from center-to-center.

The bottom part (labeled as C): This component has a height of 9.5mm and thickness of 3mm, which acts as the support for holding parts labeled as A and B. This helps provide closure and was loose in order for oxygen exchange to occur in the plate.

For connecting the 316L stainless steel bar electrode connection, the attachment is based on the buss bar model. The buss bar allows for aligned electrode placement as seen on Figure 12. In this technique of attaching wire, a metal bar has slots in order for wires to connect, which is then held together with a screw.

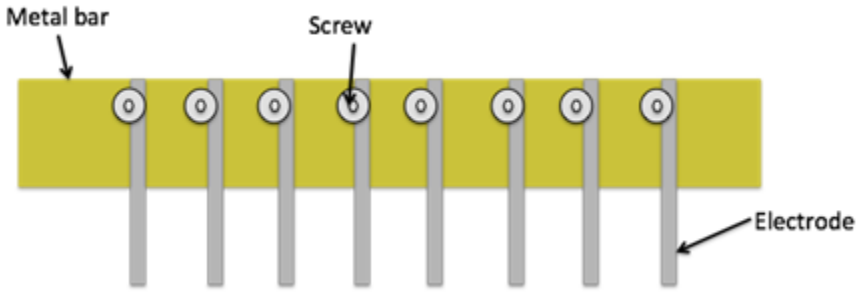

Figure 12: Shows the buss bar design. The bar was made of brass and 316L stainless steel wires were placed into slots which are held together by stainless steel screws

4.4 Final Design Selection

Tables 6, 7, and 8 represents the advantages and disadvantages of the alternative designs for the electrical stimulation, tissue construct development and lid device components. The purpose of this chart was to aid the team in determining the optimal device designs.

Table 6: Shows the electrical stimulation method with corresponding advantages and disadvantages

Design Means	**Advantages**	**Disadvantages**
Contactless: Electric Field	● User-friendly ● Work in incubator ● No Corrosion	● Difficult to control voltage applied ● Difficult to monitor voltage being delivered ● Difficult to manufacture electric current plate
Contactless: Static Magnetic Field	● User-friendly ● Working in incubatory ● No corrosion ● Able to maintain environment (i.e. no increase of temperature when current is applied)	● Difficult to control voltage applied ● Difficult to monitor voltage being delivered ● Difficult to manufacture
Contact: Metallic Electrode	● Easy to control ● Able to stimulate as tetanic contraction ● Able to solder or use epoxy	● Potential corrosion if metal is not correctly chosen, thus resulting in cytotoxicity ● Difficulty in sterilization techniques
Contact: Conductive Polymer Electrode	● Easy to control ● Able to stimulate as tetanic contraction ● Does not change environmental conditions in incubator ● Bio-functional ● If piezoelectric - able to be used for mechanical stimulation	● Depending on polymer, potential insulation material versus conductive ● Sterilization issues - Autoclaving can change properties

Table 7: Shows the tissue culture method with its corresponding advantages and disadvantages

Design Means	**Advantages**	**Disadvantages**
Ring Shaped Tissue	● Promotes alignment	● Does not mimic *in vivo* skeletal muscle formation
Micropost attachment Tissue	● Promotes alignment ● Promotes anchorage ● Mimics tendon attachment ● Research exists	● Difficulty transferring tissue/removing constructs from agarose ● Potential necrosis
Self-Assembly	● Greater alignment than micropost attachment ● No necrosis ● Reduce materials and costs	● Minimal parameters ● More precision required ● Potential holes in tissue structure ● Reduced mechanical properties of tissue

Table 8: Shows the lid design means with corresponding advantages and disadvantages

Design Means	**Advantages**	**Disadvantages**
Soldering	● Able to provide electrical stimulation ● Able to be sterilized using EtO ● Simultaneously stimulates each well of the plate ● Relatively inexpensive	● Poor method of holding electrodes, leading to contact with constructs ● Drilling holes causes polystyrene to break ● Unable to survive after multiple uses ● Inaccurate dimensions for manufacturing
Grooved Channel	● Better placement of electrodes ● Easily sterilized using EtO ● Simultaneously stimulates each well of the plate ● Able to provide electrical stimulation ● Reusable ● Survive incubator environment	● Difficult and time consuming to manufacture ● Difficulty placing wires in grooves ● Relatively expensive
Buss Bar	● Better placement of electrodes ● Easily sterilized using EtO ● Simultaneously stimulates each well of the plate ● Able to provide electrical stimulation ● Reusable ● Survive incubator environment ● Easy to manufacture ● Accuracy in dimensions	● Time consuming to manufacture ● Difficulty placing electrodes into the holes ● Relatively expensive

A selection matrix was then created to organize and evaluate how well each design feature met the necessary device functions. This matrix helped the team to determine the design features

that should be incorporated within the final design. Each feature was given a score zero to five, five being the feature perfectly meets the function. Table 9 shows the rankings of each of the designs with means of achieving them.

Table 9: Selection matrix for evaluation of design features

Selection Criteria			**Design Features**			
Post Means	**Electrodes in Top Plate**	**Piezioelectric Materials**	**Electrodes in Base Plate**	**Magnetic Fields**	**Suspended Electrodes from Top Plate**	**Contactless Electrode Array**
Provides Electrical Stimulation	5	3	3	2	2	1
Microcontrollers Means	**Arduino Board**	**Raspberry Pi**				
Provide Automation for Electrical Stimulation	4	4				
Feedback Means	**Sensor**	**Filters**	**Potentiometer**			
Obtain Electrical Stimulation Feedback	2	1	4			
Anchorage Means	**Ring Formation**	**Micro-post Attachment**	**Self-Assembly**			
Promotes Tissue Anchorage	3	5	4			
Electrode Attachment Means	**Grooved Channels**	**Soldering**	**Buss Bar**			
Provides Attachment for Electrodes	4	2	5			
Corrosion Resistance Means	**316L Stainless Steel**	**Conductive Polymer**	**Gold**	**Platinum**	**Platinum-Iridium Alloy**	**Silver**
Ensures Electrode Corrosion Resistance	4	3	5	4	4	4

For electrical stimulation, the team considered both methods of contact and noncontact. The monetary value of the contactless and contact methods were approximately the same. However, the advantages of the electrode method outweighed the static magnetoelectric field method. The team determined that the electrodes were easy to manufacture and practical, as more research has been done on this technique. Additionally, the electricity flowing through the electrodes into the media allows for the connecting tissue to contract more efficiently, which made the contactless method unappealing. Therefore, the selected material for the electrodes was 316L Stainless Steel. For more information on the selection process for the electrodes, see Appendix D.

The team determined that the ideal platform to be used to program the electrical stimulation was the Arduino Uno as seen on Figure 13. This microcontroller was relatively inexpensive, well-documented, and had a simpler programming environment than the Raspberry Pi [52][53]. Furthermore, the Arduino was sturdier and was compatible with any computer allowing for easy adaptability for the user [52]. By using the Arduino Integrated Development Environment (IDE),

the team was able to program stimulation parameters, allowing the user to plug in the desired inputs. This allowed the user to simultaneously test different regimens for muscle maturation. The source transmitted the input to the breadboard which delivers about 5V to eight rows representative of the rows in the 96-well plate. A 100KΩ potentiometer was placed in each row, and it regulated the output of the electric field for each of the rows. A set of input analog pins on the breadboard was used to plug in the connecting wires between the breadboard and the lid component. The USB directly connected to the computer, in which the stimulation regimen could be programed and updated. The microcontroller component then sent the signal to the source input, initiating the stimulation and causing the source LED light to blink through the duration of the signals emitted. The circuit diagram of the device is located in Appendix E.

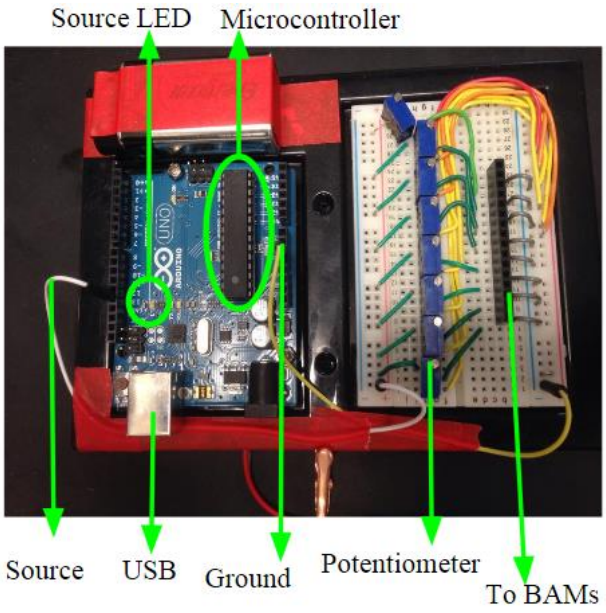

Figure 13: Platform for electrical stimulation of BAMs

The team also determined the optimal method of developing the tissue constructs was the micropost attachment method because it mimics muscle-tendon attachment. This eliminates the option of the ring shaped, which is commonly used in tissue-engineered blood vessels. The team determined that following the micropost attachment model for creating the tissue constructs would

be ideal because the tissues are formed in one location and don't need to be transferred upon formation as seen with self-assembly models. The micropost attachment method enables easy insertion of electrodes into the well, without touching or damaging the tissues.

Table 10 below displays the selection matrix that was used to evaluate alternative designs and led to the selection of the buss bar method as the final design for the device lid.

Table 10: Selection matrix for evaluation of alternative designs

		Alternative Designs	
Device Constraints/Requirements	**Grooved Channel Design**	**Soldering Design**	**Buss Bar Design**
Operational in Incubator Environment	5	5	5
Accuracy and Precision of Electrode Placement	3	1	5
Maintain Electrical Stimulation Regimen	4	3	4
Maintain Electrical Stimulation Field and Frequency Limits	4	3	4
Maintain Sterility During Operation	4	4	4
Prevent Significant Corrosion	3	2	4
Operational for Prolonged Use	4	2	5
Total	**27**	**20**	**31**

The essential design features that led to the selection of the final lid design were the following: method of electrode attachment, ease of manufacturability, ethylene oxide (EtO) sterilization capability, loose enough for oxygen and carbon dioxide exchange, non-corrosive materials, withstand incubator environment, and simultaneously stimulate each well of the plate. Overall, the selection process was focused on protecting the tissue constructs and the user. Figures 14 and 15 show the final assembly of the lid.

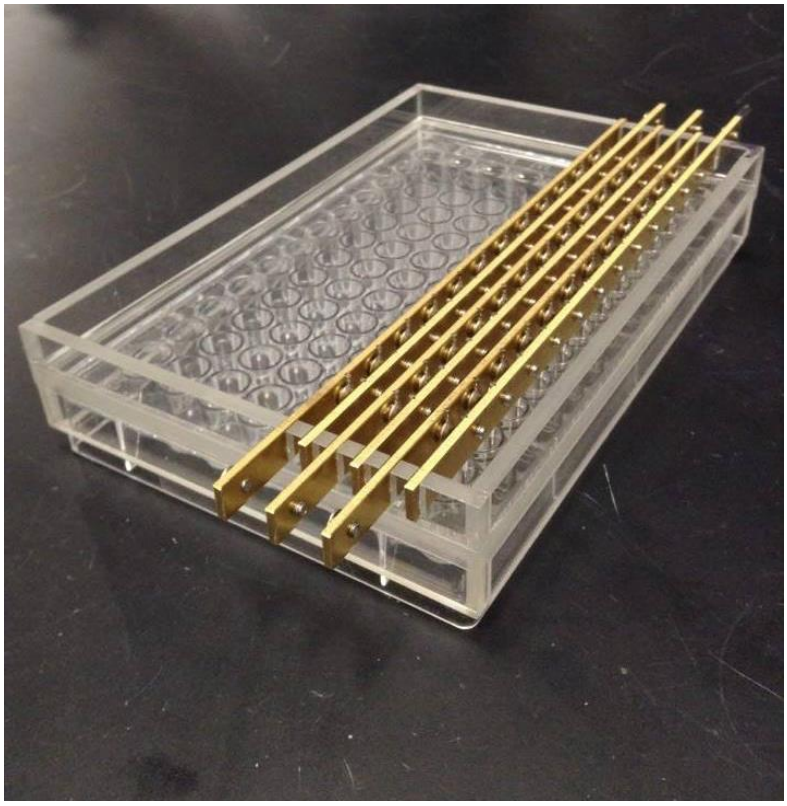

Figure 14: Isometric view of the final assembly of the buss bar lid

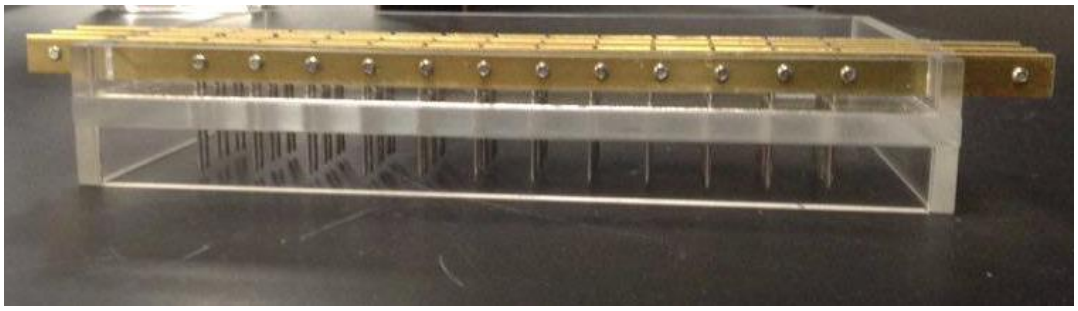

Figure 15: Side view of the final assembly of the buss bar lid

Chapter 5: Final Design Verification

This section addresses the various tests and results the team conducted to verify the functionality of the final design.

5.1 Tests for Electrical Current through Fluids

One of the first test done to evaluate the conductivity of the device was the hydrochloric acid (HCl) test. 200ul of 1M HCl was poured into a 96-well plate. The Arduino was then simply set to deliver a constant 4.8V through copper electrodes to the fluid. HCl was selected due to the free ions contained and its high reactivity upon charge acquisition. The HCl in the well began to bubble and fizz proving that the wells had received some sort of current through the wires. Figure 16 below demonstrates what the fizzing looks like when current is received.

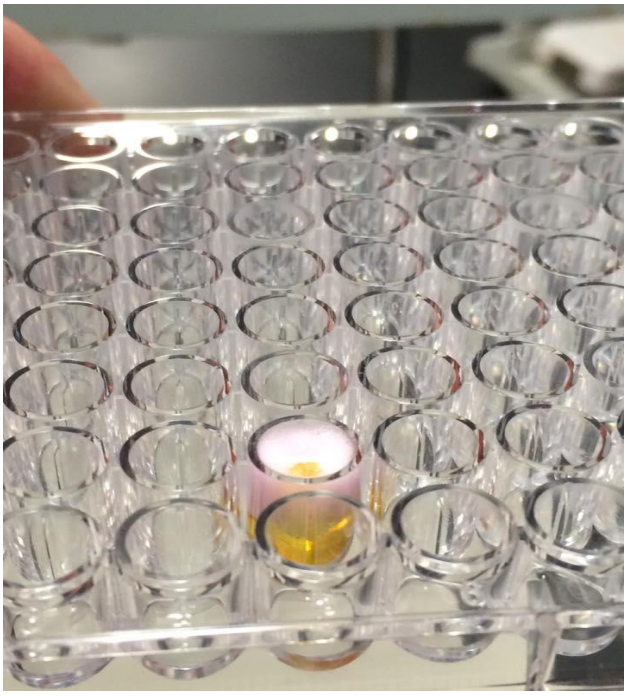

Figure 16: Shows the HCl stimulation test, where foaming provided conductivity occurred

The accuracy of this test could be improved if the electrodes were made of the Stainless Steel wire that was chosen for our final material. But at the time that this test was conducted, the team had not finalized or ordered the electrode material.

The impact of the age of the media on the voltage and current delivered to the tissue was tested. Two Stainless Steel electrodes were inserted through holes drilled 3mm apart in a single well in a 96-well plate lid. The electrodes were clipped with wires and secured to the top of the lid with electrical tape to ensure stability. Figure 17 displays the setup of this experiment. The input voltage from the Arduino (A) was connected via the red wire to one of the electrodes (B). A red wire connected from the opposing electrode connect (C) to the voltmeter to measure current (D). A green wire was attached to the ground in the Arduino and connected in series to the ground in the voltmeter. The actual lid of the plate contained two stick stirrer tools that were used to stabilize the electrodes (E).

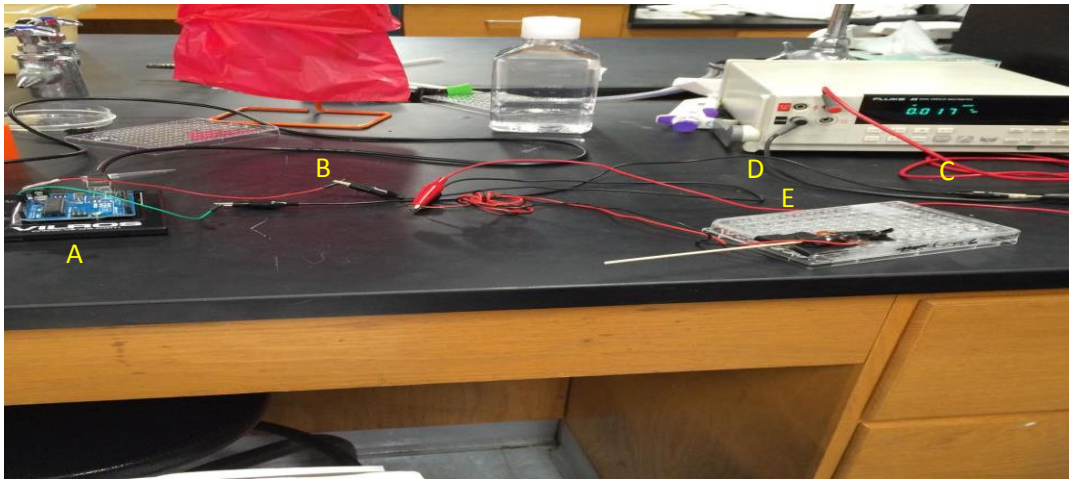

Figure 17: Shows the setup of the complete media test

Four separate groups of culture media were used; one contained fresh complete media, one was leftover media from two days after being used in culture, another was leftover media from three days after being used in culture and the last was from four days after being used in culture. Four days was the maximum aged media because our client made it clear that media changes would occur within one to three days of culturing BAMs or after previously replacing media. The data collected after four days served to show electrical current and voltage behavior in case the media change was abnormally altered after four days rather than the common maximum of three

days. Table 11 contains the results of the test. All current and voltage were expressed in amperes, A, and volts, V, respectively.

