The Effects of Electromechanical Stimulation on Myocardial Constructs

A Thesis Submitted by

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Abstract

Cardiovascular disease causes artery walls to thicken via the buildup of atherosclerotic plaque. This plaque buildup may block coronary arteries, causing myocardial infarctions and eventually heart failure. The most effective treatment for these problems is full heart transplant, but there are simply not enough resources to carry out this procedure for every case. Cardiac tissue engineering is a potential treatment that solves this resource problem. Many groups have already begun to research tissue engineered myocardium. Since the myocardium tissue of the heart is influenced by mechanical stimulation from blood that fills and stretches the ventricles and electrical stimulation from the electrical conduction system of the heart, many researchers have looked at both mechanical and electrical stimulation. They have each shown improvements in the cardiac function of constructs. The combination of electrical and mechanical stimulation better mimics the environment *in vivo*. We believe that conditioning myocardial constructs in an environment in which combined mechanical and electrical stimulation are both present in a way that mimics the environment of the heart *in vivo* will improve cardiac function further. In this report, we establish that electrical stimulation has a greater positive effect on myocardial constructs than mechanical stimulation in terms of twitch force, force per cell, cell alignment, cell connectivity, and cell communication and that there is a relationship between the twitch force of a electromechanically stimulated construct and the delay time between the applied mechanical and electrical stimulation.

Acknowledgements

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1. Introduction

1.1 Significance

Cardiovascular disease is the leading cause of death in the United States and it is becoming one of the leading causes of death worldwide (CDC 2012). It is often the result of atherosclerosis, a condition in which the artery walls thicken due to the buildup of plaque. Plaque buildup may block coronary arteries, causing myocardial infarction (MI) and eventually heart failure. Currently, the methods of treatment for these conditions are meant to be preventative or are enacted to maintain the function of the heart after it has already been damaged. To date, few successful methods have been developed to repair or replace infarcted cardiac tissue besides full heart transplant. Although heart transplants have been very successful in the past, there are a limited number of hearts available each year, limiting the people who can be helped (Vunjak-Novakovic *et al.* 2009). Alternative methods to replace or repair the infracted or failing heart must be explored to fill this growing need.

A number of groups have made tissue engineered myocardium (Zimmermann *et al.* 2002, Radisic *et al.* 2004, Black *et al.* 2009). Since the myocardial tissue of the heart is influenced by mechanical stimulation from blood that fills and stretches the ventricles and electrical stimulation from the electrical conduction system of the heart, there have been a number of groups who have looked at both mechanical and electrical stimulation. These attempts have each shown improvements in the cardiac function of the engineered tissue. However, a combination of electrical and mechanical stimulation together better mimics the environment *in vivo*. To date, only one other group has attempted to use simultaneous mechanical and electrical stimulation to condition

cardiac tissue (Liao *et al.* 2008). This study was limited, however, by the fact that stimulation wasn't applied in a physiologically appropriate way. An environment in which combined mechanical and electrical stimulation are both applied in a physiologically appropriate way better mimics the environment of the heart.

The overall goal of the project is to develop functional cardiac tissue to replace damaged tissues in the body. Our central hypothesis is that stimulating myocardial constructs with combined mechanical and electrical stimulation that mimics the systolic contractions of the heart will create constructs more similar to myocardium and better suited for myocardial repair.

1.2 Specific Aims

Specific Aim #1: Determine how mechanical and electrical stimulation individually influence the properties of the construct

We fabricated bioreactors that allowed for individual mechanical and electrical stimulation of the constructs. We then applied mechanical and electrical stimulation to the constructs to mimic the conditions of the heart *in vivo*. We manipulated the mechanical and electrical stimulation in order to determine the effects of each on the constructs. After the constructs were cultured and stimulated, we characterized their contractility, cell organization via immunohistology, cell number via DNA quantification, he cellular connectivity via measures of gap junction organization and function, and protein expression via Western Blotting to determine which conditions are optimal.

Specific Aim #2: Determine how the timing of combined mechanical and electrical stimulation influences the properties of the construct when mimicking the systolic contractions of the heart

We fabricated a bioreactor that allowed for simultaneous mechanical and electrical stimulation of the constructs. We then applied combined mechanical and electrical stimulation that mimics the systolic contractions of the heart (isovolumic contraction). We manipulated the timing of the electrical and mechanical stimulation in order to determine the parameters that best mimic isovolumic contraction *in vivo*. After the constructs were cultured and stimulated, we characterized their contractility, cell organization via immunohistology, cell number via DNA quantification, cellular connectivity via measures of gap junction organization and function, and protein expression via Western Blotting to determine which conditions are optimal.

1.3 Long Term Goals

The long term goal of cardiac tissue engineering is to develop alternative methods to repair or replace damaged cardiac tissue. The short term goal is to create functional cardiac tissue similar to tissues in the body. We hypothesize that stimulating the myocardial constructs with combined mechanical and electrical stimulation that mimics the systolic contractions of the heart will create constructs more similar to myocardium and better suited for myocardial repair. If successful, we will significantly impact the way in which people with heart problems are treated by generating new functional cardiac tissue to repair their damaged hearts. Even if this particular experiment does not produce cardiac tissue ready to be used in human patients, it will

provide useful information that is will be needed to create functional cardiac tissue in the future. Overall, it is necessary to understand the effects of mechanical stimulation and electrical stimulation on myocardial constructs individually and simultaneously before functional cardiac tissue can be produced because they are integral in the heart's function.

2. Background

2.1 Myocardial Infarction and Current Treatments

2.1.1 Myocardial Infarction:

A MI, more commonly known as a heart attack, occurs when the coronary arteries are occluded by plaque. The cardiac tissue dies because oxygen and nutrients cannot pass through the blocked coronary artery and are unable to reach the tissue to sustain it (AHA 2012). The infracted heart remodels itself by increasing the wall thickness. This increased wall thickness causes the heart to increase in size to compensate for the reduced generated force (AHA 2012). Over time, the heart becomes overworked and thus, the patient goes into heart failure. As mentioned earlier, a MI is more likely to occur when someone suffers from cardiovascular disease because it causes increased amounts of plaque to buildup in the coronary arteries. In addition to increasing the risk of MI, cardiovascular disease also puts people at risk for heart failure and other heart diseases (AHA 2012). These conditions are very serious and require effective treatments. Current methods for treating MI are meant to be preventative or are enacted to maintain the diminished function of the heart after it has already been damaged.

2.1.2 Methods for Treatment:

Preventative Methods

Preventative methods include dieting, medication, and other healthy lifestyle choices. Dieting can reduce the risks of heart disease and therefore other heart related conditions by keeping a person healthy (AHA 2012). It may also cause weight loss and therefore reduce the burden on the heart. Medicating is also an effective method in preventing serious heart related conditions. Depending on the condition, doctors may prescribe medication to prevent blood clotting, lower blood pressure, ease chest pain, or lower cholesterol to help the heart function more easily (AHA 2012). Anticoagulants antiplatelet agents are used to prevent blood clotting, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers/inhibitors, beta blocker, calcium channel blockers, and diuretics are used to lower blood pressure, vasodilators and digitalis preparations are used to ease chest pain, and statins are used to lower cholesterol (AHA 2012). Other healthy lifestyle choices like exercise and not smoking also reduce the risk of heart disease (AHA 2012). Although there are preventative methods in place, unfortunately MIs still occur.

Methods to Restore Blood Flow and Function Following MI

If preventative methods are unsuccessful and the patient experiences an MI or heart failure, there are few options for treatment. One possible treatment option is bypass surgery which redirects the flow of blood around the blocked artery. Bypass surgery is very invasive and requires cutting the heart to excise or redirect arteries to

bypass the occluded artery. This can be dangerous and potentially harmful to the patient. If the MI is severe enough, then the patient will likely experience heart failure. The options for treating heart failure are pretty much limited to total heart transplantation, which is the gold standard for heart failure treatment and completely restores the function of the heart. However, the patient is always on immunosuppresants which causes other autoimmune issues. In addition, there are a limited number of hearts available to the increasing number of people who need them each year, making heart transplantation a limited solution for heart failure patients (Vunjak-Novakovic *et al.* 2009). Thus, other innovative methods are needed.

Thus, cell therapy has been explored as another viable treatment, but it is still in the stages of development. It is believed that if healthy stem cells are injected into a damaged area, they may be able to help repair the damaged area (Laflamme *et al.* 2007). Many studies are currently being conducted, but the results have been very inconclusive. Current studies have shown improvement in heart function after the injection of stem cells into the heart (Beitnes *et al.* 2011, Schächinger *et al.* 2009). However, studies have shown that the majority of cells die before adhering to the damaged region, and those that do adhere, do not differentiate correctly into myocytes (Terrovitis *et al.* 2010). Thus, current cell therapy results are typically believed to be a transient effect due to the paracrine signaling from the stem cells (D'Alessandro *et al.* 2010). Thus, researchers have been interested in encapsulating the cells in an attempt for better retention.

2.2 Tissue Engineering of Myocardium

2.2.1 Cell Sources in Cardiac Tissue Engineering:

Tissue engineering is another potential option for repairing or replacing infarcted heart tissue in the future. Tissue engineering constructs are created by seeding cells onto scaffolds and growing the construct *in vitro* before implanting it into the body. A variety of cells can be seeded into these scaffolds. Neonatal rat cardiomyocytes (Barash *et al.* 2010, Black *et al.* 2009, Radisic *et al.* 2004, and Zimmermann *et al.* 2002) and embryonic stem cells (Schmelter *et al.* 2006) have become two of the most popular cell types used in cardiac tissue engineering, in part because human cardiomyocytes are not easily obtained. Neonatal rat cardiomyocytes are preferred over adult rat cardiomyocytes because they have more potential to proliferate in culture (Zimmermann *et al.* 2002). This is because neonatal rat hearts contain myoblasts that are still able to proliferate and generate more myocytes during the first couple of days after birth.

Embryonic stem cells are another preferred stem cell source because they have the potential to proliferate in culture and differentiate into functional cardiac cells. Differentiation of the stem cells into cardiac tissue is not without issues because it is not yet completely understood how to obtain specific functional tissues using stem cells. Therefore, neonatal rat cardiomyocytes are ideal to use when studying cardiac tissue because they do not need to be differentiated, but they still have the potential to proliferate in culture and generate more myocytes.

2.2.2 Fibrin Gels in Cardiac Tissue Engineering:

In addition to having a variety of cell sources available for cardiac tissue engineering, there are also a number of different materials that are commonly used as scaffolds for the cells. Materials like collagen and fibrin have been used as hydrogel scaffolds in the past. Hydrogels are useful scaffolds because they are able to entrap cells throughout the entire construct and oxygen and nutrients are able to travel through the pores easily, allowing growth deep within the scaffold (Tee *et al.* 2010). Other materials that have been used as scaffolds include PLGA (Boerboom *et al.* 2008), alginate (Barash *et al.* 2010), and electrospun polyurethane (Liao *et al.* 2008). Fibrin has proven to be more useful than collagen in recent studies because cells are able to degrade fibrin more easily than collagen and replace it with their own extracellular matrix (Ahmed *et al.* 2008). In addition, because it is one of the main clotting components of blood and is heavily involved in the natural wound healing response of the body, fibrin is capable of being broken down safely if necessary and can be autologously sources from the patient (Ye *et al.* 2011). Fibrin has also been used in other materials used in the body like tisseel, a surgical sealant approved by the FDA. Additionally, fibrin is flexible and adhesive. Cells are able to adhere to its surface while withstanding mechanical strain. Lastly, fibrin is pro-angiogenic, which is very important for the heart since it is made up of various veins and arteries.

2.3 Mechanical and Electrical Stimulation of Engineered Cardiac Tissue

2.3.1 Mechanical Stimulation:

While the effects of both mechanical and electrical stimulation on engineered cardiac tissue have been studied, more is known about the effects of mechanical stimulation on the development of cardiac tissue constructs. Various bioreactors have been used to apply cyclic strain to myocardial constructs. The first attempts at applying cyclic strain in a bioreactor involved clamping both ends of the construct and stretching it by oscillating the clamps with a crank (Kim *et al.* 2000). Although Kim *et al.* showed that this method improved the function of smooth muscle constructs; it also caused an uneven distribution of stress that could potentially tear them. Kim *et al.* ultimately concluded that an elastic scaffold was necessary to engineer tissue in a mechanically dynamic environment.

The current standard for applying cyclic strain to myocardial constructs was developed by Zimmermann *et al.* in 2002. They used a ring structure that was molded with silicon tubing and Teflon disks and they stretched the constructs by using a device that pulled them apart with two rods that were inserted in the middle of the constructs. Zimmermann *et al.* reported that this method produced constructs with similar structure, composition, and mechanical properties to myocardium. Furthermore, when implanted, the constructs improved systolic and diastolic function in an infracted rat heart (Zimmermann *et al.* 2006). Syedain *et al.* used a similar ring structure in 2008 to mechanically stimulate fibrin based constructs seeded with porcine valve interstitial cells, but they applied cyclic strain by using air pressure to distend latex tubing and the ring constructs that had been placed around it. Using this method, constructs stimulated

with constant strains had improved mechanical properties while constructs stimulated with increasing strains had further improved mechanical properties (Syedain *et al.* 2008). Mechanical stimulation has been repeatedly shown to improve the function of myocardial constructs and therefore is an integral part in creating myocardial constructs to repair or replace dead or damaged heart tissue in the future.

2.3.2 Electrical Stimulation:

A fair amount of studies have been completed on the effects of electrical stimulation on the development of cardiac tissue constructs. Most studies use a pair of carbon rods as electrodes to pulse myocardial constructs using an electric field in the hopes of pacing the cells (Radisic *et al.* 2004 and Barash *et al.* 2010). In 2004, Radisic *et al.* pre-cultured constructs without electrical stimulation and electrically stimulated them in wells evenly spaced in between 2 carbon electrodes for 5 days. They found an increased level of cell alignment and coupling, increased amplitude of construct contractions by a factor of 7, and an increased level of ultrastructural organization. In 2010, Barash *et al.* used a similar approach, but they also incorporated a perfusion reactor to push media through their constructs while they electrically stimulated them. They were able to stimulate more constructs at one time using this method. Barash *et al.* found that combined electrical stimulation and media perfusion promoted cell elongation and striation in the cell constructs and increased the expression of Connexin-43, the gap junction protein responsible for cell to cell coupling. Just like mechanical stimulation, electrical stimulation has been shown to improve the function of myocardial

constructs and therefore is an integral part in creating myocardial constructs to repair or replace dead or damaged heart tissue in the future.

2. 4 Simultaneous Stimulation: Electromechanical Timing

Many attempts have been made to improve the development of cardiac tissue constructs with mechanical or electrical stimulation, but only one attempt has been made to improve the development of constructs with simultaneous mechanical and electrical stimulation (Liao *et al.* 2008). Since the effects of separate mechanical and electrical stimulation on the development of cardiac tissue are not fully understood, most studies do not experiment with simultaneous stimulation. Liao *et al.* found that their electromechanical stimulation created smooth muscle constructs with more cell alignment, better elongation, and more contractile proteins. However, their attempts to study the effects of the stimuli had two major design flaws. First, Liao *et al.* used an electrospun scaffolding system in which they embedded cells. Electrospinning allows researchers to create scaffolds with large surface-to-volume ratios, high permeability, and an interconnecting pore structure (Szentivanyi *et al.* 2011). However, due to the thinness of the fibers, the scaffolds created are not truly three dimensional scaffolds, rather, they more closely mimic a two dimensional system. Second, when stimulating the cardiac tissue construct, the group applied mechanical stimulation and electrical stimulation in turns so that both stimuli would never be acting at once. The electric pulses were being passed through the constructs while they were not under mechanical stress and once the mechanical stress was applied, the electric pulse stopped. This does not model the behavior of the heart.

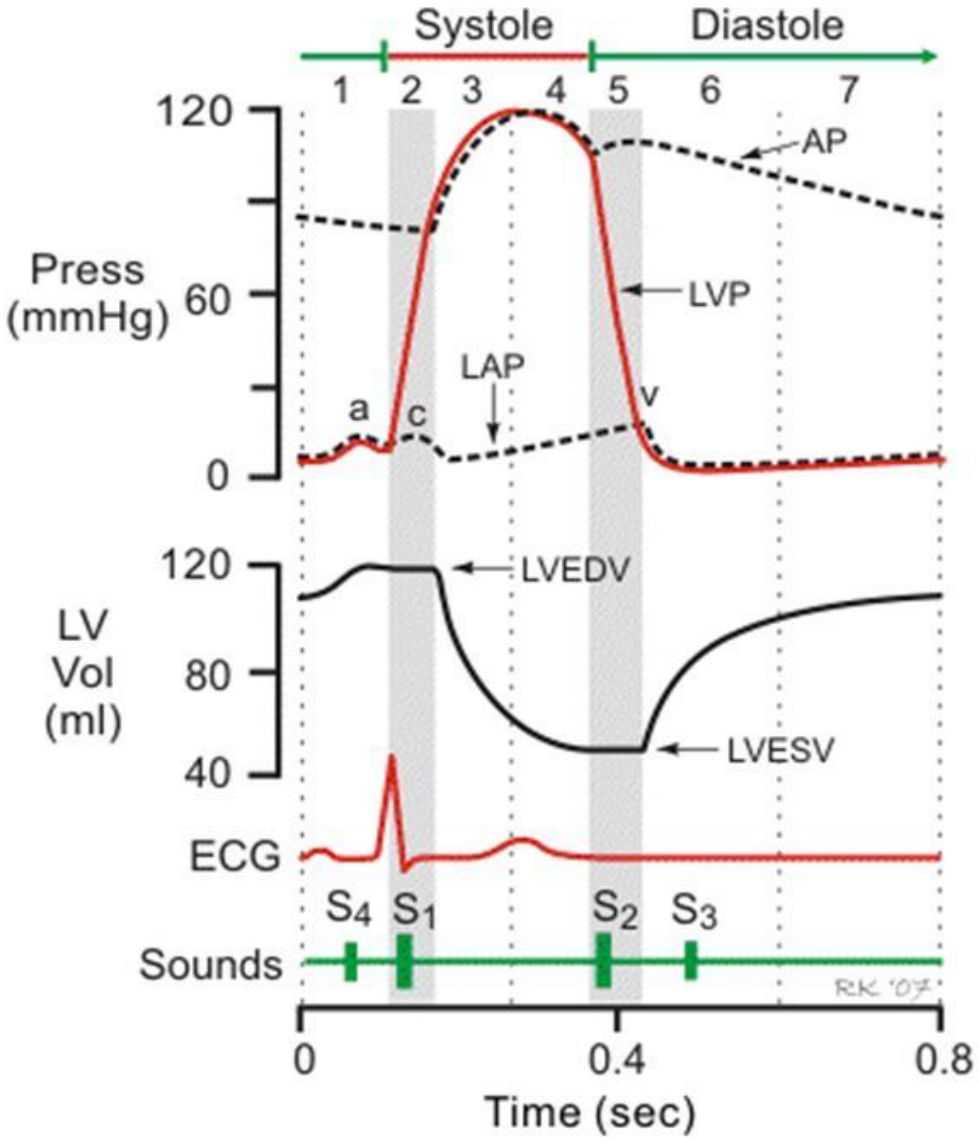

Figure #1 – This is a simplified Wigger's Diagram. It depicts the pressure, volume, and electrical activity in the ventricle during the course of the cardiac cycle. One can see that once the ventricle is filled with blood it expands. It is then electrically stimulated to cause a contraction. The pressure in the ventricle increases, but the volume stays the same. This phenomenon is known as an isovolumic contraction. Once the ventricle reaches a certain pressure, the blood is ejected and the cycles start over again.

