ABSTRACT

Title of Document:	ARRHYTHMOGENESIS AND CONDUCTION PROPERTIES OF CARDIOMYOCYTES IN RESPONSE TO DYSSYNCHRONOUS MECHANICAL AND ELECTRICAL STIMULATION
	Dulciana Dias Chan, Master of Science, 2010
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Many cardiac therapeutic modalities, including pacemakers, implantable cardioverter defibrillators, and cardiac resynchronization therapy devices, are used to treat abnormalities in cardiac function and conduction. Both electrical and mechanical dyssynchrony can have deleterious effects including reduced cardiac output and an increased susceptibility to cardiac arrhythmias. It is postulated that electro-mechanical dyssynchrony may contribute to the susceptibility of the heart to cardiac arrhythmias. In this study, a novel system was developed to study these effects by altering the electro-mechanical activation sequence in cultured neonatal rat cardiomyocyte monolayers by dyssynchronously stimulating the monolayers with applied electrical fields and pulsatile mechanical strain. Specifically, optical mapping was utilized to compare action potential duration and quantify arrhythmia susceptibility of cardiomyocytes subjected to pulsatile mechanical strain, electrical stimulation, and dyssynchronous electrical and mechanical stimulation. This system provides a method to evaluate changes in cardiomyocyte conduction properties due to altered electro-mechanical coupling and the subsequent impact on arrhythmogenesis.

ARRHYTHMOGENESIS AND CONDUCTION PROPERTIES OF

CARDIOMYOCYTES IN RESPONSE TO DYSSYNCHRONOUS MECHANICAL

AND ELECTRICAL STIMULATION

By

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Thesis submitted to the Faculty of the Graduate School of the
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Professor William E. Bentley, Chair
Professor Felipe Aguel
Professor Sameer Shah

Dedication

To my family and friends for their support

To my siblings Celina and Melina for their encouragement

To my husband Chris, for his patience and love

And in honor of their dedication to my education, my parents

W.C. Raymond and Sonia Chan

Dad, you were the first to introduce me to engineering and

your memory and lessons will never be forgotten.

Mom, you taught me how to strive and

are my constant supporter.

"How great are your works, Lord,

how profound your thoughts!"

Psalm 92:5

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Table of Contents

Chapter 1: Introduction

Cardiac function is derived from cellular electro-mechanical coupling where an electrical impulse elicits a mechanical contraction in cardiac tissue. The electrical impulse, known as an action potential, is propagated from cell to cell through electrically coupled low-resistance gap junction proteins. The initial period of an action potential consists of a depolarizing current, immediately followed by an influx of Ca^{2+}, which then triggers a cellular contraction which is a decrease in cell length. An electrical impulse followed by mechanical contraction with a delay on the order of tens of milliseconds is the natural activation sequence of the heart.

Many cardiac therapeutic modalities, including devices such as pacemakers, implantable cardioverter defibrillators, and cardiac resynchronization therapy devices, are used to treat abnormalities in cardiac function and conduction. Electrical dyssynchrony, or the lack of coordinated electrical activation throughout the heart, has been shown to reduce cardiac pump function and induce myocardial stress.[1] Mechanical dyssynchrony is discoordination of mechanical interactions within and between ventricles which leads to inefficient mechanical forces that are necessary for normal cardiac contraction.[2] Mechanical dyssynchrony is often a by product of electrical dyssynchrony, but can occur even with normal electrical activation.[3] Both electrical and mechanical dyssynchrony can have deleterious effects including reduced cardiac output and an increased susceptibility to cardiac arrhythmias.[4] Electro-mechanical dyssynchrony, also known as mechano-electrical feedback or contraction-excitation feedback, is a phenomenon in which cardiac electrical activity

is modulated by mechanical stretch.[5] The changes electrophysiological properties develop according to the timing of mechanical strain (during different phases of the action potential).[6] It is also postulated that electro-mechanical dyssynchrony may contribute to the susceptibility of cardiac arrhythmias and rhythm disorders.[7]

Cardiomyocyte monolayers have been used as a simplified model for examining various properties of cardiac tissue.[8-10] In particular, the effects of mechanical strain[11-22] and electrical stimulation[23-27] have been examined previously in separate studies using cultured cardiomyocyte monolayers. However, it is unknown if any system has been designed to study simultaneous mechanical and electrical stimulation in cardiomyocytes. The goal of this research is to implement a system with the ability to alter the electrical and mechanical activation sequence of cardiomyocytes, specifically electrical stimulation in synchrony (in phase) or dyssynchrony (out of phase) with mechanical strain. The aim of this study is to contribute to the understanding of cardiac electro-mechanical dyssynchrony and its impact on cardiac remodeling and cardiac conduction properties, as well as to measure its ability to promote arrhythmogenesis.

Chapter 2: Background

Cardiac Remodeling

Cardiac remodeling can be defined as a change in tissue properties due to an initiation or termination of a stimulus. Cardiomyocyte remodeling can occur due to stimuli, including disease (such as heart failure), injury (such as myocardial infarction), or therapy (such as pacing).[28] Remodeling can be evident through cardiomyocyte adaptation, such as protein production or alteration of ion channel responses.[29] While initial improvements, such as synthesis of new contractile proteins and new sarcomere assembly,[28] can be apparent due to remodeling, adverse functional effects, due to changes in electrophysiological properties such as action potential prolongation, may contribute to arrhythmia formation. For example, it has been suggested that remodeling may contribute to the development of an arrhythmogenic substrate in the failing human heart.[22, 30, 31] Electrical and or mechanical stimulation can be used to elicit a remodeling response in cultured cardiomyocytes.[13, 14, 21-23, 26] Electro-mechanical dyssynchrony alters the activation sequence of cardiomyocytes which induces remodeling, which is characterized by changes in protein synthesis, cellular organization, and cell signaling pathways.

Ventricular Arrhythmia

Arrhythmia is defined as a cardiac rhythm disorder which can be further categorized by the location of the affected cardiac region in the whole heart. In cardiac tissue, the formation of an arrhythmia can occur when the leading edge or wavefront of the

3

action potential (AP) propagation wave interacts with the repolarization phase of a preceding wave.[32, 33] Reentry describes repetitive ventricular arrhythmias in which the activation front of an action potential never meets refractory tissue, causing the rhythm to persist.[33] Reentrant arrhythmias can be classified as either anatomical or functional. An anatomical reentry is a repetitive propagation wave that circulates around an inexcitable obstacle, such as a cardiac structure including blood vessels or scar in cardiac tissue or gaps in cardiomyocyte monolayers.[34] A functional reentry, which is the focus of this study, also known as a spiral wave is a reentry that circulates freely and is not necessarily anchored to an anatomical obstacle. The reentry occurs due to functional changes such as excitability, fiber orientation, or conduction properties of the cardiomyocytes or myocardium. These arrhythmias can manifest in 2D substrates in several ways including rotating single-arm spirals, multi-arm spirals (spiral waves that rotate in the same direction around a common center), or unstable spirals (which continuously form and break up).[35] Rotating spirals can also be characterized by chirality, or direction of rotation, and are usually denoted as clockwise or counterclockwise.

Reentry also can be caused by a region of cardiac tissue exhibiting slow conduction, short refractory periods, large dispersion of refractory periods, or unidirectional blocks.[36] Other changes in functional electrophysiological properties, such as action potential duration restitution (APDR) and conduction velocity, can also lead to an arrhythmic conduction pattern such as reentry.

4

As stated earlier, ventricular arrhythmias are commonly seen in heart failure patients.[33, 37] The mortality rate of severe heart failure patients correlates with depressed left ventricular function and the presence of ventricular arrhythmias.[33] The combination of left ventricle dilation and low ejection fraction is linked to ventricular arrhythmias, which may indicate that chronic strain can cause electrophysiological remodeling which in turn can be a factor of arrhythmia formation in heart failure patients.[33] As seen in myocytes isolated from failing hearts, action potential duration prolongation can also cause monolayers to be highly arrhythmogenic.[38]

Proteins

There are several significant proteins which are commonly studied in cardiomyocyte research and commonly observed during cardiac remodeling. Specifically, F-actin, connexin, and cadherins are proteins that aid in the organization, function, and electro-mechanical coupling of cardiomyocytes. F-actin is a cytoskeletal protein with a filamentous polymer subunit structure that can easily be identified by specific molecular probes. The F-actin polymers form bundles and networks that not only provide a structural framework for the plasma membrane but are also necessary for cardiac contraction. Bundles of repeating arrays of actin filaments, called sarcomeres, undergo shortening during cardiac contraction.[39]

Connexin is a transmembrane protein which provides the gap junction structure in cardiomyocytes. Gap junctions, intercellular couplers, are channels between cells that permit the exchange of ions and a low-resistance pathway for electrical current to flow between cells. These are critical in fast conduction of action potentials in that

conduction velocity is dependent on the magnitude of the intercellular resistance which is largely determined by gap junctions. Specifically, Connexin 43 (Cx43) is most abundant in the heart[32] and the location and amount of Cx43 impacts cardiomyocyte membrane electrical properties.[40]

Cadherins are transmembrane proteins that function as cell adhesion molecules by linking cells together and attaching cells to extracellular matrix proteins. N-cadherin is located in the intercalated disks of cardiomyocyte membranes and is necessary for myofibril (chain of sarcomeres) organization and stabilization,[41, 42] as well as aiding in the assembly of Cx43.[42]

During cardiac remodeling, the alteration of these proteins, including their location and production, is commonly observed and measured.[43, 3, 44]

Cardiomyocyte Cell Culture

When myocytes from hearts early in development are isolated and plated in a high enough density on an appropriate substrate, they adhere to the substrate. They randomly disperse and spread in multiple directions, and when they encounter other myocytes, form gap junctions. The result is a two dimensional monolayer or continuous cell network that behaves like native cardiac tissue, in that cells are excitable, remain mechanically active such that they contract following electrical stimulation, and APs can propagate from cell to cell. Cardiomyocytes typically exhibit spontaneous contractile activity (beating) at 1-2 Hz.[23] Since monolayers

behave similarly to native tissue, they have been used to study hypertrophy,[12, 16] arrhythmias,[8, 35] intracellular communication,[22] protein expression,[23-26] and the effects of static[22, 45] and pulsatile strain.[13, 14, 16-18, 20-22, 22]

Cardiac tissue is naturally anisotropic which means the properties of the tissue vary according to the direction of measurement.[46] Therefore the spatial alignment of myocytes and location of intercellular junctions such as gap junctions affect the electrical conductivity of the tissue.[46] Anisotropy allows for sequential electrical and mechanical activity which aids in efficient pumping of the heart.[46] However, cultured myocytes form two dimensional monolayers that are isotropic because individual cells are randomly oriented and therefore are not aligned to enhance end-to-end connections;[46] therefore, intercellular connections are not necessarily representative of native cardiac tissue. Myocytes transform electrophysiologically over time in that cell size, morphology, and action potential duration (APD) all change during development.[23] Since cardiomyocyte monolayers are well established for use in cardiac remodeling studies,[22-26, 46] consist of a homogeneous cell type, and are a preparation that is easily sustainable and reliable, they were chosen as the ideal preparation of cardiac tissue for these experiments.

Mechanical Strain

In this study, mechanical strain will be used to initiate cardiac remodeling. However, mechanical strain can also induce alignment of myocytes, called anisotropy which is a characteristic of cardiomyocytes in vivo. Alignment of cells in myocyte

monolayers can be induced using several techniques including microabrasion,[46, 47] micropatterning and photolithographic patterning,[12] microfabrication including mictrotexture with grooved or pegged surfaces,[48] or external mechanical force on a deformable membrane.[14, 22] The application of mechanical strain to cultured neonatal cardiomyocytes is a method that induces alignment of the myocytes and promotes protein synthesis, which ultimately impacts the electrical properties of the myocytes and monolayers. In order to more accurately simulate native cardiac tissue in vivo, mechanical strain of a pliable silicone substrate was the technique chosen for this study in order to produce cardiomyocyte alignment while at the same time mechanically stimulating the cardiomyocytes.