Table 11: Shows the results of media's current and voltage over four days

Media	Current (A)	Voltage (V)
Fresh Complete Media	0.01 ± 0.01	4.8 ± 0.1
1 Day Old Media	0.01 ± 0.01	4.8 ± 0.1
2 Day Old Media	0.01 ± 0.01	4.8 ± 0.1
3 Day Old Media	0.01 ± 0.01	4.8 ± 0.1
4 Day Old Media	0.01 ± 0.01	4.8 ± 0.1

The current did not fluctuate much in the span of fresh media to four day old media. From the data obtained, the voltage also remained consistent as the media aged. As a result, the media did not drastically affect the current and voltage applied and the voltage and current administered were likely to be the same (or very close) to the voltage and current that was received. These tests were conducted to show that the system was administering a current as well as testing the conductivity of the media used to develop the BAMs since the device would be used over the course of multiple days.

5.2 Potentiometer Resistance Tests

This test was performed to determine the resistance and corresponding voltages obtained from the potentiometers. The tests were performed on 100kΩ CT-94W potentiometers to determine what BAMs would be experiencing for '#' number of turns of the potentiometer shaft. Pin3 of the potentiometer was used as input and Pin2 was used as the output, so anti-clockwise spinning of shaft increased the resistance of the potentiometer and vice versa. This would enable a user to control the strength of the electric field for an electrical stimulation regimen. Table 12 below contains the results obtained for this test.

Table 12: Potentiometer parameters as it is turned # number of times

#	**Measured Resistance ±0.1 x 10^4Ω**	**Measured Current Supplied ±0.01 A**	**Measured Voltage Supplied ±0.1 V**
1	3.4 x 10^{-3}	0.08	4.8
2	4.3 x 10^{-3}	0.08	4.8
3	0.4	0.01	4.7
4	1.0	0.50 x 10^{-2}	4.5
5	1.6	0.30 x 10^{-2}	4.2
6	2.4	0.20 x 10^{-2}	3.8
7	3.2	0.13 x 10^{-2}	3.5
8	4.0	0.11 x 10^{-2}	3.1
9	4.7	0.09 x 10^{-2}	2.8
10	5.5	0.08 x 10^{-2}	2.4
11	6.2	0.07 x 10^{-2}	2.1
12	6.8	0.06 x 10^{-2}	1.7
13	7.5	0.05 x 10^{-2}	1.4
14	8.1	0.04 x 10^{-2}	1.2
15	8.7	0.04 x 10^{-2}	0.9
16	9.3	0.03 x 10^{-2}	0.6
17	10.0	0.03 x 10^{-2}	0.2
18	10.6	0.03 x 10^{-2}	0.0

From Figure 18, the number of turns of the potentiometer was linearly related to the resistance and essentially the voltage output. This meant the voltage output from the potentiometer and electric field could be varied in an approximately linear manner. The applied voltage was the reciprocal of applied current, which removed the exponential relationship. This was shown in

Figure 19.

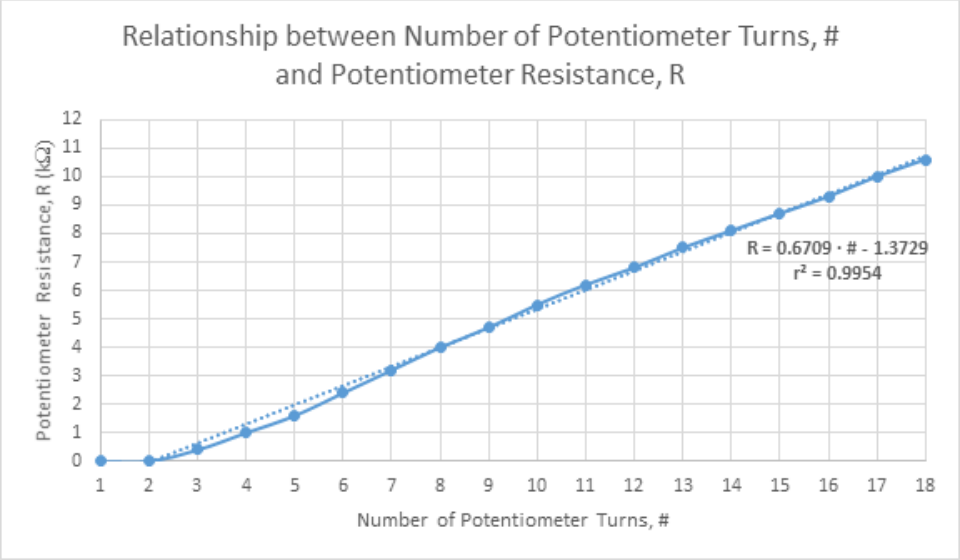

Figure 18: Linear relationship between potentiometer turns and potentiometer resistance

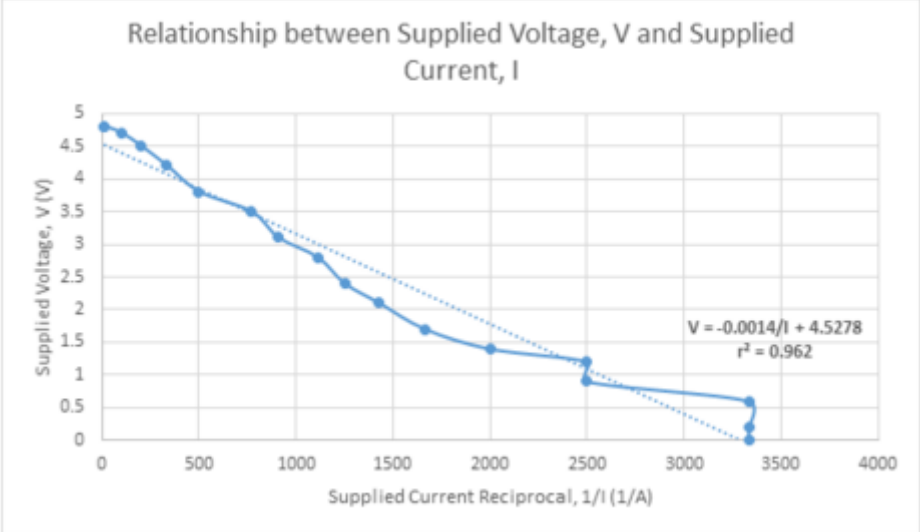

Figure 19: Relationship between the supplied voltage from potentiometer and the supplied current

5.3 Electrical Design Validation

The voltage output from the device in culture media was also measured and compared to the voltage output directly from the electrodes while the device supplied an electrical stimulation. To measure supplied voltage, the electrodes were connected to a multimeter and the voltage was read. To measure received voltage, the electrodes were placed in a culture well of a 96-well plate

containing 200ul of culture media. A custom-made probe was then placed in the culture media very close to the electrodes. The probes were connected to a multimeter and the voltage was read. The average voltage received in culture media was 4.7V, compared to the 4.8V measured directly from the electrodes. This gave a 2% variation in the voltage outputs with ±0.1 standard deviation. This insignificant variation shows that differentiation media did not significantly affect the voltage being delivered and the electrical stimulation regimen programmed by the device would provide accurate stimulation to the tissue.

5.4 Electrode Integrity and Corrosion Test

This test was to determine if there were chemical influences from the electrodes that would have a chemical effect on the culture media and therefore harm the BAMs. Two 10mm length Stainless Steel-AISI 316L electrodes were placed 3mm apart within a well of a 96-well plate. The well contained 200µl of differentiation media. The voltage output and culture media resistance were measured with a multimeter before and after an electrical stimulation regimen was passed through the media. After twenty-four hours of stimulation, the resistance of the media removed from the BAMs was measured. The result would show if non-visual factors caused corrosion, which would change the resistance. The voltage output was measured directly from the electrodes and the culture media resistance was measured using custom-made Stainless Steel measurement probes in the media. This test showed whether media would affect the applied voltage, 4.8V, in comparison to the voltage received by the electrodes in media. The stimulation parameters from Donnelly et al. were used and the electrodes were analyzed after one day of stimulation [28]. The results after one day of continuous electrical stimulation are summarized in Table 13 below.

Table 13: Results of electrode integrity and corrosion test before and after an electrical stimulation regimen was applied

	Before Stimulation Regimen	**After Stimulation Regimen**
Electrode voltage output	4.8V	4.8V
Culture media resistance	500 kΩ	500 kΩ

Both the voltage output and culture media resistance did not change after the stimulation regimen. Additionally, the culture media did not change color or look contaminated and so the team accepted the results to mean that the electrodes did not corrode over the testing period. These results mean the electrodes were safe to use with actual BAMs and would not harm the BAMs by causing toxicity in the culture media.

5.5 Constant Electrical Current Test

The device was also tested to determine the maximum constant current that the Arduino can deliver before triggering the internal fuse. The internal fuse protects the Arduino microprocessor from damage upon drawing too much current at a given time. This test was done by connecting the Arduino 5V and GND pins to a multimeter directly with no resistance or load. After reaching about 0.8A, the current dropped steeply to a steady-state of about 0.25A. This value showed that the maximum safe constant current draw from the Arduino is 0.8A. This result was important to determine in case the device was to be used in a tetanus application, where the system would apply a consistent current and voltage to BAMs to trigger tetanic contractions. Figure 20 below shows a graphical representation of the results obtained.

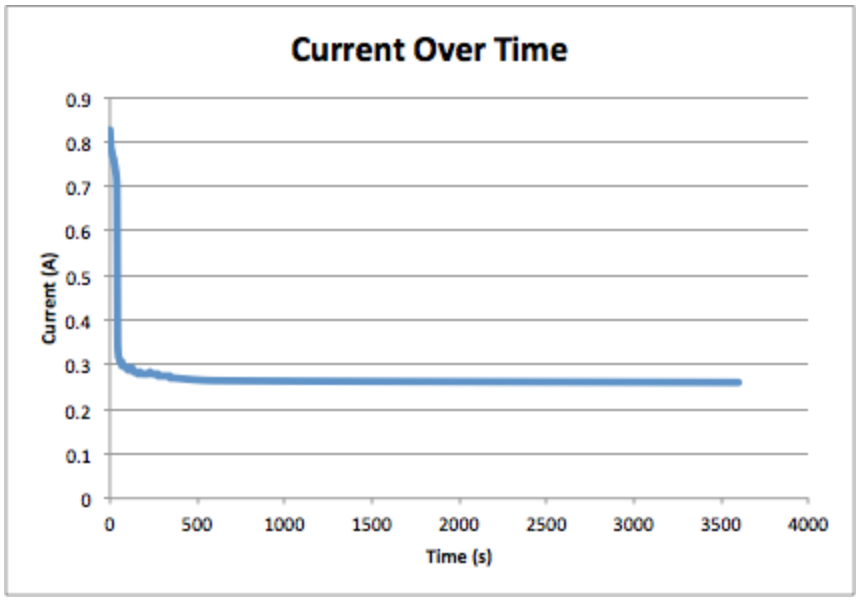

Figure 20: Shows the graphical representation of the change of current output from the Arduino program over time

5.6 BAM Electrical Stimulation Regimen

The device was tested on eight three different groups of BAMs to study the effects of electrical stimulation using our device. Two BAM groups were experimental groups (2V/mm and 1V/mm were applied to constructs), whereas the third group was the control with no electrical stimulation. The Arduino was tested to determine if the stimulation regimen inputted by a user was in fact being delivered to tissues, in the sense that the device was producing real pulses, before the electrical stimulation was performed with BAMs. Based on the stimulation parameters from Donnelly et al., this test was done by measuring four pulses delivered by the device using an oscilloscope [28]. Figure 21 below shows the readings obtained from the oscilloscope.

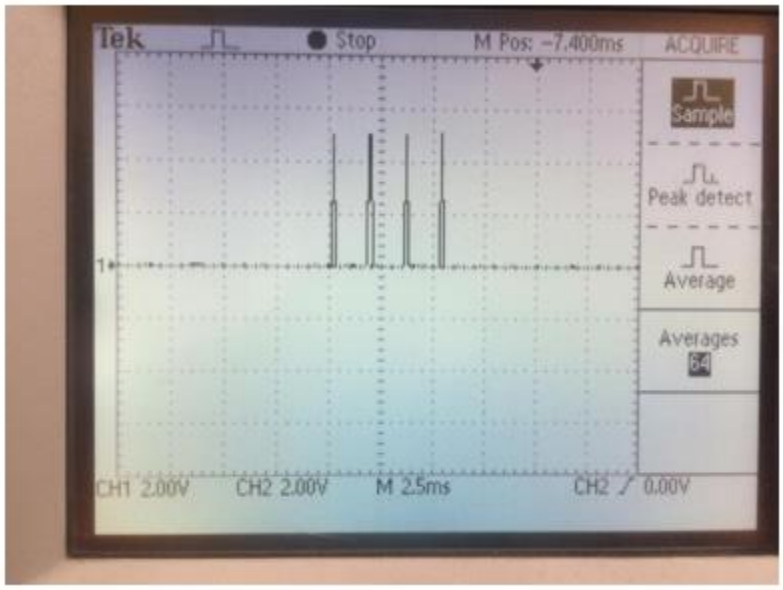

Figure 21: Four 0.1ms wide pulses recorded from the device by the oscilloscope

The next test involved timing the 3.6s rest interval between four successive pulses to make sure they were 3.6s apart. After successful results, the system was connected to the customized device lid, where the cell culture plate had one row of three constructs of BAMs (Row A), the second row of two wells with BAMs (Row B) and a third row of three wells (Row C) with BAMs that received no electrical stimulation served as the control. The Arduino was programmed to stimulate BAMs with 4 x 0.1ms pulses at a frequency of 10 Hz with a rest of 3.6s [28]. There was an electric field of 2V/mm applied to Row A, 1V/mm applied to Row B and 0V/mm applied to Row C. This electrical stimulation was administered continuously for three hours for ten days total, where every twenty-four hours the plate was removed from the incubator to change the differentiation media and resume stimulation. Table 14 shows a summary of the stimulation parameters used for this regimen.

Table 14: Summary of BAM electrical stimulation parameters [28]

Stimulation Frequency	4 pulses
Stimulation Interval	0.4 seconds
Pulse Width	0.1 milliseconds
Train Recovery	3.6 seconds
Train Cycles	2700 per day
Stimulation Time	3 hours continuous
Recovery time	21 hours
Total Duration	10 days

Figure 22 below shows a graphical representation of the pulses delivered by the device to the BAMs for that particular electrical stimulation regimen.

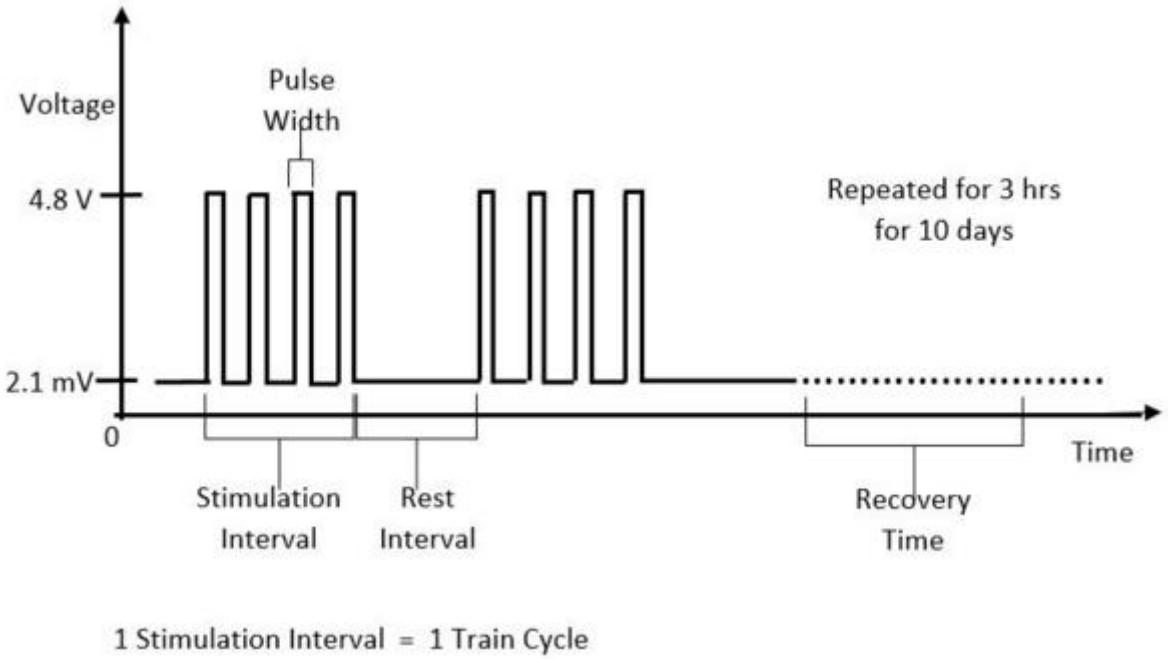

Figure 22: Graphical representation of pulses delivered by the device to the BAMs

5.7 BAM Maturation Results

After ten days of stimulation the BAMs were fixed and stained with hematoxylin and eosin (H&E) stains. The hematoxylin stains for the nucleus of the cells (dark blue), while the eosin stains the cytoplasm (pink). Figure 23 shows the results of the unstimulated compared to the stimulated BAMs in Figure 24.