Diagram From: http://www.cvphysiology.com/Heart%20Disease/HD002b.htm

The heart undergoes mechanical stress when blood fills the ventricles and stretches the walls of the chamber. An electrical pulse is applied when the ventricle is full of blood and experiencing mechanical stress. The electric pulse induces isovolumic contraction, where the ventricle contracts and increases pressure in the chamber while

remaining at the same volume. Once a certain pressure is reached while the ventricle is still contracting, the blood is ejected from the ventricle into the pulmonary artery or the aorta. The ventricle is no longer experiencing mechanical strain and thus is allowed to shorten. The ventricle relaxes and the process starts all over again. A diagram of this process can be seen in Figure #1 on the previous page. In order to model this process, an electrical impulse must be applied when the myocardial construct is mechanically stressed, but end before the construct is no longer mechanically strained. This would model the process of filling, isovolumic contraction, and ejection accurately.

3. Research Design and Methods

Rationale: We hypothesized that electromechanical stimulation will create stronger constructs than mechanical or electrical stimulation alone since it more closely mimics physiological conditions. Currently, no research has been done on electromechanical stimulation of hydrogel cardiac tissue engineering constructs. In the heart, electrical stimulation occurs while the ventricle or atrium is filled with blood. Therefore, we stretched the construct with mechanical stimulation and then electrically stimulated the construct causing the construct to contract while stretched. This sequence of events mimics the conditions of the heart when it undergoes isovolumic contractions. By varying the times that the construct is mechanically stretched and electrically stimulated, we were able to closely mimic the cardiac environment and find the optimal delay time. In particular we modified the delay between the mechanical and electrical stimulation to determine the optimal delay time.

Approach Overview: We found the optimal delay time that mimics the cardiac environment as it undergoes isovolumic contraction by 1) isolating neonatal rat cardiomyocytes, 2) creating myocardial constructs, 3) stimulating the constructs in bioreactors using various delay times between mechanical and electrical stimulation, and 4) analyzing the contraction force and composition of the constructs.

3.1 Neonatal Rat Cardiomyocyte Isolation

The cardiomyocytes embedded in the myocardial constructs were harvested from 3-day-old neonatal Sprague-Dawley rats. In order to isolate the cardiomyocytes, a hemostat, #5 forceps, large scissors, micro-scissors, and a scalpel handle (#4), all sterilized via autoclaving, were used. To maintain a sterile environment in the hood during the procedure, the procedure was done on an absorbent bench underpad with a sterile drape placed on top of it. The surgical instruments, a sterile #20 scalpel blade, and a 4 x 4 gauze pad were kept sterile by dumping them onto the sterile drape without touching them before putting on the sterile gloves. Before bringing the pups in the hood and putting on the sterile gloves on, betadine was brought in the hood in a beaker and PBS-glucose was brought into the hood in Petri dishes on ice. Once the pups were in and the sterile gloves were on, the hemostat was clamped onto the folded gauze pad and placed in the Betadine and the scalpel was assembled.

To begin the isolation, a pup was then picked up by pinching the skin between its shoulder blades and decapitated with one cut of the large scissors, ensuring that the spinal cord was cut. Then the pup's chest was swabbed with the betadine soaked gauze and its chest was cut open with the scalpel to expose the heart. Pressure applied to the

pup's shoulder blades was increased to force the heart past the ribs so that it could be removed with the scalpel. The heart was removed from the pup and placed in the Petri dish containing PBS-glucose that is on ice. The isolation was repeated for every pup in the litter.

Once all the hearts were isolated, residual blood and connective tissue was removed by rinsing the heart with ice-cold PBS glucose solution. The top third of each heart was also removed to isolate the ventricular tissue and the ventricular tissue was placed into a fresh petri dish with ice-cold PBS glucose. The hearts were then minced into 1 mm^3 pieces using the micro-scissors and the forceps. All of the tissue pieces and all of the solution were transferred into a 50 ml conical using a pipette and placed on ice.

Once all of the tissue pieces settled to the bottom of the conical, the supernatant was removed until only 10 mL of it was left and 7 mL of 37°C type II collagenase solution was added to the conical. The conical was placed on an orbital shaker going 60 rpm in a 37°C incubator for 7 minutes. After 7 minutes the conical was removed from the incubator and titrated 5 to 7 times. Once the tissue pieces settled to the bottom of the conical again, the supernatant was aspirated off and 7 mL of 37°C type II collagenase solution was added to the conical again. The conical was then placed on an orbital shaker going 60 rpm in a 37°C incubator for 7 minutes again. After 7 minutes go by, the conical was removed from the incubator and titrated 10 times, After the tissue pieces settle to the bottom again, the supernatant was removed and collected in another conical. 10 mL of stop media was added to the supernatant to stop the collagenase. We added 7 mL of 37°C type II collagenase solution to the tissue pieces

again, placed the conical on an orbital shaker going 60 rpm in a 37°C incubator for 7 minutes, titrated the solution 10 times, collected the supernatnent, and added 10 mL of stop media to the supernatant another 6 times before we ran out of collagenase solution. After the final digestion step, the cell solutions were filtered through 70µm cell sieve into fresh conical. The cells were spun down, resuspended, counted using a hemocytometer and Trypan Blue solution (75 µl Trypan Blue, 125 µl PBS), and placed on ice.

3.2 Construct Creation

The constructs were made by adding the isolated native cardiac cells from 3-day-old neonatal Sprague-Dawley rats at a final concentration of 5 x 10^6 cells/mL to a fibrin-forming solution (33 mg/mL bovine fibrinogen, 2 U/mL bovine thrombin, and 2 M $CaCl_2$). The resulting constructs had an initial volume of about 0.9 mL with a 3.3 mg/mL final fibrin concentration. The constructs were cast in Teflon tubular molds which were placed in outer mold casings (Kendall monoject 6 cc syringes) and capped with red

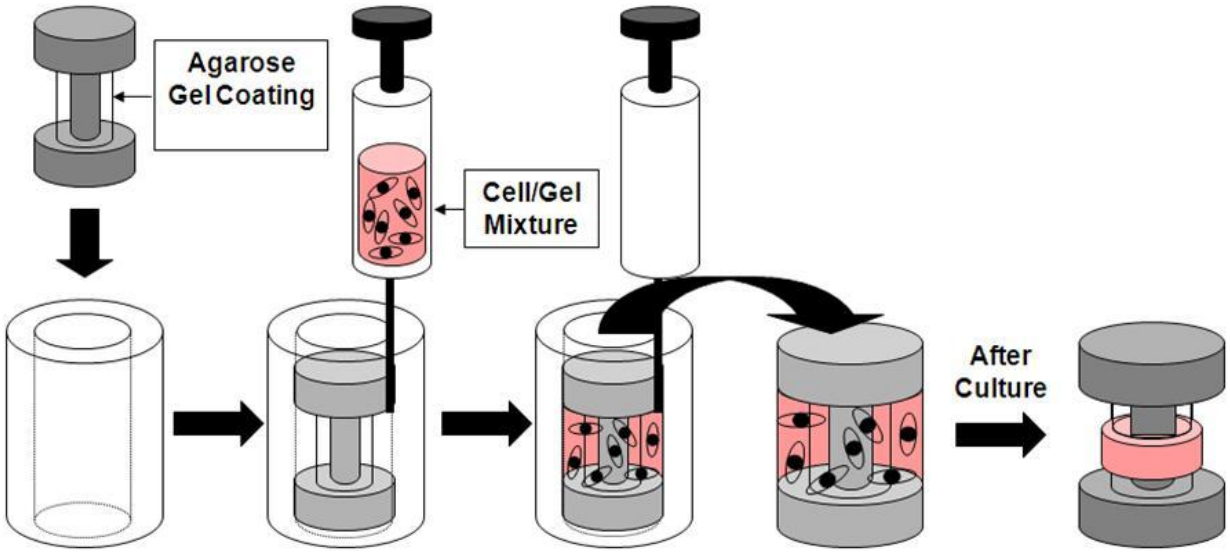

Figure #2 - A diagram of the construct creation process.

silicone O-rings to prevent the construct solution from leaking out of the mold when it was injected into the mold using a syringe. The constructs were allowed to set in the outer mold casings for 15 minutes at 37 °C before ejecting them into a jar containing myocardial construct media. The myocardial construct media contained Dulbecco's Modified Eagle Medium (DMEM), 10% horse serum, 2% fetal bovine serum, 1% penicillin/streptomycin, 50 µg/mL of ascorbic acid, and 2µg/ml of insulin. Insulin and ascorbic acid were used to promote ECM production in the constructs. The non-adherent Teflon mandrels allowed the constructs to align by compacting in the axial direction. The constructs were cultured on the molds for two days to allow for initial compaction before they were moved to the latex tubing or Teflon rod of the bioreactors for stimulation. This process is shown in Figure #2 on the bottom of the previous page and constructs ready for stimulation can be seen in Figure #3 on the top of this page. First the constructs were removed from the mold and put into warmed DMEM. The constructs were then slid onto the latex tubing or Teflon rod for mechanical and/or electrical pacing. The static, no stimulation constructs were be kept on the manifold for the duration of the experiment.

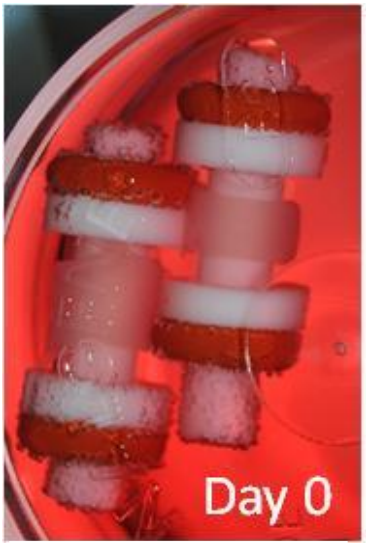

Figure #3 – A picture of constructs ready to be stimulated in culture.

Though the initial constructs were spread to fill the whole gap between the two Teflon rings, once the cells compacted in the constructs, the rings shortened to a fraction of their original size. However, the rings tended to be reasonably thick (~300-400 microns). Due to a diffusion limitation of nutrients and oxygen to the cells, the

constructs could have had an increase in apoptosis of cells which would have greatly influence the results of these experiments as well as the overall goal of implanting the constructs into the heart for repair (Black *et al.* 2009).

3.3 Mechanical and Electrical Stimulation in Bioreactor

3.3.1 Bioreactor Design:

In order to analyze the effects of mechanical and/or electrical stimulation on a myocardial construct, we created bioreactor systems that allow for combined and separate stimulation of the construct. The mechanical bioreactor was made from a 1 ounce glass jar and PTFE-faced caps containing important inserts. The electrical and electromechanical bioreactors were made from hard plastic jars with plastic tops. A leur-lock connector was added on top of each bioreactor to allow for a 22 µm filter to be

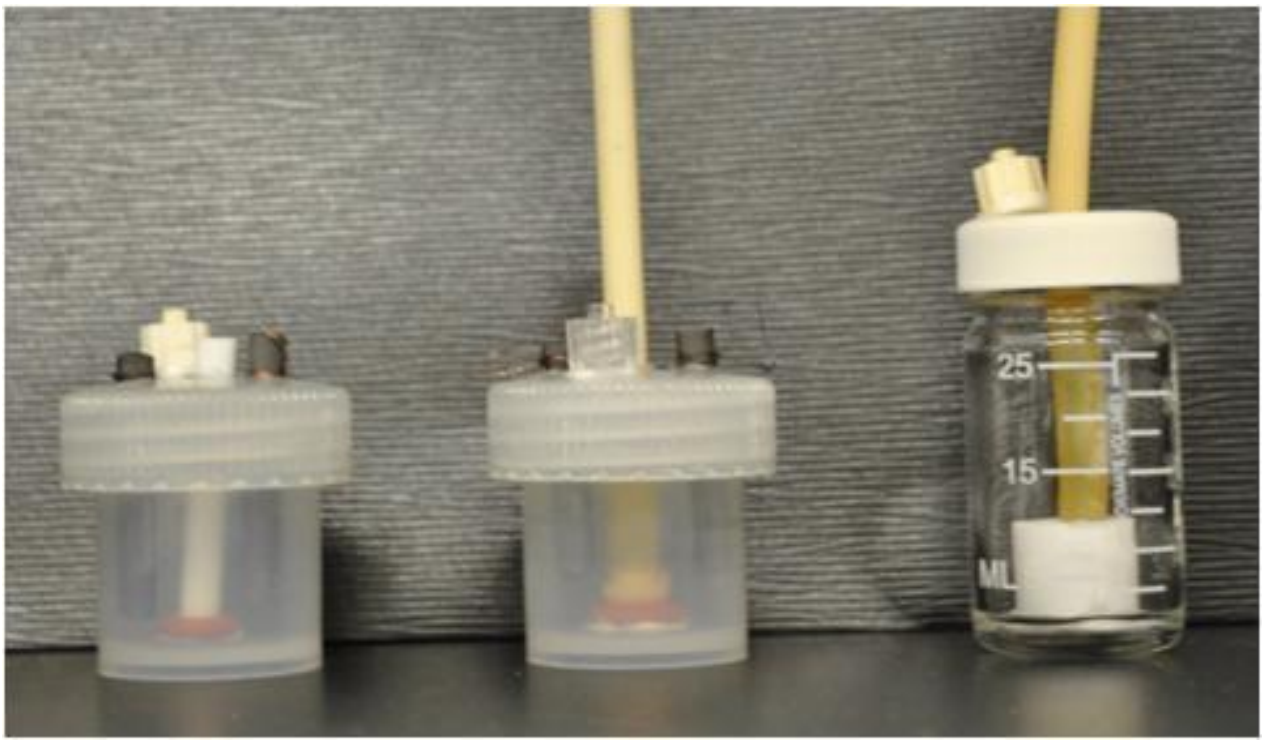

Figure #4 – A picture of the bioreactors we designed. Pictured from left to right: electrical bioreactor, electromechanical bioreactor, and mechanical bioreactor.

attached, maintaining sterility in the system with air exchange. The tubing in the center of the mechanical and electromechanical bioreactors, used to mimic the filling of the ventricles with blood, was made from latex, and expands with increased air pressure from the pressurized air outlet regulated by a custom made LabVIEW code. The end of the tubing was epoxied with a small Teflon rod to block the opening. A Teflon rod was used as the center piece of the electrical bioreactor because mechanical stimulation did not need to be applied. In the electrical and electromechanical bioreactors two thin carbon rods, which were used as the electrodes for the electrical stimulation, were placed on opposite sides of the Teflon rod/latex tubing. The carbon rods were connected to silver wires which were attached to the leads of a cardiac stimulator (S88X, Astro-Med Inc.) to allow a current to be carried across the carbon rods. The electrical signal caused the myocytes in the construct to contract. A Teflon spacer or rubber o-ring was glued to the bottom of each bioreactor to keep the tubing or Teflon rod straight and aligned, while limiting the quantity of media needed in the system. All components were glued in place with an autoclavable sealant. Figure #4 on the bottom of the previous page depicts all three types of bioreactos used in this project.

The constructs were placed around the middle of the latex tubing or Teflon rod and in between the two carbon rods to allow maximum contractions. 21 ml of myocardial construct medium (DMEM, 10% horse serum, 2% fetal bovine serum, 1% penicillin/streptomycin, 50 µg/mL of ascorbic acid, 2µg/ml of insulin) was added to each bioreactor and changed every two days. The mechanical, electrical, and electromechanical stimulation for a number of bioreactors were connected in parallel to allow for multiple samples of the same conditions to be run at the same time. The

constructs were stimulated for 2 weeks before they were removed from the bioreactors and analyzed.