The ability of mechanical strain to induce cell alignment in culture is dependent upon the initiation time of strain exposure.[21] It has been shown that mechanical strain induces cell alignment when the applied strain was started three (3) hours after seeding, while no alignment was evident when strain was initiated 24 hours after seeding.[13] Although time of strain initiation was not indicated, Zhuang, et al. showed that alignment occurred after six (6) hours of continuous pulsatile strain.[22] It has also been shown that applied strain was able to induce alignment in monolayers plated at a low density, where there were fewer cell-cell contacts, as opposed to high density monolayers where alignment did not occur.[13] As a result of the applied mechanical strain, cell orientation in the monolayer is promoted parallel to the direction of strain.[14]

The effect of mechanical strain on protein synthesis has also been studied extensively in myocyte cultures and it has been shown that strain increases the synthesis of gap junction proteins.[22] Specifically, strain causes an overall increase in Cx43,[20] an increase in Cx43 protein area,[22] an increase in Cx43 mRNA,[19] and localization of Cx43 at the longitudinal cell termini.[14]

Other proteins are impacted by mechanical strain in myocyte monolayers. For example, strain causes an increase in the density of cytoskeletal filaments[20] and activates RhoA, which is a protein that promotes actin formation.[14, 21] Applied strain also causes an increase in N-cadherin,[20, 22] which has been shown to be a vital component for the alignment and arrangement of myocytes.[13] Additionally, strain promotes the localization of N-cadherin at the longitudinal cell termini.[14]

Strain induces alignment and protein changes which consequently impact the electrical properties of the myocytes. For example, propagation velocity and the velocity of the action potential upstroke have been shown to increase with pulsatile strain.[22] It has also been shown that static strain increases the conduction velocity of myocytes.[22]

Electrical Field Stimulation

Temporal synchrony of cardiomyocyte activation is imperative for proper cardiac contraction and function. In cell culture, electrical field stimulation with periodic pulses stabilize the time dependant developmental changes in functional electrical properties of myocytes, induces morphological changes in monolayers, including cell

alignment, and increases gap junction connections, thereby increasing conduction velocity.[23, 26, 27] Electrical field stimulation also stabilizes electrophysiological properties of cardiac cells such as APDR and conduction velocity restitution curves.[23] Electrical field stimulation of myocyte monolayers at 2 Hz has been shown to maintain and stabilize APDR and maximum capture rate (MCR) over a time period of days 4 to 8 (post plating) when compared to non-stimulated monolayers which showed a downward shift in APDR over the same time period.[23] Conduction velocities have been shown to increase over the time of culture (from day 4 to day 6) in both electrically stimulated and non-stimulated monolayers although the rate of increase was greater in electrically stimulated monolayers.[23] Electrical field stimulation has also been shown to suppress spontaneous myocyte beating.[23]

Similarly, electrical field stimulation increases the quantity of gap junction connections between cells. In one study, after five days of continuous electrical stimulation, cell connections via gap junctions, including Cx43, were more prevalent.[26] This indicates that these proteins, which were previously disorganized during digestion in the cell culture process, recovered and reorganized.[26] The Cx43 levels in the aforementioned study were measured using multiple methods, including molecular expression levels, amounts, distribution and gene expression. Cx43 levels in electrically stimulated monolayers treated with Verapamil, a L-type Ca^{2+} channel blocker, were found to be comparable to electrically stimulated drug-free controls and showed that electrical stimulation maintains the level of functional gap junctions without myocyte contraction.[26] Applied electrical field stimulation increases gap

junction quantity which causes an increase in conduction and overall functionality of cardiac tissue.

Electrical field stimulation also causes morphological changes in myocytes, which is indicative of a positive myocyte response. Electrical stimulation (1Hz, 5V/cm, 2 ms rectangular pulses) applied continuously to monolayers for five days increased intercellular coupling, alignment (evidenced by myofibers aligning in the direction of electrical field lines), elongation, and synchronous contractions.[26] The time between plating of monolayers and initiation of electrical stimulation is important because enzyme digestion temporarily inhibits electrical signal transmission and in turn contraction.[26] Optimal cellular activity have been found to occur when stimulation was initiated on days 3-5 of the culture.[26]

Optical Mapping

Optical mapping is an imaging technique that utilizes voltage-sensitive dyes to visualize the electrical activity of the heart. The voltage sensitive dye molecules bind to the cellular membrane of the myocytes and, when excited with light of a certain wavelength, emits light of another wavelength (emission wavelength) with an intensity that is proportional to the transmembrane potential of the cells.

There are several advantages that make optical mapping ideal for these types of experiments. Optical mapping allows simultaneous events to be recorded from many sites and depicts the spatial information of a monolayer unlike electro-cardiographic

techniques. The use of voltage-sensitive dyes allows transmembrane potentials to be

recorded during the application of electrical pulses while keeping the recorded data

free of electrical stimulus artifacts.[8] It also has been a well documented and widely

used technique for research involving cardiomyocyte cultures at microscopic[9] as well

as macroscopic length scales.[9, 49]

Chapter 3: Dyssynchronous Mechanical and Electrical Stimulation

Objectives

The objective of this research was to develop a system to electrically stimulate cardiomyocyte monolayers dyssynchronously (out of phase) with pulsatile mechanical strain, validate this system by measuring electrophysiological changes of cardiomyocyte monolayers resulting from dyssynchronous mechanical and electrical stimulation, and finally quantify the susceptibility of monolayers to arrhythmias due to dyssynchronous mechanical and electrical stimulation. Until now, cardiomyocyte studies have investigated the morphological and protein expression changes due to mechanical strain or electrical field stimulation separately.[12-14, 19-22, 26] However, no studies have explored stimulating cardiomyocytes with both mechanical strain and electrical fields, either synchronously or dyssynchronously. The arrhythmogenic tendency of cardiomyocytes due to dyssynchronous mechanical and electrical stimulation has similarly not been fully explored. It is the goal of this research to develop a system to induce cardiac remodeling through dyssynchronous mechanical and electrical stimulation and to quantify the changes in electrophysiological properties of cardiomyocyte monolayers.

Materials and Methods

All animals were treated according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the White Oak Animal Facility of the Food and Drug Administration (FDA) in accordance with the Guide for the Care and Use of Laboratory Animals.[50]

Cell Culture

Cardiac cells were removed from ventricles of 2-3 day old neonatal Sprague-Dawley rats (Harlan, Indianapolis, IN). The ventricles were minced in to 8-12 pieces and digested in a 0.7 mg/mL 3x trypsin solution (~614,000 USP/g) (US Biological, MA) while being gently swirled at 75 rpm overnight at 4°C. The trypsin solution was then aspirated and the ventricles were quenched with 25 mL of warm growth media. The growth media consisted of Dulbecco's Modification of Eagle's Media (DMEM) supplemented with L-glutamine, 2mg/mL vitamin B12, 10% Fetal Bovine Serum (FBS) to remove fibroblasts, 0.1 mM Bromodeoxyuridine (BrdU) to inhibit the growth of non-myocardial cells, 10mM HEPES to maintain physiological pH, and 50 µg/mL streptomycin/penicillin to inhibit bacterial growth (all obtained from Fisher Scientific).[8, 46, 51-53] The ventricles and media were rotated at 75rpm in a 37°C water bath for 4 minutes. The ventricles were then dissociated into a suspension of single cells using a four step digestion using 45mg/mL (254 u/mg) collagenase (Worthington Biochemical Corporation, NJ). The first digestion was discarded. The other three digestion phases consisted of pipetting the collagenase-tissue solution to help dissociate the cells. The collagenase tissue solution was then centrifuged at

14

1000 rpm. The supernatant was discarded and the cell pellet was resuspended in

Hank's buffered salt solution (HBSS) (Fisher Scientific). After the cells were well

suspended in solution, the cell suspension was filtered through a 40μm cell strainer

(Fisher Scientific). The suspension was then centrifuged again at 1000 rpm. The

supernatant was discarded and the cell pellet was resuspended in warm media and

pre-plated in a tissue culture flask in a 5% CO_2, 37°C incubator for one 15 minute

period to reduce the fibroblast content.[22]

After a cell count, the cells were plated on a 3.2 cm x 3.2 cm area (10.2 cm^2) of the

silicone strain chamber (STREX, B-Bridge International, Inc., CA) with a total of

~2.6 million cells. The silicone surfaces were coated with 25μg/1mL human

fibronectin (BD Biosciences, MA) to ensure adhesion of the cells to the chambers and

growth of dense monolayers without large intercellular spaces. Since the square

silicone chambers were 10.2 cm^2 and the dish where the monolayers would be placed

for experimentation was a circular region of 23 mm in diameter, fibronectin was

placed in the center of the silicone chamber and then covered with a circular glass

coverslip (23 mm in diameter) to pattern the fibronectin in a circular shape in the

center of the silicone chamber. Cells adhered to the fibronectin coated area and not

the outlying non-fibronectin coated silicone membrane area. A monolayer plated on

a circular substrate was necessary to fit in the cell dish system used during the

experimental protocol (described in Experiment Protocol section). Confluent

monolayers were then grown in a CO_2 incubator at 37˚C.

Chambers with confluent monolayers, assessed under phase contrast microscopy, were used for experimentation. All experiments were done 4-5 days after culturing. Each series of experiments was performed on 5 to 12 cultures derived from one cell isolation. Prior to experimentation, the fibronectin and monolayer coated circular area was cut from the center of the silicone chamber. The circular pattern of the monolayer was necessary so that when this cut was made, it prevented cellular injury or disruption in monolayer confluence.

Mechanical Strain Unit

A mechanical strain unit (STREX Cell Strain Instrument, B-Bridge International, Inc., CA) (Figure 1.) was used to subject cardiomyocyte monolayers to uniaxial pulsatile mechanical strain while in culture. This mechanical strain unit was adapted to have an analog signal sensor output which produced a voltage waveform that was proportional to the cycle of strain and expressed the changes in length of silicone chamber due to the strain (Figure 2.). The 8% uniaxial pulsatile strain cycle (0.5 Hz) consisted of a strain period of 0.25 sec, a hold period of 0.5 sec, a relax period of 0.25 sec and hold period of 1 sec. Cell strain in the y direction (ε_Y) is defined as

$$\varepsilon_Y = \frac{\ell - L}{L} x100 \qquad \text{(Equation 1.1)}$$

where L is the length of the silicone chamber before strain and ℓ is the length of the silicone chamber at maximum strain (Figure 3.).
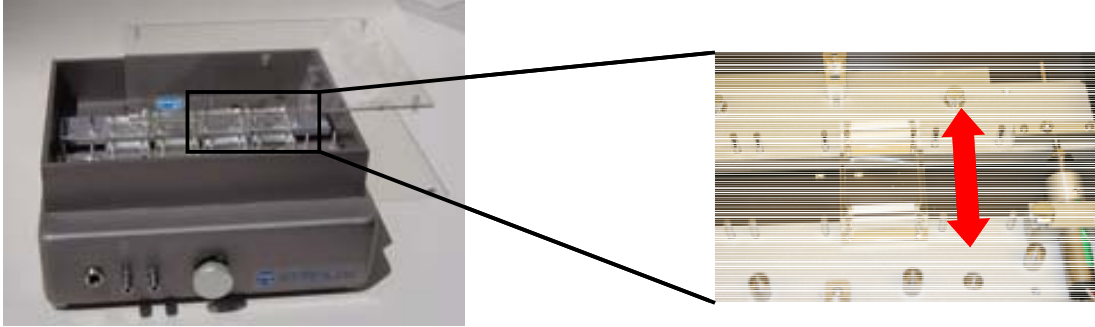
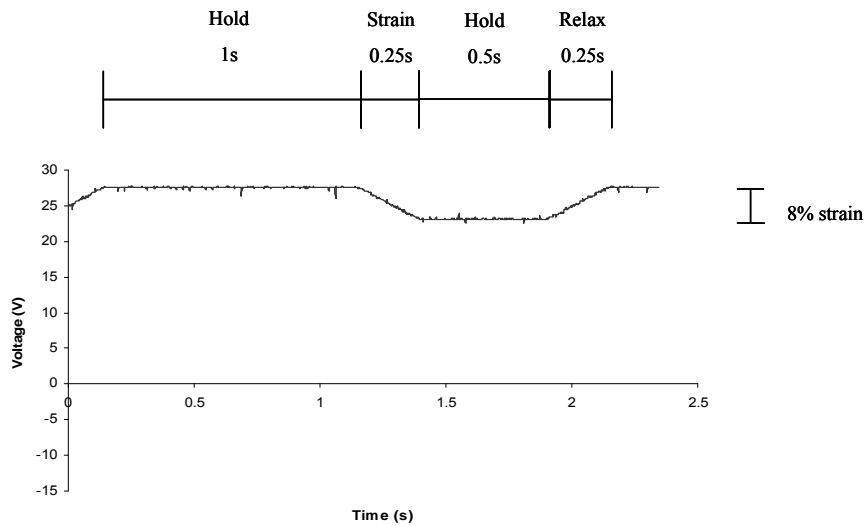Fig. 1. Mechanical strain unit and silicone chamber (inset). The arrow indicates the direction of strain.