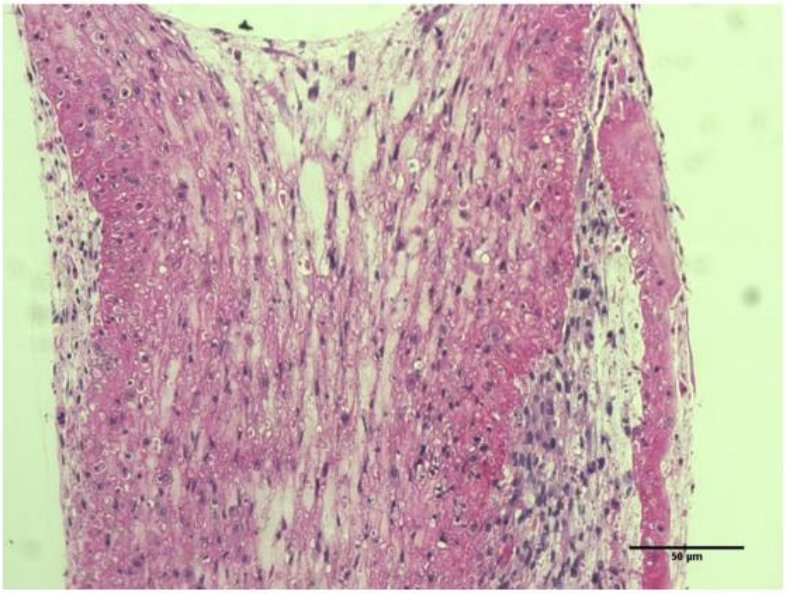

Figure 23: Hematoxylin and eosin stain of non-stimulated BAMs (0V/mm) at magnification of 10X

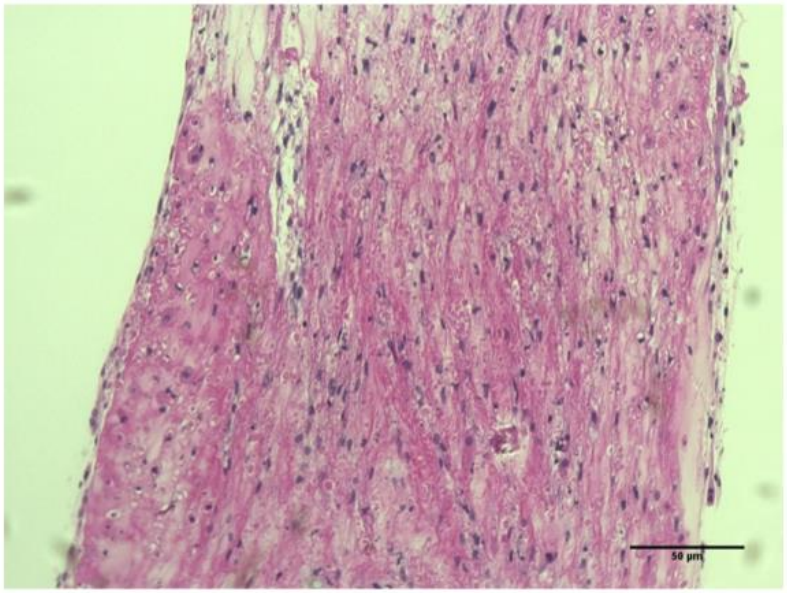

Figure 24: Hematoxylin and eosin stain of stimulated BAMs (2V/mm) at magnification of 10X

Non-stimulated BAMs showed tissue disintegration, where the white spots throughout the tissue indicated tissue corrosion and therefore tissue necrosis. Additionally, the right side of the tissue contains a cluster of cells, showing that it was not uniformly distributed throughout the whole tissue. Stimulated BAMs had limited tissue necrosis, and cells were evenly distributed throughout the tissue. Additionally, the stimulated tissue showed the beginning of myotube alignment, while the BAMs without stimulation did not show any alignment. Therefore, these results indicated that the electrical stimulus delivered by the device did not harm the tissue, but helped it maintain its integrity. It also indicated that further testing was needed to identify the regimen needed to allow muscle tissue to mature properly.

Chapter 6: Final Design Validation

This section addresses how the device operates as it met the desired design objectives. Additionally, the team discusses the final standards needed in the development of the final design for it to be manufactured. Finally, the team discusses impact of the design on the economy, environment, and its influence on the society, political ramifications, ethical concerns, safety issues, manufacturability, and sustainability.

6.1 Device Procedure for Operation

The operation of the device was a two-part process, which included the preparation of the BAMs and setting up the device for stimulation. BAM development would precede the device operation as previously described in prototype manual system, which doubled the cellular seeding density from 200,000 to 400,000 cells [55]. Three days after seeding, the NIPAAM was removed, which allowed time for the contraction of the fibrinogen-thrombin matrix structure on the micropost attachment. Upon the removal of NIPAAM, differentiated media contained tranexamic acid (TEX) and aprotinin is added to the wells. The cells are allowed 5-10 days for differentiation before being electrically stimulated [28]. During this process the BAMS were developed with a regular tissue culture cap fitting a 96-well plate.

Electrical stimulation of BAMs started three days after the initial seeding. Observation of the shape of the BAMS in the micropost attachment method enabled for spontaneous contractions to the micropost, as in Figure 25.

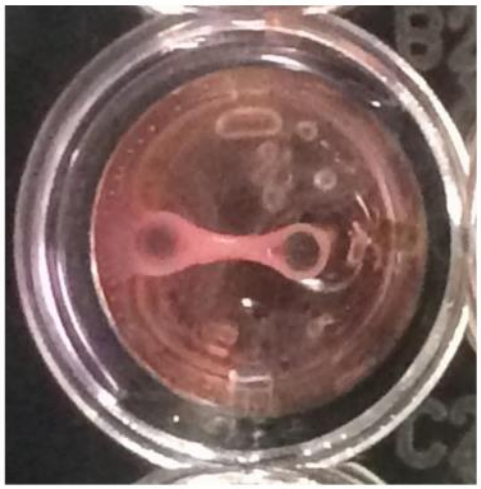

Figure 25: Bioartificial muscle (BAM) orientation using the micropost attachment method

To begin electrical stimulation, the customized designed device lid was placed on the tissue 96-well plate in a sterile setting to prevent tissue contamination. The plate was placed in the incubator, where it could attached to the device's wires that leave through a custom plug in the back of the incubator, where this prevented contamination of the incubator. The wires passing through the plug were connected to the device containing the Arduino Uno and electrical circuitry. Each component of the device operation aimed to satisfy the project objectives as mentioned in section 3.2.1. The device could function within an incubator due to the selection of anti-corrosive materials. Sterility was not compromised in this device operation as the modified rubber stopper (plug) allowed the wires to connect the well plate to the Arduino outside of the incubator as these electric components would not be able to function within the incubator environment. The Arduino Uno would be connected to a PC for programming of the desired parameters. The other wires would be connected to the breadboard which allowed the use of the potentiometer for voltage adjustment of the device.

The Arduino Uno was programed outside of the incubator utilizing the program "stimulation.ino". Upon loading of the code on the microcontroller, the user would see two

flashing LED lights indicating the device was ready to function. At this time, the user would be prompted to input the desired parameters such as pulse width, electrical stimulation frequency, number of pulse cycles, number of train cycles and rest interval between pulse cycles for the device to run a desired stimulation regimen. These parameters could be adjusted per experiment and each row would receive different electric fields. This electric field adjustment is simply done by turning the potentiometers to obtain the corresponding voltage according to Table 12. The Arduino would display the time the electrical stimulation was expected to be done, therefore the user knows when to get back to the Arduino Uno. This enabled a user friendly operation of the device, as the user simply programs the regimen and could rely on the system to provide the desired stimulation on its own without user involvement until the regimen was complete. The whole procedure can be visualized Figure 26.

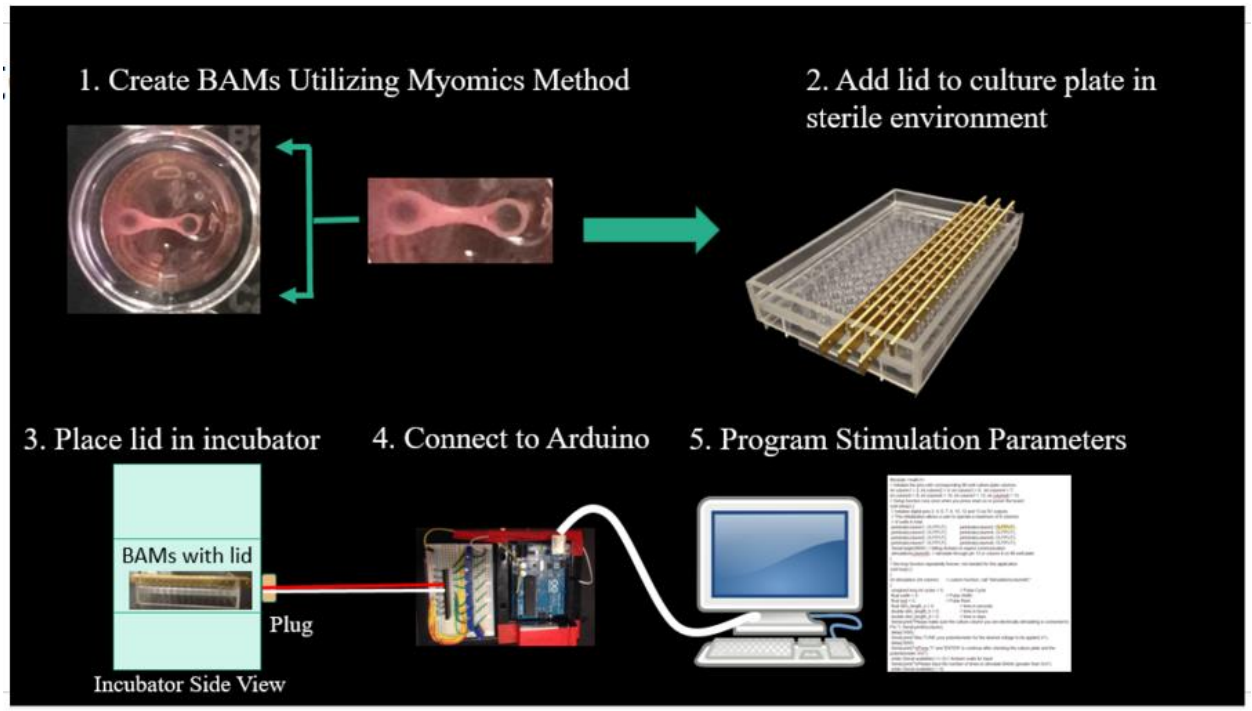

Figure 26: Procedure for BAM stimulation using final device

The final set up of the device can be seen in Figure 27 below.

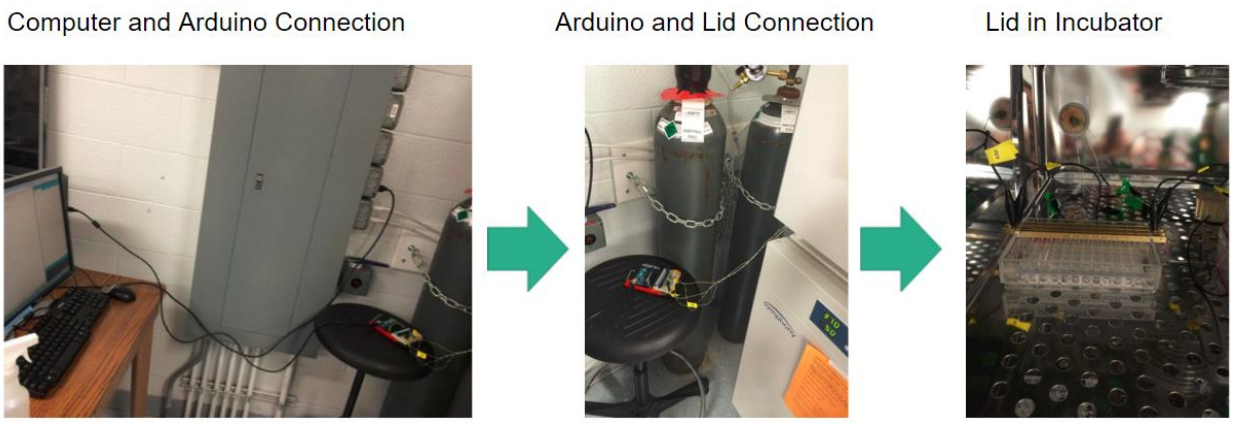

Figure 27: Set up of the final device

6.2 Industry Standards

Section 3.3 mentions the particular ISO Standards that were considered in the development of the team's design. Although the team researched these standards, the majority were not directly incorporated within the team's design. The ANSI standards for a 96-well plate were followed as the team used the bottom portion of a 96-well plate as part of their final design. In addition 96-well plates are the standards used in industry for the development of BAMs for drug screening. The remaining standards would therefore need to be incorporated into the team's final device if it was to be manufactured and marketed in industry.

6.3 Project Impact

This device would have a large impact in biomedical engineering, yet its impact does not end there. This device has effects outside the scope of this project, but will also have social, environmental and political ramifications. These aspects were important as they show what would be needed to be considered in the device's transition from research to clinical application.

6.3.1 Economic Impact

A skeletal muscle stimulator had a high economic impact for patients, pharmaceutical companies, and research centers in the field. The cost of production of this device was estimated to be approximately $400. The use of this device would be to decrease the extensive use of live animal models. This device will reduce costs of live animal models and its regular care such as

food, shelter, and general up keeping. Additionally, extensive toxicity testing for muscular dystrophy drugs *in vivo* is expensive, averaging about $8,000-25,000 per test. A stimulator device provides a fast cell-based method, which can quickly and efficiently test various drugs, making it easier for regulators to evaluate potential effects as well as decrease costs of running the study. Additionally, this device will economically benefit the end-user because it will allow for treatments to be more affordable and more readily available for use and implementation. The bill of materials for the operational use of the device is located in Table 15.

Table 15: Shows the Bill of Materials for the overall project and final device

Item	Quantity	Cost	Where Purchased From
Lab Fee	1	$150.00	WPI BME Labs
Stainless Steel Wire	1	$177.59	Sigma Aldrich
1 x Feather Header Kit - 12-pin and 16-pin Female Header Set[ID:2886]	1	$9.50	Adafruit Industries
HWS5160 1x2 Female Header Machined .1"sp	1	$13.04	Phoenix Enterprises
1MOhm Potentiometers	12	$0	In Stock In Lab
Arduino Boards	2	$0	Owned
Clear Cast Acrylic Sheet 3/16" thick, 12" X 12"	1	$10.13	McMaster Carr
Brass Bar 1/16" X 1/4"	2 (2 feet long each)	$6.62	McMaster Carr
Stainless Steel Button Head Screws	2-56 x 1/8"	$4.86	McMaster Carr
Shipping	1	$12-$15	McMaster Carr
Total		**$386.74**	

6.3.2 Environmental Impact

The device was designed to be reliable and reusable. The materials were selected because they withstand repetitive electrical stimulation experiments as well as function within an incubator for long periods of time. This reusability is beneficial to the environment because the device will not need to be disposed of after each use and therefore will not contribute to waste products in the environment. The device will need to be sterilized by ethylene oxide (EtO) so that it can be used repeatedly.

The device consists of an acrylic top lid as well as a standard polystyrene 96 well plate as the bottom plate. This well plate would be replaced after each application as to not contaminate the contents of the culture. The act of replacing the lid after each application could be potentially harmful to the environment as plastic is non-biodegradable and could cause build up in the

environment after it is disposed of. In addition, some of the contents of the plate and media could potentially be harmful to the environment and organisms if not properly removed before the disposal of the device. Although the polystyrene well plate has the potential to be harmful, the usage of this plate greatly reduced the total cost of the device for the team. A future improvement to this project could be to manufacture a customized well plate that could also be reused, similar to the top lid. In the meantime, the environmental factors of the device can be increased by the team providing instructions on proper disposal of each aspect of the device to ensure that proper procedures can be followed that will protect the environment as best as possible.

6.3.3 Sustainability

It was important that the device was reusable as this can save energy. If EtO sterilization is not done properly, this gas has the potential to be fatal or extremely harmful to organisms. The reusable device lid will reduce the amount of energy needed to manufacture the lid as well as the amount of resources needed as it will not need to be reproduced for each use. This device can be used in a variety of settings for many applications and therefore not only saves energy but also promotes efficiency as modifications would not be needed in order to use the device for a different application. The device lid was made from acrylic and machined to customized dimensions. This process would require additional energy and increase waste products to manufacture the lid. A future improvement to increase sustainability would be to find ways to harness renewable energy for the device.

6.3.4 Societal Influence

This device would have a large impact in society because of its improvements of current research techniques for the drug screening of many drugs and therapies for incurable diseases. There is a limited availability of effective pharmaceuticals and research platforms for degenerative muscle disease, therefore this product will have a promising influence on furthering research

developments which would quicken the process of discovering treatments for muscular disease. This device also could stimulate other contractile muscle tissues such as the *in vitro* tissue engineered myocardium. Therefore this device could be used for other studies besides those just pertaining to muscular disease. This applicability would decrease the cost of research and development of drugs, and pharmaceutical companies can save time and money for their discoveries. These discoveries would improve the quality of life for the end-user and therefore have a beneficial societal impact.

6.3.5 Political Ramifications

This device could be controversial due to the usage of human skeletal muscle cells in creating BAMs for testing. Political and ethical concerns may exist because of humans being the source for the development of the BAMs. There could be potential cultural and religious reservations towards this new method of testing. This device would create a new platform for drug testing that will provide an opportunity to increase the sample size without increase budgets. Therefore, this increased throughput would directly affect the global therapeutic market. It will also reduce costs of animal testing as well as costs associated with an unviable drug, especially since the pharmaceutical companies would be testing directly on bioartifical human tissue without the need for animal models.

6.3.6 Ethical Concerns

This product may improve the lives by providing scientists with a novel research tool for muscle tissue abnormality studies that have the possibility for a scientific breakthrough. Since the product will not be used directly by the patients, who are the benefiters in the long run, there are no expectations of violating major ethical boundaries in the device manufacture or use. There could be resistance in some cultures as the model is designed to do testing on samples of human skeletal muscle cells. Yet the same living tissue used for such experimentation has already been in use in

current studies and should pose no major ethical threats to scientists or society. However, if patient-specific cells were to be used, the studies would have to adhere to Health Insurance Portability and Accountability (HIPPA) laws of patient confidentiality. Precautions and fail-safes would be needed to protect patient identity in the use of the device and dissemination of resultant information would need to be addressed.