3.3.2 Bioreactor Controls:

The mechanical and electrical stimulation were controlled by a computer through National Instruments' LabVIEW program and a NI data acquisition (DAQ) device (Figure #5). For the mechanical stimulation, the DAQ device sent an ON signal to a solenoid valve that allows for air to expand into the latex tubing, and an OFF signal to allow for air to be exhausted out of the latex tubing, causing it to relax. The air used in the system was from the high pressured air outlet in the laboratory. Duty cycle describes the ratio of the ON signal to the OFF signal, i.e. a duty cycle of 50% describes a system where the latex tubing is expanded, stretching the constructs for 50% of the time, while

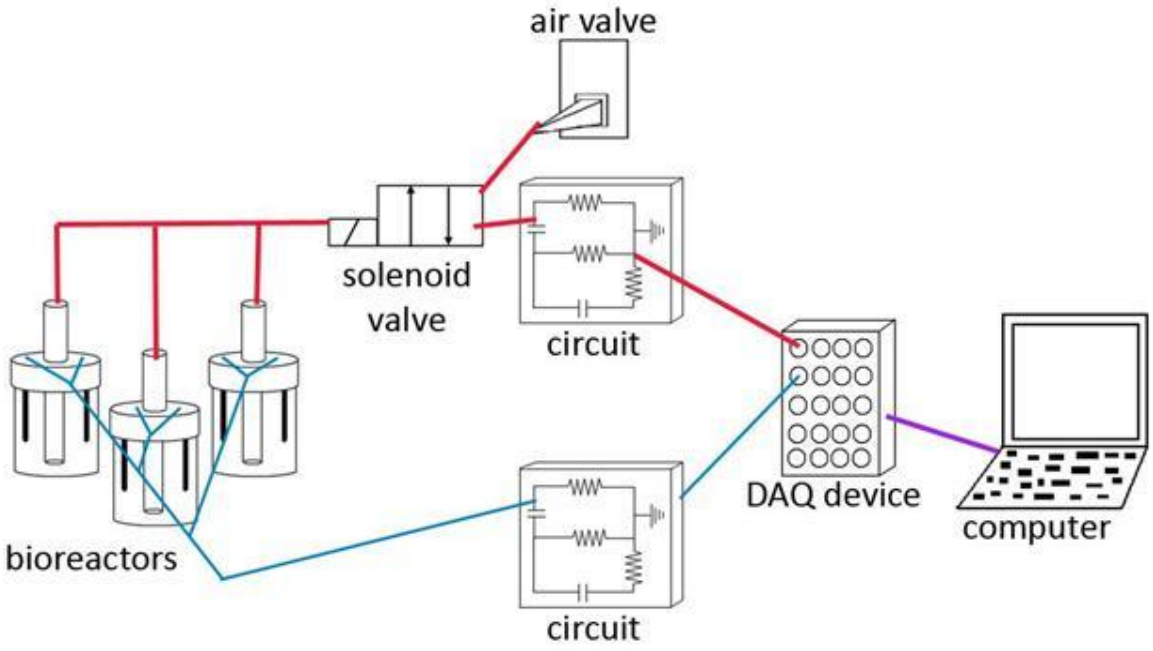

Figure #5 – A diagram of the system controls and how the individual components interface with each other. The DAQ device and the custom made LabVIEW program are able to control both mechanical and electrical stimulation at the same time through electrical connections.

the constructs are relaxed for the other 50% of the time. An average rate of 1 hz was maintained, to mimic a human pulse rate of approximately 60 beats per minute. The program allowed for multiple test cases to be run simultaneously, allowing for better control and comparison of the constructs.

For the electrical stimulation, the cardiac stimulator (S88X, Astro-Med Inc.) sent a voltage for a given short duration to an electrical circuit that was connected to a series of bioreactors in parallel. We were interested in sending a uniform voltage across the carbon rods so the circuit was designed to maintain uniform voltage across many bioreactors. The electrical stimulation for each electrical bioreactor was controlled very easily by setting the parameters for the stimulation on the cardiac stimulator itself. This allowed high levels of control and flexibility to be maintained over the timing of stimulation seen by the construct system.

Electromechanical stimulation is a combination of the mechanical and electrical control systems. The electrical stimulation for each electromechanical bioreactor was also controlled by setting the parameters for the stimulation on the cardiac stimulator

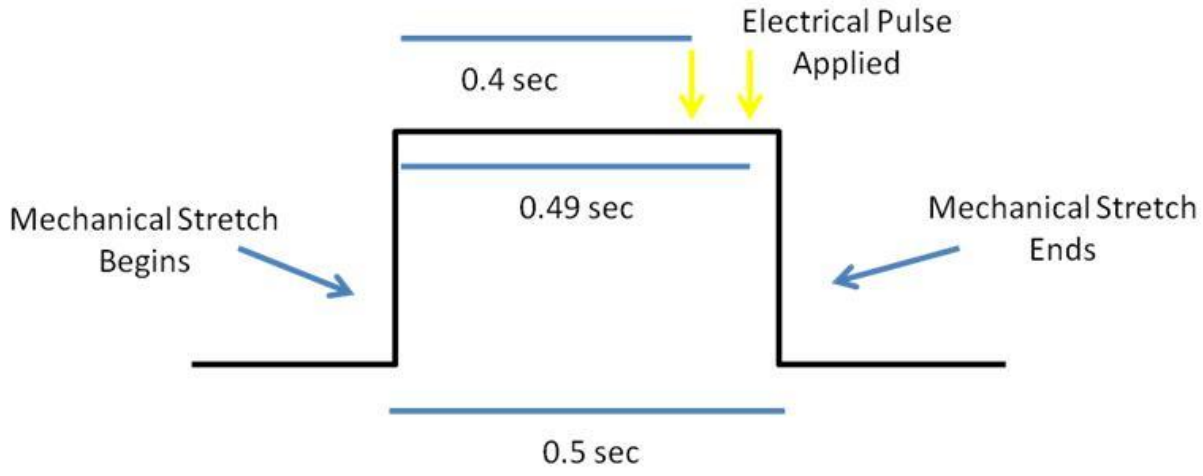

Figure #6 – A diagram of how electromechanical stimulation was applied. This system allows for electrical stimulation to be triggered by or occur a set amount of time after mechanical stimulation began. That set amount of time is called the delay time.

itself. By connecting the cardiac stimulator to the computer and the mechanical control system in LabVIEW, the electrical stimulation could be set to be triggered by or occur a set amount of time after the mechanical stimulation occurred. Figure # 6 on the previous page shows how electromechanical stimulation was applied to the constructs. The flexibility and control of this system will help us find the optimal time delay between mechanical and electrical stimulation very easily.

3. 4 Construct Analysis

3.4.1 Contraction Force:

The constructs were tested to determine the mechanical properties of the construct. To test the twitch force of the constructs, they were placed on a custom-built testing system that uses a force transducer, which converts force into a voltage that can be acquired using a custom-made LabVIEW program. The constructs were placed around the two poles of the custom-built test system and stretched to a preload of 10 mN before pacing. The constructs were then paced via general field stimulation using a cardiac stimulator (S88X, Astro-Med Inc.). The twitch force of the construct was in response to a 1 Hz pulse train of 10 ms, monopolar square waves with amplitudes of 8, 8.8, and 9.6 V/cm. The data was analyzed offline using MATLAB (The Mathworks Inc.). The twitch force was defined as the peak force achieved during pacing minus the baseline force immediately before the pacing stimulus. The twitch force for the construct was the average twitch force of multiple pacing events after the twitch force reached steady state. The contraction time was defined as the time it takes for the twitch force to reach the maximum over the average peak force achieved. The relax

time was defined as the time it takes for the construct to relax to half of the peak force. These measurements are also defined in Figure #7 below.

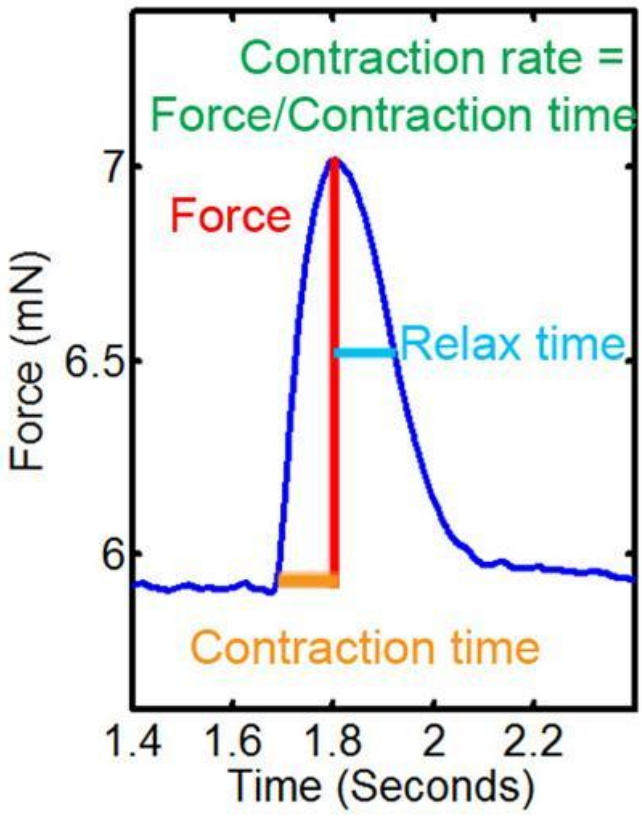

Figure #7 – A diagram depicting the measurements taken from the twitch force data using a custom made MATLAB program. The twitch force is the average force of multiple pacing events after the twitch force reached steady state. The contraction time is defined as the time it takes for the twitch force to reach the maximum over the average peak force achieved. The relax timeis defined as the time it takes for the construct to relax to half of the peak force.

3.4.2 DNA Quantification:

DNA content was quantified with a modified Hoechst assay. The samples were digested with 0.5 mg/mL proteinase K in a digestion buffer of 100 mM tris and 50 mM EDTA at pH 7.4 overnight at 56°C. 100 µl of the digested sample and 100 µL of the Hoechst dye solution were placed in each well in a 96-well microplate. The samples were read in a microplate reader at 360/460 nm excitation and emission spectrum. By

plugging these results into a standard curve obtained by testing standard concentrations of calf serum in the same assay, the cell count numbers were obtained by assuming that there are approximately 7.6 pg of DNA per cell.

3.4.3 Immunohistology:

Constructs were fixed in 4% paraformaldehyde, frozen in embedding medium, and cut into 7 μm sections using a cryotome. The sections were labeled for cardiomyocytes with myosin heavy chain (MHC) and α-actin. The nuclei were labeled with a Hoechst stain (DAPI). Connexin 43 (Cxn 43) was also labeled to determine how connected the cardiomyocytes were to each other. The images were viewed on an epifluorescent microscope (Olympus IX 81) to determine the alignment and connectivity of the cells.

3.4.4 Western Blotting:

Western blot analysis was also conducted on the constructs. The constructs were lysed using 30 seconds of sonication in ice-cold lysis buffer. A BCA total protein assay was used to determine the total protein of each lysate. Cell lysates with equal amount of total protein were separated with a denaturing SDS PAGE, and transferred to a nitrocellulose membrane where they were probed with antibodies. The antibodies were used to probe for the expression of β-actin, connexin 43 (Cxn 43), phosphorylated connexin 43 (pCxn 43), Akt, ERK, phosphorylated ERK (pERK), and calcineurin.

4. Results

4.1 Experiment Parameters

Over the course of this project, myocardial constructs were created and stimulated for a 2 week period four different times. Each of the experiments was run with a different set of parameters for the stimulation applied to the constructs. Figure #8 below defines all of the parameters for the various stimulations applied

Condition	Experiment #1	Experiment #2	Experiment #3	Experiment #4
Static	Left on Teflon molds	Left on Teflon molds	Left on Teflon molds	Left on Teflon molds
Mechanical	1 Hz, 50% duty cycle, **5% stretch**	1 Hz, 50% duty cycle, **5% stretch**	1 Hz, 50% duty cycle, **10 to 5% stretch**	1 Hz, 50% duty cycle, **7.5% stretch**
Electrical	1 Hz, 6 ms pulse, **4.80 V, Monophasic**	1 Hz, 6 ms pulse, **4.80 V, Biphasic**	1 Hz, 6 ms pulse, **2.40 V, Biphasic**	1 Hz, 6 ms pulse, **2.40 V, Biphasic**
EM Sync	No delay between stimulations	No delay between stimulations	No delay between stimulations	No delay between stimulations
EM Delay	**0.4 s delay** between stimulations	**0.49 s delay** between stimulations	**0.49 s delay** between stimulations	**0.45 s delay** between stimulations
EM Offset	None Run	None Run	None Run	**0.5 s delay** between stimulations

Figure #8 – A table defining all of the parameters of all of the experiments run. The major differences between the stimulations applied to the constructs in all of the experiments are highlighted in bold. Note that not all conditions were run in each experiment.

Constructs used in the static condition were not stimulated at all and were left on the Teflon molds. Constructs used in the mechanical condition were placed around the latex tubing of mechanical bioreactors and stretched at a frequency of 1 Hz for 0.5 seconds (50 % duty cycle) at various percent strains. Constructs used in the electrical

condition were placed around the Teflon rod of electrical bioreactors and electrically paced at a frequency of 1 Hz for 6 milliseconds with voltages. Constructs used in the electromechanical synchronized (EM sync) condition were placed around the latex tubing of electromechanical bioreactors and stimulated with both the mechanical and electrical stimulation from the corresponding experiment at the same time. Constructs used in the electromechanical delayed (EM delay) condition were placed around the latex tubing of electromechanical bioreactors and stimulated with both the mechanical and electrical stimulation from the corresponding experiment at various delay times. Constructs used in the electromechanical offset (EM offset) condition were placed around the latex tubing of electromechanical bioreactors and stimulated with both the mechanical and electrical stimulation from the corresponding experiment at a delay time of 0.5 seconds (completely offset).

4.2 Contraction Force

The contraction force measurements of each construct were taken using a force transducer and a custom made MATLAB program. These measurements were taken to understand exactly how strong the cardiomyocytes in the constructs were after stimulation. Figure #9 on the next page compares the mean twitch forces of the constructs from each stimulation condition in every experiment.

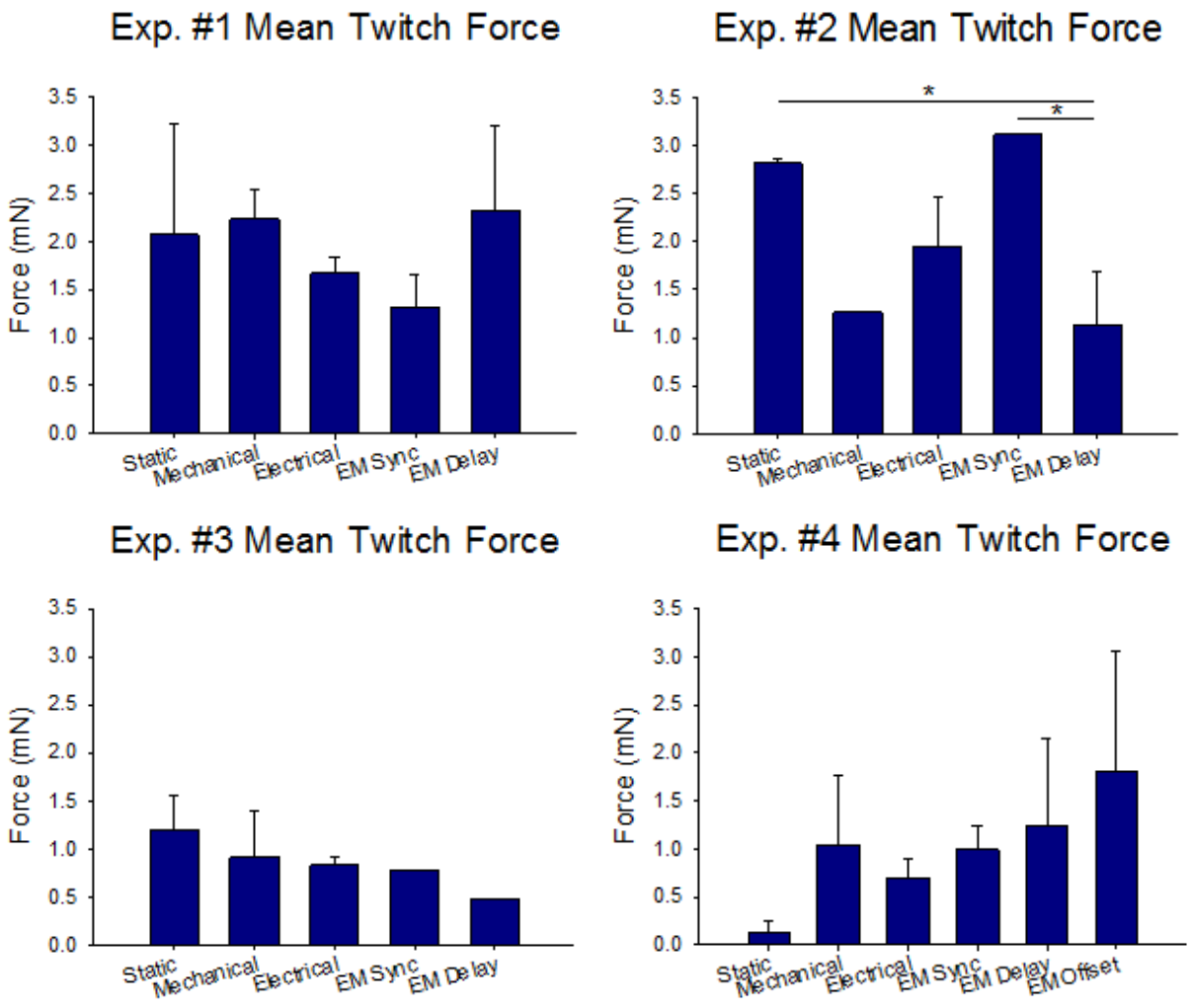

Figure #9 – A series of graphs comparing the twitch force of the constructs from each stimulation condition in every experiment run throughout the project.

The twitch forces of the constructs from experiments #1 and #2 are much higher than the twitch forces from experiments #3 and #4. The twitch forces of the constructs from experiments #3 and #4 are all very similar and show few differences. Constructs from experiment #4 also experienced a contamination. The twitch forces of the constructs from experiments #1 and #2 show some differences. In experiment #2, the mean twitch force of the EM delay condition is statistically significant when compared to the EM sync and static conditions, but the EM sync condition has an n=1. The mean

twitch force of the electrical condition in experiment #2 is also higher than the mean twitch force of the mechanical condition. In experiment #1, the mean twitch force of the mechanical condition is higher than the mean twitch force of the electrical condition and the mean twitch force of the EM delay condition is higher than the mean twitch force of the EM sync condition. The mean twitch force of the electrical condition in experiment #2 is higher than the mean twitch force of the electrical condition in experiment #1.

The contraction force measurements taken by the force transducer and the custom made MATLAB program included contraction time. Using the twitch force and the contraction time, one can calculate the contraction rate of the constructs by dividing the twitch force by the contraction time. Figure #10 on the next page compares the mean contraction rates of the constructs from each stimulation condition in every experiment.