Fig. 2. Equivalent voltage waveform for 8% mechanical strain. The mechanical strain unit was adapted to have analog signal sensor output which produced a voltage waveform that was proportional to the cycle of strain and expressed the changes in length of the silicone chamber due to the strain. The uniaxial pulsatile strain cycle (0.5 Hz) consisted of a strain period of 0.25 sec, a hold period of 0.5 sec, a relax period of 0.25 sec and hold period of 1 sec. The pulsatile mechanical strain cycle depicted above the equivalent voltage output from mechanical strain unit. The amplitude of the voltage waveform represents the percent strain.
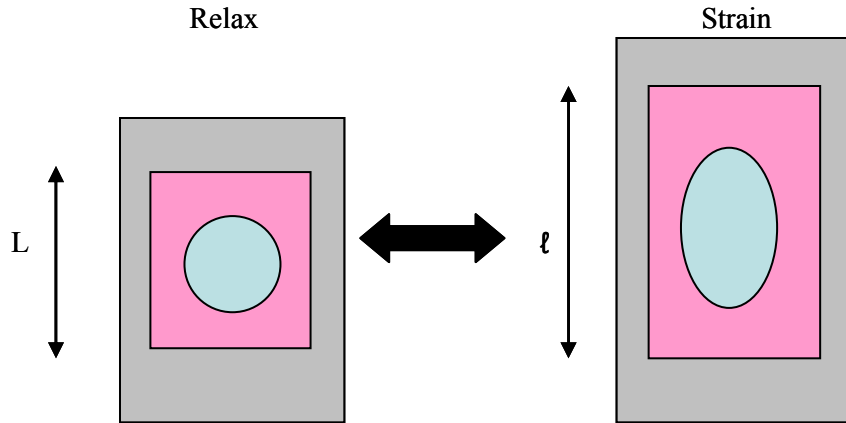
Fig. 3. Depiction of strain on a silicone chamber in the y direction. The grey rectangle depicts the silicone chamber. Pink represents the well of the silicone chamber. Blue represents the cardiomyocyte monolayer. The image on the left depicts a silicone chamber during the hold phase immediately after the relation phase of mechanical strain cycle. L is the length of the silicone chamber before strain. The image of the right represents the silicone chamber during the hold phase immediately after the strain phase of the mechanical strain cycle. ℓ is the length of the silicone chamber at maximum strain or 8% strain. (The images are not to scale).

Electrical Field Stimulation System

In order to subject cardiomyocytes monolayers in culture to electrical field stimulation by a field stimulus, a stimulation setup was adapted from experiments by Sathaye et al., 2006 and Johnson et al., 1994[23, 24] and is shown in Figure 4. Square-wave pulses were delivered by a stimulator (Grass SD9) through 0.12 inch diameter parallel carbon rod electrodes (Ladd Research, VT). The carbon electrodes were submerged in media at opposite sides of each chamber in the strain system to form an electric field within each chamber across the monolayer of cells. The carbon rods were held by a custom-built polycarbonate scaffold. The chambers were electrically connected in series via platinum wire adhered to the carbon rods by biocompatible epoxy (Epoxy Technology, MA). In order to reduce ion buildup, the polarity of the

stimulus for every other pulse was reversed using an A385 stimulus isolator (World Precision Instruments, FL).

Stimulus threshold and capture of the cultures at the stimulus rate of 0.5 Hz was determined by visual inspection of the cardiomyocytes under phase contrast microscopy and was consistent across cultures from different isolations. To standardize the experiments, the stimulation current was set to 30mA with a 4 ms pulse duration, which resulted in an output voltage of 9 V and a 0.56 V/cm field stimulus across each monolayer.

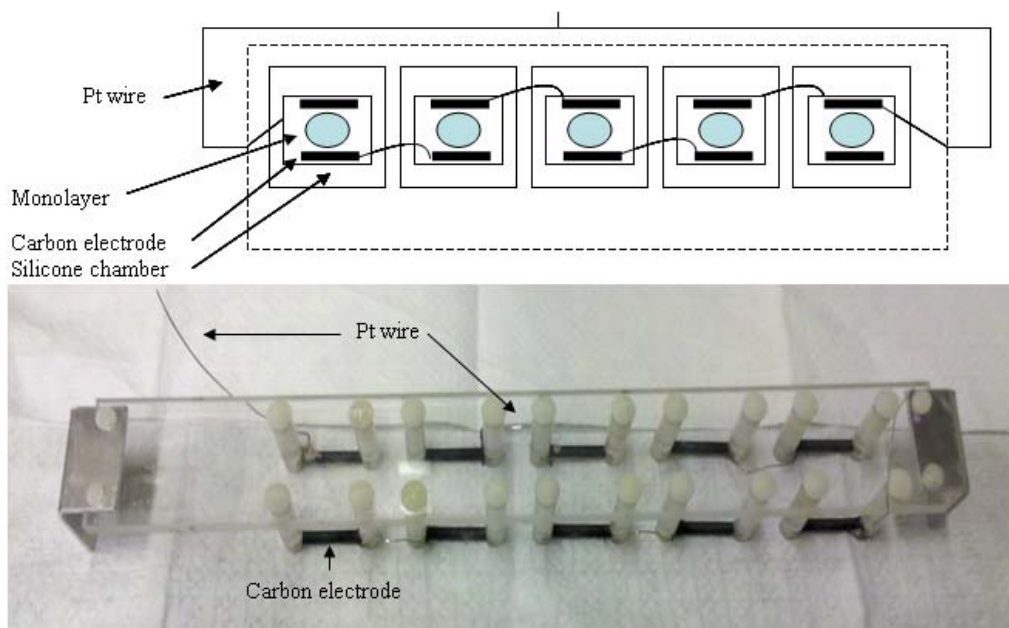Fig. 4. Electrical Field Stimulation Setup. The upper panel is a schematic of the electrical field stimulation setup. The cardiomyocyte monolayers are represented in blue. The entire setup is placed in an incubator which is represented by a dotted line. The lower panel is the scaffold that holds the carbon electrodes used to electrical stimulate cardiomyocyte monolayers in the silicone chamber during incubation.

Mechanical and Electrical Stimulation Timing

In order to dyssynchronously, mechanically and electrically stimulate cardiomyocyte monolayers, in culture, a signal conditioning circuit was used for timing electrical field pulses from the electrical field stimulation system to be dyssynchronous with mechanical strain from the mechanical strain unit, such that the electrical stimulus rate was determined according to the frequency of strain. The mechanical strain control unit containing the strain settings (% strain and frequency) was connected to the strain instrument which consists of a step motor that physically moves the brackets holding the silicone chambers on either end. The strain instrument was custom designed to convert an analog signal sensor, which detects the distance between the brackets (or change in distance that the brackets move in the y direction) to an analog voltage output that is proportional to the applied strain in the system. This voltage output was then processed using a signal conditioning circuit in order to time the electrical stimulation relative to the applied mechanical stimulation.

The signal conditioning circuit converted the position signal from the sensor in the strain unit to a signal that could be used for triggering a Grass SD9 stimulator. The stimulator was used to set the timing of the electrical pulses and the A385 stimulus isolator was used to convert the signal into a constant current output with alternating polarity. Details of the signal conditioning circuit can be found in Appendix A. The equivalent mechanical strain waveform and corresponding electrical signal can be seen in Figure 5.

The normal pattern for activation of cardiac cells follows a sequence where an electrical impulse propagates through the tissue, followed by a mechanical contraction. In order to mechanically and electrically stimulate cardiomyocytes dyssynchronously, the electrical stimulation was applied to the cardiomyocytes immediately prior to, the onset of mechanical strain. The timing of the electrical field stimulus was set to be 1075 ms after the onset of strain from the mechanical strain unit. In this way, the native activation sequence is reversed. The frequency of both mechanical and electrical stimulation was set to 0.5 Hz.
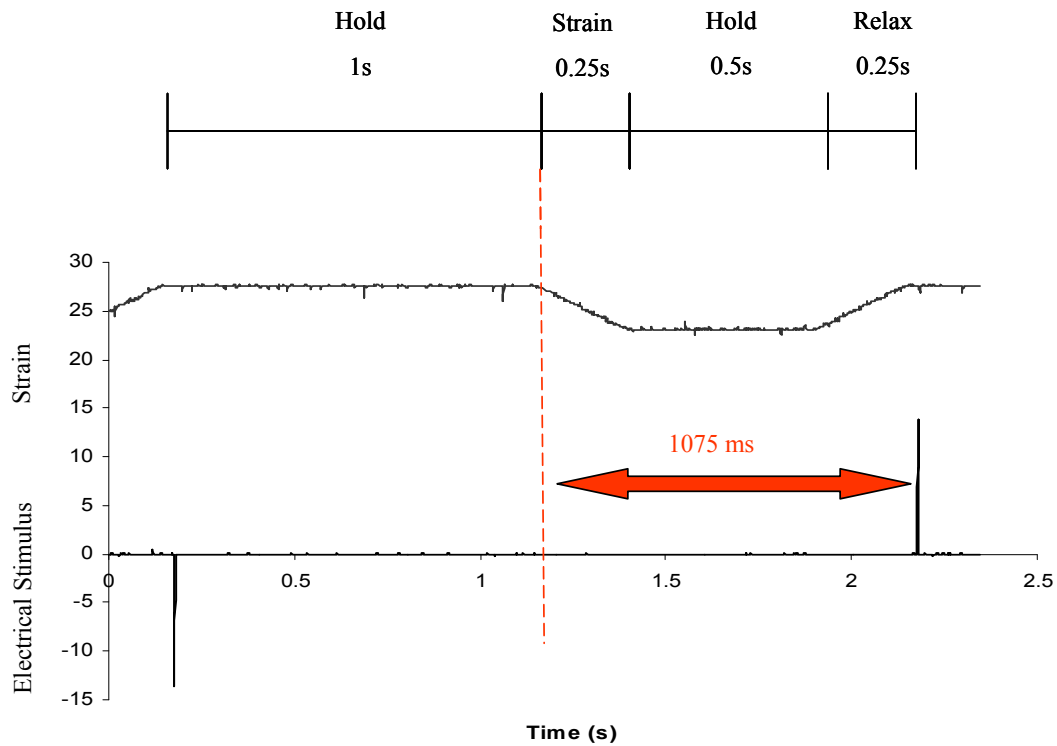

Fig. 5. Mechanical and electrical timing. The uniaxial pulsatile strain cycle (0.5 Hz) consisted of a strain period of 0.25 sec, a hold period of 0.5 sec, a relax period of 0.25 sec and hold period of 1 sec. The pulsatile mechanical strain cycle is depicted above the equivalent voltage output from mechanical strain unit. The amplitude of the voltage waveform represents the percent strain. The timing of the electrical stimulus is also represented below the voltage output from the mechanical strain unit. The electrical stimulation was delayed 1075 ms from the onset of mechanical strain in order to induce dyssynchronous mechanical and electrical stimulation. The dotted line represents the location of the electrical stimulation prior to mechanical stimulation during normal or synchronous cardiomyocyte activation.