6.3.7 Health and Safety Issues

The electrical stimulation product will serve to improve the maturity and development of tissue engineered BAMs used in therapeutic drug screening. The accuracy of these therapeutic tests or screening will be improved because this device will create better matured BAMs than those currently in use. This improvement would increase the reliability of results from tests and would accelerate the development of new treatments for muscle tissue related abnormalities. These abnormalities include Duchenne Muscular Dystrophy, FSHD, and muscle fatigue; to positively influence the health of the general product. The final manufacturing and testing of the device would have to address user safety and protection from high voltage through proper insulation and grounding the components for user contact during operation. Adherence to standards for making electrical connections with medical devices would prevent improper assembly and operation of the device.

6.3.8 Manufacturability

The device was designed and developed with simple components as well as with well-document guidelines. The team created Computer Aided Designs (CAD) files of the device with descriptions of materials that could be used to manufacture the lid in other labs. In addition, the Arduino code and circuit board configuration is well documented with diagrams and pictures in order to maximize ease of reproducibility. This device could be manufactured anywhere which would increase its market value and increase the accessibility of the device for researchers.

Chapter 7: Discussion

As mentioned in section 5.7 H & E stain of stimulated and unstimulated cells showed that electrical stimulation did have a positive impact on BAM maturation. The team had to be cautious of overstimulating the BAMs and referred to literature to select the appropriate stimulation regimen that would not harm the tissues. As previously mentioned, the team used the stimulation regimen from the Dennis group for the verification tests [28]. The team used C2C12s to conduct their tests because these myoblasts are widely documented for BAM development when prototyping electrical stimulation devices [7][28]. It was assumed that C2C12s would be representative of how human cell would respond to the stimulation regimen, in respect to tissue maturation. Using these cells influences the team's results as C2C12s do have slightly different physiology than human cells, which in future applications would pose differences in the response of C2C12 BAMs to drugs opposed to that of human cells. Yet the C2C12s worked well as a proof of concept to verify that the team's device can apply an electrical stimulation regimen and impact the cells.

In addition, since both the Dennis and Vandenburgh groups used C2C12s in their BAMs, this allowed the team to compare their results to existing models to see how each system affected BAM development [7][28]. In comparison, the IonOptix device the Dennis group used, only stimulates BAMs in large 6-well plate culture plates opposed to the smaller sized BAMs created in 96-well plates [8]. The BAMs Dennis group created were too large subsequently causing the middle of the tissue to become necrotic because of not enough nutrient supply [30]. Since the team's device worked with a 96-well plate the tissue developed represented the small functional unit of the muscle, therefore being small enough to benefit from surrounding nutrient supply without vascularization. Additionally, stimulated tissues showed minimal to no signs of necrosis. Furthermore, the tissue stimulated by the team's device showed more alignment in comparison to

unstimulated tissue which supports and hints strongly at the fact that that this device provides enough electrical activity for BAM maturation.

The team's device had several advantages in comparison to models on the market. This system enabled eight different electrical stimulation regimens to occur simultaneously within the same well plate. This feature will be beneficial for future applications of the device as different treatments or regimens can be tested within the same plate which will create more consistency among tests and therefore more accurate results. There were no other models that enable varying stimulation parameters within the same plate and test. In addition, no other model allowed for electrical stimulation to be applied while the well plate was in the incubator. This factor gave the team's device an advantage over current models because BAMs need an incubator environment to develop properly. The longer the BAMs are outside the incubator, there will be a more harmful impact on the tissue. The team's model in only outside of the incubator for about five minutes once a day to change the media, while the Myomics model is removed from the incubator for about twenty minutes each time a stimulation cycle is applied to the tissues [7]. The ten day study the team conducted would have took the Vandenburgh group about ten months to replicate using the Myomics model [7]. The team's device greatly reduced user involvement and made the stimulation regimen more efficient so that more experimental tests could be conducted.

A few assumptions were made in the development and testing of their device. It was assumed that rod electrodes would deliver the same electric field as an electric plate would, because of the well to electrode size ratio. An electric plate has the potential to provide a more uniform electric field than rod electrodes, but regardless the plate or rod would be programmed to deliver the same field strength. The plate would provide a greater extent of electrical field uniformity, but the rods provide greater convenience for use in small culture wells of 96-well

plates. In addition the Electrode Integrity and Corrosion Test displayed no visible signs of corrosion on the electrodes and the team therefore assumed that the device does not cause any corrosion to its components. Furthermore, there were no noticeable signs of color change on the electrode, material disintegration or some other permanent change to the material's appearance. In addition the electrode voltage output and culture media resistance remained the same before and after the test corrosion test. If there had been any corrosion it is likely that the voltage or resistance would have been altered due to changes in the electrode material and media composition. Lastly, the team assumed that different Arduino boards would provide the same electrical output. There is the possibility that precision and accuracy would vary among different Arduino boards which could result in slightly different electric outputs being applied in comparison to what is being programmed and also when comparing different boards. Yet, this system was built using an Arduino Uno and therefore these boards were programmed to work consistently and should be the same quality among different devices. Moreover, these boards were durable, making the Arduino board to maintain its functionality for long periods of time.

Despite these assumptions, the team's device was able to meet all project constraints. The team created a rubber stopper to feed wires into the incubator and acrylic lid that fits on a standard 96-well plate that was able to function in an incubator environment (37°C, 5% CO_2, and 95% humidity) while maintaining the integrity and functionality of each component. In addition, the materials used in this device are biocompatible so that the tissues will still be able to develop and mature over time. The device lid that functions in the incubator and comes into contact with the BAMs was EtO sterilized, which prevents contaminations from harming the tissues. The incubator was a sterile environment for the BAMs to develop. Lastly the Electrode Integrity and Corrosion Test did not show any signs of corrosion so therefore the device is safe from foreign body effects

on BAMs. The media still remained in the well after stimulation and there was minimal to no necrosis in the tissue constructs, which shows there was no toxicity or damage to the tissues. Therefore, for the duration of the test, there was no electroplating and the device did not cause corrosion.

The device also did not overstimulate the cells. Our electrical stimulation regimen followed the guidelines outlined by our constraints. We applied train stimulations or train cycles to compensate for stimulating the BAMs over 3Hz, and the maximum electric field applied was 2V/mm [33]. The stimulation pulse width was less than 0.25ms to promote surface propagation of action potentials in case this provides important developmental cues [28]. This was because *in vivo*, skeletal muscle contracts when an action potential is propagated along the surface of the muscle cell membrane causing depolarization of the t-tubular membrane and consequent calcium release from the sarcoplasmic reticulum. Pulse widths longer than 0.25ms were known to result in direct calcium release from the sarcoplasmic reticulum and not surface membrane propagation using the t-tubule system [28]. The test regimen did not harm the tissue which further supported that the team met all the electrical stimulation constraints.

The team did create a successful device that matured BAMs, yet there were a few limitations that could be further improved upon. One limitation of the team's project was that tests were only conducted using C2C12s and not human cells. C2C12s eventually could reach a point where there would be a loss in myogenic potential. In addition, human cells would be more representative of pathophysiology, which be helpful for future applications of the device. The decision to use C2C12s was made due to time and budget restrictions. Moreover, both the Dennis and Vandenburgh groups made BAMs using C2C12s and therefore the team could have a standard of comparison to see how their device matured tissue in respect to current gold standards

[30][55]. In addition the device is not programmed for different pulse widths and frequencies among each row of the plate. Yet the device does allow for customized electric fields which does allow for multiple tests to occur within the same plate. Each row has the same pulse width and frequency so that these features can serve as controls in experiments and the electric field can be changed to see how different field strengths effect the development of the tissues. The device was capable of maturing BAMs which was the ultimate goal of the device regardless of whether the plate allowed for various pulse widths and frequencies; these features were not required but would be supplemental features.

Another limitation was that the device must be located near a computer. The USB cord must be plugged into a computer and the wires must be long enough to extend from the Arduino board through the back of the incubator. This was not a major limitation as wires were purchased in many different sizes, yet it was still an important aspect to consider in the device set-up. The last limitation of the device was that the voltage cannot be controlled directly through the Arduino but instead it would be controlled by the physical potentiometers located on the circuit board. Incorporating voltage control through the Arduino would increase accuracy and user friendliness. With the use of potentiometers, the device was still able to apply eight different electric fields to a 96-well plate to meet the original functional intent.

Chapter 8: Conclusion and Future Recommendations

This chapter will provide the team's conclusion and future recommendations regarding the final device for their Major Qualifying Project.

8.1 Conclusion

Ultimately, the team's device functioned as intended and was able to meet each of the device objectives and functions as mentioned in section 3.2. The team was able to successfully manufacture a lid that fits on a 96-well plate which is the standard used in industry for BAM development for high content screening. This lid was made of materials that would not corrode or lose their sterility while functioning in an incubator. The electrical components of the device remained outside of the incubator and the wires connected the Arduino Uno to the device lid through a customized stopper in the back of the incubator.

The team was able to create a system that provided an programmable, controllable and accurate electrical stimulation regimen. The electrical stimulation system was created using an Arduino board. The coding of the Arduino Uno created a user friendly interface that prompts to fill out specific stimulation parameters. This interface also provided helpful reminders for the operation of the device and told the user how long the regimen will take to complete. The user simply needed to adjust the regimen as desired per experiment and then leave the device unattended as the code would provide the stimulation automatically without user involvement.

This electrical stimulation system was capable of providing eight different electric fields within the same device and simultaneously stimulate each well of a 96-well plate. The team conducted multiple device verification tests that showed that the device produced a reliable electrical stimulation in the sense that the applied electric field from the system was the same as that received by the tissue; more specifically the culture media resistance and electrode voltage

output remained unchanged before and after testing. There were also no significant signs of electrode corrosion or contaminated culture media.

The team performed an H & E stain on BAMs made from C2C12s that were matured using the device. The unstimulated control tissue showed large areas of necrosis and clustering of cells in comparison to the stimulated tissue. In addition the stimulated tissue maintained its integrity and had a uniform composition, which showed signs of the beginning of myotube alignment. BAM anchorage and attachment was achieved by incorporating the Myomics micropost attachment method within a standard 96-well plate [29].

The team initially was faced with the need to create a system that would improve BAM models in order to provide more accurate testing of various drugs and therapies to treat muscular diseases. The team established a buss bar model to work in conjunction with an electrical stimulation system composed of an Arduino board and circuit board. This system overall met the team's objectives. In addition the team was able to use this system to develop BAMs that mimicked the 3D human muscle physiology while also adding a 4D component which is dynamic maturation in respect to time. Since this device enabled adjustable and automatic electric stimulation to be applied to an incubated 96-well plate, this system could be used for indefinite culture of muscle tissue which will enable long term studies of muscular diseases.

8.2 Recommendations

After the completion of this project, the team determined a few future recommendation that would further improve the functionality of the team's device. The team would like to conduct more tests to further show that this system improves BAM development. BAM development would be shown through improved myofiber alignment and maturation. This could be done through myosin staining of tissues that are exposed to various stimulation parameters and also tissues developed by current gold standards compared to those developed by our system. In addition, the team would

like to conduct tests using human myoblasts because it would be more representative tissue needed for therapeutic applications.

Another future recommendation would be to incorporate a mechanical stimulation component within the same plate. Muscle technically needs both mechanical and electrical stimulation in order to properly mature and develop. With the addition of mechanical stimulation, the overall system would further improve the quality of the BAMs to better replicate how muscles mature in the body.

Additional improvements to the electrical stimulation system would allow it to be more versatile and user friendly. One recommendation includes the usage of Arduino analog pins instead of potentiometers within the circuit board in order to have better control of the electrical stimulation being applied to the tissue. These analog pins would make the device more user friendly. Incorporation of amplifiers within the circuity would allow for increased voltage output and electric field strength range of the system which would widen the device's applications. The team only manufactured six buss bars which would cover three rows of the eight row plate. For further testing, the team would like to manufacture additional buss bars that could cover all eight rows of the plate. Another recommendation would be to add more customizable factors to the electrical stimulation regimen. Currently, each row of the plate receives different electric fields at the same time, but all eight rows received the same pulse width, rest and cycles. An improvement to the device would be to modify the code so that each row could have customized pulse width, rest and cycles. This would save time and money and increase efficiency of testing as multiple experiments could be completed within the same well plate and very easily compared to one another. With all of these changes the team could conduct their own experiments and test different

stimulation parameters in comparison to accepted regimens published in literature to see the effects on the BAMs.

References

[1] S. Al-Zaidy, L. Rodino-Klapac and J. Mendell, "Gene Therapy for Muscular Dystrophy: Moving the Field Forward", *Pediatric Neurology*, vol. 51, no. 5, pp. 607-618, 2014.

[2] S. Sacconi, L. Salviati, and C. Desnuelle, "Facioscapulohumeral muscular dystrophy", *Biochimica Et Biophysica Acta - Molecular Basis Of Disease*, vol. 1852, no. 4, pp.607-614, 2007.

[3] N. Sanyal. (2015). "Global Duchenne Muscular Dystrophy Treatment Market to See Spectacular Growth by 2019" in *Drug Discovery & Development.* [Online]. Available: http://www.dddmag.com/articles/2015/04/global-duchenne-muscular-dystrophy-treatment-market-see-spectacular-growth-2019.

[4] K. Donnelly, A. Khodabukus, A. Philp, L. Deldicque, R. Dennis and K. Baar, "A Novel Bioreactor for Stimulating Skeletal Muscle In Vitro", *Tissue Engineering Part C: Methods*, vol. 16, no. 4, pp. 711-718, 2010.

[5] T. Braun and M. Gautel, "Transcriptional mechanisms regulating skeletal muscle differentiation, growth and homeostasis", *Nature Reviews Molecular Cell Biology*, vol. 12, no. 6, pp. 349-361, 2011.

[6] K. Cole, N. Henao, T. Miller, and K. Pawelski, "Mechanical and Electrical Stimulation Device for the Creation of a Functional Unit of Human Skeletal Muscle in Vitro." Major Qualifying Project, Worcester Polytechnic Institute, Worcester, MA, 2015.

[7] H. Vandenburgh, J. Shansky, F. Benesch-Lee, K. Skelly, J. Spinazzola, Y. Saponjian and B.S. Tseng, "T.P.4.04 Automated drug screening with contractile muscle tissue engineered from dystrophic myoblasts", *Neuromuscular Disorders*, vol. 19, no. 8-9, p. 614, 2009.

[8] IonOptix. (2015). *Culture Dish for Culture Pacer Systems* [Online]. Available: http://www.ionoptix.com/product/culture-dish-for-culture-pacer-systems/.

[9] A. Mayeuf-Louchart, B. Staels and H. Duez, "Skeletal muscle functions around the clock", *Diabetes Obes Metab*, vol. 17, pp. 39-46, 2015.

[10] R. Romitti, K. Mathews, G. Zamba, C. Cunniff, J. Andrews, and D. Matthews. (2009).

"Prevalence of Duchenne/Becker Muscular Dystrophy Among Males Aged 5--24 Years--Four States, 2007" in *Morbidity and Morality Weekly Report*, 1119-1122.

[11] M. Guglieri and K. Bushby, "Recent developments in the management of Duchenne muscular dystrophy", *Paediatrics and Child Health*, vol. 21, no. 11, pp. 501-509, 2011.

[12] A. Fiorillo, C. Heier, J. Novak, C. Tully, K. Brown, K. Uaesoontrachoon, M. Vila, P. Ngheim, L. Bello, J. Kornegay, C. Angelini, T. Partridge, K. Nagaraju and E. Hoffman, "TNF-α-Induced microRNAs Control Dystrophin Expression in Becker Muscular Dystrophy", *Cell Reports*, vol. 12, no. 10, pp. 1678-1690, 2015

[13] Centers for Disease and Control. (2009). "Prevalence of Duchenne/Becker Muscular Dystrophy Among Males Aged 5-24 Year: Four States" in *Morbidity and Mortality Weekly Report* 1119-1122.

[14] J. Statland and R. Tawil, "Facioscapulohumeral Muscular Dystrophy", *Neurologic Clinics*, vol. 32, no. 3, pp. 721-728, 2014.

[15] C. Bentzinger, Y. Wang and M. Rudnicki, "Building Muscle: Molecular Regulation of Myogenesis", *Cold Spring Harbor Perspectives in Biology*, vol. 4, no. 2, pp. a008342-a008342, 2012.

[16] K. Moore, T. Persaud, and M. Torchia, *The Developing Human: Clinically Oriented Embryology*, Philadelphia: Elsevier, Inc., 2013.

[17] A. Gillies and R. Lieber, "Structure and function of the skeletal muscle extracellular matrix", *Muscle & Nerve*, p. n/a-n/a, 2011.

[18] M. Das, J. Rumsey, N. Bhargava, M. Stancescu and J. Hickman, "Skeletal muscle tissue engineering: A maturation model promoting long-term survival of myotubes, structural development of the excitation–contraction coupling apparatus and neonatal myosin heavy chain expression", *Biomaterials*, vol. 30, no. 29, pp. 5392-5402, 2009.

[19] J. Mertens, K. Sugg, J. Lee and L. Larkin, "Engineering muscle constructs for the creation of functional engineered musculoskeletal tissue", *Regenerative Medicine*, vol. 9, no. 1, pp. 89-100, 2014.

[20] S. Hinds, W. Bian, R. Dennis and N. Bursac, "The role of extracellular matrix

composition in structure and function of bioengineered skeletal muscle", *Biomaterials*, vol. 32, no. 14, pp. 3575-3583, 2011.

[21] M. Koning, M. Harmsen, M. van Luyn and P. Werker, "Current opportunities and challenges in skeletal muscle tissue engineering", *J Tissue Eng Regen Med*, vol. 3, no. 6, pp. 407-415, 2009.

[22] S. Rengenathan, S. Lindow, S. Keilich, and S. Hussain, "Mechanical stimulator for tissue-engineered skeletal muscle", Major Qualifying Project, Worcester Polytechnic Institute, Worcester, MA, 2015.

[23] A. Barat, R. Chin, A. Gribble, and J. Konowitch, "Mechanical stimulator for *in vitro* skeletal tissue," Major Qualifying Project, Worcester Polytechnic Institute, Worcester, MA, 2014

[24] O. Adebayo, T. Hookway, J. Hu, K. Billiar and M. Rolle, "Self-assembled smooth muscle cell tissue rings exhibit greater tensile strength than cell-seeded fibrin or collagen gel rings", *Journal of Biomedical Materials Research Part A*, vol. 101, no. 2, pp. 428-437, 2012.