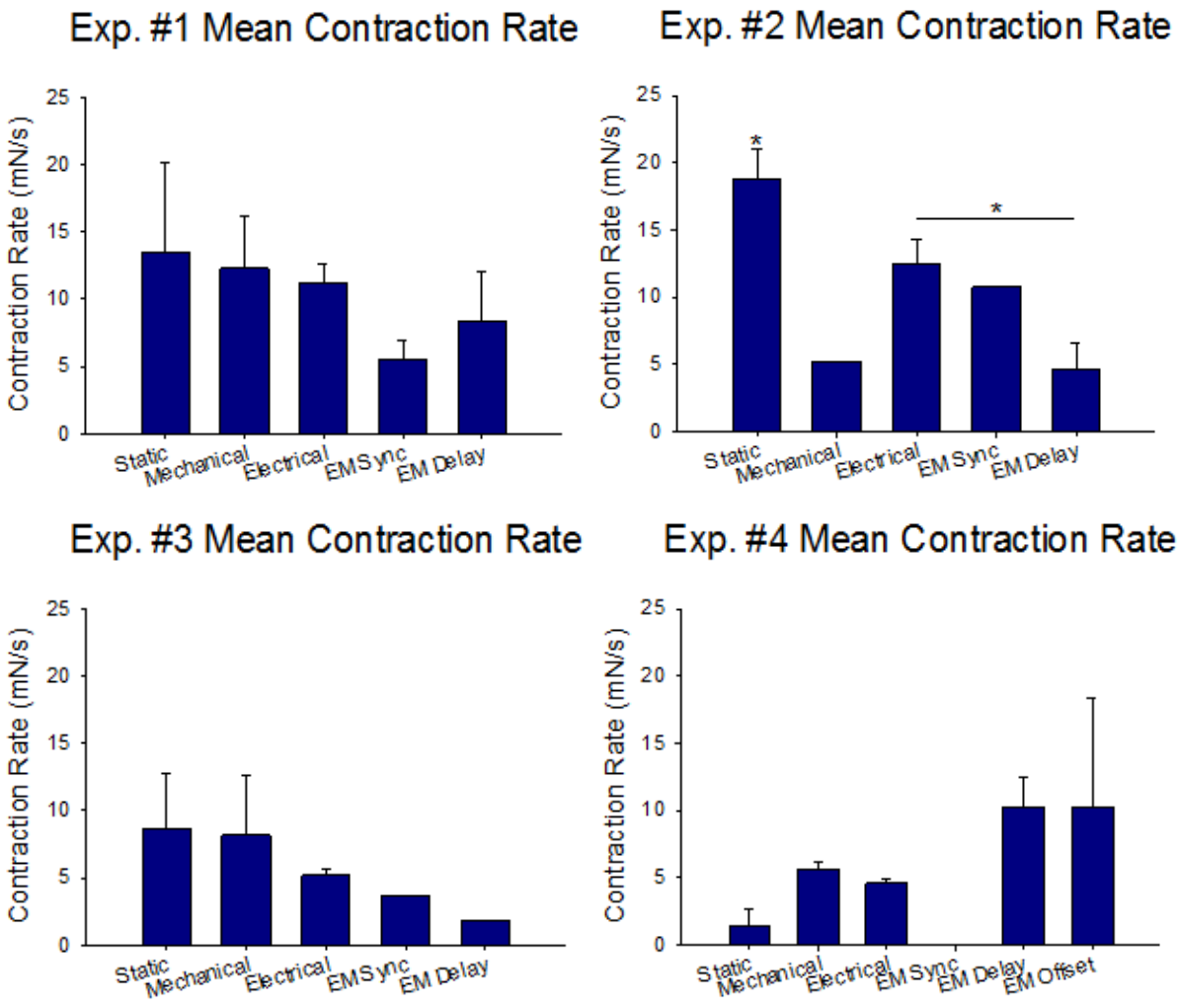

Figure #10 - A series of graphs comparing the mean contraction rate of the constructs from each stimulation condition in every experiment run throughout the project.

The contraction rates of the constructs from experiments #1 and #2 are much higher than the contraction rates from experiments #3 and #4. The contraction rates of the constructs from experiments #3 and #4 are all very similar and show few differences. Constructs from experiment #4 also experienced contamination. The contraction rates of the constructs from experiments #1 and #2 show some differences. In experiment #2, the mean contraction rate of the electrical condition is statistically significant when compared to the EM delay condition and the static condition is

statistically significant when compared to all other conditions. Although the mean contraction rate of the constructs from the EM sync condition is higher than the mean contraction rate of the constructs from the EM delay condition, it should be noted that the EM sync condition has an n=1. The mean contraction rate of the electrical condition in experiment #2 is also higher than the mean contraction rate of the mechanical condition. In experiment #1, the mean contraction rate of the mechanical condition is higher than the mean contraction rate of the electrical condition and the mean contraction rate of the EM delay condition is higher than the mean contraction rate of the EM sync condition. The mean contraction rate of the electrical condition in experiment #2 is similar to the mean contraction rate of the electrical condition in experiment #1.

The contraction force measurements taken by the force transducer and the custom made MATLAB program included relax time. Using the twitch force and the relax time, one can calculate the relax rate of the constructs by dividing the twitch force by twice the relax time. Figure #11 on the next page compares the mean relax rates of the constructs from each stimulation condition in every experiment.

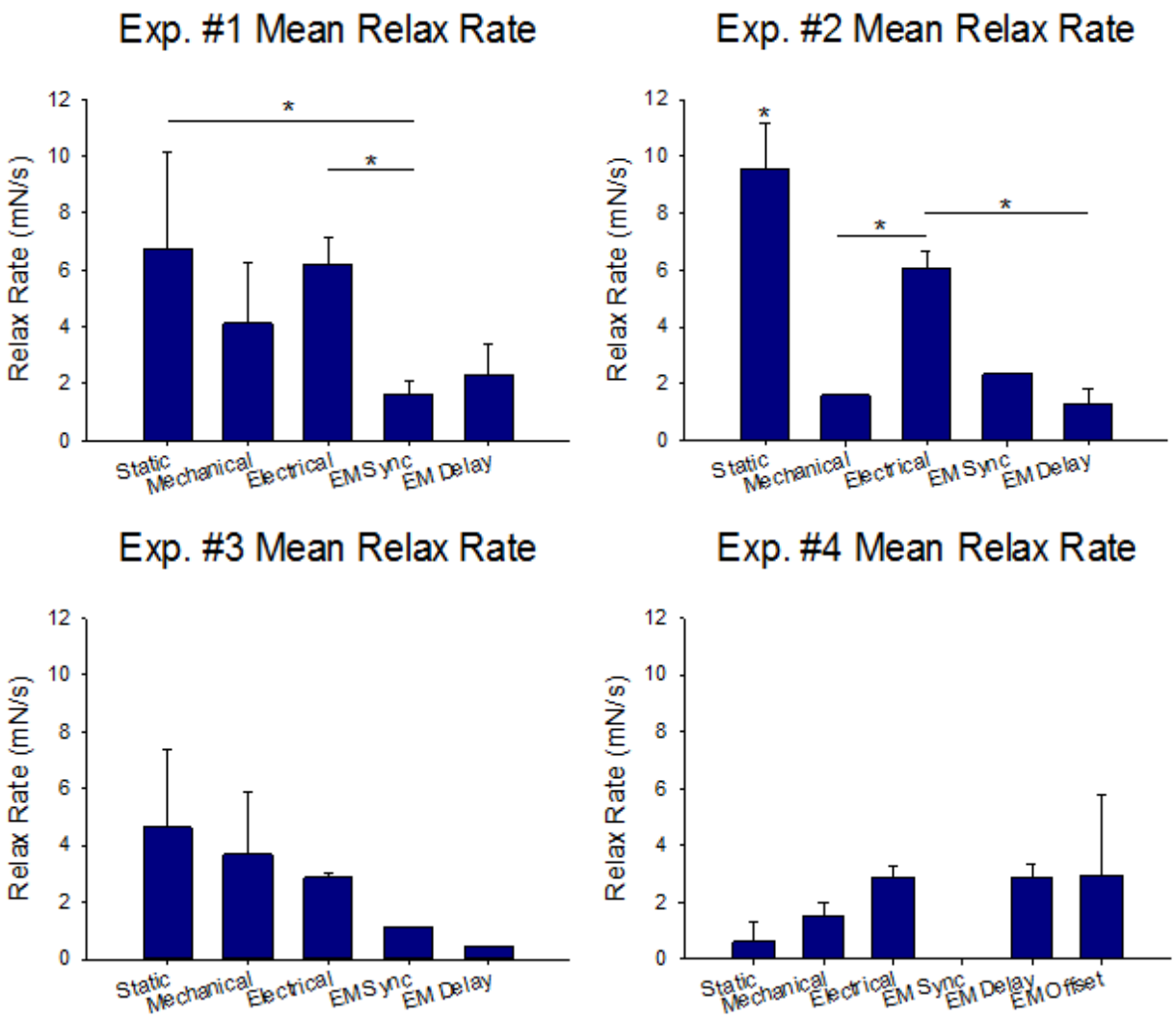

Figure #11 - A series of graphs comparing the mean contraction rate of the constructs from each stimulation condition in every experiment run throughout the project.

The relax rates of the constructs from experiments #1 and #2 are much higher than the relax rates from experiments #3 and #4. The relax rates of the constructs from experiments #3 and #4 are all very similar and show few differences. Constructs from experiment #4 also experienced contamination. The relax rates of the constructs from experiments #1 and #2 show some differences. In experiment #2, the mean relax rate of the electrical condition is statistically significant when compared to the mechanical and EM delay conditions. The static condition is also statistically significant when compared

to all other conditions. Although the mean relax rate of the constructs from the EM sync condition is higher than the mean relax rate of the constructs from the EM delay condition, it should be noted that the EM sync condition has an n=1. The mean relax rate of the electrical condition in experiment #2 is also higher than the mean relax rate of the mechanical condition. In experiment #1, the mean relax rate of the EM sync condition is statistically significant when compared to the static and electrical conditions .In experiment #1, the mean relax rate of the electrical condition is higher than the mean relax rate of the mechanical condition and the mean relax rate of the EM delay condition is higher than the mean relax rate of the EM sync condition. The mean relax rate of the electrical condition in experiment #2 is similar to the mean relax rate of the electrical condition in experiment #1.

4.3 DNA Quantification

In order to get more information about the viability and the strength of the constructs, the amount of DNA in each construct was quantified to calculate the number of cells in each constructs. By calculating the concentration of DNA that was in the construct via a modified Hoechst assay, multiplying by the volume of the construct that was used, and assuming that there is 7.6 picograms in a cell, one can get a cell count. Figure #12 on the next page compares the mean amount of cells in each construct from each condition in every experiment.

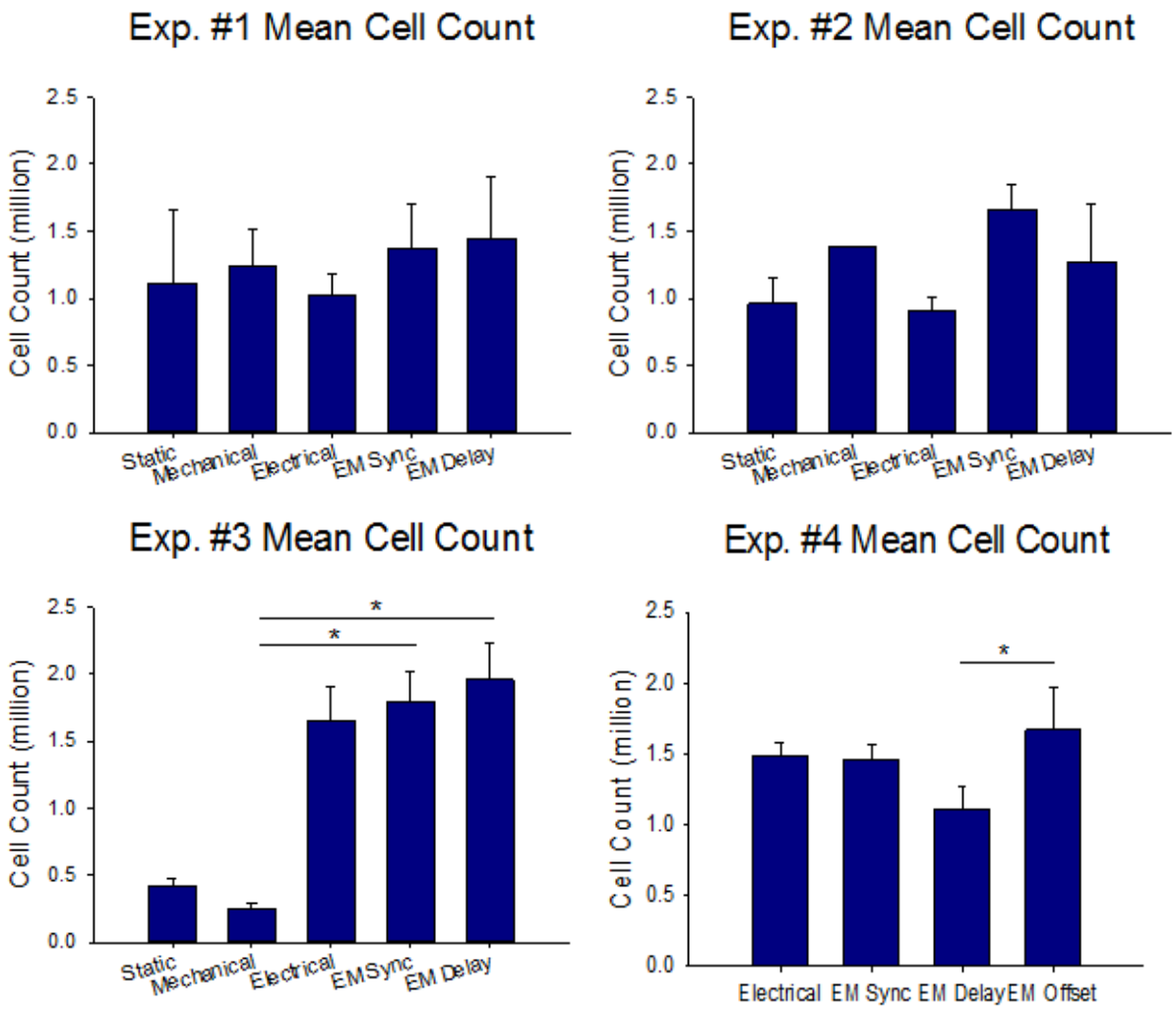

Figure #12 – A series of graphs that compare the mean amount of cells in each construct from each condition in every experiment throughout the project.

The number of cells in each construct in experiments #1 and #2 are all very similar and show little difference. There is no statistically significant data in these experiments. Experiments #3 and #4, however, do have statistically significant data. In experiment #3, the mean cell count of the constructs in the EM sync and EM delay conditions are statistically significant when compared to the mean cell count of the constructs from the mechanical condition. In experiment #4, the mean cell count of the constructs in the EM offset condition is statistically significant when compared to the

mean cell count of the constructs from the EM delay condition. Constructs in experiment #4 experienced a contamination and the static and mechanical constructs all died before they could be analyzed this far.

The cell count of the constructs could also be compared by the order in which they were made (or batch number) to test the quality of the cell isolation and construct creation process. Figure #13 below compares the mean amount of cells in each construct in every experiment according to the order in which they were made.

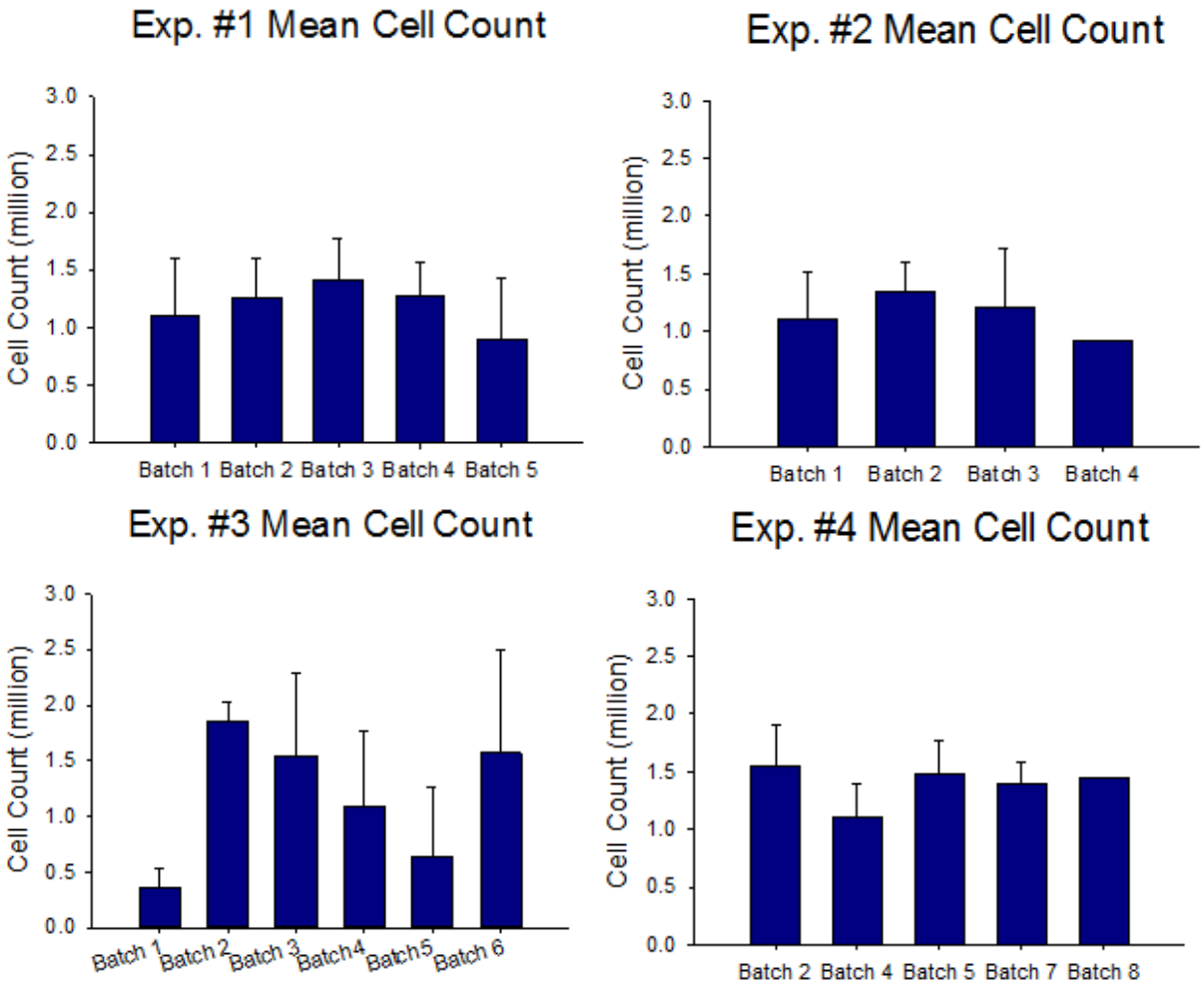

Figure #13 - A series of graphs that compare the mean amount of cells in every experiment throughout the project according to the order in which they were made.