Experimental Groups

After isolation and plating, the monolayers were divided into four experimental
groups during the incubation period; Control (no stimulation), Mechanical strain,
Electrical stimulation, and Dyssynchronous mechanical strain and electrical
stimulation. The schedule for stimulation for these groups is shown in Figure 6.
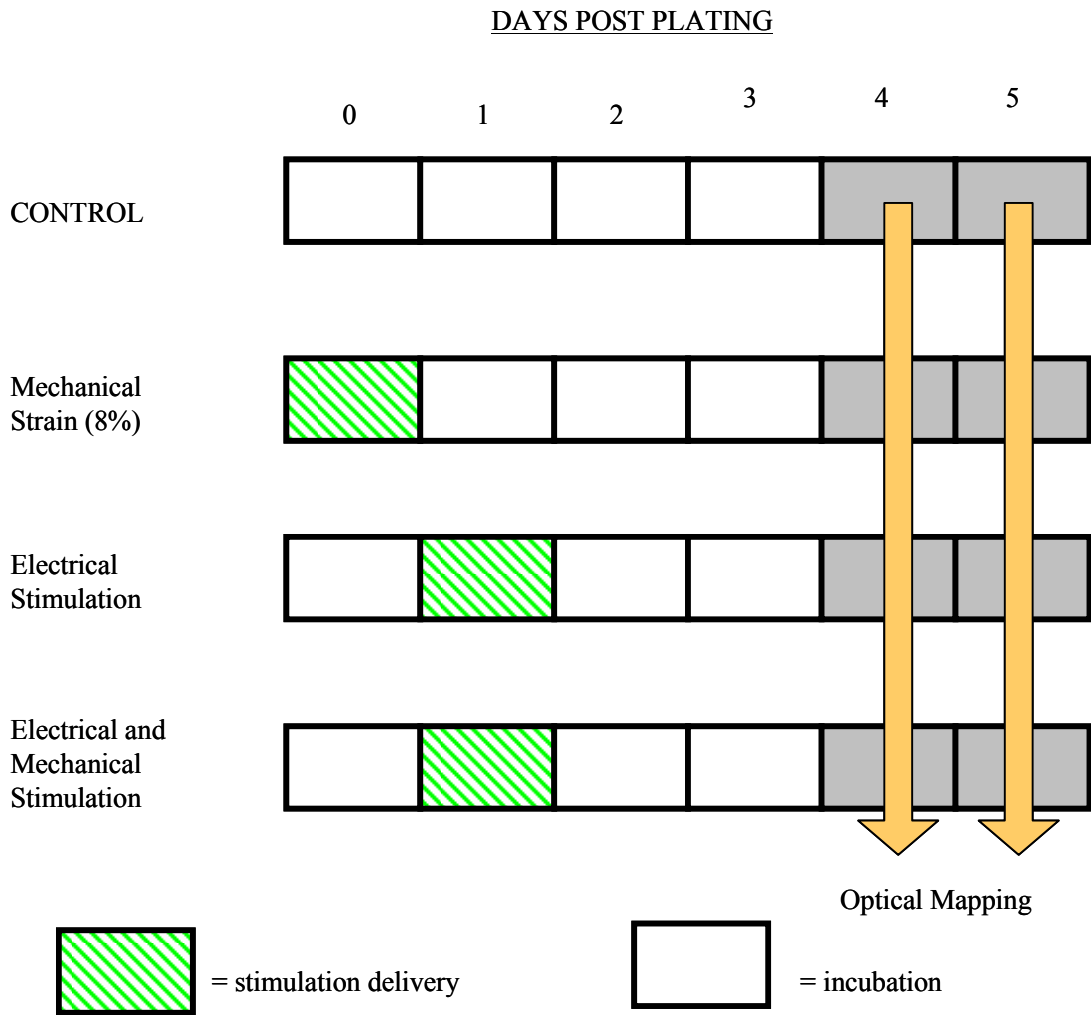
Fig. 6. Overview of experimental protocol. At 3 hours post plating, a subgroup of
monolayers was mechanically strained at 0.5 Hz and 8% strain for 24 hours. At day 1 post
plating, a subgroup of monolayers underwent electrical field stimulation at 0.5 Hz for 24
hours. At day 1 post plating, a subgroup of monolayers underwent dyssynchronous
mechanical strain and electrical stimulation for 24 hours. All groups underwent optical
mapping between day 4 and 5 post plating.

The control group consisted of monolayers grown on silicone chambers without any type of stimulation. The post plating incubation period was 1-5 days.

A second group consisted of monolayers subjected to mechanical stimulation during the incubation period using the cell mechanical strain system. Cardiomyocyte monolayers underwent uniaxial pulsatile mechanical strain from a computer-controlled stepping motor (STREX Cell Strain Instrument, B-Bridge International, Inc., CA). This group was subjected to 8% uniaxial pulsatile strain at 0.5 Hz that began three hours after plating until 24 hours after plating. These stimulation parameters were chosen to reduce the amount of cell death that occurred and to replicate the frequency of strain of several previous studies. [13, 14, 21] The percentage strain was also chosen to be consistent with previous studies.[12, 54, 55] The start time of mechanical strain was also chosen to be consistent with previous studies.[13, 14, 21]

The third group was the electrically stimulated group that underwent 24 hours of electrical field stimulation during the incubation period at 0.5 Hz starting one day after plating. Although previous studies have electrically stimulated myocytes at 1 and 3 Hz,[23-27] it was found that the cardiomyocytes were healthier when strained at a rate 0.5 Hz and therefore the same frequency was chosen for the electrical stimulation frequency. The start time of electrical stimulation strain was also chosen to be consistent with previous studies.[23, 26, 27]

The fourth group consisted of monolayers subjected to both mechanical and electrical stimulation during the incubation period. This group underwent the 24 hours of dyssynchronous 8% pulsatile mechanical strain and field electrical stimulation at 0.5 Hz starting one day after plating.

Optical Mapping System

The optical recording system consisted of a Nikkor Model NI8514/1450 (85 mm focal length, 1.4 numerical aperture) (Japan) tandem lens setup to transfer the monolayer image onto a 16x16 element Hamamatsu Model C4675-102 photodiode array (PDA) (Figure 7).[9, 56] A Quartz Tungsten Halogen lightsource (Newport Corporation, CA) trans-illuminated the monolayers after passing through a heat filter, an Oriel Model 76994 shutter, and a 510 nm excitation filter (Chroma, VT). The voltage-sensitive dye fluoresced through a 610 nm emission filter (Chroma, VT) and was detected by the PDA. (The staining procedure for the voltage-sensitive dye will be discussed in the section entitled Experimental Protocol.) The 256 optical signals were collected, passed through a current-to-voltage converter and amplified using 256 custom designed amplifiers for up to 100x amplification. Signals were filtered using a switched capacitor Bessel filter. A monochrome charged coupled device (CCD) camera was used to visualize the preparation to ensure the monolayer image was focused on the PDA. The PDA imaged a 17.5 x 17.5 mm^2 area. The dimensions of the individual diodes were 0.95 x 0.95 mm with an interdiode (center-to-center) distance of 1.1 mm. The signals were then digitized at 1 kHz using a data acquisition board (Sheldon Instruments). Data acquisition software was written in LabVIEW

(National Instruments) which allowed data acquisition at 1000 samples per second

per diode. Additionally, LabVIEW was programmed to control the timing of the

stimulators during experimentation and control the shutter between the preparation
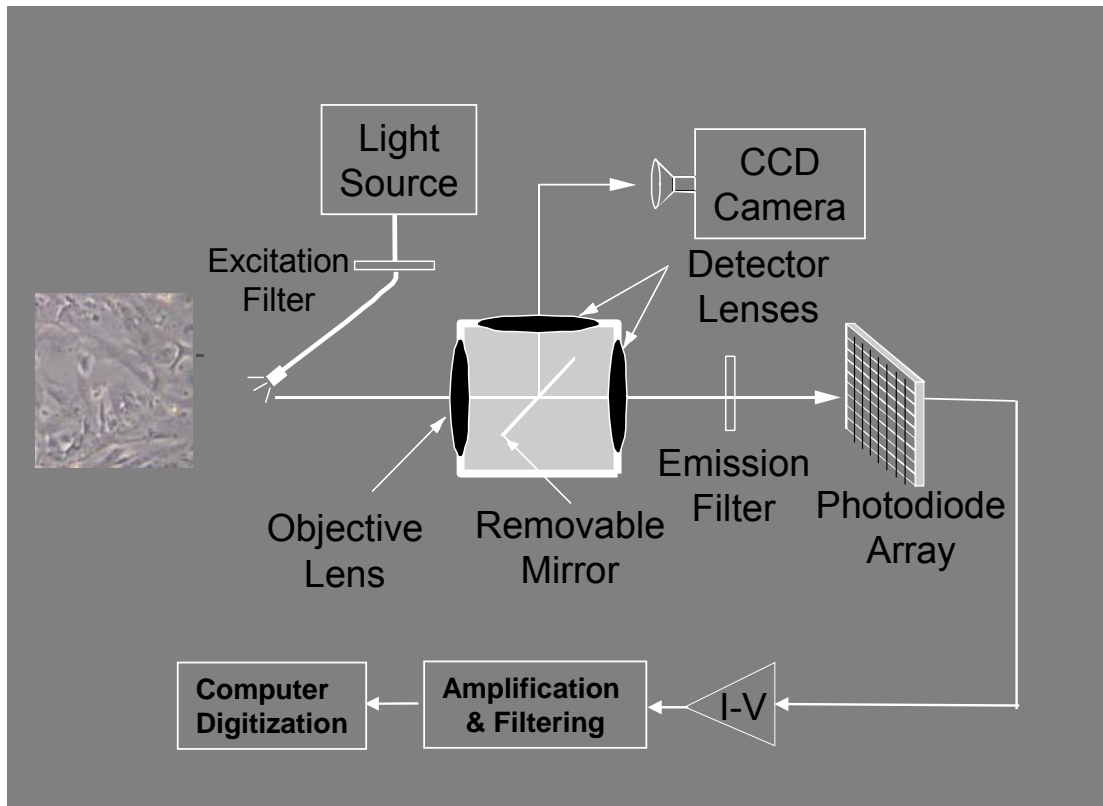
and the light source during data acquisition.

Fig. 7. Optical mapping system schematic. A lightsource passes through an exaction filter to trans-illuminated the monolayers The light passes through tandem lens through an emission filter and onto PDA. The optical signals are passed through a current-to-voltage converter (I-V) and amplified using 256 custom designed amplifiers for up to 100x amplification and filtered using a switched capacitor Bessel filter. The signals were then digitized at 1 kHz using a data acquisition board (Sheldon Instruments). A monochrome charged coupled device (CCD) camera was used to visualize the preparation to ensure the monolayer image was focused on the PDA.