[25] F. Guilak, D. Butler, S. Goldstein, and D. Mooney. n.d. "Functional Tissue Engineering" in *Springer Science & Business Media*, pp. 378-388, 2013.

[26] H. Vandenburgh, S. Hatfaludy, P. Karlisch and J. Shansky, "Mechanically induced alterations in cultured skeletal muscle growth", *Journal of Biomechanics*, vol. 24, pp. 91-99, 1991.

[27] J. Andersen, M. Juhl, T. Nielsen, J. Emmersen, T. Fink, V. Zachar and C. Pennisi, "Uniaxial cyclic strain enhances adipose-derived stem cell fusion with skeletal myocytes", *Biochemical and Biophysical Research Communications*, vol. 450, no. 2, pp. 1083-1088, 2014.

[28] K. Donnelly, A. Khodabukus, A. Philp, L. Deldicque, R. Dennis and K. Baar, "A Novel Bioreactor for Stimulating Skeletal Muscle In Vitro", *Tissue Engineering Part C: Methods*, vol. 16, no. 4, pp. 711-718, 2010.

[29] T. Nedachi, H. Fujita and M. Kanzaki, "Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle", *AJP: Endocrinology and Metabolism*, vol. 295, no. 5, pp. E1191-E1204, 2008.

[30] R. Dennis, P. Kosnik II, M.Gilbert, J. Faulkner, "Excitability and Contractility of Skeletal Muscle Engineered From Primary Cultures and Cell Lines", *American Journal of Physiology - Endocrinoloty and Metabolism*, vol. 280, no.2, C288-C295, 2001.

[31] C. Dym, P. Little, and E. Orwin, *Engineering Design: A Project-Based Introduction.* Hoboken: John Wiley & Sons, Inc, 2014.

[32] S. Ambady. (2010). *Cell Culture Procedures and Protocols - A Manual for Biomedical Engineering MQP Module*. [Online]. Available: https://my.wpi.edu/bbcswebdav/pid-427696-dt-content-rid-2160621_1/courses/BME3813-C15-MASTER/Cell%20culture%20procedures%20and%20protocols.pdf .

[33] H. Park, R. Bhalla, R. Saigal, M. Radisic, N. Watson, R. Langer and G. Vunjak-Novakovic, "Effects of electrical stimulation in C2C12 muscle constructs", *J Tissue Eng Regen Med*, vol. 2, no. 5, pp. 279-287, 2008.

[34] Sigma-Aldrich Co. LLC. (2015). "Corning Carbo-BIND 96 Well Plates." [Online], Available at: http://www.sigmaaldrich.com/catalog/product/sigma/cls2507?lang=en&region=US

[35] M. Thelen, W. Simonides and C. Hardeveld, "Electrical stimulation of C2C12 myotubes induces contractions and represses thyroid-hormone-dependent transcription of the fast-type sarcoplasmic-reticulum Ca2+-ATPase gene", *Biochem. J.*, vol. 321, no. 3, pp. 845-848, 1997.

[36] H. Park, R. Bhalla, R. Saigal, M. Radisic, N. Watson, R. Langer and G. Vunjak-Novakovic, "Effects of electrical stimulation in C2C12 muscle constructs", *J Tissue Eng Regen Med*, vol. 2, no. 5, pp. 279-287, 2008.

[37] D. Pedrotty, "Engineering skeletal myoblasts: roles of three-dimensional culture and electrical stimulation", *AJP: Heart and Circulatory Physiology*, vol. 288, no. 4, pp. H1620-H1626, 2004.

[38] H. Fujita, T. Nedachi and M. Kanzaki, "Accelerated de novo sarcomere assembly by electric pulse stimulation in C2C12 myotubes", *Experimental Cell Research*, vol. 313, no. 9, pp. 1853-1865, 2007.

[39] A. Ito, Y. Yamamoto, M. Sato, K. Ikeda, M. Yamamoto, H. Fujita, E. Nagamori, Y. Kawabe and M. Kamihira, "Induction of functional tissue-engineered skeletal muscle constructs by defined electrical stimulation", *Sci. Rep.*, vol. 4, 2014.

[40] K. Yamasaki, H. Hayashi, K. Nishiyama, H. Kobayashi, S. Uto, H. Kondo, S. Hashimoto and T. Fujisato, "Control of myotube contraction using electrical pulse stimulation for bio-actuator", *Journal of Artificial Organs*, vol. 12, no. 2, pp. 131-137, 2009.

[41] International Organization for Standardization. (2015). *ISO* [Online]. Available: http://www.iso.org/iso/home.html.

[42] International Organization for Standardization. (2012). *ISO 13022:2012 Medical products containing viable human cells — Application of risk management and requirements for processing practices.* [Online]. Available: https://www.iso.org/obp/ui/#iso:std:iso:13022:ed-1:v1:en.

[43] International Organization for Standardization. (2009). *ISO 10993-5:2009 Biological evaluation of medical devices -- Part 5: Tests for in vitro cytotoxicity* [Online]. Available: http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=36406.

[44] International Organization for Standardization. (2004). *ISO 17664:2004 Sterilization of medical devices -- Information to be provided by the manufacturer for the processing of Resterilizable medical devices*. [Online]. Available: http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=31456.

[45] International Organization for Standardization. (1995). *ISO 11845:1995 Corrosion of metals and alloys -- General principles for corrosion testing.* [Online]. Available: http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=3208.

[46] Society for Laboratory Automation and Screening. (2014). *Microplate Standards* [Online]. Available: http://www.slas.org/resources/information/industry-standards/.

[47] International Organization for Standardization. (2013). *ISO 13688:2013 Protective clothingGeneralrequirements.*[Online].Available: http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=51449.

[48] International Organization for Standardization. (2012). *ISO/IEC Guide 63:2012 Guide to*

the development and inclusion of safety aspects in International Standards for medical devices[Online].Available: http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=50729.

[49] IonOptix. (2015). *Culture Dish for Culture Pacer Systems* [Online]. Available: http://www.ionoptix.com/product/culture-dish-for-culture-pacer-systems/.

[50] A. Coletti, O. Baulaigue, P. Cara, R. Coletti, A. Ferro, E. Gaio, M. Matsukawa, L. Novello, M. Santinelli, K. Shimada, F. Starace, T. Terakado and K. Yamauchi, "JT-60SA power supply system", *Fusion Engineering and Design*, vol. 86, no. 6-8, pp. 1373-1376, 2011.

[51] R. Balint, N. Cassidy and S. Cartmell, "Conductive polymers: Towards a smart biomaterial for tissue engineering", *Acta Biomaterialia*, vol. 10, no. 6, pp. 2341-2353, 2014.

[52] Arduino. 2015. About Us. Available: https://www.arduino.cc/en/Main/AboutUs

[53] Raspberrypi. (2015). Raspberry Pi Blog. Retrieved from: Al. Alasdair. (2015). *ESP8266: This $5 microcontroller with Wi-Fi is now Arduino-compatible* [Online]. Available: http://makezine.com/2015/04/01/esp8266-5-microcontroller-wi-fi-now-arduino-compatibl/

[54] R. Salgado-Rodriguez, A. Licea-Claverie, A.Zizumbo-Lopez, and K.Arndt, “Smart pH/temperature sensitive hydrogels with tailored transition temperature”, *J. Mex. Chem. Soc.*, vol. 53, no. 2, pp. 118-126, 2013.

[55] H. Vandenburgh, J. Shansky, F. Benesch-Lee, V. Barbata, J. Reid, L. Thorrez, R. Valentini and G. Crawford, "Drug-screening platform based on the contractility of tissue-engineered muscle", Muscle & Nerve, vol. 37, no. 4, pp. 438-447, 200