The number of cells in each construct in experiments #1 and #2 are all very similar and show little difference. There is no statistically significant data in these experiments. Although there is no statistically significant data, experiments #3 displays a large range of cells in each batch. In experiment #3, the mean cell count of the constructs made in batch 1 and batch 5 are much lower when compared to the mean cell count of the other construct. In experiment #4, the mean cell counts of the constructs are all similar. Constructs in experiment #4, however, experienced a contamination and the static and mechanical constructs all died before they could be analyzed this far.

The mean cell count of the constructs is also useful when compared to the constructs' mean twitch force. By dividing the mean twitch force by the mean cell count for each condition, one can get the mean force per cell for each stimulation condition. Figure #14 on the next page compares the mean force per cell of the constructs from each stimulation condition in every experiment.

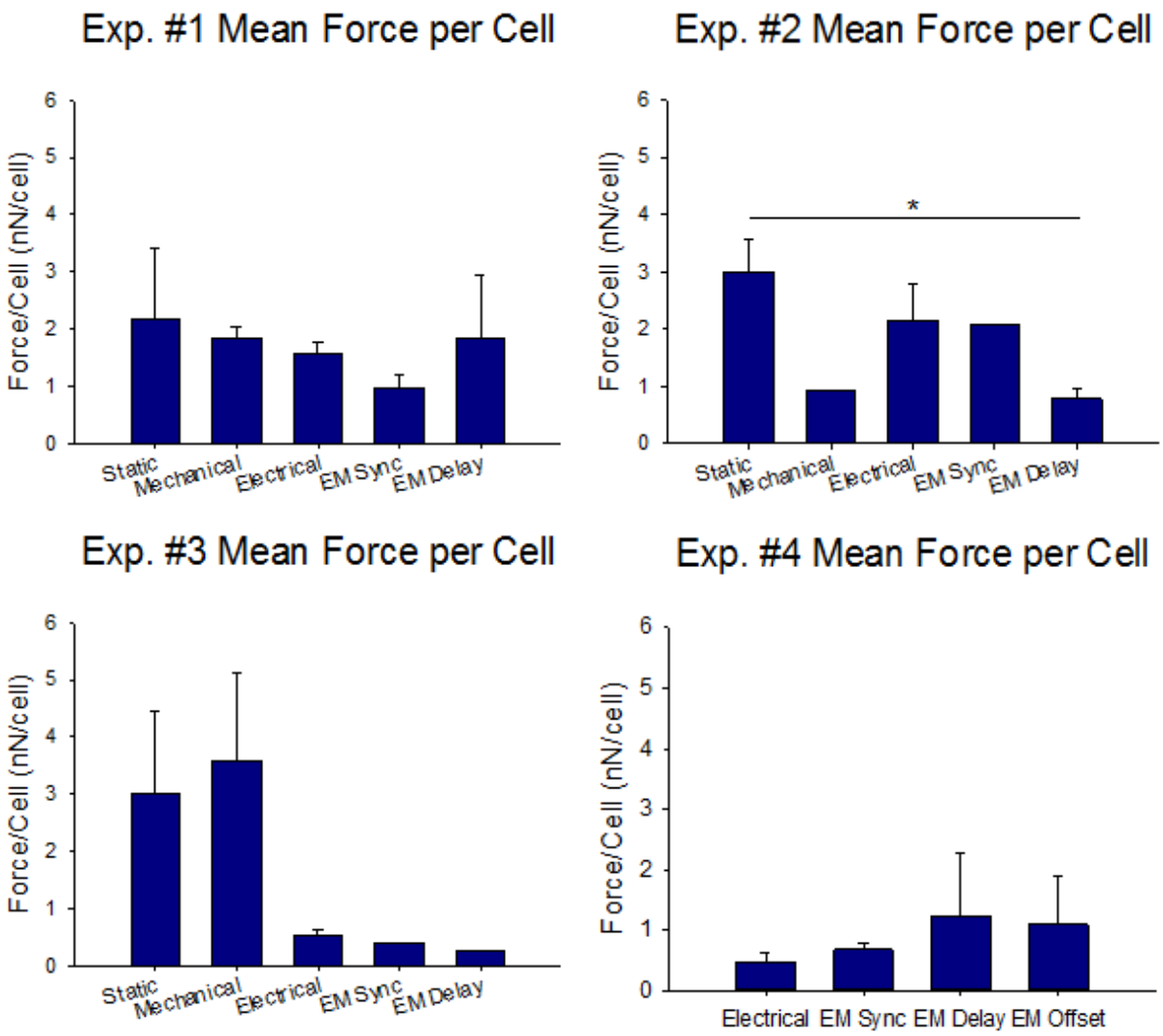

Figure #14 – A series of graphs that compare the mean force per cell of the constructs from each stimulation condition in every experiment throughout the project.

The mean force per cell of the constructs from experiments #1 and #2 are much higher than the mean force per cell from experiments #3 and #4. The mean force per cell of the constructs from experiments #3 and #4 are all very similar and show few differences. Constructs from the static and mechanical conditions in experiment #3 have very high force per cell measurements because of low cell counts, not high twitch forces. Constructs from experiment #4 experienced a contamination. The mean force per cell of the constructs from experiments #1 and #2 shows some differences. In

experiment #2, the mean force per cell of the static condition is statistically significant when compared to the EM delay condition. The mean force per cell of the electrical condition in experiment #2 is also higher than the mean force per cell of the mechanical condition. Although the EM Sync condition has a higher mean force per cell than the EM delay condition, it must be noted that the EM sync condition has an n=1. In experiment #1, the mean force per cell of the mechanical condition is higher than the mean force per cell of the electrical condition and the mean force per cell of the EM delay condition is higher than the mean force per cell of the EM sync condition. The mean force per cell of the electrical condition in experiment #2 is higher than the mean force per cell of the electrical condition in experiment #1.

4.4 Immunohistology

In order to get more information about the viability and the strength of the constructs, the histology of the cells in the constructs was analyzed to look for cell alignment and cell connectivity. Antibodies were used to probe for protein markers MHC, Cxn 43, and α-actin. MHC and α-actin are both cardiac specific structural proteins that are involved with muscle physiology. Cxn 43 is a membrane protein that is involved in cell communication. By seeing if the cells in a construct were more aligned via MHC and α-actin or connected via Cxn 43, one could make better decisions on why certain constructs had higher twitch forces. Figure #15 on the next page compares the histology of representative constructs from each stimulation condition in experiment #1.

Immunohistology – MHC and Cxn 43

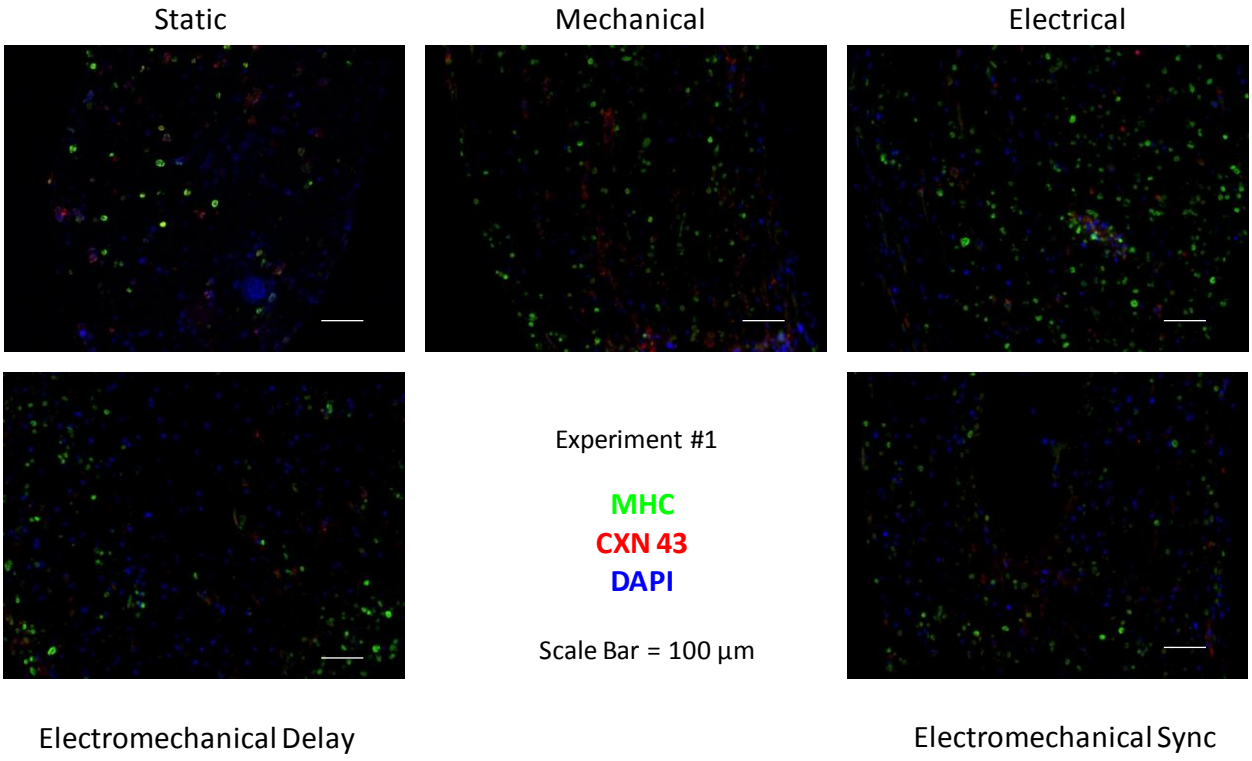

Figure #15 – A series of pictures comparing the histology of representative constructs from each stimulation condition in experiment #1. The scale bar in each image equals 100 microns. The green stain is MHC, the red stain is Cxn 43, and the blue stain is DAPI.

All of the constructs from each stimulation condition in experiment #1 are populated with nuclei as seen by the blue stain. All of the electrically stimulated constructs seem to have a more uniform distribution of nuclei throughout the construct than non-electrically stimulated constructs. MHC is expressed as the bright green dots and the faint green lines seen in all of the constructs. Cxn 43 is expressed as the small red dots seen in all of the constructs, but the mechanically stimulated construct seems to express more Cxn 43.

Figure #16 below compares the histology of representative constructs from each stimulation condition in experiment #2.

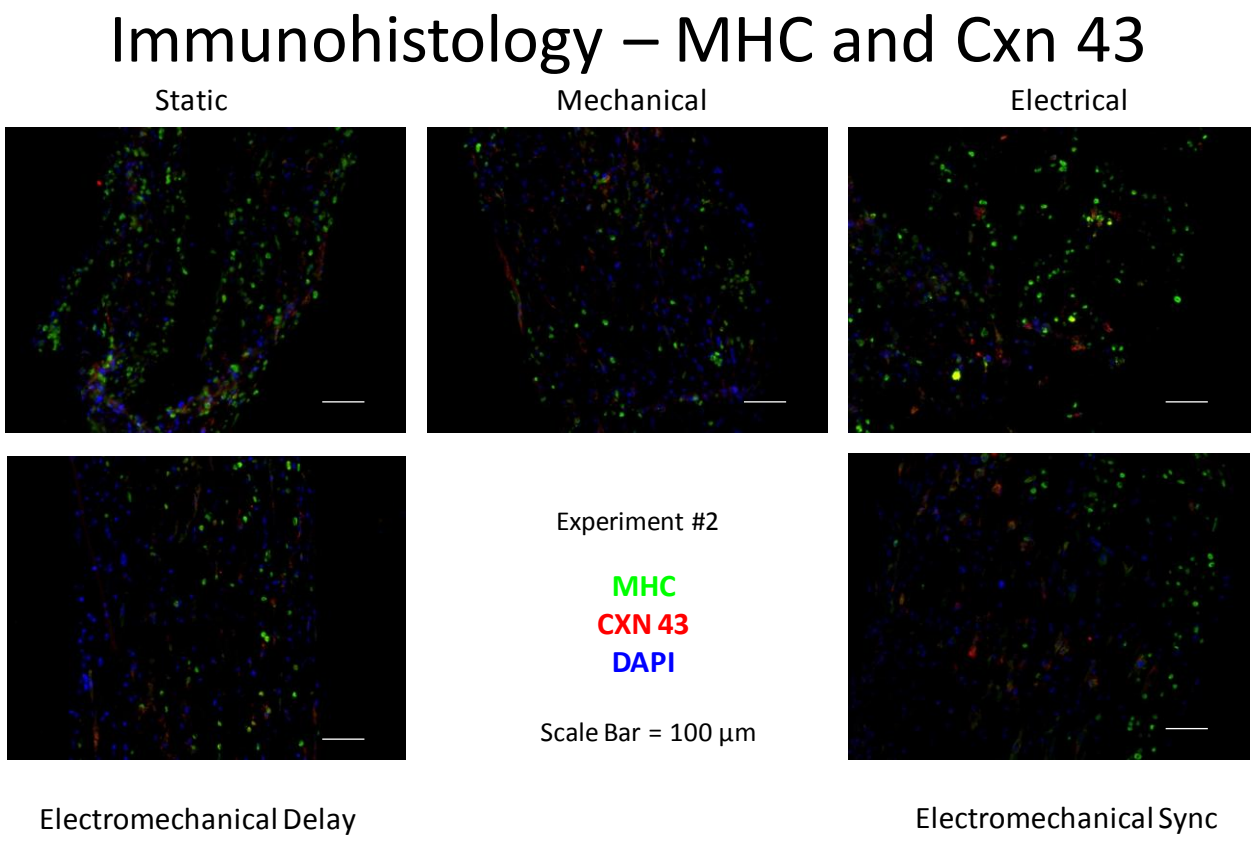

Figure #16 - A series of pictures comparing the histology of representative constructs from each stimulation condition in experiment #2. The scale bar in each image equals 100 microns. The green stain is MHC, the red stain is Cxn 43, and the blue stain is DAPI.

The constructs from experiment #2 are similar to those from experiment #1. All of the constructs from each stimulation condition in experiment #2 are populated with nuclei as seen by the blue stain. All of the electrically stimulated constructs seem to have a more uniform distribution of nuclei throughout the construct than non-electrically stimulated constructs. MHC is expressed as the bright green dots and the faint green lines seen in all of the constructs. Cxn 43 is expressed as the small red dots seen in all

of the constructs, but the mechanically stimulated construct seems to express more Cxn 43.

Figure #17 below compares the histology of representative constructs from each stimulation condition in experiments #1.

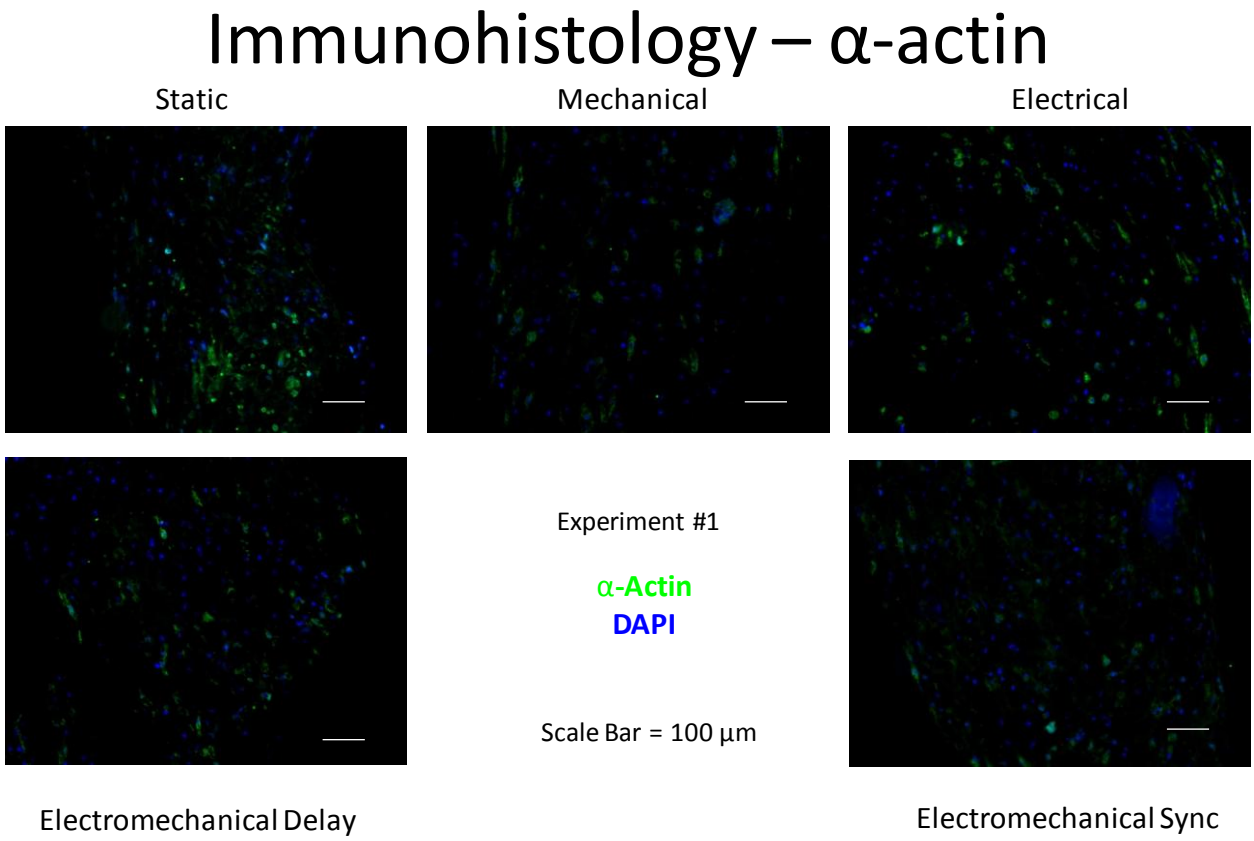

Figure #17 - A series of pictures comparing the histology of representative constructs from each stimulation condition in experiment #1. The scale bar in each image equals 100 microns. The green stain is α-actin and the blue stain is DAPI.