Experimental Protocol

First, the cardiomyocyte monolayers were stained with 24μL of 4.2 mM voltage-

sensitive dye (di-8-ANEPPS) (Invitrogen) for 30 minutes. Cardiomyocyte

monolayers underwent a steady state pacing protocol while being optically mapped in order to record their activity in response to a pacing stimulus. Monolayers were paced with a point electrode at predetermined intervals and a decrementing pacing frequency from 1 to 10 Hz (1000 to 100 ms cycle length) with an electrical pulse stimulus at 1.5 times voltage threshold (at which capture first occurred for each monolayer) and a 10 ms pulse duration. Monolayers were paced for a minimum of 30 seconds prior to data collection. Additionally, between changes in stimulation interval, 30 seconds was allowed for cell recovery. Stimulation intervals were decreased until the cardiomyocyte monolayers were no longer capturing or induction of an arrhythmia occurred. Capture is defined as cardiomyocyte activation being equal to the frequency of the pacing stimulus, also called 1:1 capture (Figure 8). A recording of the optical measurements of the electrical activity of the monolayers was made for six to eight seconds while monolayers were stimulated at each pacing frequency. During experimentation, the preparation was maintained at 37°C using a culture dish system (Bioptechs, PA) in which thermal transfer occurs from the stage, through the glass dish that lies beneath the monolayers. The monolayers are superfused with warm CO_2-infused DMEM via a customized adaptor (Bioptechs, PA) and micro-perfusion pump set (Bioptechs, PA) at a rate of 0.5 mL/min.

A

Stimulus

Pacing
Rate

$$\frac{\Delta F}{F} \alpha\ V_m$$

Time

B

Stimulus

Pacing
Rate

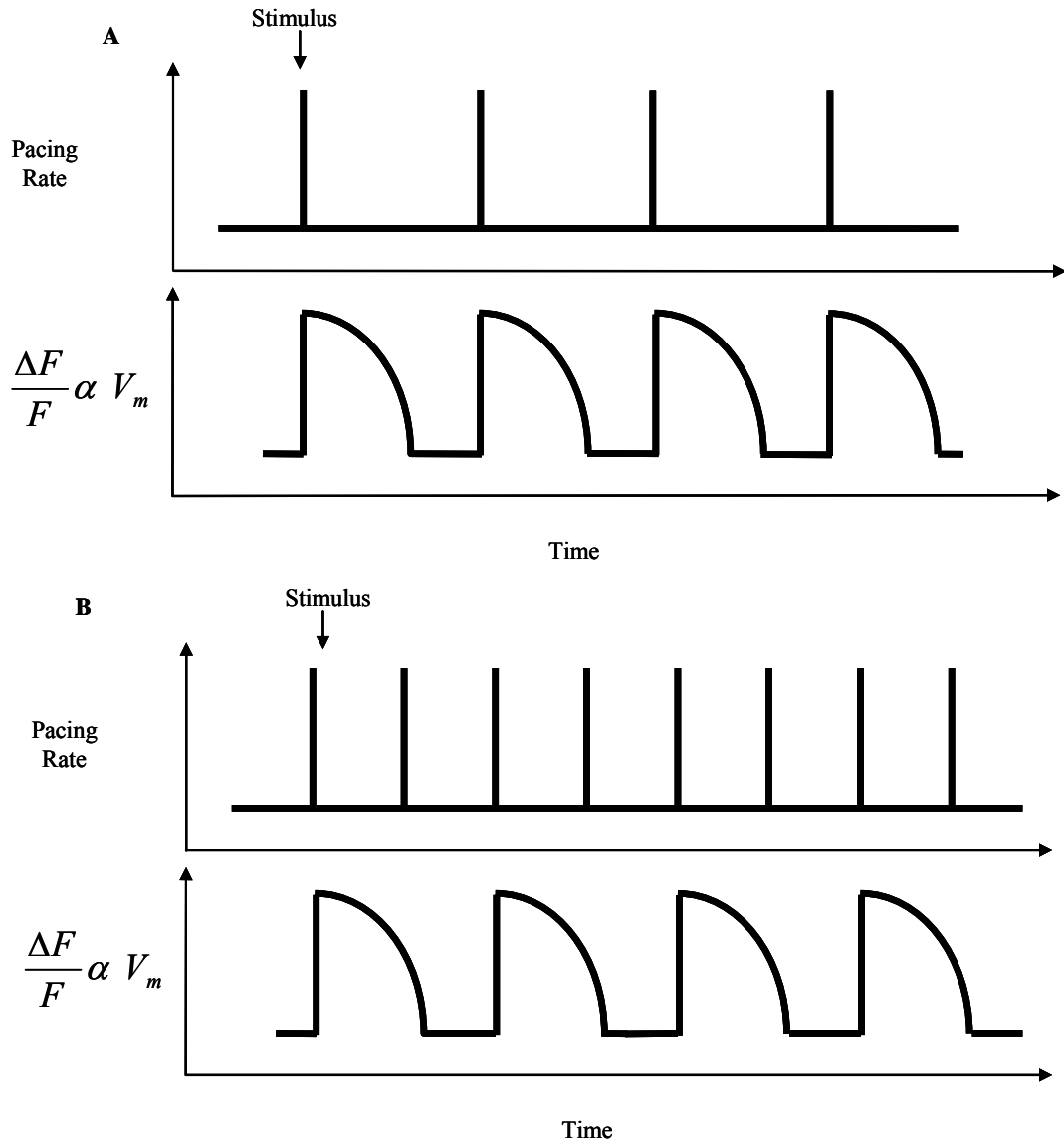$$\frac{\Delta F}{F} \alpha\ V_m$$

Time

Fig. 8. Pacing Protocol. The upper panel represents an electrical pacing stimulus. The lower panel is a schematic of the activation of action potentials in response to the pacing stimulus. (A) 1:1 capture. The activation of the action potentials is equal to the frequency of the pacing stimulus. (B) loss of 1:1 capture. The activation of the action potential is not equal to the frequency of the pacing stimulus.

If an arrhythmia was induced during the pacing protocol, the electrical stimulation

from the point electrode was stopped to see if the arrhythmia would terminate without

an electrical stimulus. A sustained arrhythmia was considered an arrhythmia that did

not spontaneously terminate for at least 16 seconds. A shock protocol was applied to

terminate sustained arrhythmias. The shock protocol consisted of applying a high

voltage stimulus from a Grass S88 stimulator starting with 20V (25 Ω) and 20 ms via

the perfusion tubes of the customized adaptor (Figure 9). If a shock failed to

terminate the arrhythmia, the shock voltage and duration was increased by 10V and

10 ms. If the arrhythmia still would not be terminated by a 60V and 60 ms shock, the
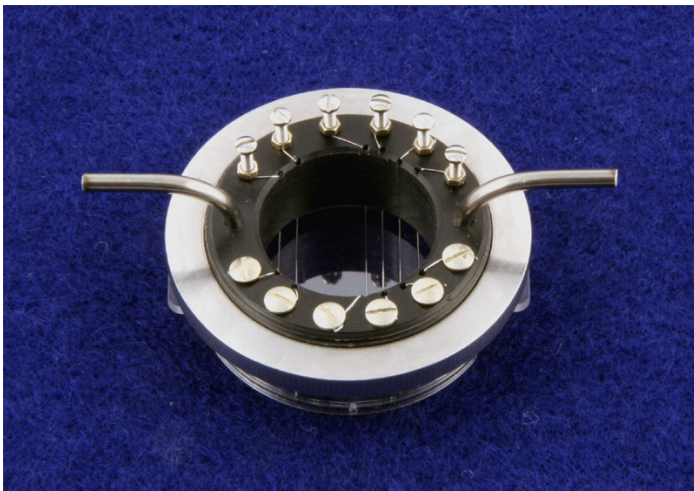
experiment was ended.

Fig. 9. Customized Adaptor. An enlarged view of the customized adapter with perfusion
tubes. This adaptor is placed over the temperature controlled culture dish system on which
the monolayers is placed.

Immunohistochemistry

Immunohistochemistry was performed in order to confirm alignment and orientation

changes in cardiomyocyte monolayers due to 8% pulsatile mechanical strain.

Cardiomyocyte monolayers adhered on silicone chambers were fixed in 4% formalin

in Dulbecco's Phosphate Buffered Saline (PBS) (Fisher Scientific) for 10 minutes and

rinsed two times with PBS. Each chamber was blocked with 0.2% Triton-X and 2%

Bovine Serum Albumin and immunostained for F-actin using monoclonal anti-F actin (Invitrogen). Other monolayers were immunostained for both F-actin and Cx43 using monoclonal anti-F actin (Invitrogen) and Alexa fluor 488 goat anti-rabbit (Invitrogen), respectively. Immunostained monolayers were mounted on glass slides and examined by an Olympus IX70 confocal microscope.

Data Analysis

Data preparation and initial filtering was performed in LabVIEW. Data preparation included identifying which pixels from the PDA contained fluorescence data and interpolating pixels with missing data as needed. Typically 5 pixels, but no more than 10 pixels, were interpolated due to damaged pixels or motion artifacts. Filtering included a seventh order median filter, a detrend filter, and if necessary an eighth order low pass Bessel filter with a cutoff frequency of 60 Hz to reduce signal noise. The low pass filter was verified to ensure that while noise was reduced, the quantitative analysis of the data was not affected. A detrend filter was used to deduct baseline drift by subtracting a third order polynomial, fit to the signal, from the original optical signal. Data measurement and quantification was preformed with PV-Wave software (Visual Numerics, TX). For each optical recording, all data was normalized to set the minimum and maximum fluorescence at each pixel to the same scale.

APD80 is the time interval between depolarization and 80% repolarization of the action potential, which is defined as 80% of the maximum amplitude

(Figure 10).[46, 47, 49] APD_{80} calculations were performed on an averaged action potential from each optically mapped recording for each pixel such that APD_{80} was an aggregate APD_{80} for the coverslip at a given cycle length. Restitution curves were generated by plotting APD_{80} as a function of each pacing interval (or basic cycle length). Average APD_{80} of reentrant arrhythmias were measured by taking the average of three action potentials during the arrhythmia, at the time and pacing rate of arrhythmia onset. Maximum capture rate (MCR), defined as the maximum pacing frequency during the steady state pacing protocol at which capture occurs, was identified and recorded for each cardiomyocyte monolayer for comparison.[26, 51]

$$\frac{\Delta F}{F} \alpha\ V_m$$

Basic Cycle Length

80% Max Amplitude

APD_{80}

Time
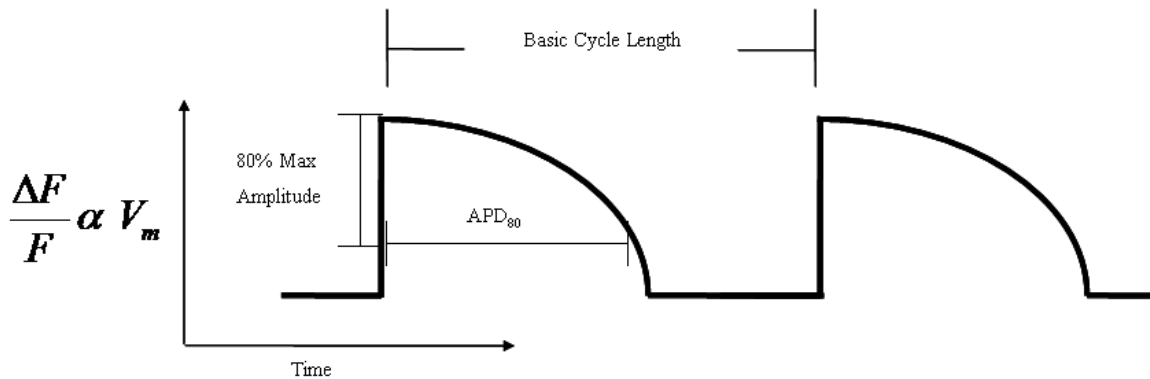
Fig. 10. APD80 calculation method. This is a schematic of action potentials obtained from optical mapping image. ΔF/F is the fluorescence signal, which is proportional to Vm which is membrane potential.

Statistical Analysis

Data was presented as a mean ± standard deviation (SD) and analyzed using one-way ANOVA followed by post hoc student's t-test. Correlation and regression analysis (Microsoft Excel) were used to evaluate trends in data sets. Differences of $P<0.05$ were considered significant.