Appendix A: Drawings of Lid Design

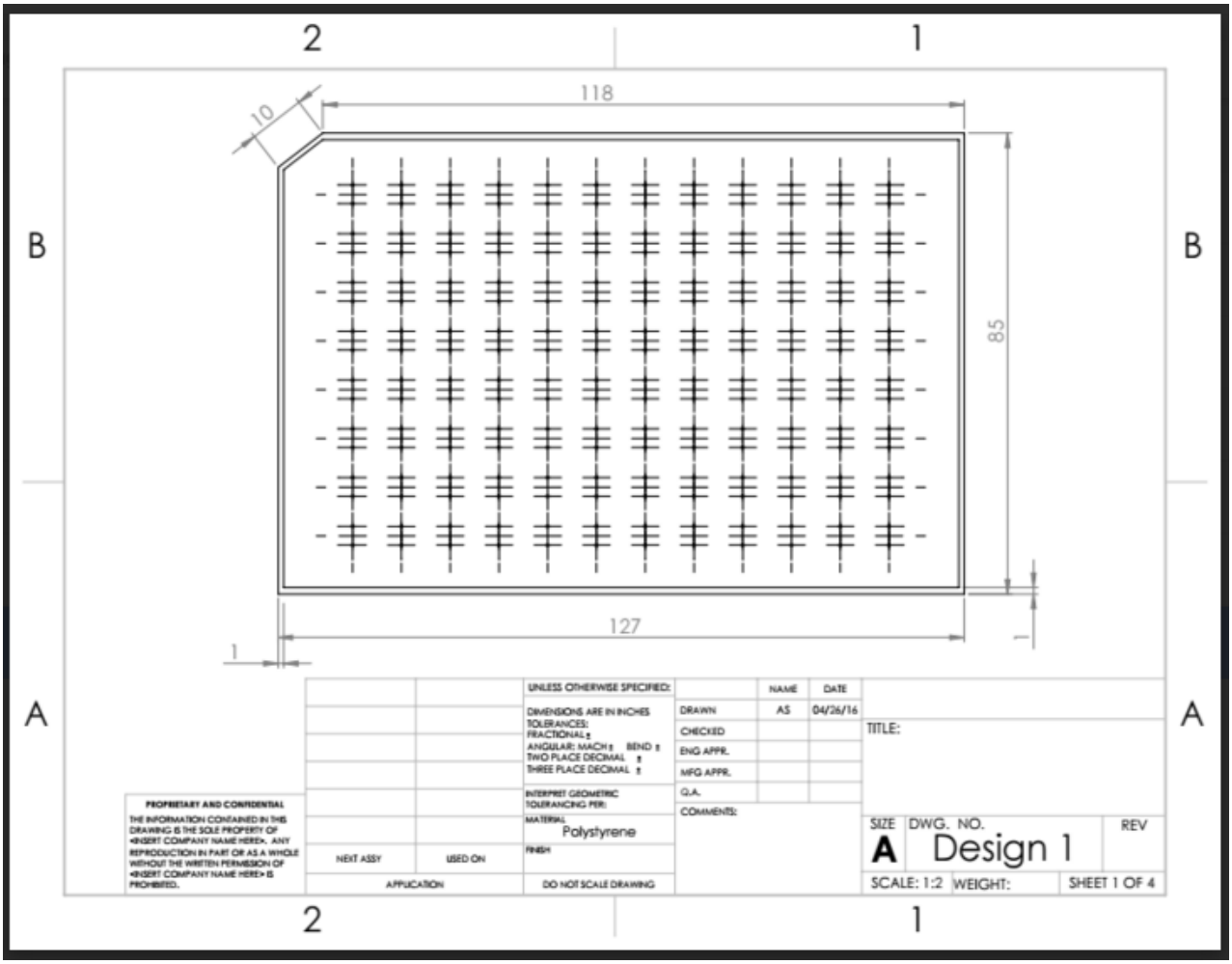

Figure 28: Drawing of lid design - sheet 1

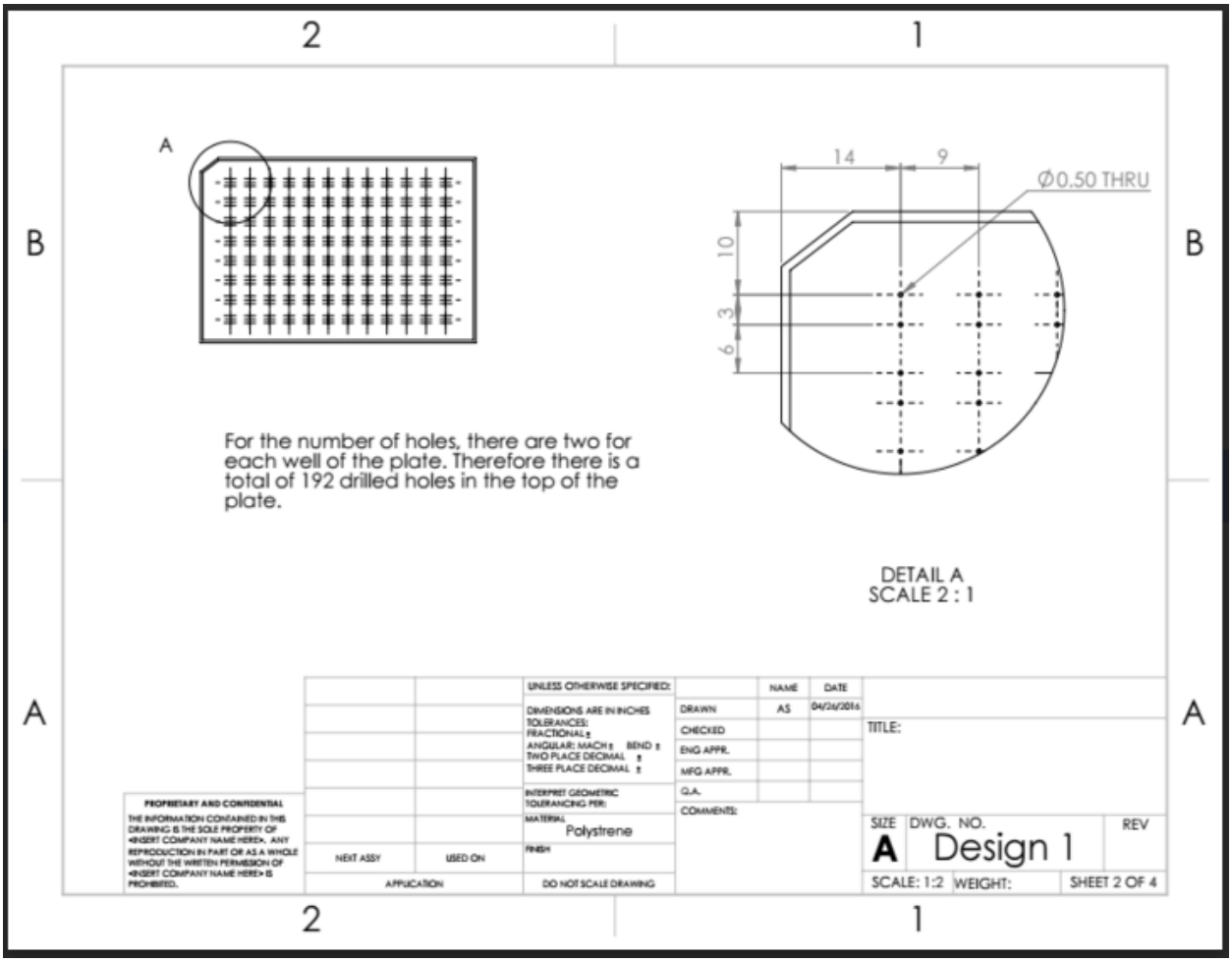

Figure 29: Drawing of lid design - sheet 2

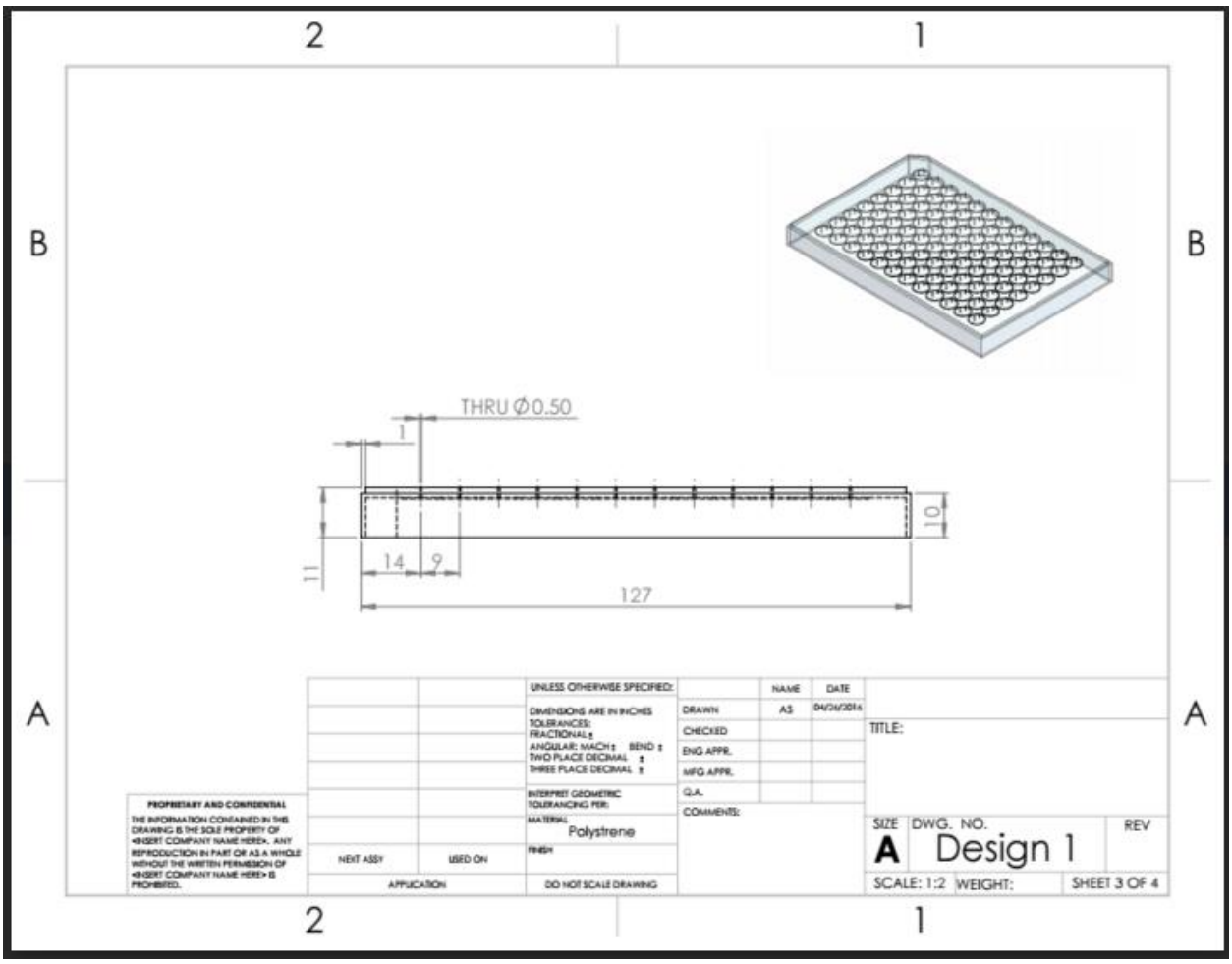

Figure 30: Drawing of lid design - sheet 3

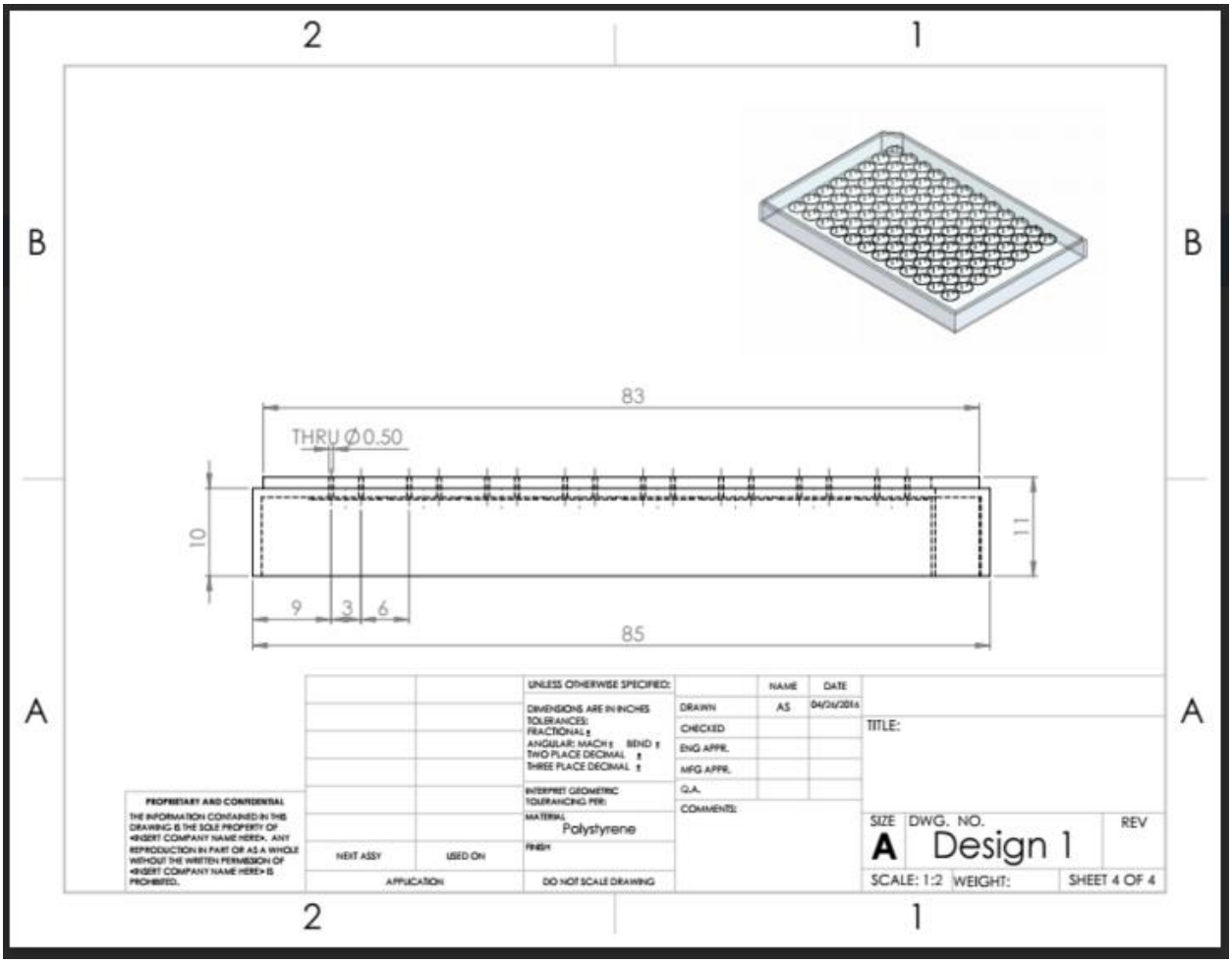

Figure 31: Drawing of lid design - sheet 4

Appendix B: Drawings of Grooved Channel Design

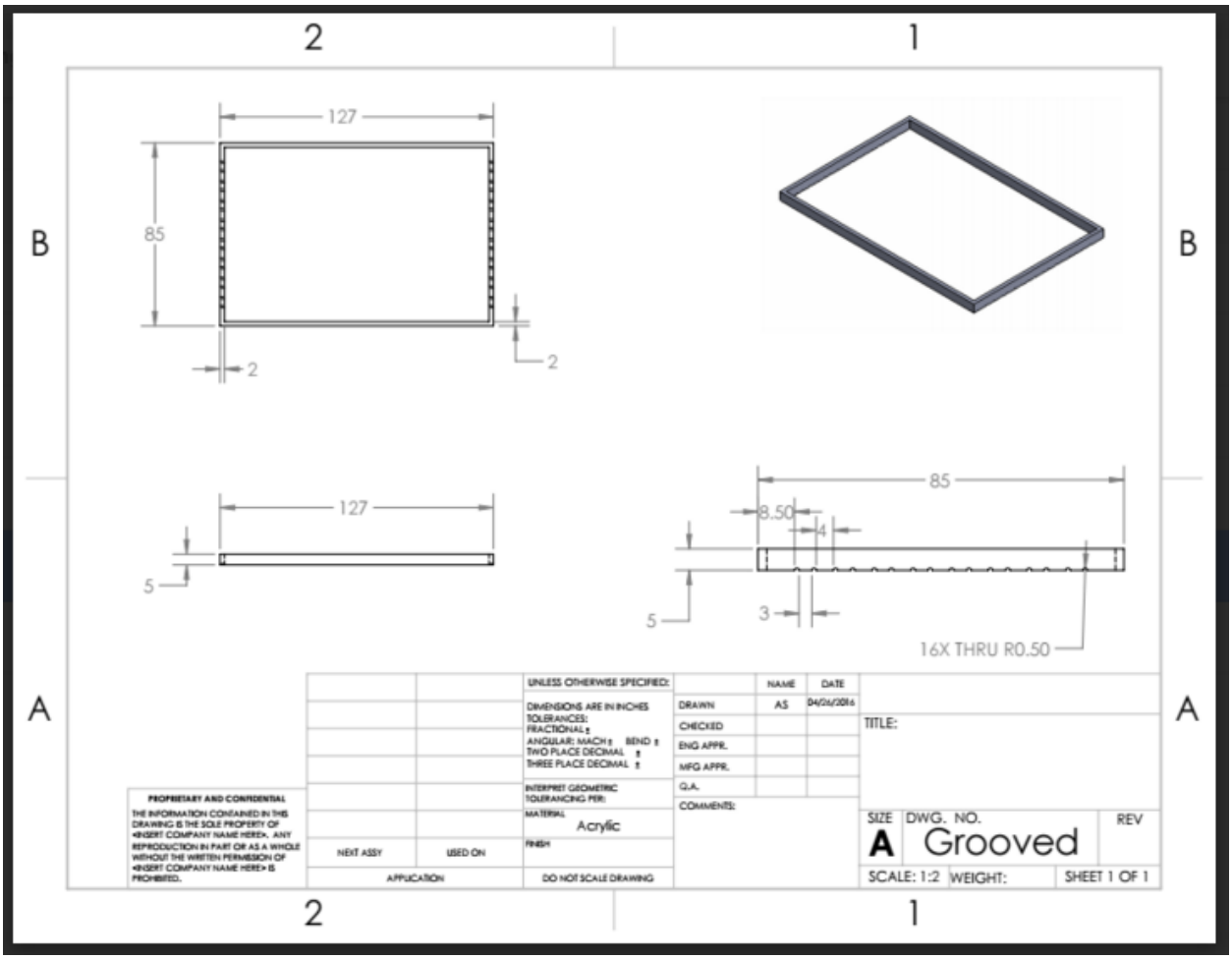

Figure 32: Drawing of grooved channel design - top part

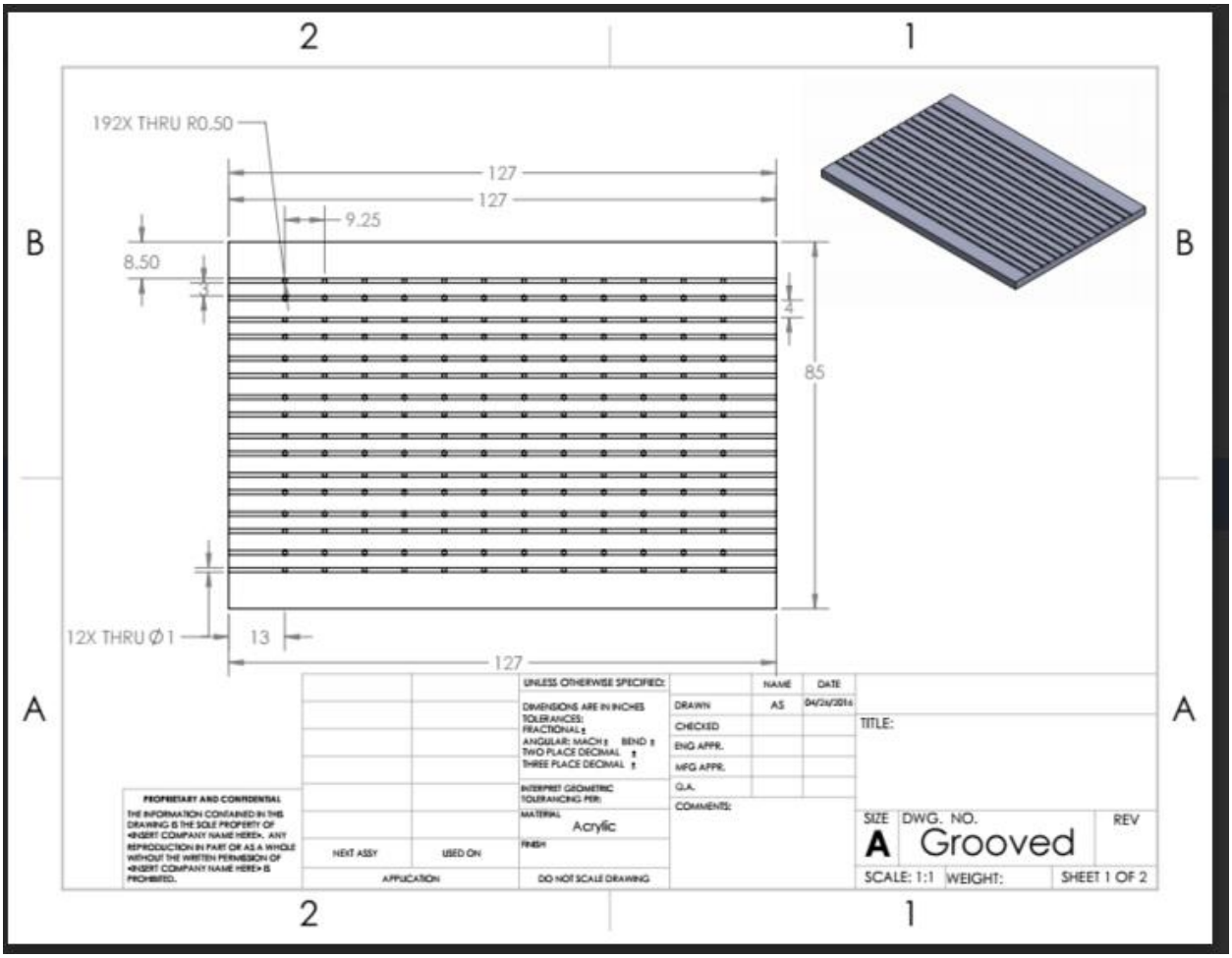

Figure 33: Drawing of grooved channel design - middle part sheet 1

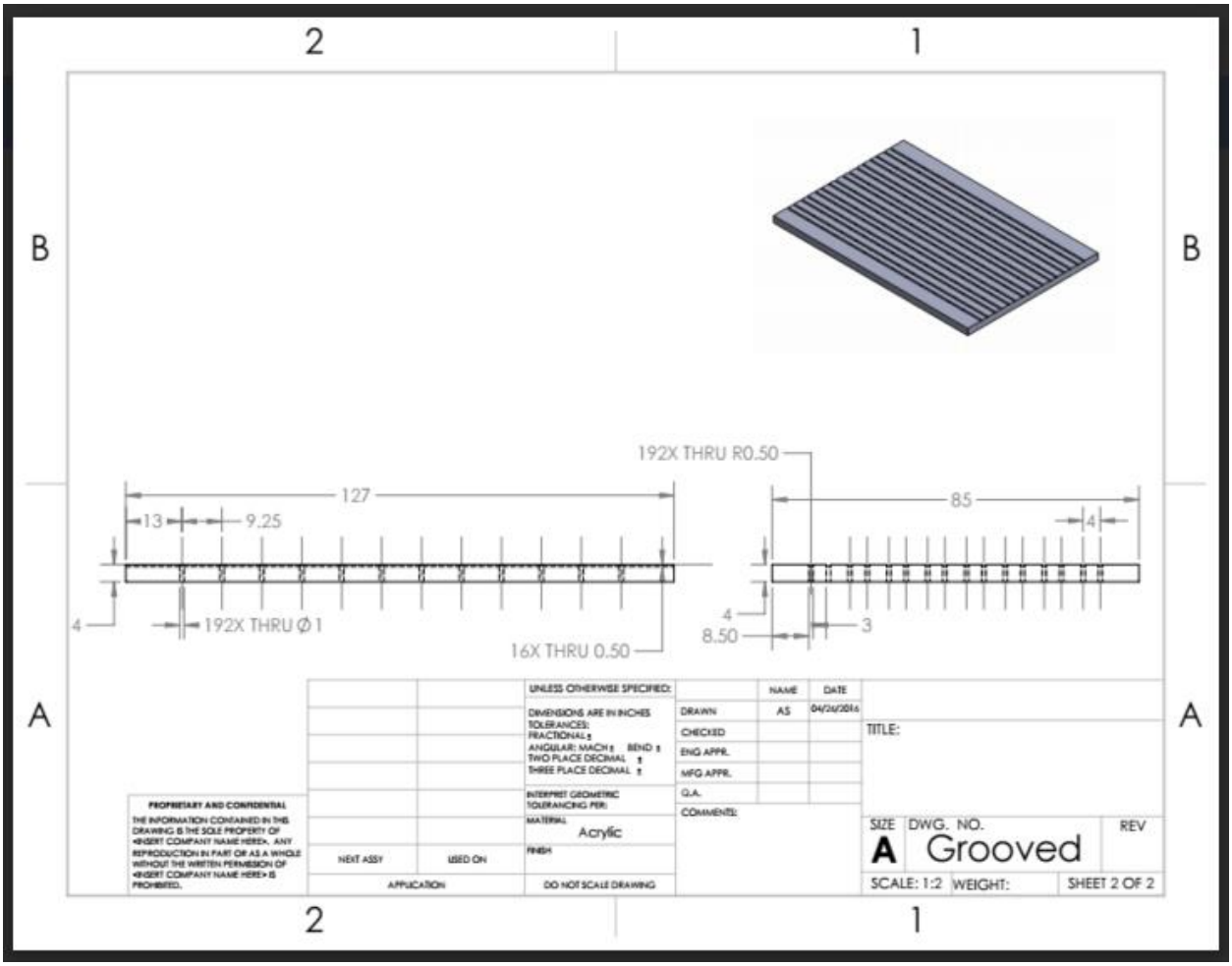

Figure 34: Drawing of grooved channel design - middle part sheet 2

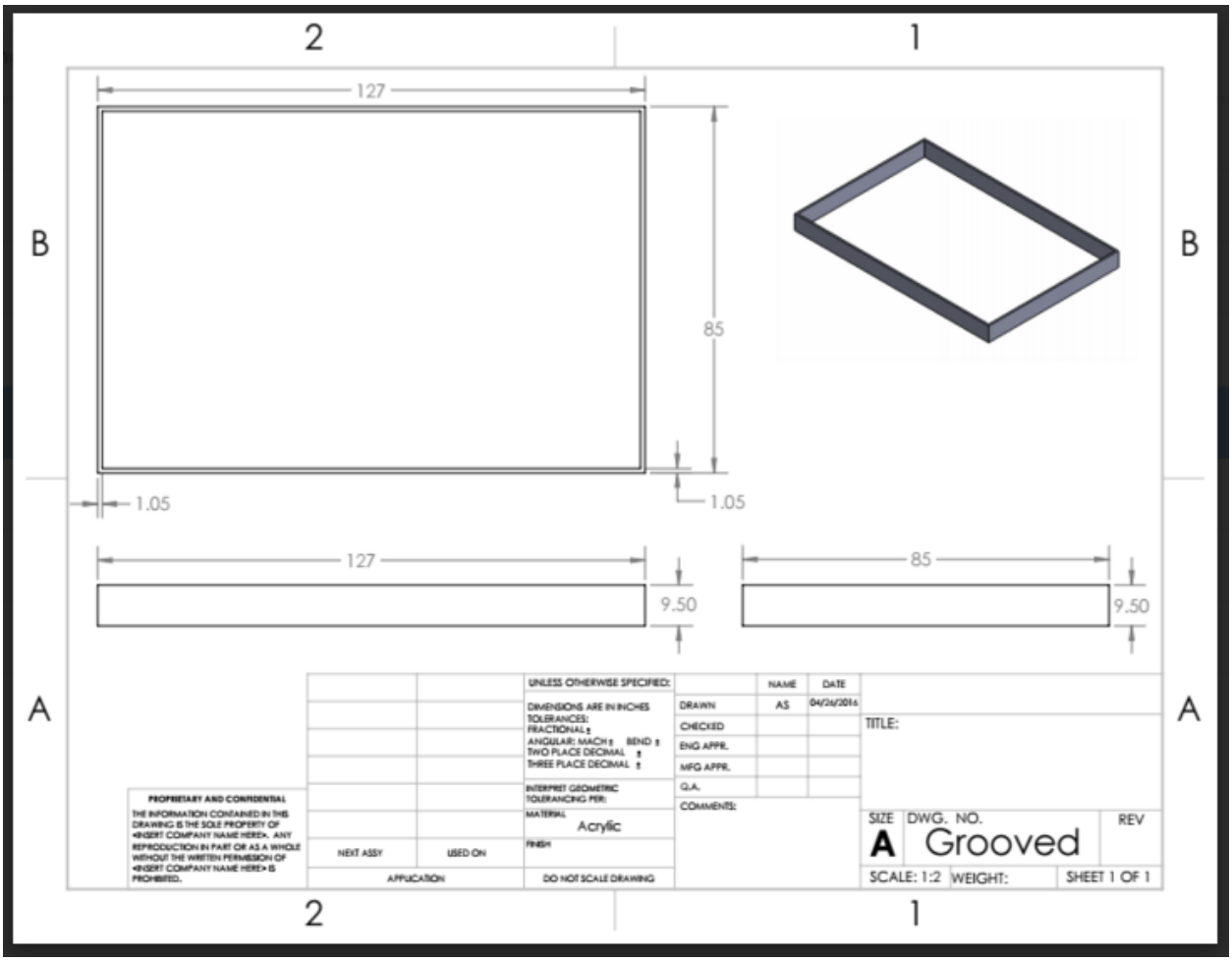

Figure 35: Drawing of grooved channel design - bottom part

Appendix C: Drawings of Buss Bar Design

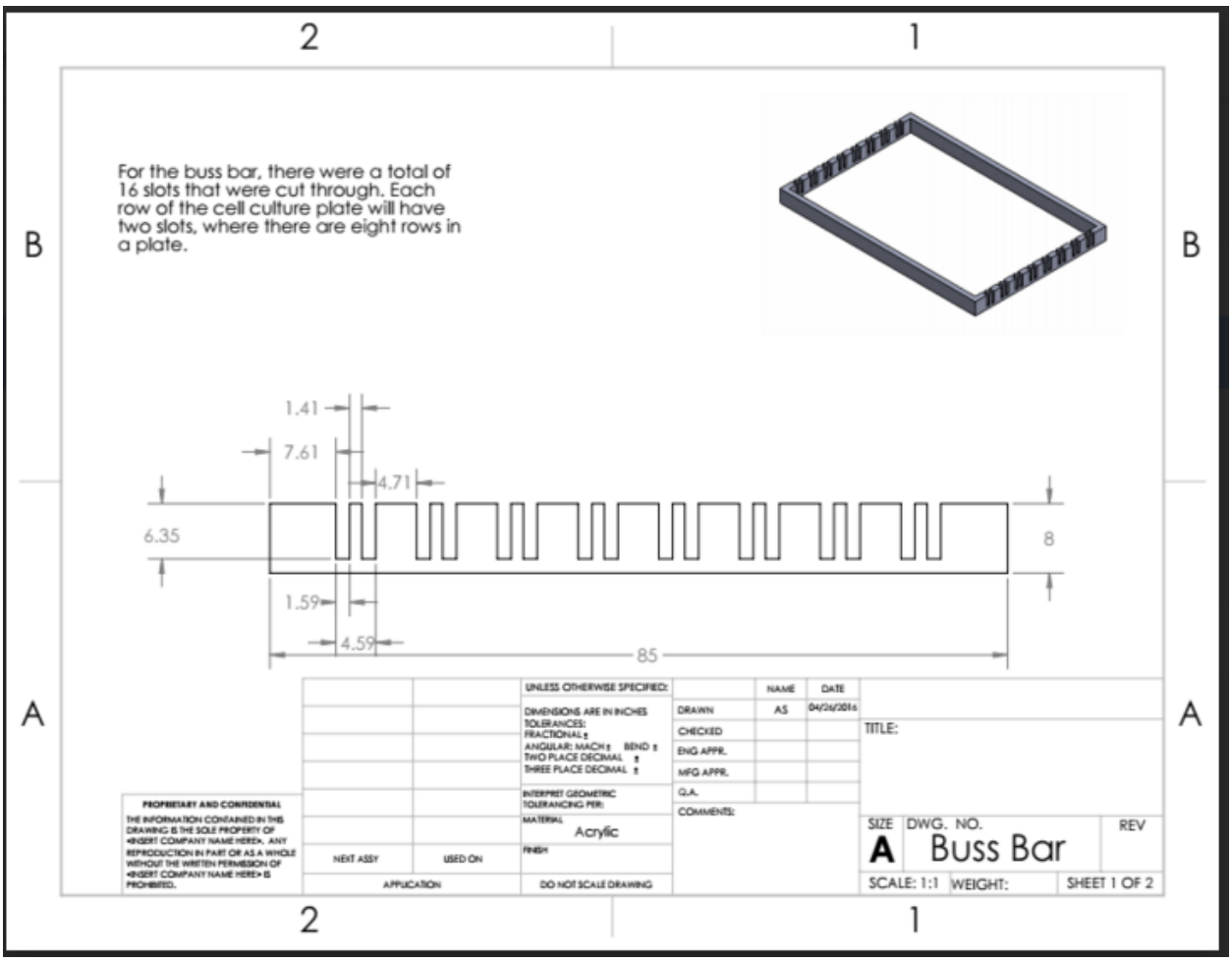

Figure 36: Drawing of buss bar design - top part sheet 1

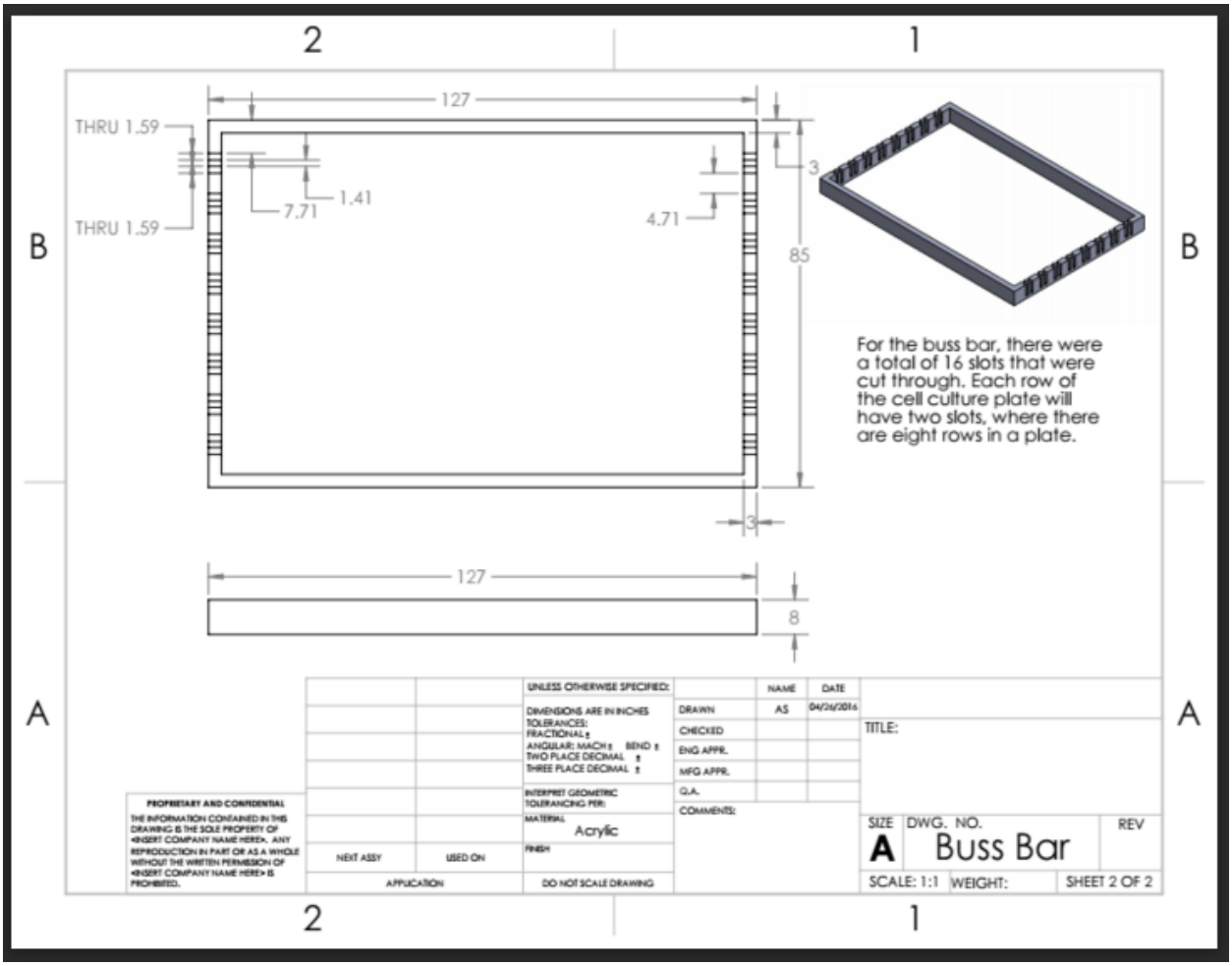

Figure 37: Drawing of buss bar design - top part sheet 2

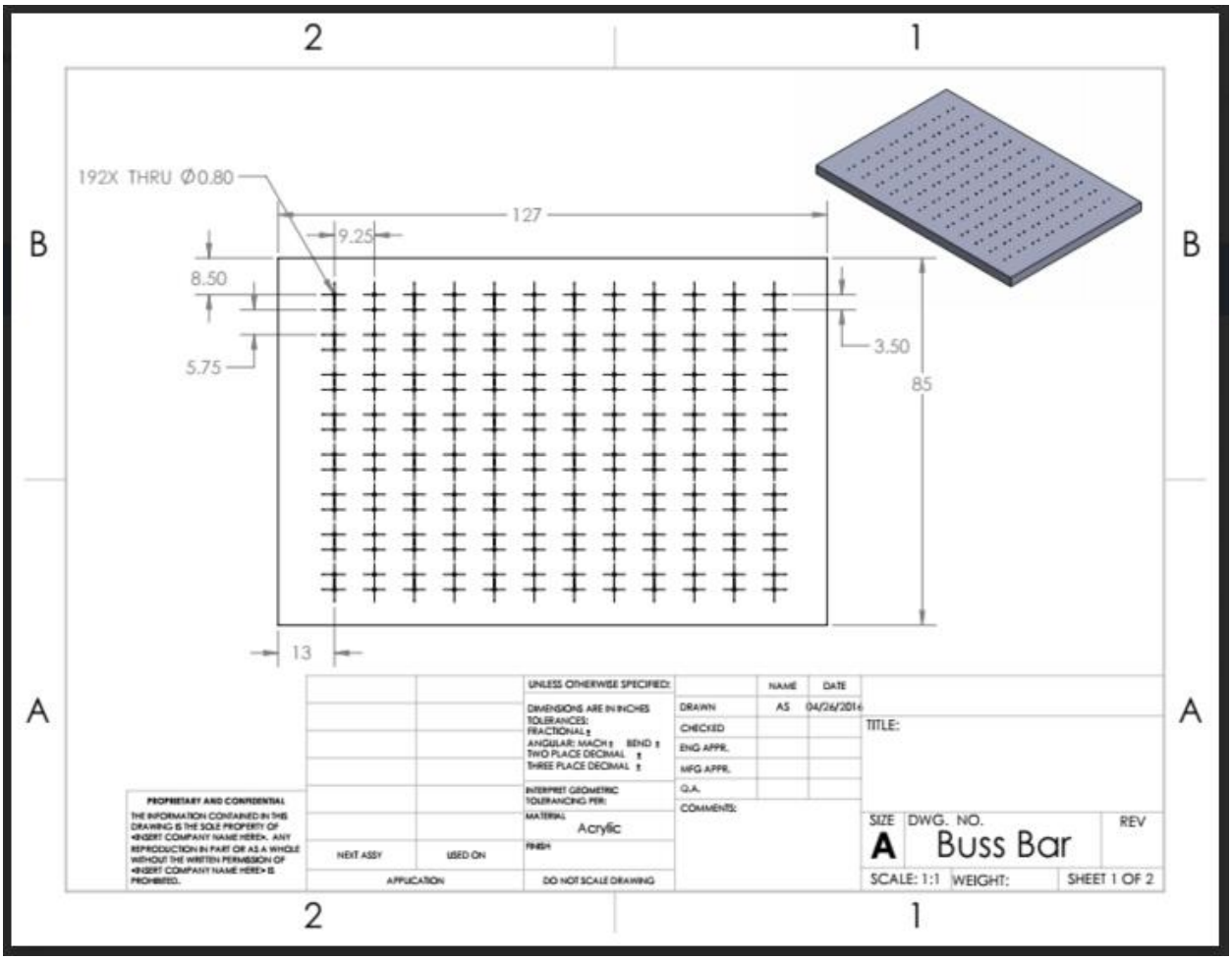

Figure 38: Drawing of buss bar design - middle part sheet 1

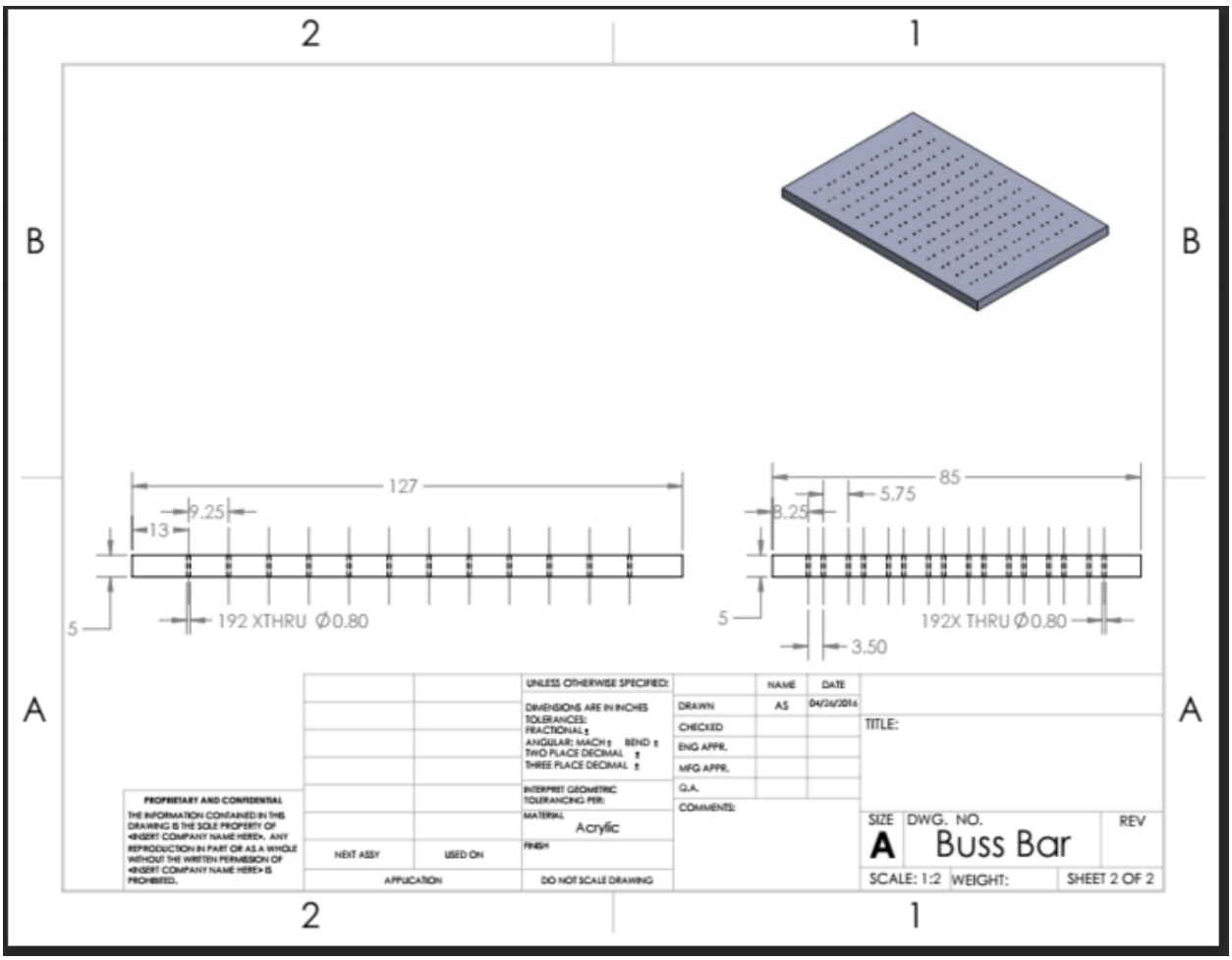

Figure 39: Drawing of buss bar design - middle part sheet 2

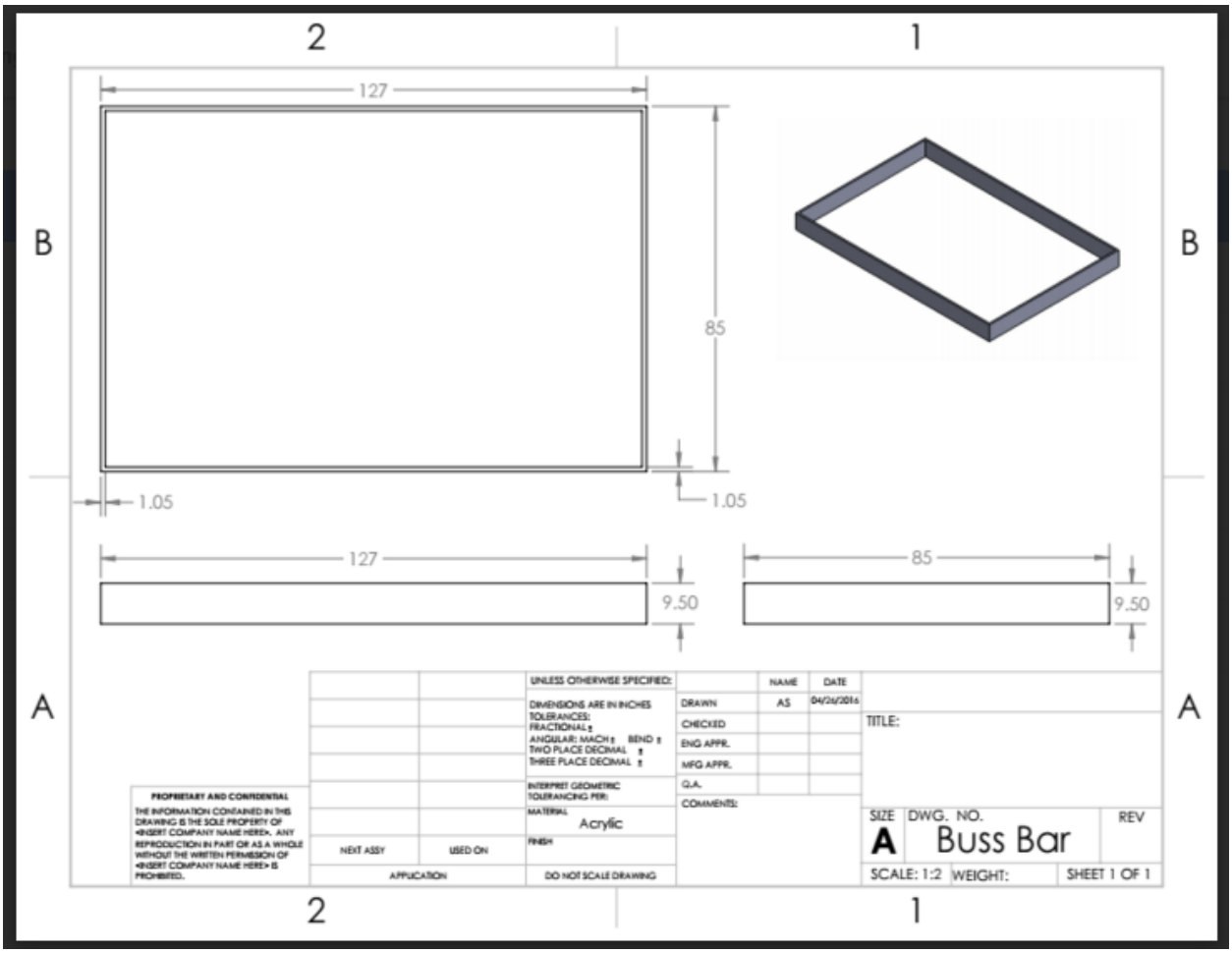

Figure 40: Drawing of buss bar design - bottom part

Appendix D: Electrodes and Wires

In this section, it is dedicated to the process of determining the material of choice for the final design, choosing the final electrode material, and describing the size, length and attachment method.

Potential Electrode Materials

To determine the best material for the electrodes, the team researched current *in vitro* models on the market. This gave insight on commonly used metal electrodes used in devices. The characteristics that were necessary for the metals to be used in the 96-well plate include having high corrosion resistance, electrically conductive, thermally and chemically stable. Table 16 shows the materials researched with their corresponding advantages and disadvantages, while Table 17 shows the material with its corresponding prices, diameter and length. All materials were quoted by American Elements, except for the Stainless Steel – AISI 316L, which was quoted from Sigma Aldrich.

Table 16: Shows the materials researched with corresponding advantages and disadvantages

Material	Advantages	Disadvantages
Platinum Wire	-High corrosion resistance -Easy to manufacture -Thermally, chemically and electrically stable	-Expensive
Titanium Wire	-High corrosion resistance -Biocompatible	-Needs to be oxidized or nitride for *in vitro* applications -Low electrical and thermal conductivity
Platinum Iridium Wire	-Chemically stable -High hardness -Good electrical qualities -High corrosion resistance -Commonly used for *in vitro* devices	-Hard to manufacture -Expensive
Silver Metal Wire	-High corrosion resistance -Good electrical qualities	-Expensive
Stainless Steel - AISI 316L Alloy	-High corrosion resistance -Easily manufactures -High heat resistance -Commonly used for *in vitro* applications -Electrically conductive	-Needs to be handled correctly as corrosion can occur

Table 17: Shows the materials with corresponding price, length and diameter

Material	**Length (ft)**	**Diameter (mm)**	**Unit Price ($)**
Platinum wire	1	0.5	584.37
Titanium wire	3.28	0.63	571.98
Platinum Iridium wire	1	0.4	582.94
Silver Metal Wire	1	0.5	497.44
Stainless Steel - AISI 316L alloy wire	16.4	0.8	160.00

Final Electrode Material

From research, the team determined the best method of applying electrical stimulation to be through contact stimulation. The electrode method can easily provide an electric field once electrical current is applied. Using Tables 16 and 17, the team determined the best option for the device. Based on the estimations provided by the companies, this drew the team to use 16.4ft of 316L stainless steel as sufficient for creating the electrodes. This cost the team $160.00 without shipping and handling. Additionally, a major consideration for choosing this material was its high corrosion resistance. Having corrosion resistance prevents ion leakage from the electrodes during electrical stimulation in an incubator setting, as a means to avoid cytotoxicity.

Electrode Size