All of the constructs from each stimulation condition in experiment #1 are populated with nuclei as seen by the blue stain. All of the electrically stimulated constructs seem to have a more uniform distribution of nuclei throughout the construct than non-electrically stimulated constructs. α-actin is expressed as the bright green lines

seen in all of the constructs. Although α-actin is expressed in all of the constructs, it seems that the α-actin is more aligned in electrically stimulated constructs.

Figure #18 on the next page compares the histology of representative constructs from each stimulation condition in experiments #2.

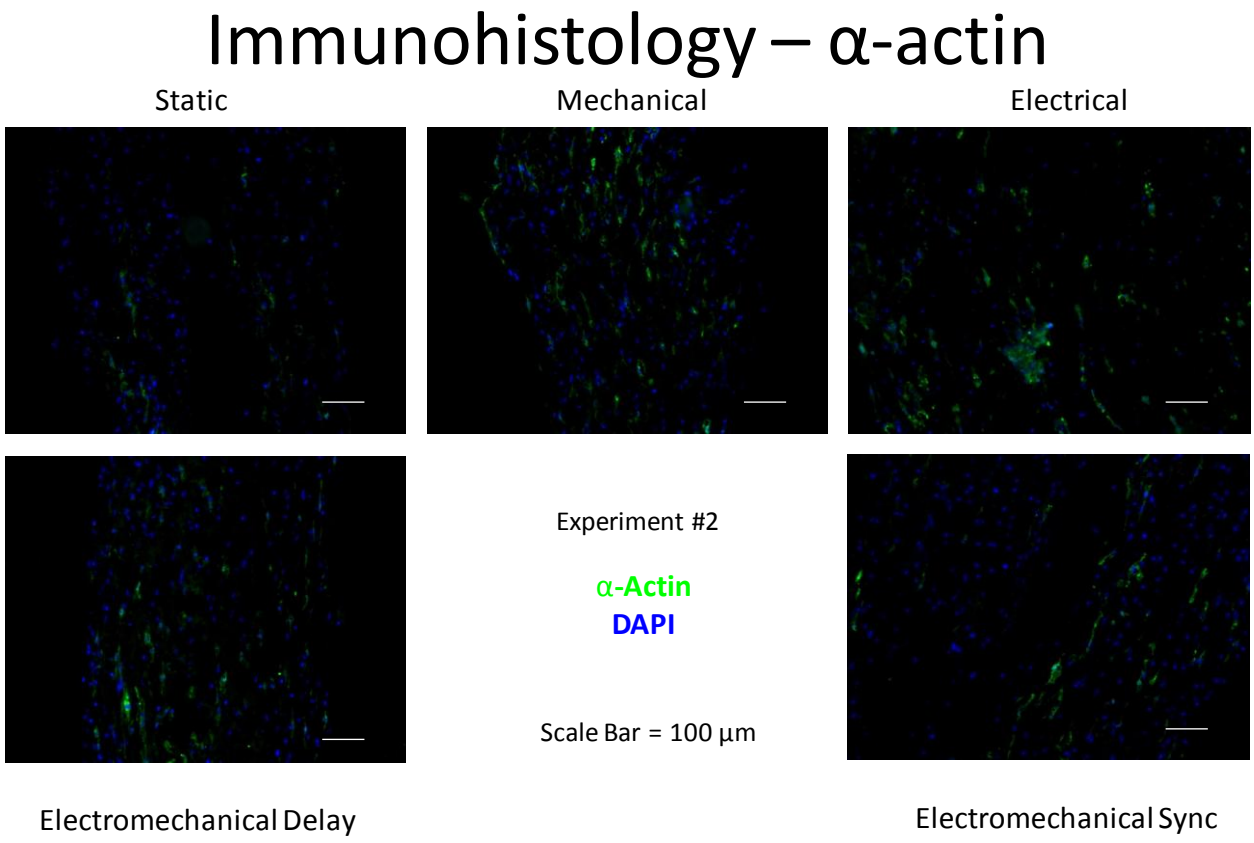

Figure #18 - A series of pictures comparing the histology of representative constructs from each stimulation condition in experiment #2. The scale bar in each image equals 100 microns. The green stain is α-actin and the blue stain is DAPI.

The constructs from experiment #2 are similar to those from experiment #1. All of the constructs from each stimulation condition in experiment #2 are populated with nuclei as seen by the blue stain. All of the electrically stimulated constructs seem to have a more uniform distribution of nuclei throughout the construct than non-electrically

stimulated constructs. α-actin is expressed as the bright green lines seen in all of the constructs. Although α-actin is expressed in all of the constructs, it seems that the α-actin is more aligned in electrically stimulated constructs.

4.5 Western Blotting

In order to get more information about the cell alignment and cell connectivity of the constructs, a Western blot analysis of all the constructs from experiments #1 and #2 was carried out. Figure #19 below shows a standard β-actin blot as a reference point.

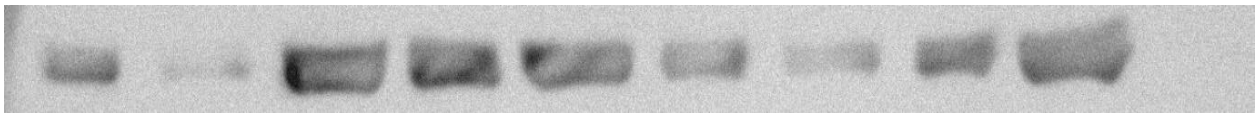

Figure #19 – A picture of a standard β-actin blot. It is shown so as only to serve as a reference.

Antibodies were used to probe for protein markers β-actin, Cxn 43, and pCxn 43. β-actin is a standard structural protein found in most cells. Cxn 43, as mentioned earlier is a membrane protein that is involved in cell communication. pCxn 43 is a less functionalized from of Cxn 43 which is less useful for cell communication. By normalizing Cxn 43 and pCxn 43 to β-actin and comparing them to each other, one can make better assumptions on how connected cells in the constructs are. Figure #20 on the next page compares the expression of β-actin, Cxn 43, and pCxn 43 of the constructs from each stimulation condition in experiment #1.

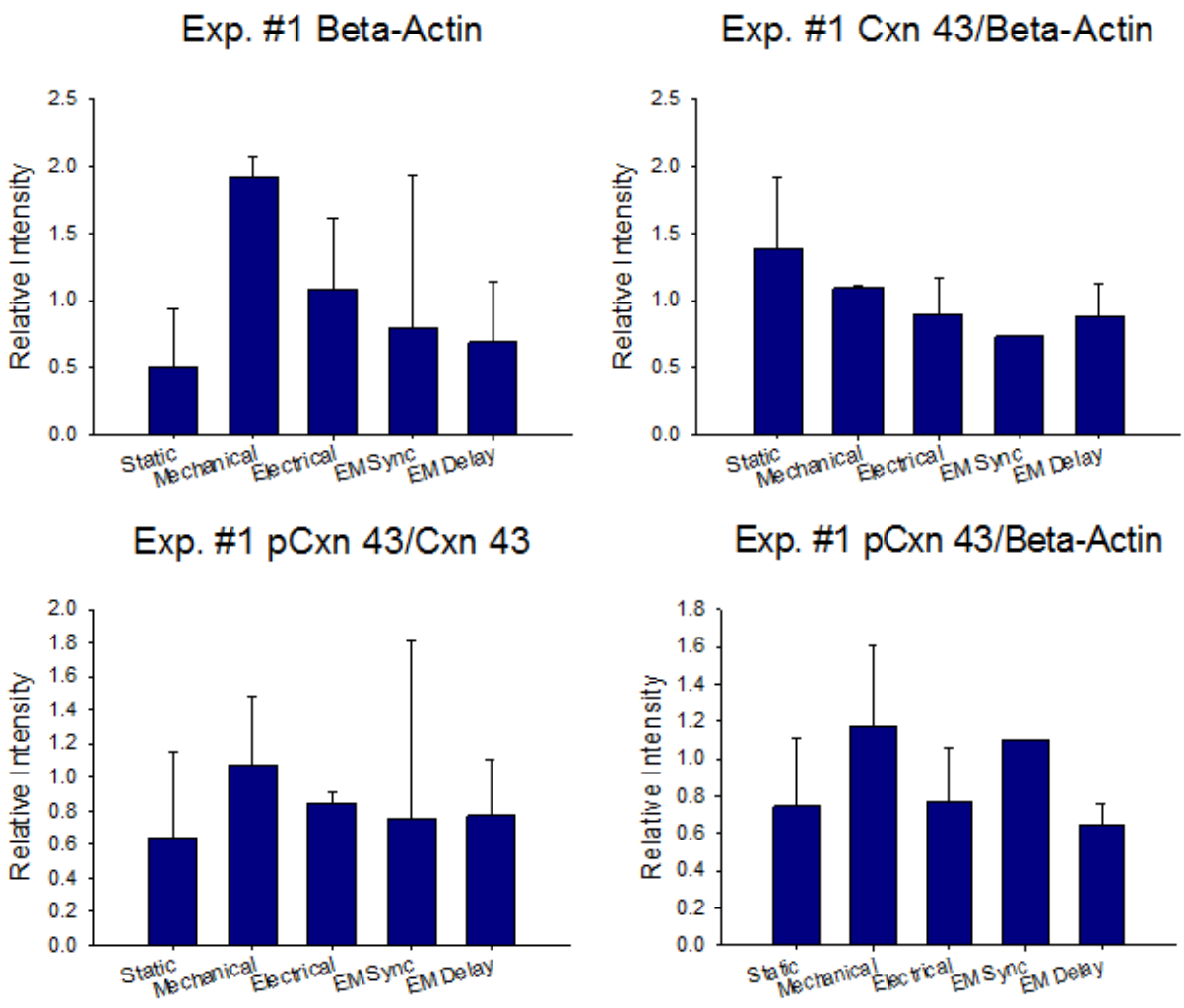

Figure #20 – A series of graphs that compare the expression of β-actin, Cxn 43, and pCxn 43 of the constructs from each stimulation condition in experiment #1.

The expression of β-actin, Cxn 43, and pCXN 43 is variable and does not seem to be following any trends in experiment #1. All of the constructs expressed the same amount of Cxn 43. Constructs from the mechanical and EM sync conditions expressed higher levels of pCxn 43 than the other stimulation conditions. When pCxn 43 was compared to Cxn 43, only constructs from the mechanical stimulation showed elevated levels of expression.

Antibodies were used to probe for protein markers Akt, calcineurin, ERK, and pERK. These proteins are all markers of muscular hypertrophy. By normalizing Akt, calcineurin, ERK, and pERK to β-actin and comparing them to each other, one can make better assumptions about their relative expression. Figure #21 below compares the expression of Akt, calcineurin, ERK, and pERK of the constructs from each stimulation condition in experiment #1.

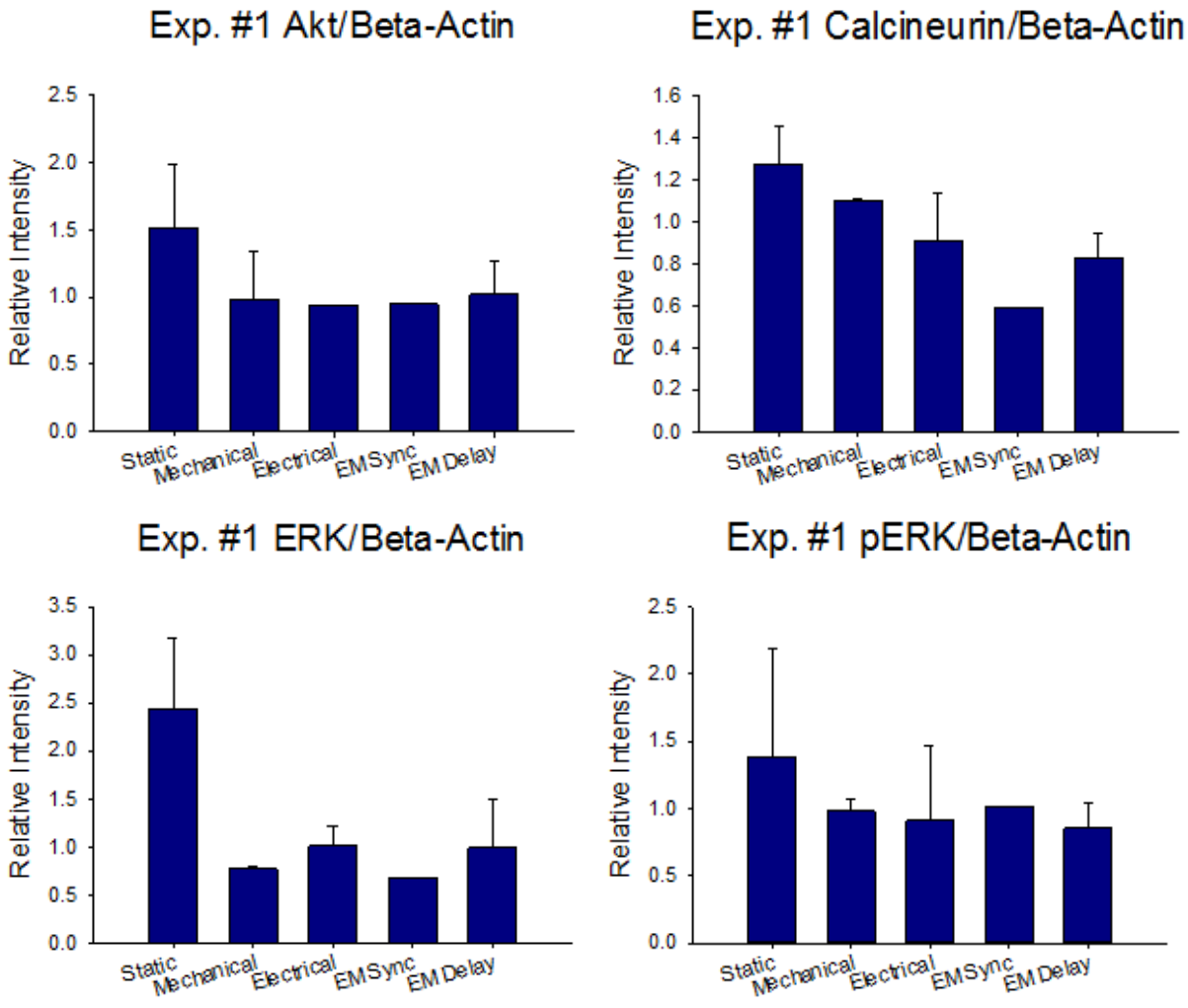

Figure #21 - A series of graphs that compare the expression of Akt, calcineurin, ERK, and pERK of the constructs from each stimulation condition in experiment #1.

The expression of Akt, calcineurin,ERK, and pERK is variable and does not seem to be following any trends in experiment #1. The static constructs expressed the highest levels of Akt, calcineurin, ERK, and pERK. The rest of the conditions all expressed similar, lower levels of AKT, ERK, and pERK. Although the rest of the conditions differed in their expression of calcineurin, they were all lower than the static controls.

By normalizing Akt, calcineurin, ERK, and pERK to β-actin and comparing them to each other, one can make better assumptions about how they affect muscular hypertrophy. Figure #22 on the next page compares the expression and ratios of Akt, calcineurin, ERK, and pERK of the constructs from each stimulation condition in experiment #1.

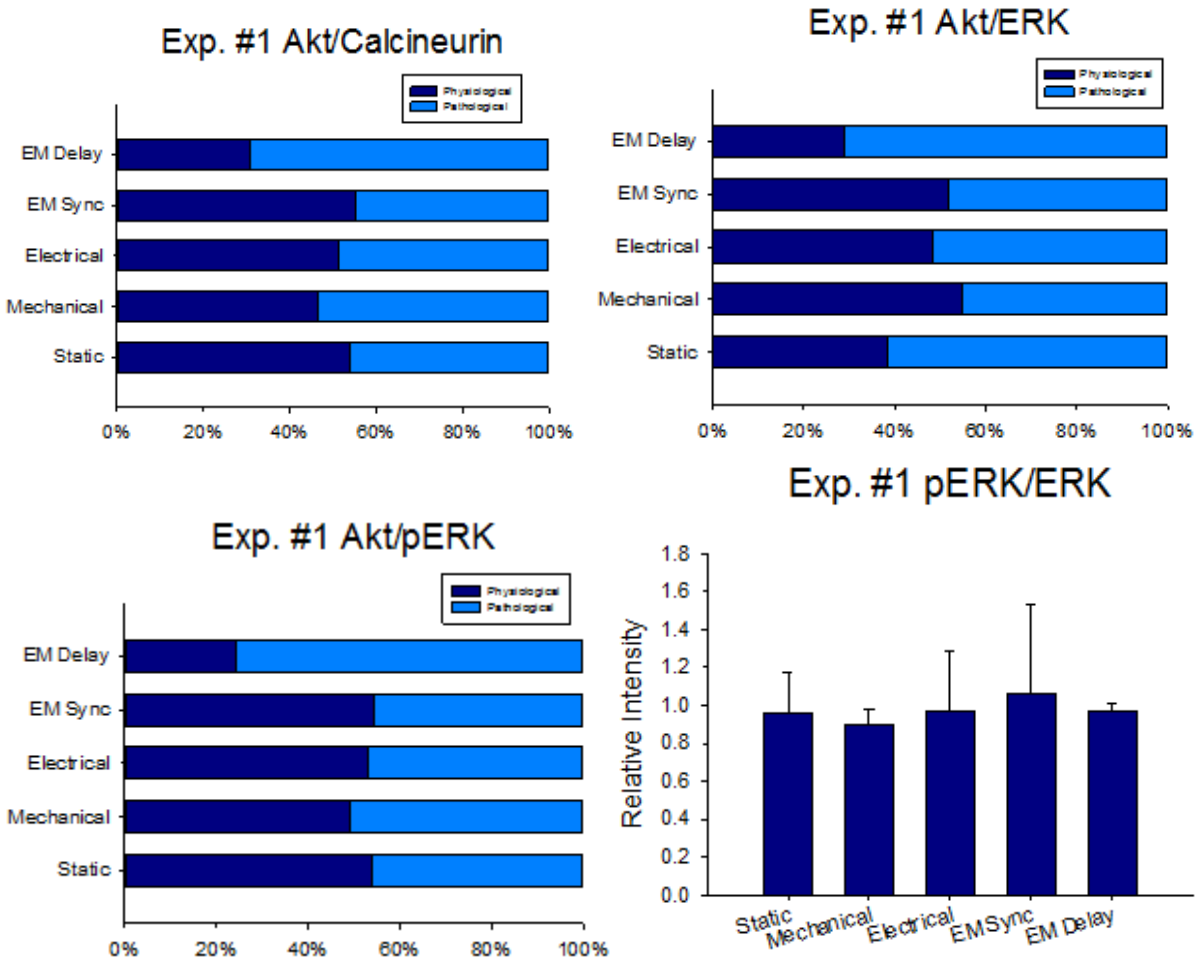

Figure #22 - A series of graphs that compare the expression and ratios of Akt, calcineurin, ERK, and pERK of the constructs from each stimulation condition in experiment #1.