Results

Morphology

In order to confirm that the chosen mechanical strain parameters in these experiments induced cellular alignment, immunofluorescent microscopy was performed on the control group and mechanical strain induced group. Immunofluorescent imaging of F-actin confirmed that the 8% mechanical strain, using the mechanical strain unit, induced cell alignment in the direction of strain as seen in Figure 11. It also demonstrated that Cx43 was diffusely distributed around the cytoplasmic membrane in the control group, while localized at longitudinal cell termini in the mechanically strained group.
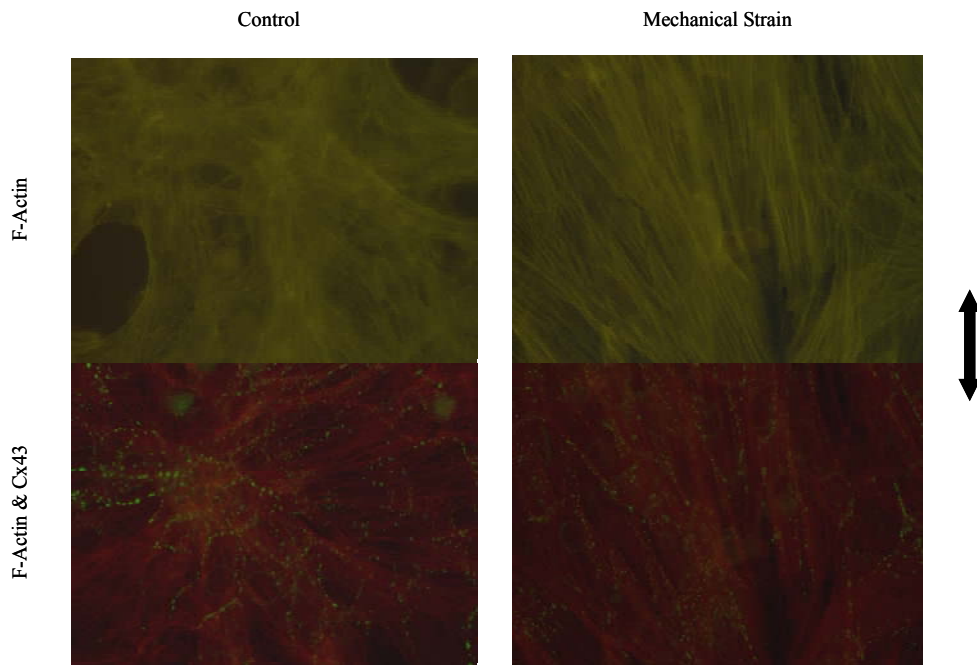
Fig. 11. Expression and distribution of F-actin and Cx43 in cultured cardiomyocyte monolayers. Representative immunofluorescent micrographs (at 60x magnification) of cardiomyocyte monolayers with and without mechanical strain are shown. Cardiomyocyte monolayers were stained for F-actin (with phalloidin in green) in the upper panel. Cardiomyocyte monolayers were stained for F-actin (with phalloidin in red) and Cx43 (Alexa fluor 488 goat anti-rabbit in green) in the lower panel. Arrow indicates strain direction. Both F-actin and Cx43 aligned in the direction of strain.

APD$_{80}$

The average APD$_{80}$ of control monolayers (n=10), mechanically strained monolayers (n=11), electrically stimulated monolayers (n=11), and mechanically and electrically stimulated monolayers (n=6) was measured at cycle lengths, 1000 ms, 500 ms, 333 ms, 250 ms, and 200 ms and reported in Figure 12. Previous studies usually report APD$_{80}$ measured at a 2 Hz pacing rate.[23, 47, 49] Therefore, in addition to the figure depicting APD$_{80}$ at different pacing frequencies, the average APD$_{80}$ at a pacing frequency of 2 Hz for control monolayers, mechanically strained monolayers, electrically stimulated monolayers, and dyssynchronously mechanically and electrically stimulated monolayers was 214.34 ± 32.56, 201.09 ± 37.56, 179.47 ± 23.34, and 179.39 ± 22.14 ms respectively. At a cycle length of 1000 ms, there was significant difference between all stimulation groups compared with control. At a cycle length of 500 ms, there was a significant difference between the electrical stimulation group and the combined electrical and mechanical stimulation groups compared with the control group. At a cycle length of 500 ms, there was also a significant difference between the mechanical stimulation and electrical stimulation groups. There was no significant difference observed between any groups at cycle lengths 333 ms, 250 ms and 200 ms. The sample APs of all experimental groups are also shown in Figure 13.
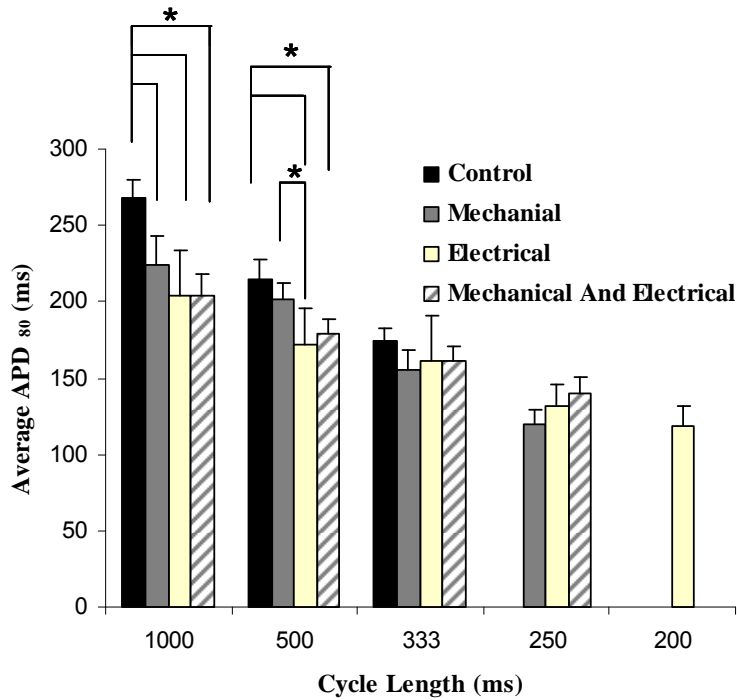
Fig. 12. Average APD80. The average APD80 of each experimental group measured at different cycle lengths. (* indicates $p < 0.05$)

Fig. 13. Representative cardiomyocyte monolayer action potentials in experimental groups showing changes in action potential shape. The electrical stimulation and mechanical and electrical stimulation groups have shortened action potential durations and different rates of repolarization compared to the control and mechanical strain group.

Arrhythmia Inducibility

Arrhythmias developed in 12 of 27 control monolayers, six (6) of 18 mechanically strained monolayers, seven (7) of 21 electrically stimulated monolayers and four (4) of 15 mechanically and electrically sti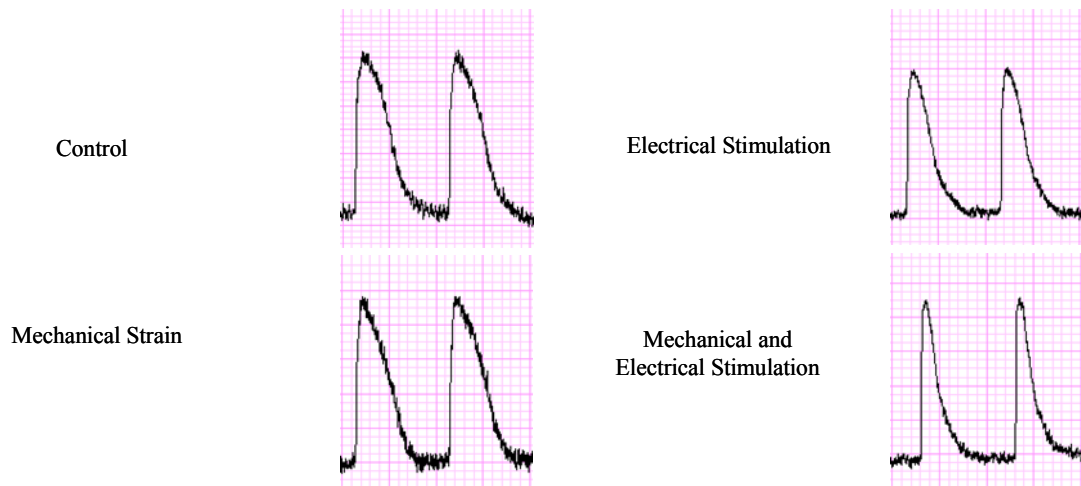mulated monolayers for total inducibilities of 44.4%, 33.3%, 33.3%, and 26.7%, respectively (Figure 14). No significant difference in inducibility between experimental groups was observed, however a trend was observed where the stimulated groups seemed to be less inducible when subjected to increasingly rapid pacing from a point electrode than the control group.
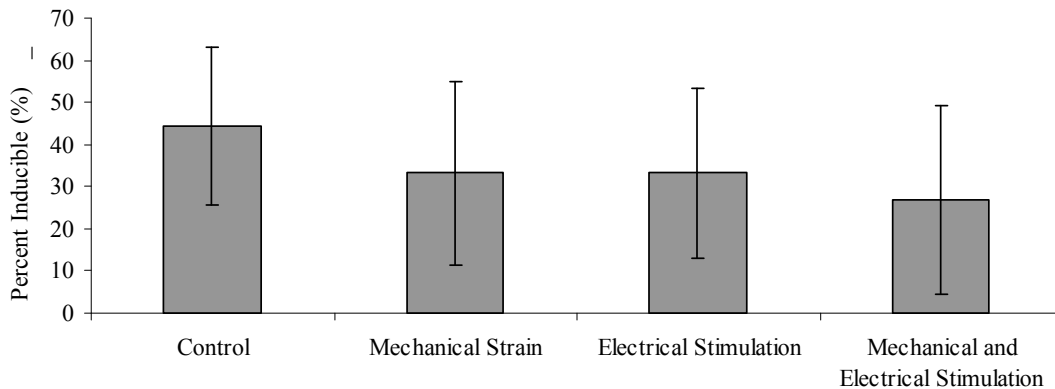
Fig. 14. Arrhythmia inducibility in cardiomyocyte monolayers. Percent inducibility of each experimental group is plotted showing a trend in which groups with any type of stimulation seem to be less inducible than control. (The whiskers represent 95% confidence intervals).

APD$_{80}$ of Reentrant Arrhythmias

The average APD$_{80}$ of arrhythmias, measured at arrhythmia onset at the pacing frequency at which the arrhythmia occurred, in control monolayers (n=12), mechanically strained monolayers (n=6), electrically stimulated monolayers (n=7) and mechanically and electrically stimulated monolayers (n=4) was 206.48 ± 59.811 ms, 198.33 ± 45.23 ms, 270.67 ± 71.05 ms and 195.96 ± 51.07 ms,

34

respectively (Figure 15). The average APD_{80} of arrhythmias in each experimental

group was not found to be significantly different.
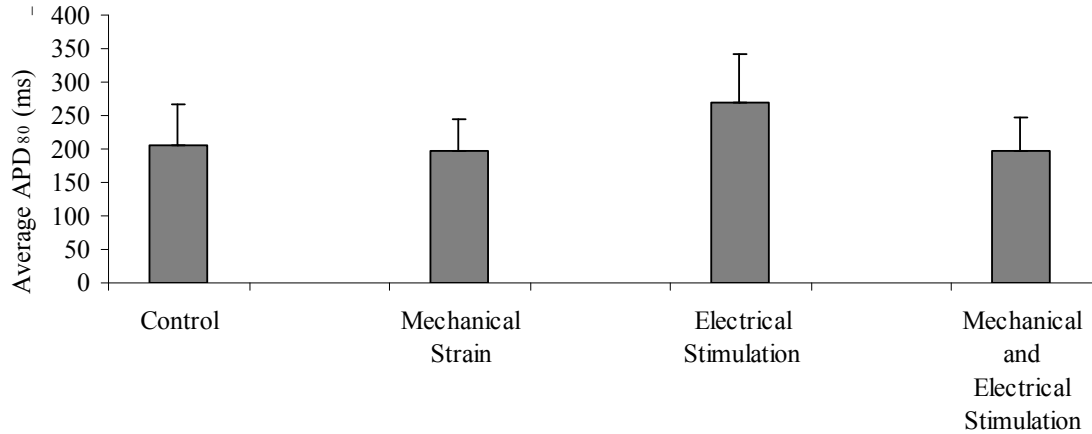
Fig. 15. APD80 of reentrant arrhythmias. Average APD80 of reentrant arrhythmias for all experimental groups. No significant difference between the groups was found.