In the Page Lab, the electrodes used are approximately 1mm in diameter. Also from literature, other device models used electrodes that ranged between 0.5 to 1mm. The team therefore used selected the size of 0.8mm from this range as a size that would be thin enough to work with in small culture wells, but thick enough to remain rigid upon applied pressure. The length of the 316L stainless steel wire was determined by its ability to be inserted into the customized lid, as well as making sure it did not touch the bottom of the plate and fitted into the brass buss bar. As a result, the length of the wire was 20mm.

Connecting Wires

For connecting the 316L stainless steel bar electrode connection, the attachment is based on the buss bar model. In this technique of attaching wire, a metal bar has slots in order for wires to connect, which is then held together with a screw. Figure 12 shows the buss bar design.

Appendix E: Circuit Diagram of the Device

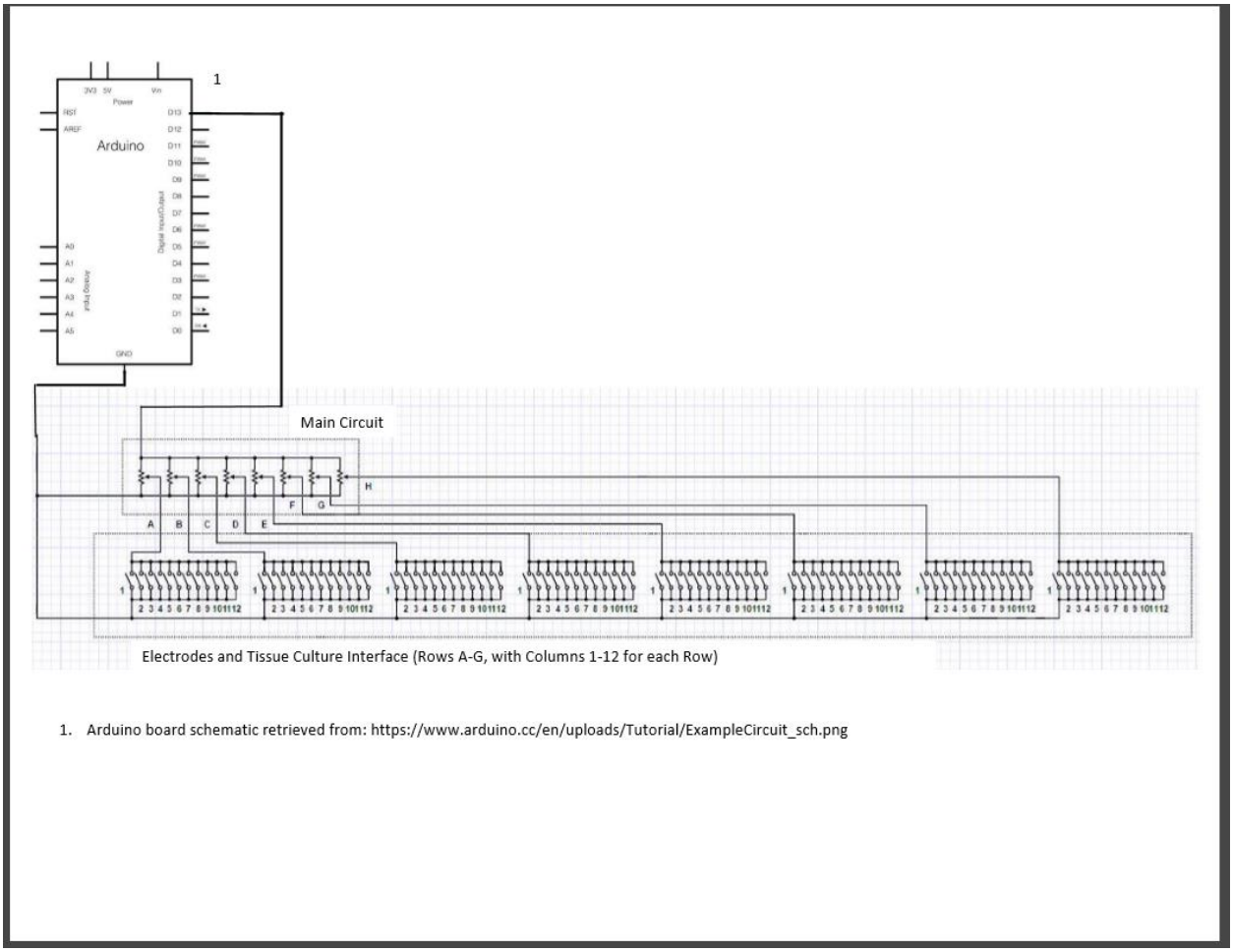

Figure 41: Circuit diagram of the device

Appendix F: Arduino Uno Code for Electrical Stimulation System

```
/*
  Stimulation
  Passes 5V square wave voltages from Arduino through pin 13 to 8 rows
  of 96-well culture plate. Voltage will be tuned with potentiometers
  between 0V and 5V. The stimulation device includes Arduino, Potentiometers,
  Battery(backup power), Wires and USB cord for power and computer communication.

  Pulse Width refers to the amount of time ~5V is flowing through pin (pwidth);
  Stimulation Frequency refers to how fast the pulses will be delivered (pfreq);
  Pulse Cycles refers to the number of times the Pulse Period (pwidth + prest = 1000.0/pfreq) is
repeated (pcycles);
  Rest Interval refers to the amount of time between trains before next Pulse Cycle (trecovery);
  Train Cycles refers to the number of times the Train Period (twidth +trecovery) is repeated
(tcycles);

  Pulse Rest refers to the amount of time ~0V is flowing through pin (prest) will be a calculated
output from pfreq;
  Pulse Period refers to the amount of time to complete Pulse Cycles;
  Stimulation Interval refers to the amount of time to complete Pulse Cycles. This is also the
Pulse Period. -- output (twidth);
  Train Period refers to the amount of time to complete a Train Cycle. (Train Width + Train
Recovery);


  "stimulation();" function is coded into Arduino and so to begin stimulation,
  go to the Menu bar above, click Tools -> click Serial Monitor.
  Serial Monitor allows the user to communicate with Arduino using the keyboard.

  Simply follow the instructions that pop up. As a safety measure, Arduino
  settings should be the last thing you do to electrically stimulate BAMs.
  Make sure BAMs are intact in incubator, and correctly connected to Arduino
  BEFORE opening the Serial Monitor to command Arduino.

  Stimulation is timed and so check your BAMs as soon as the stimulation
  is over; the Arduino will tell you how long every stimulation regimen will
  take. In case you make a mistake in typing in the regimen parameters,
  just close the Serial Monitor window and reopen it to restart the stimulation
  process. That should fix the problem.
```

```
  created 10 December 2015
  modified 04 April 2016
  by MQP RLP 1502
    Worcester Polytechnic Institute
    get_swole@wpi.edu
*/

#include <math.h> // Include Mathematical library for Mathematical computation or formulas

// Setup function below runs once when you power on the board or press reset on the board
void setup()
{
  pinMode(13, OUTPUT); // Initialize digital pin 13 on Arduino as 5V output
  Serial.begin(9600); // Tell Arduino to expect communication

  stimulation(); // Stimulate through Arduino pin 13 to culture rows A to H on 96-well plate
}

// Loop function runs repeatedly forever; not needed for this application
void loop(){
}


// The function below is the custom function that allows for user-defined electrical stimulation
regimen
// This function is called using "stimulation();" in the Setup function above
int stimulation()
{

  float pwidth = 0;                    // Pulse Width: input
  unsigned long int pfreq = 0;         // Pulse Frequency: input
  float prest = 0;                     // Pulse Rest (calculated from pfrequency): output
  unsigned long int pcycles = 0;       // Number of Pulse Cycles in 1 train( how many pwidth +
prest = 1000.0 / pfreq): input

  float twidth = 0;                    // Train Width or Pulse Period will be how long it will take to
complete a train = (pwidth + prest) * pcycles = (1000.0 / pfreq) * pcycles: output
  float trecovery = 0;                 // Train Recovery will be how long between trains: input
  unsigned long int tcycles = 0;       // Number of Train Cycles for the whole regimen Frequency
= how many (twidth + trecovery) for the whole regimen: input