The expression of Akt/calcineurin, Akt/ERK, Akt/pERK, and pERK/ERK is variable and does not seem to be following any trends in experiment #1. The results of this experiment are incorrect due to what may be procedural errors. Further investigation is needed to analyze these results

Antibodies were used to probe for protein markers β-actin, Cxn 43, and pCxn 43. β-actin is a standard structural protein found in most cells. Cxn 43, as mentioned earlier is a membrane protein that is involved in cell communication. pCxn 43 is a less functionalized from of Cxn 43 which is less useful for cell communication. By

normalizing Cxn 43 and pCxn 43 to β-actin and comparing them to each other, one can make better assumptions on how connected cells in the constructs are. Figure #23 below compares the expression of β-actin, Cxn 43, and pCxn 43 of the constructs from each stimulation condition in experiment #2.

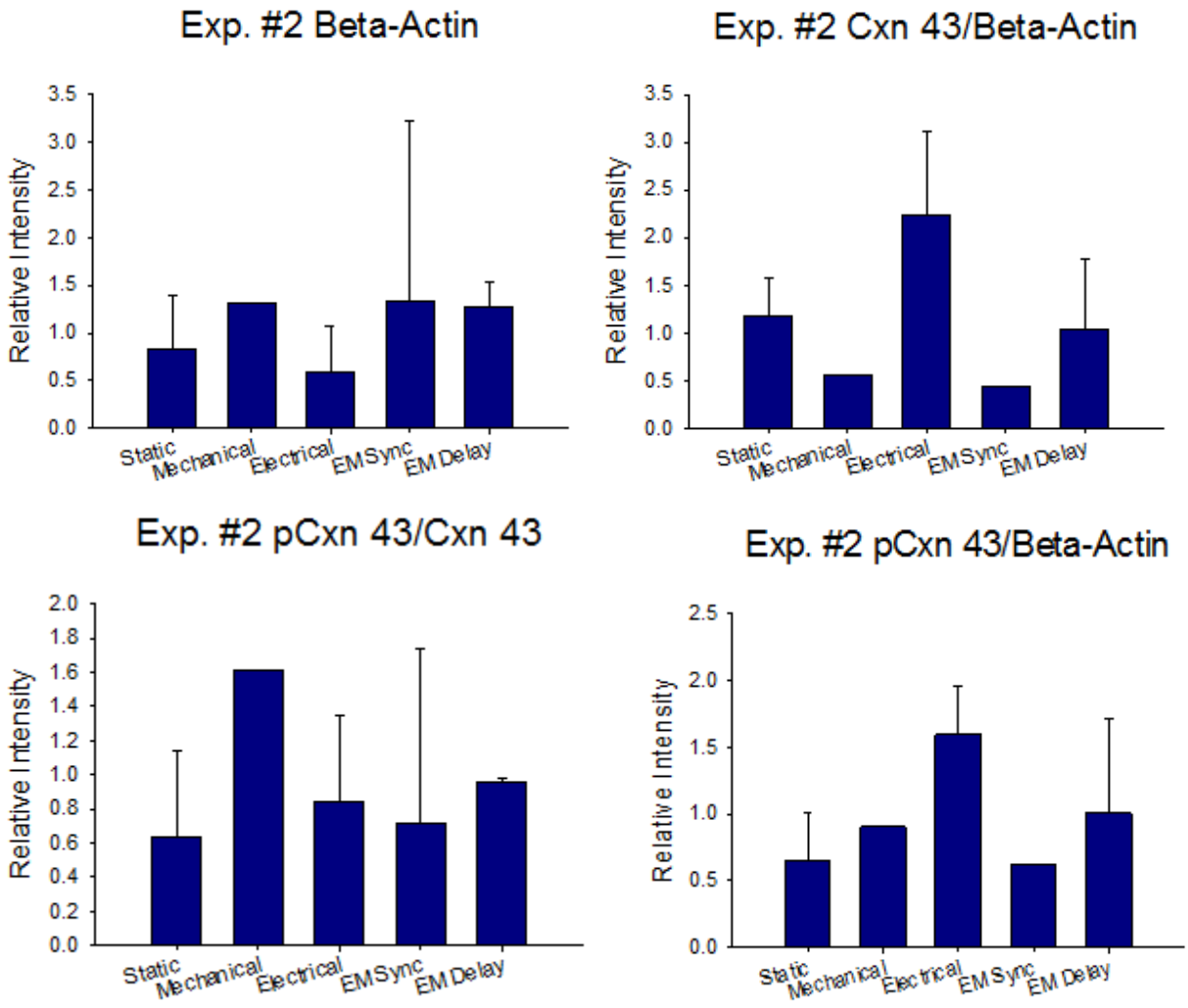

Figure #23 - A series of graphs that compare the expression and ratios of β-actin, Cxn 43, and pCxn 43 of the constructs from each stimulation condition in experiment #2.

The expression of β-actin, Cxn 43, and pCXN 43 is variable in experiment #2, but it does seem to be following some trends. The constructs from the electrical condition expressed an elevated amount of Cxn 43 when compared to the static constructs. The

mechanical and EM sync constructs expressed a lower level of Cxn 43 than the static constructs and the EM delay constructs expressed a similar level of Cxn 43 when compared to static constructs. Constructs from the electrical and EM delay conditions expressed higher levels of pCxn 43 than the static constructs while the mechanical and EM sync constructs expressed similar levels of pCxn 43 when compared to the static constructs. When pCxn 43 was compared to Cxn 43, only constructs from the mechanical stimulation showed elevated levels of expression.

Antibodies were used to probe for protein markers Akt, calcineurin, ERK, and pERK. These proteins are all markers of muscular hypertrophy. By normalizing Akt, calcineurin, ERK, and pERK to β-actin and comparing them to each other, one can make better assumptions about their relative expression. Figure #24 on the next page compares the expression of Akt, calcineurin, ERK, and pERK of the constructs from each stimulation condition in experiment #2.

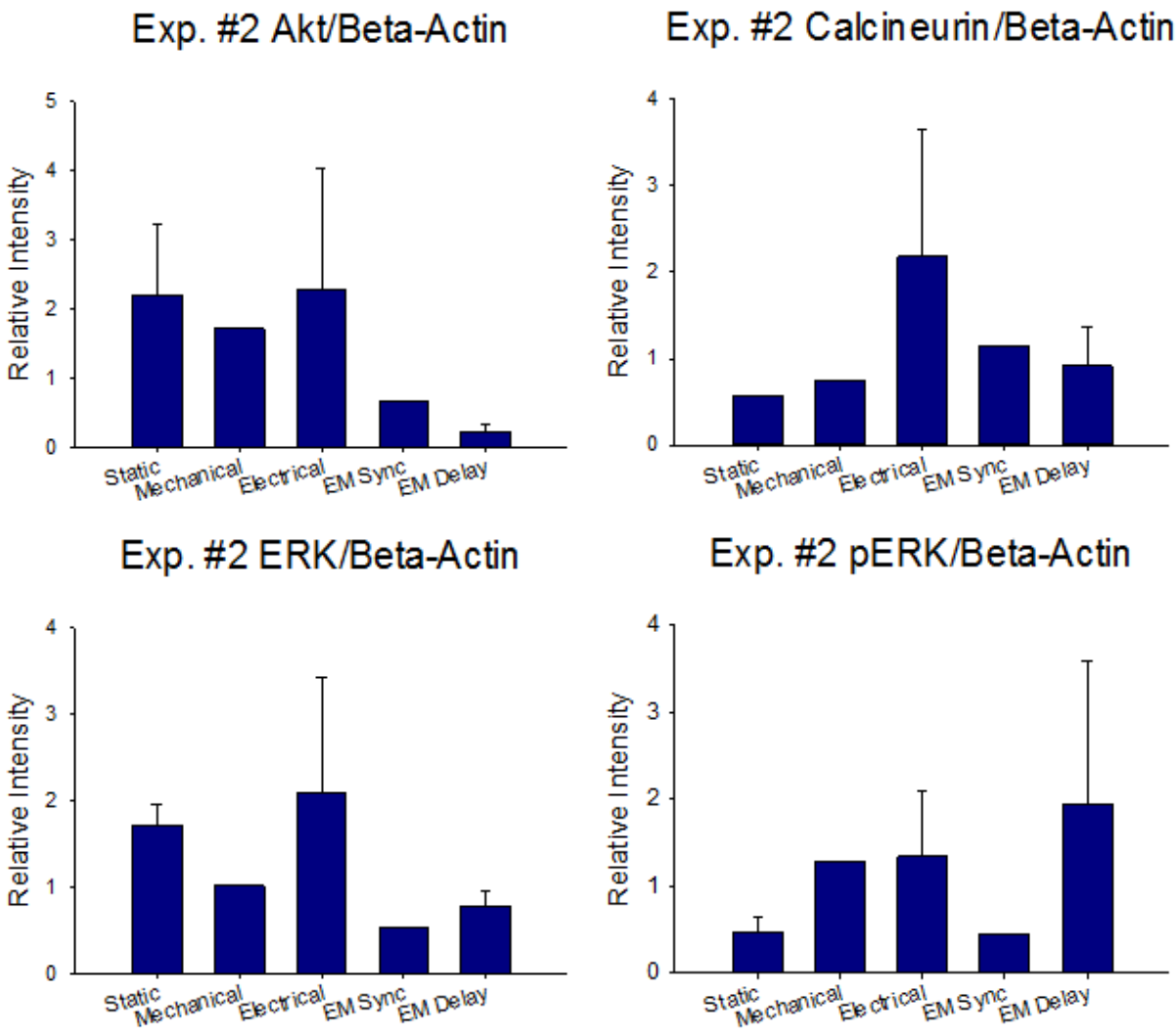

Figure #24 - A series of graphs that compare the expression of Akt, calcineurin, ERK, and pERK of the constructs from each stimulation condition in experiment #2.

The expression of Akt, calcineurin,ERK, and pERK is variable and does not seem to be following any trends in experiment #2. The results of this set of experiments are so variable it is not useful to write any observations.

By normalizing Akt, calcineurin, ERK, and pERK to β-actin and comparing them to each other, one can make better assumptions about how they affect muscular hypertrophy. Figure #25 on the next page compares the expression and ratios of Akt,

calcineurin, ERK, and pERK of the constructs from each stimulation condition in experiment #2.

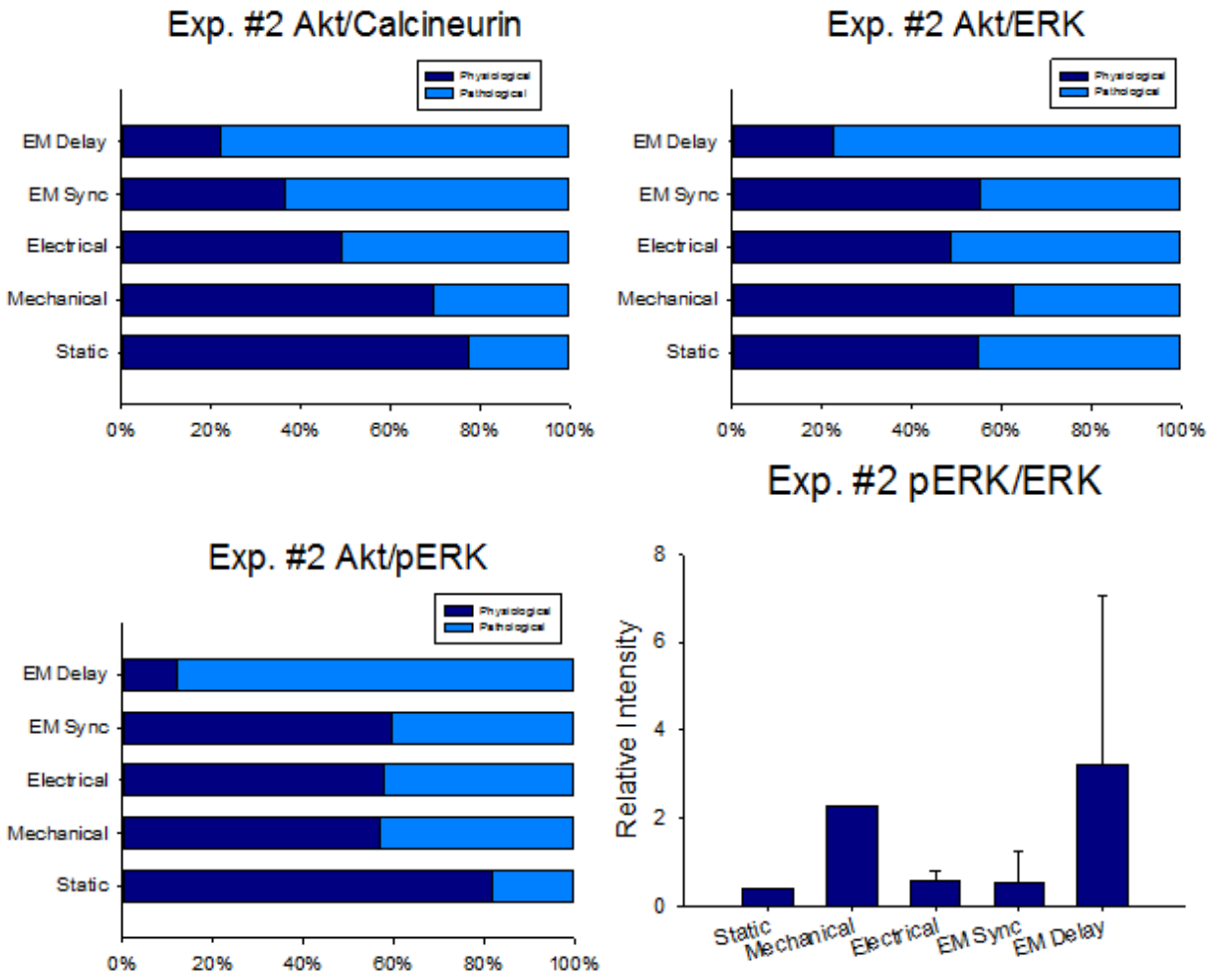

Figure #25 - A series of graphs that compare the expression and ratios of Akt, calcineurin, ERK, and pERK of the constructs from each stimulation condition in experiment #2.

The expression of Akt/calcineurin, Akt/ERK, Akt/pERK, and pERK/ERK is variable and does not seem to be following any trends in experiment #1. The results of this experiment are incorrect due to what may be procedural errors. Further investigation is needed to analyze these results

5. Discussion

5.1 Contraction Force

The contraction force data is extremely variable and very limited. The data collected from experiments #3 and #4 were unable to be used because experiment #3 had low measurements due to a below average isolation and experiment #4 experienced a contamination. However, experiments #1 and #2 were able to be compare. Although mechanical stimulation did not consistently produce constructs with higher twitch force than electrical stimulation or vice versa, the biphasic electrical stimulation of experiment #2 did produce constructs with a higher twitch than the monophasic stimulation of experiment #1. It should also be noted that the shorter delay time for the EM delay condition in experiment #1 did produce constructs with a higher twitch force than the longer delay time for the EM delay condition in experiment #2. The EM sync condition in experiment #2 had an extremely high twitch force because n=1. The rest of the constructs in that condition died due to the stimulation.

5.2 DNA Quantification

The cell count data sheds some more light onto the contraction force results obtained earlier. The cell counts for the different stimulation conditions and the different batch numbers are variable in experiments #3 and #4. This is probably because experiment #3 had a below average cell isolation and construct creation process and experiment #4 experienced a contamination. This means that data from experiments #3 and #4 must be thrown out. Experiments #1 and #2, however, showed no variablilty in cell count across stimulation or batch number. They can be used as reliable data from

this study. The contraction force data is extremely variable which means that the force per cell data will be as well if cell count is constant across stimulation like it is in experiments # 1 and #2. Although mechanical stimulation did not consistently produce constructs with higher force per cell than electrical stimulation or vice versa, the biphasic electrical stimulation of experiment #2 did produce constructs with a higher force per cell than the monophasic stimulation of experiment #1. It should also be noted that the shorter delay time for the EM delay condition in experiment #1 did produce constructs with a higher force per cell than the longer delay time for the EM delay condition in experiment #2. The EM sync condition in experiment #2 had an extremely high force per cell because n=1. The rest of the constructs in that condition died due to the stimulation.