Maximum Capture Rate

The average MCR of control monolayers was 180.5 ± 80.3 beats/minute (range of 60

to 300 beats/minute). The average MCR of mechanically strained monolayers was

184.8 ± 46.5 beats/minute (range of 120 to 240 beats/minute). The average MCR of

electrically stimulated monolayers was 187.6 ± 60.4 beats/minute (range of 60 to 300

beats/minute). The average MCR of mechanically and electrically stimulated

monolayers was 160.9 ± 64.8 beats/minute (range of 60 to 240 beats/minute)

(Figure 16). The average MCR of each experimental group was not found to be

significantly different and no trend was apparent.
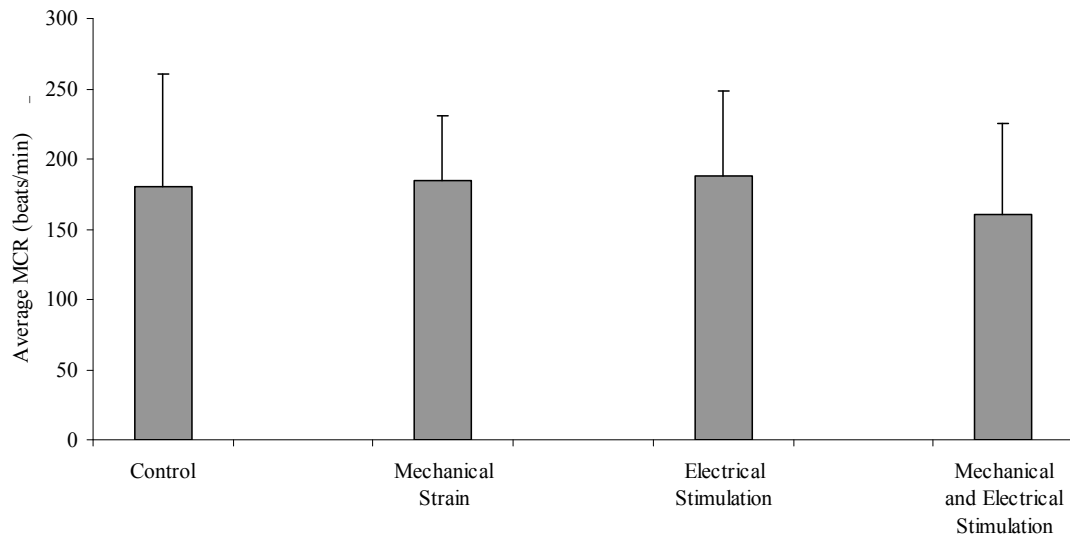
Fig. 16. Maximum capture rates of cardiomyocyte monolayers during pacing protocol. The average maximum capture rates of all experimental groups was plotted. No significant difference between groups was observed.

Average Arrhythmia Rate of Rotation and Type of Arrhythmia

Of the 12 control monolayers that resulted in arrhythmias, the average rate of rotation was found to be 3.5 ± 1.0 Hz. Eight (8) of these monolayers were single arms spirals, while three (3) monolayers had two-arm spirals with the same chirality and one (1) had a three-arm spiral with different chirality. Of the six (6) mechanically strained monolayers that resulted in arrhythmias, the average rate of rotation was found to be 3.5 ± 0.8 Hz. Three (3) of these monolayers exhibited single spirals, while one (1) monolayer had a two-arm spiral with the same chirality and two (2) monolayers had two-arm spirals with different chirality. Of the seven (7) electrically stimulated monolayers that resulted in arrhythmias, the average rate of rotation was found to be 2.7 ± 0.8 Hz. All seven (7) of these arrhythmias were single spirals. Of the four (4) mechanically and electrically stimulated monolayers that resulted in arrhythmias, the

average rate of rotation was found to be 3.7 ± 0.8 Hz (Figures 17 and 18). All four

(4) of these arrhythmias were single spirals. The average rate of rotation for each

experimental group was not found to be significantly different.
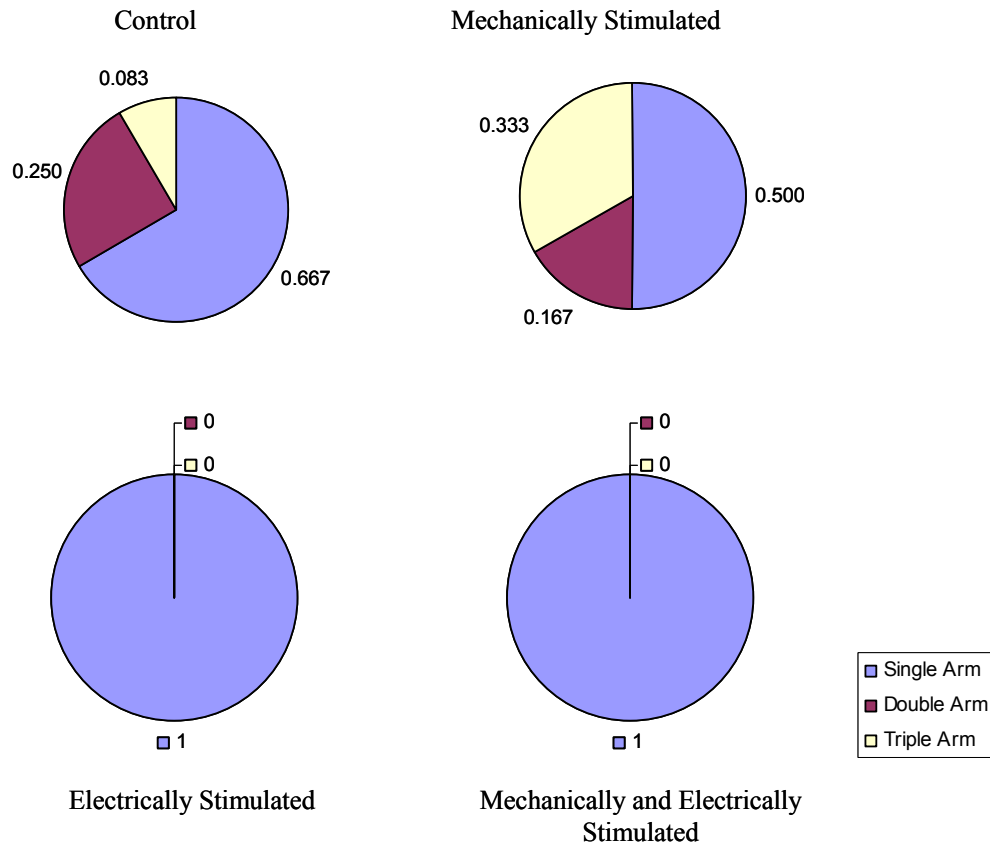

Fig. 17. Types of Arrhythmias. The number of single, double, and triple arm arrhythmias were plotted in each experimental group. The majority of arrhythmias in all groups were single arm spirals. The electrical stimulation and mechanical and electrical stimulation groups only had single arm spirals.
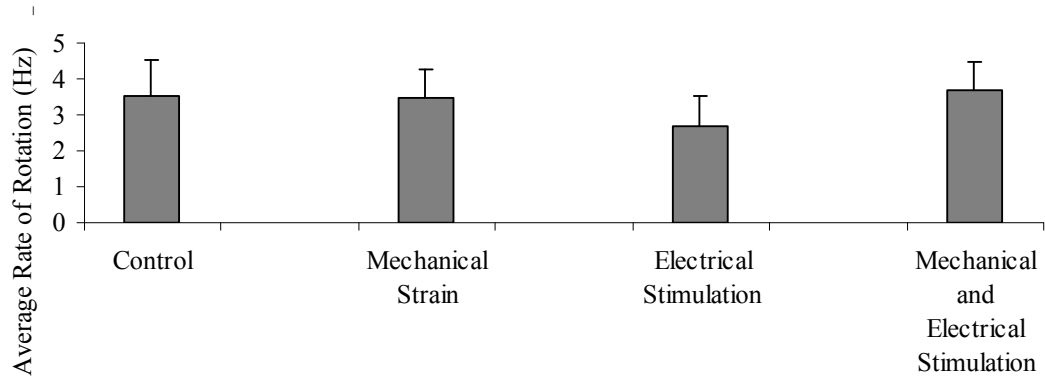
Fig. 18. Average rate of rotation of reentrant arrhythmias in cardiomyocyte monolayers. The average rate of rotation of arrhythmias was measured in each experimental group. No significant difference was found between each group.

Arrhythmia Sustainability and Termination Voltage

Of the 12 control monolayers with inducible arrhythmias, 10 of these arrhythmias were sustained. Nine (9) control monolayers that exhibited sustained arrhythmias were subjected to the shock protocol, of which six (6) were successfully shocked out of arrhythmia. Termination shocks required an average voltage of 22.5 ± 3.5 V with an average duration of 22.5 ± 3.5 ms.

Of the six (6) mechanically strained monolayers in which arrhythmias were induced, three (3) of these arrhythmias were sustained. These three (3) monolayers with sustained arrhythmias were subjected to the shock protocol of which two (2) were successfully shocked out of arrhythmia. Termination shocks required an average voltage of 25.0 ± 7.1 V with an average duration of 25.0 ± 7.1 ms.

Of the seven (7) electrically paced monolayers in which arrhythmias were induced, four (4) of these arrhythmias were sustained. Two (2) of these monolayers with sustained arrhythmias were subjected to the shock protocol but neither were successfully shocked out of arrhythmia.

Of the four (4) mechanically and electrically stimulated monolayers in which arrhythmias were induced, three (3) of these arrhythmias were sustained. Three (3) of these monolayers with sustained arrhythmias were subjected to the arrhythmia termination protocol of which two (2) were successfully shocked out of arrhythmia. Termination shocks required an average voltage of 35.0 ± 7.1 V with an average duration of 35.0 ± 7.1 ms

Discussion

A system to apply dyssynchronous mechanical and electrical stimulation to cardiomyocyte monolayers was successfully developed. This is the first system to apply this combined stimulation to cardiomyocyte monolayers for the purpose of investigating the consequences of dyssynchronous electrical and mechanical stimulation on cardiac electrophysiology. This system was used to characterize the changes in conduction properties including APD_{80}, APD_{80} of reentrant arrhythmias, rate of reentry rotation, and maximum capture rate. The overall inducibility of cardiomyocytes subjected to dyssynchronous mechanical and electrical stimulation was also compared.