```

```
  float tperiod = 0;                  // Train Period will be how long it will take to complete a train
cycle = (twidth + trecovery)

  float stim_length_s = 0;            // Time in seconds to complete train cycles (whole regimen for
day)
  double stim_length_h = 0;           // Time in hours
  double stim_length_d = 0;           // Time in days
  int columns = 0;                    // Number of columns to be stimulated

  Serial.print("Please input the number of culture rows (1-8) you wish to electrically stimulate
and hit 'ENTER' to continue.\n");
  while (Serial.available() == 0); // Arduino waits for input
  columns = Serial.parseInt();
  Serial.print("Double-check that all connections to culture rows are correct before
proceeding.\n\n");
  delay(3000);

  Serial.print("\nPlease input how long you want the pulse ON in milliseconds (greater than or
equal to 0)\n");
  while (Serial.available() > 0)
  {
    int junk = Serial.read(); // Empties whatever is in the buffer into the junk variable, making
room for another input
  }
  while (Serial.available() == 0);// Arduino waits for input
  pwidth = Serial.parseFloat();
  Serial.print("Pulse Width = "); Serial.print(pwidth); Serial.println(" milliseconds");
  delay(1000);

  Serial.print("\nPlease input the frequency of the pulses during 1 train in Hz (greater than 0)\n");
  while (Serial.available() == 0);// Arduino waits for input
  pfreq = Serial.parseInt();
  Serial.print("Stimulation Frequency = "); Serial.print(pfreq); Serial.println(" Hz");
  delay(1000);

  if (pwidth > (1000.0 / pfreq))
  {
    Serial.print("\nFor this particular regimen, Pulse Width CANNOT be more than ");
Serial.print(1000.0 / pfreq); Serial.println(" milliseconds. Please try again.");
  }
```

```
  else
  {
    Serial.print("\n");
    prest = (1000.0 / pfreq) - pwidth;
    Serial.print("Pulse Rest = "); Serial.print(prest); Serial.println(" milliseconds");
    delay(1000);

    Serial.print("\nPlease input the number of times to stimulate BAMs during 1 train (greater
than 0)\n");
    while (Serial.available() == 0);// Arduino waits for input
    pcycles = Serial.parseInt();
    Serial.print("Pulse Cycles = "); Serial.println(pcycles);
    delay(1000);

    Serial.print("\n");
    twidth = (1000.0 / pfreq) * pcycles;
    Serial.print("Train Width = Pulse Period = "); Serial.print(twidth); Serial.println("
milliseconds");
    delay(1000);

    Serial.print("\nPlease input how long you want BAMs to rest between trains in milliseconds
(greater than or equal to 0)\n");
    while (Serial.available() == 0);// Arduino waits for input
    trecovery = Serial.parseFloat();
    Serial.print("Rest Interval = "); Serial.print(trecovery); Serial.println(" milliseconds");
    delay(1000);

    Serial.print("\n");
    tperiod = twidth + trecovery;
    Serial.print("Train Period = "); Serial.print(tperiod); Serial.println(" milliseconds");
    delay(1000);

    Serial.print("\nPlease input the number of trains you want for this regimen (greater than
0)\n");
    while (Serial.available() == 0);// Arduino waits for input
    tcycles = Serial.parseInt();
    Serial.print("Train Cycles = "); Serial.print(tcycles);
    delay(1000);
```

```
    stim_length_s = ((twidth + trecovery) / 1000.0 ) * tcycles;  // Mathematical formula that needed "math.h" library
    stim_length_h = stim_length_s / 3600.0;             // Mathematical formula that needed "math.h" library
    stim_length_d = stim_length_s / 86400.0;            // Mathematical formula that needed "math.h" library

    Serial.print("\n\nYour stimulation regimen will be complete in ");
Serial.print(stim_length_s); Serial.print(" seconds ");
    Serial.print("OR "); Serial.print(stim_length_h); Serial.print(" hours ");
    Serial.print("OR "); Serial.print(stim_length_d); Serial.print(" days.");
    Serial.print("\nDon't forget to check on it.\n");

    for (int i = 1; i <= tcycles; i++)   // Ask Arduino to repeat the trains for the set number of cycles you wanted
    {
      for (int j = 1; j <= pcycles; j++) // Ask Arduino to repeat the pulses for the set number of cycles you wanted
      {
      digitalWrite(13, HIGH);   // Send HIGH pulse out of pin 13; this is the voltage level of ~5V
      delayMicroseconds(pwidth * 1000.0);
      digitalWrite(13, LOW); // Send LOW pulse out of pin 13; this is the voltage level of ~0V
      delayMicroseconds(prest * 1000.0);
      }
    delay(trecovery);
    }
    delay(1000);
    Serial.println("\nElectrical stimulation regimen you specified has ended successfully.\n\n");
  }
}
```