5.3 Immunohistology

After analyzing the histology of the cells in the constructs for cell alignment and cell connectivity in experiments #1 and #2, there are some promising results. Antibodies were used to probe for protein markers MHC, Cxn 43, and α-actin. MHC and α-actin are both cardiac specific structural proteins that are involved with muscle physiology. These proteins can be used to look for cell alignment in a construct or tissue. Cxn 43 is a membrane protein that is involved in cell communication. Cxn 43 can be used to determine how much cells are communicating and how certain cells are connected. Similar results were seen in both experiment #1 and experiment # 2. Although all of the constructs had nuclei present, electrically stimulated constructs seemed to have a more even distribution of nuclei. In addition, electrically stimulated constructs also seemed to

have more cell alignment as seen through α-actin expression. This may be because electrical stimulation creates more cellular connectivity (Radisic *et al.* 2004) and allows nutrients to enter the scaffold and nourish cells on the inside. The static and mechanically stimulated constructs on the other hand, had more nuclei present on one side of each construct probably because this was the side exposed to culture medium while in the bioreactor.

A higher level of expression of Cxn 43 was seen in mechanically stimulated constructs. This is strange because a higher level of Cxn 43 usually means more cell connectivity and more cell alignment, but electrically stimulated constructs had more cell connectivity and cell alignment. In order to clear up these conflicting results, a Western blot analysis was run.

5.4 Western Blotting

After analyzing the cell alignment and cell connectivity via Western blotting in experiments #1 and #2, there are some Interesting results. Antibodies were used to probe for protein markers β-actin, Cxn 43, and pCXN 43. β-actin is a standard structural protein found in most cells. Cxn 43, as mentioned earlier is a membrane protein that is involved in cell communication. pCxn 43 is a less functionalized from of Cxn 43 which is less useful for cell communication. By normalizing Cxn 43 and pCxn 43 to β-actin and comparing them to each other, one can determine how connected certain cell areas well as how functional connected cells are. In experiment #2, the constructs from the electrical stimulation condition expressed an elevated amount of Cxn 43 when compared to the static constructs. Constructs from the electrical and EM delay

conditions expressed higher levels of pCxn 43 than the static constructs. These electrically stimulated constructs are most likely expressing elevated levels of Cxn 43 and pCxn 43 because they are more aligned and well connected. Since pCxn 43 is a less functionalized form of Cxn 43 and not as useful for cell communication, a comparison of pCxn 43 to Cxn 43 determines how functional those cells are. Constructs from the mechanical stimulation showed elevated ratios of pCxn 43 to Cxn 43 meaning they have more pCxn 43 which is less functional. Constructs from the electrical and EM delay stimulation showed a 1:1 ratio of pCxn 43 to Cxn 43 meaning they have just as much less functional pCxn 43 as functional Cxn 43.

The rest of the results about the expression Akt, calcineurin, ERK and pERK is not completed. The results obtained are incomplete and believed to be incorrect. While Western blotting, problems occurred with stripping antibodies off of the proteins in the nitrocellulose paper. With leftover antibodies on the nitrocellulose paper, signals interfere with each other and one cannot determine what the true signal is. More process development must be carried out and the analysis must be redone.

If there had been any successful Akt, calcineurin, ERK, or pERK data, it could have been used to measure the amount of hypertrophy occurring in the constructs. All of the proteins are markers of hypertrophy or muscle growth in size and number. Akt is a marker for physiological hypertrophy meaning growth from stimulation like exercise. Our stimulation of the constructs is aimed to trigger physiological hypertrophy. Calcineurin, ERk, and pERK are pathological markers of hypertrophy meaning growth to compensate other shortcomings like a myocardial infarction. Our stimulation of the constructs is not aimed to trigger physiological hypertrophy. Figure #26 on the next

page demonstrates how these proteins interact in the physiological and pathogical hypertrophy pathways. By comparing the expression of Akt to the expression of calcineurin, ERK, or pERK, one could have gotten an idea of how much physiological vs. pathological hypertrophy was occurring. pERK is the activated version of ERK. In addition, one could compare the expression of pERK to the expression of ERK to how much pathological hypertrophy was occurring as well.

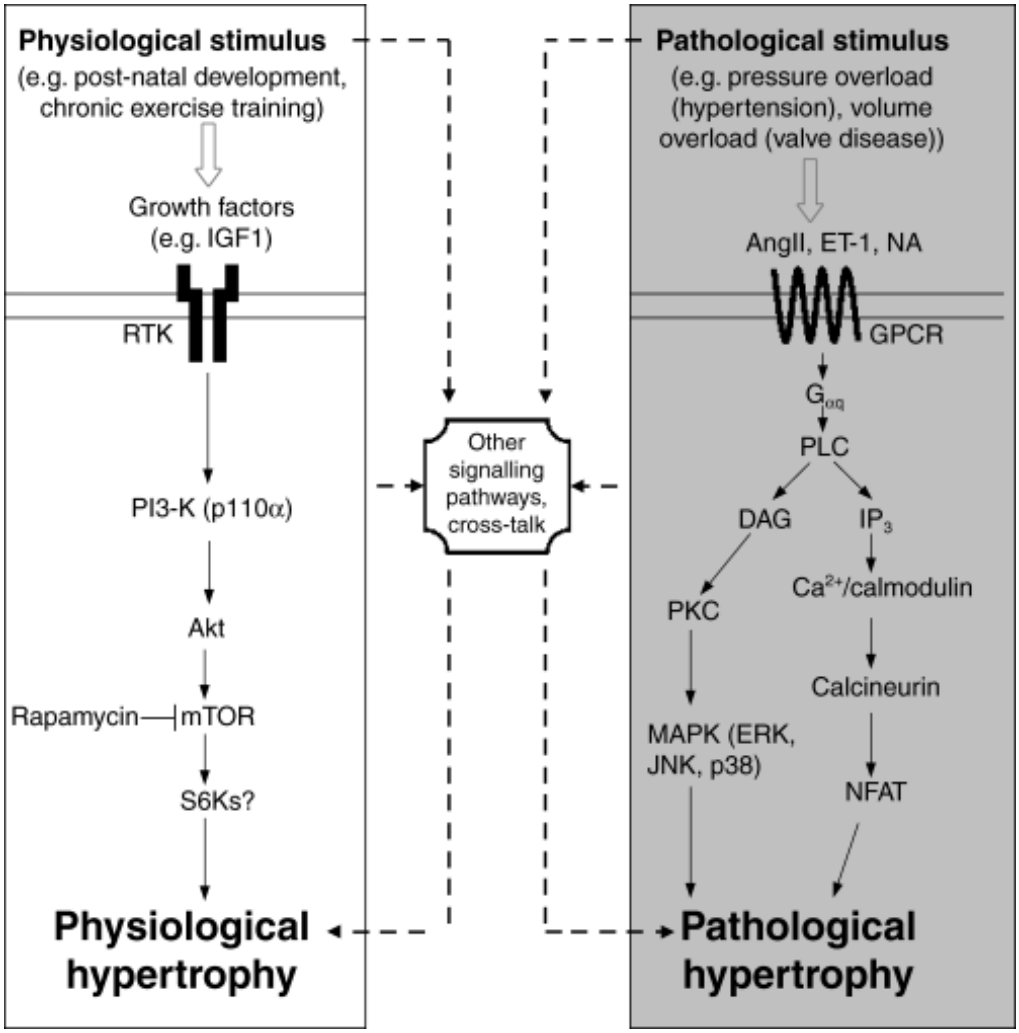

Figure #26 – A diagram showing how physiological hypertrophy and pathological hypertrophy occur.

McMullen JR et al., *Clinical And Experimental Pharmacology & Physiology* 2007. 34 (4): 255-62.

5.5 Conclusions

Biphasic electrical stimulation produced constructs with a higher mean twitch force and a higher mean force per cell than monophasic electrical stimulation. Electrically stimulated constructs also had a more even distribution of nuclei throughout their constructs when compared to static and mechanically stimulated constructs. This may be true because electrical stimulation induces more cell alignment, cell connectivity, and expression of Cxn 43 (Radisic *et al.* 2004) so nutrients are able to travel through the constructs and nourish cells deep within while mechanically stimulated cells only survive if they are exposed to media because mechanical stimulation does not induce cell communication and connectivity. Electrically stimulated constructs were also shown to have more cell alignment via the expression of α-actin. Electrically stimulated constructs were also shown to express higher levels of Cxn 43 at a functional ratio with pCxn 43. These results point to the fact that electrical stimulation has a greater positive effect on myocardial constructs than mechanical stimulation in terms of twitch force, force per cell, cell alignment, cell connectivity, and cell communication. Electrical stimulation is very important part of cardiac tissue engineering.

For the EM delay condition, a shorter delay time was found to produce constructs with a higher twitch and force per cell than constructs stimulated with a longer delay time. There is a reaction in twitch force to the change in delay time between mechanical and electrical stimulation. More runs at more delay times must be run to determine how changes in the delay time affect the development of the constructs. This is crucial understanding how electromechanical stimulation affects myocardial constructs.

6. Future Directions

6.1 Short Term Future Directions

More experiments will be run to further study the effects of mechanical, electrical, and electromechanical stimulation on myocardial constructs. Since initial research focused on understanding the effects of individual mechanical and electrical stimulation on myocardial constructs, short term future studies will move on to further investigating how the timing of combined mechanical and electrical stimulation affects myocardial constructs now that we have seen response to change. Using the flexibility of the control system, we will be able to easily manipulate the time delay between the mechanical and electrical stimulation so that we can find the optimal time delay. Hopefully these studies on the timing of electromechanical stimulation will be completed shortly.

6.2 Long Term Directions

Once the effects of the timing of electromechanical stimulation is completely understood, variability can be added to mechanical, electrical, and electromechanical stimulation. Future studies will probably focus on variability of the amplitude or frequency of the stimulation. Variability in the frequency and amplitude of stimulation models the variability of heart function throughout the day. When one exercises, their heart beats faster and stronger. When one sleeps, their heart beats slower and shallower. The variability studies will be very interesting because they will very closely model the *in vivo* conditions of the heart. Again, using the flexibility of the control

system, we will be able to easily manipulate the mechanical and electrical stimulation so that we can mimic the variability of the heart.

7. Appendix

Due to the variability of the twitch force data obtained, tables of the twitch force data for every construct tested in every experiment have been included for reference. The tables are organized by experiment and condition.

Experiment #1: Seeded 10/20/11, Harvested 11/2/11						
Sample	Batch	Mean Force (mN)	Mean Up Time (s)	Up Rate (mN/s)	Mean Relax Time (s)	Relax Rate (mN/s)
Static 1	1	1.4784	0.159	9.298113208	0.1747	4.231253578
Static 2	2	3.5883	0.1586	22.62484237	0.1579	11.36257125
Static 3	3	3.1201	0.1615	19.31950464	0.1585	9.842586751
Static 4	4	2.4044	0.1557	15.44251766	0.1523	7.893630991
Static 5	5	1.0371	0.1417	7.318983769	0.1303	3.979662318
Static 6	1	0.8014	0.1182	6.780033841	0.122	3.28442623
Mechanical 1	1	1.7696	0.194	9.121649485	0.3367	2.627858628
Mechanical 2	2	2.1102	0.286	7.378321678	0.7473	1.411882778
Mechanical 3	3	2.5312	0.1977	12.80323723	0.2898	4.367149758
Mechanical 4	4	2.441	0.1478	16.51556157	0.2248	5.429270463
Mechanical 5	5	2.3099	0.149	15.50268456	0.1712	6.746203271
Electrical 1	1	1.4988	0.1502	9.978695073	0.1355	5.530627306
Electrical 2	2	1.6264	0.1594	10.20326223	0.1446	5.623789765
Electrical 3	3	1.8889	0.1492	12.66018767	0.152	6.213486842

Electrical 4	4	1.6745	0.1377	12.16049383	0.1117	7.495523724
Electrical 5	2	Poor Compaction – Dead				
EM Sync 1	1	1.5095	0.2282	6.614811569	0.3565	2.117110799
EM Sync 2	2	1.7239	0.2473	6.970885564	0.4487	1.920993983
EM Sync 3	3	1.1893	0.229	5.193449782	0.398	1.494095477
EM Sync 4	4	1.3111	0.2353	5.572035699	0.3863	1.696997152
EM Sync 5	3	0.8087	0.232	3.485775862	0.401	1.008354115
EM Delay 1	1	2.8772	0.2707	10.6287403	0.476	3.022268908
EM Delay 2	2	2.6857	0.2262	11.87312113	0.3975	3.378238994
EM Delay 3	3	2.7533	0.312	8.824679487	0.5517	2.495287294
EM Delay 4	4	2.5315	0.3057	8.280994439	0.5895	2.147158609
EM Delay 5	4	0.7384	0.3085	2.393517018	0.669	0.55186846

Experiment #2: Seeded 11/3/11, Harvested 11/16/11						
Sampe	Batch	Mean Force (mN)	Mean Up Time (s)	Up Rate (mN/s)	Mean Relax Time (s)	Relax Rate (mN/s)
Static 1	1	2.7933	0.1345	20.768029774	0.125	11.1732
Static 2	2	2.7473	0.1734	15.843713966	0.1868	7.353586724
Static 3	3	2.8665	0.1547	18.529411766	0.1487	9.638533961
Static 4	4	2.8474	0.142	20.052112688	0.1423	10.00491918
Mechanical 1	1	Died in Reactor – Dead				
Mechanical 2	2	1.2622	0.2407	5.24387204	0.3937	1.602997206
Mechanical 3	3	Died in Reactor – Dead				
Mechanical 4	4	Died in Reactor – Dead				

Electrical 1	1	2.6831	0.1805	14.86481994	0.217	6.182258065
Electrical 2	2	1.933	0.1535	12.59283388	0.1425	6.78245614
Electrical 3	3	1.6549	0.1372	12.06195335	0.1388	5.961455331
Electrical 4	1	1.5249	0.1494	10.20682731	0.1436	5.30954039
EM Sync 1	1	Too Much Noise - Program Can't Read				
EM Sync 2	2	Too Much Noise - Program Can't Read				
EM Sync 3	3	Too Much Noise - Program Can't Read				
EM Sync 4	2	3.1123	0.289	10.769204115	0.6615	2.352456538
EM Delay 1	1	0.5491	0.22	2.495909091	0.477	0.57557652
EM Delay 2	2	1.2242	0.236	5.187288136	0.36	1.700277778
EM Delay 3	3	1.6367	0.26	6.295	0.552	1.482518116
EM Delay 4	3	Too Much Noise - Program Can't Read				

Experiment #3: Seeded 12/1/11, Harvested 12/13/11						
Sample	Batch	Mean Force (mN)	Mean Up Time (s)	Up Rate (mN/s)	Mean Relax Time (s)	Relax Rate (mN/s)
Static 1	1	1.0372	0.1768	5.866515837	0.1913	2.710925248
Static 2	3	0.9951	0.1553	6.407598197	0.1575	3.159047619
Static 3	4	1.0129	0.1358	7.458762887	0.1248	4.058092949
Static 4	5	1.742	0.1183	14.72527473	0.101	8.623762376
Mechanical 1	1	0.5329	0.1285	4.147081712	0.1638	1.626678877
Mechanical 2	4	1.4515	0.1158	12.534542311	0.1167	6.218937446
Mechanical 3	5	1.1844	0.1025	11.555121915	0.1243	4.764279968
Mechanical 4	6	0.4972	0.114	4.361403509	0.1182	2.10321489
Electrical 1	3	0.8159	0.177	4.60960452	0.155	2.631935484
Electrical 2	4	0.9581	0.1647	5.81724347	0.173	2.769075145

				3		
Electrical 3	5	0.7637	0.1552	4.920747423	0.1263	3.023357086
Electrical 4	6	0.8047	0.1493	5.389819156	0.1328	3.029743976
EM Sync 1	2	Too Much Noise - Program Can't Read				
EM Sync 2	3	0.7798	0.2145	3.635431235	0.336	1.160416667
EM Sync 3	4	Too Much Noise - Program Can't Read				
EM Sync 4	6	Too Much Noise - Program Can't Read				
EM Delay 1	2	Too Much Noise - Program Can't Read				
EM Delay 2	3	Too Much Noise - Program Can't Read				
EM Delay 3	4	0.4698	0.254	1.849606299	0.5	0.4698
EM Delay 4	6	Too Much Noise - Program Can't Read				

Experiment #4: Seeded 1/26/12, Harvested 2/8/12						
Sample	Batch	Mean Force (mN)	Mean Up Time (s)	Up Rate (mN/s)	Mean Relax Time (s)	Relax Rate (mN/s)
Static 1	1	0.0315	0.0693	0.454545455	0.117	0.134615385
Static 2	2	0.2085	0.0912	2.286184211	0.0952	1.095063025
Static 3	4	Construct Tested - No Response				
Static 4	6	Construct Tested - No Response				
Static 5	8	Construct Tested - No Response				
Static 6	8	Construct Tested - No Response				
Mechanical 1	1	Construct Tested - No Response				
Mechanical 2	2	1.1327	0.1893	5.983623877	0.302	1.875331126
Mechanical 3	4	0.2977	Too Much Noise - Program Can't Read			
Mechanical 4	6	1.7098	0.318	5.37672956	0.731	1.169493844
Electrical 1	2	0.925	0.212	4.363207547	0.186	2.48655914
Electrical 2	8	0.5961	0.132	4.515909091	0.098	3.041326531

Electrical 3	5	Contaminated – Dead				
Electrical 4	7	0.5656	0.1137	4.974494283	0.0896	3.15625
EM Sync 1	2	0.9801	Too Much Noise - Program Can't Read			
EM Sync 2	3	Contaminated – Dead				
EM Sync 3	5	1.244	Too Much Noise - Program Can't Read			
EM Sync 4	7	0.76	Too Much Noise - Program Can't Read			
EM Delay 1	2	1	Too Much Noise - Program Can't Read			
EM Delay 2	4	2.3666	0.2005	11.80349127	0.368	3.21548913
EM Delay 3	5	1.4169	0.1632	8.681985294	0.2834	2.499823571
EM Delay 4	7	0.2	Too Much Noise - Program Can't Read			
EM Offset 1	2	1.6655	0.1845	9.027100271	0.3892	2.139645427
EM Offset 2	4	1.0231	Too Much Noise - Program Can't Read			
EM Offset 3	5	0.8754	0.303	2.889108911	0.715	0.612167832
EM Offset 4	7	Died in Reactor – Dead				
EM Offset 5	5	3.6187	0.1913	18.91636174	0.2972	6.087987887

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