Morphology and Protein alignment

Using immunohistochemistry techniques, it has been confirmed that 8% mechanical strain induces alignment of F-actin and Cx43 in cardiomyocytes. This agrees with previous studies in which cell orientation, F-actin, and Cx43 distribution was promoted in the direction of strain.[13] The movement of Cx43 is also evidence of gap junction remodeling in cardiomyocytes as shown previously.[22, 30, 31]

APD$_{80}$

APD$_{80}$ in non-inducible monolayers paced at 2 Hz has been previously reported to range from ~106-225 ms.[8, 47, 49] The APD$_{80}$ values in all groups in this work are consistent with these previous studies. Specifically, APD$_{80}$ (measured during 2 Hz pacing) in electrically stimulated and non-stimulated cardiomyocyte monolayers has been previously reported by Sathaye et al. (2006) to be 210.6 ± 9.9 ms and 197 ± 11.8 ms respectively.[23] In this study, APD$_{80}$ results differed from the Sathaye study. Sathaye et al. (2006) compared APD$_{80}$ of electrically stimulated and non-stimulated monolayers and found no significant difference between groups at any cycle length.[23] In this study, a significant difference between the electrically stimulated group and the control group at the 1000 ms and 500 ms cycle lengths was found. This difference could be due to differences between electrical stimulation protocols during the incubation period. Sathaye et al. (2006) electrically stimulated their monolayers for three days while this study stimulated the monolayers for 24 hours.[23] The length of stimulation period could make a difference since it has been shown that chronic pacing stabilized APD over time.[23] Sathaye et al. reported

that six days of electrical stimulation was less effective at stabilizing APD than eight days of electrical stimulation. In the Sathaye control group, APD_{80} decreased over time. However, in the Sathaye electrical stimulation group, APD_{80} remained constant over time. It is hypothesized that electrical stimulation causes continual contraction of the cells which promotes activation of stabilizing gap junction proteins and reduces the variability of stress response protein expression.[23, 57] Continual electrical stimulation could potentially keep the conduction properties across a monolayer constant and thereby reduce the variability of APD_{80}.

There also seems to be a trend in which any form of stimulation causes a decrease in average APD_{80}. It may be possible that stimulation whether mechanical, electrical, or combination may shorten APD_{80} which could contribute to arrhythmia resistance. Since APD_{80} is only one factor contributing to cardiac conduction, further experimentation needs to be performed to identify the impact of shortened APD_{80} on culture cardiomyocytes arrhythmogenesis.

Arrhythmia Inducibility

It is difficult to compare the arrhythmia inducibility results in cardiac recordings of this study with previous studies due to differences in pacing protocols used to induce arrhythmias. In a 2006 study, by Bursac et al., anisotropic monolayers were initially subjected to a rapid pacing protocol similar to this study;[47] however, in the Bursac study, if spontaneous reentries did not occur when 1:1 capture was absent, reentry was induced by rapid point pacing trains from peripheral sites to perturb reentries.

Bursac et al. (2006) did not clarify the number of spontaneously developing arrhythmias versus perturbed arrhythmias. They reported a total inducibility rate of 84.5% with the average rapid pacing rate to be 6.2 ± 1.4 Hz. The percent inducibility in this study is significantly lower than reported by Bursac et al. (2006) but only includes arrhythmias occurring due to rapid pacing.

Although no significant difference in arrhythmia inducibility was found between experimental groups, a trend of the stimulated groups to be less inducible than the control group was observed. This trend, combined with the reduced APD_{80} in the stimulated monolayer groups at higher cycle lengths, may warrant further experimentation with a larger sample size to determine if any form of stimulation in monolayers may reduce arrhythmia susceptibility. Since elongated APD_{80} is not the only parameter responsible for arrhythmia inducibility, other parameters such as culture homogeneity (fiber orientation), conduction velocity, excitability, and refractory period may need to be quantified to identify different factors which may contribute to arrhythmia susceptibility.

APD_{80} Reentrant Arrhythmias

Iravanian et al. (2003) reported APD_{80} of reentrant arrhythmias to range between ~92-117 ms.[49] The arrhythmia APD_{80} values from this study range from ~195-271 ms are larger than the Iravanian study which may be due to their measurements being taken at days 4-7 after plating while in these experiments measurements were taken at days 4-5 after plating.

Maximum Capture Rate

The MCRs found in this work are consistent with MCRs reported in previous studies.[23, 26, 51] Sathaye et al. (2006) reported MCRs in monolayers electrically stimulated for four (4) days and non-stimulated monolayers to be 264 ± 54 beats/min and 288 ± 54 beats/min, respectively and did not show a significant difference between groups.[23] My results are consistent with the Sathaye study, as a significant difference between the electrically stimulated and control group was not found. Radisic et al. (2004) also compared MCRs of monolayers electrically stimulated for five days to control monolayers and showed a significant difference in MCRs when measured on day eight.[26] The average MCR was reported to be 550 ± 20 beats/min and 400 ± 10 beats/min in electrically stimulated and non-stimulated monolayers, respectively, with a significant difference found between these groups. Since no significant difference between electrically stimulated and non-stimulated groups was found, the results of this study disagreed with the Radisic study. However, this discrepancy in results could be due to the difference in applied electrical stimulation protocol duration. In this study, electrical stimulation was applied to monolayers for a shorter time period, 24 hours, as opposed to the Radisic study which electrically stimulated myocytes for five days. Radisic et al.(2004) also plated their cardiomyocytes on an alternate substrate and measured MCRs on a different day after culture. Overall, a significant difference in average MCR between all experimental groups was not shown.

The MCR control values in previous studies[23, 26, 51] are much higher than those in this study. It may be postulated that these differences are due to culture variability, substrate differences, and measurement method. For example, the MCRs of neonatal ventricle wall tissue (1.5-2.5 mm thickness) was reported by Bursac et al. (1999) to be 475.0 ± 25.0 beats/min. In the same study, the MCRs of neonatal rat cardiomyocytes plated on a polyglycolic acid (PGA) mesh scaffold construct (2 mm thickness) was 111.7 ± 9.5 beats/min and 175.0 ± 21.3 beats/min when plated with a higher fraction of myocytes.[51] The MCRs found by this study are most consistent with the values reported in the three dimensional (3D) constructs by Bursac et al. (1999). Although my experiments are on a two dimensional (2D) construct, the cell preparation for both the 2D monolayers and the 3D constructs was identical. Additionally, the microelectrode array used to stimulate and record signals was in the same plane and essentially was a 2D recording of the signals similar to my monolayer recordings. Sathaye et al. (2006) used a point electrode to stimulate monolayers which was identical to my preparation. Radisic et al. (2004) used field electrodes to stimulate the myocytes but measured electrical activity with a separate electrode. However, in general it seems that ventricular wall tissue and myocytes plated on constructs or scaffolds that mimic 3D tissue produce higher MCRs than cardiomyocytes plated on 2D substrates, as was seen this experiment.

Average Arrhythmia Rate of Rotation and Type of Arrhythmia

The average rate of rotation of reentries found in this study are consistent with rate of rotations reported in previous studies such as Bursac, et al. (2004) who reported the

average rate of rotation of single-arm spirals to be 4.6 ± 1.2 Hz.[35] Bursac et al., 2006 also reported the average rate of rotation of single-arm spirals to be 5.2 ± 1.7 Hz.[47] Bursac et al. (2006) did not report the average rate of rotations in multi-arm spirals. In this study, both single arm and multi-arm spirals in the control and mechanical strain groups were observed. However, when compared, no significant difference between the average rate of rotation of the single-arm and multi-arm spirals within each group was found. Although no significant difference in the average rate of rotation in reentrant arrhythmias was found between experimental groups, it seems that arrhythmias in monolayers subjected to electrical stimulation alone during culture have a slower rate of rotation. Since the rate of rotation may be influenced by many factors including conduction velocity, monolayer confluency, cellular alignment or meandering of the spiral, further experimentation to measure these parameters should be performed.

Arrhythmia Sustainability, and Termination Voltage

As stated previously, it is difficult to compare the arrhythmia characteristics in cardiac recordings of this study with previous studies due to the difference in pacing protocol.[47] Sustained and non-sustained reentry rates are reported in previous studies,[35, 49] but the categorization of arrhythmias occurring due to a steady state pacing protocol or due to a dynamic pacing protocol was not distinguished.[47] Arrhythmia termination voltages were also not reported in previous studies. Nonetheless, the termination voltages observed in this study were able to end arrhythmias.

Study Limitations

In this research, a model has been developed to study the effects of mechanical strain and electrical stimulation on cardiomyocyte monolayers and the impact of these perturbations on arrhythmia formation. However, this model has some limitations, including being a 2D preparation, the electrophysiological transformation of cardiomyocytes over time, the physiological differences of the monolayer model, and using a steady state pacing protocol during experimentation.

Cardiomyocyte monolayers cannot represent the complex 3D structure of intact cardiac muscle. However, there are several advantages for using a 2D cell culture model to study cardiac electrophysiology. Monolayers, as opposed to single cell preparations allow the study of more complex phenomena such as wavefront propagation and cell-to-cell communication.[23] The 2D nature of this model allows one to study reentrant arrhythmias which cannot exists in anything less than a 2D system while eliminating the complexity of a 3D system, such as whole heart models where tracking the path of conduction can be difficult.[8] Monolayers are composed of a homogeneous cell type that does not include connective tissue or blood vessels which can interfere with optical recordings or the propagation of action potentials.[8]

A source of variability in this preparation is that cardiac cells transform electrophysiologically over time.[23] The cell size, morphology, and APD all change during development.[23] To reduce the electrophysiological changes with time, experiments are performed on the same day of cellular development for each culture.

There are several physiological disadvantages for using a monolayer model. For example, the action potentials of the neonatal rate are inherently different than the human. Secondly, it is not physiological for cells to be attached to a substrate. However, one advantage is that the attachment of the monolayer to a substrate eliminates motion artifacts and obviates the need for electro-mechanical uncouplers, needed in ex-vivo tissue preparations, that affect the electrophysiology of the cells.[8] Thirdly, the electrical behavior of the cardiomyocyte monolayer is dependent on the confluency of the monolayer and the isotropy of the cardiomyocytes which are parameters that are not factors in cardiac tissue. In this study, monolayers were plated with a high density of cardiomyocytes in order to ensure the confluency of the monolayers. As a byproduct of mechanical strain, anisotropy was induced in mechanically strained monolayers, which is more physiologic.

Another limitation of this study is the selection of a steady state pacing protocol to induce arrhythmias in the cardiomyocyte monolayers. There are several other pacing protocols that can be used to induce arrhythmias including paired stimulus (S1S2),[49, 58] strong electric shocks[59] and burst[35, 47] pacing protocols. The S1S2 pacing protocol consists of a series of paced beats (S1) to which a premature stimulus (S2) is applied at various intervals relative to the end of the S1 stimulus. The burst pacing protocol consists of a rapid (usually ≥ 2 Hz) burst of 10-20 pulses from a point electrode. The purpose of these protocols is to consistently induce arrhythmias in cardiomyocyte monolayers in order to measure arrhythmia inducibility, arrhythmia characteristics, mechanism of formation of arrhythmia, or even measure the success

of the arrhythmia inducing pacing protocol. There is no standard method to induce

arrhythmias in experimental preparations. Therefore, a limit to this study was that

arrhythmia inducibility using a steady state pacing protocol has not been previously

performed and therefore is not easily compared. It would be useful in future

experiments to measure arrhythmia inducibility using a S1S2 pacing protocol that

seems to be more common in published literature.

Another limitation is that the stimulus threshold and capture of the cultures that was

determined for electrical field stimulation during the incubation period was 0.5 Hz

and determined by visual inspection of the cardiomyocytes under phase contrast

microscopy at room temperature. Although the stimulus threshold and capture rate

consistent across cultures from different isolations, it would be advantageous to

determine the stimulus threshold and capture rate at physiological temperature.

Conclusion

Electro-mechanical dyssynchrony is a significant phenomena that can change the

electrophysiological properties of the heart.[6] The altered electrical and mechanical

timing in this dyssynchrony can also contribute to anomalies in action potential

depolarization which can increase arrhythmogensis. It is important to study electro-

mechanical dyssynchrony because it is a phenomena that can occur during increased

afterload or preload in vivo as is seen in patients with heart failure.[7] Since this

phenomena is difficult to investigate in vivo, a system that subjects cultured

cardiomyocyte monolayers to mechanical strain, electrical stimulation, or a

combination of mechanical strain and electrical field stimulation in culture with variable levels of synchrony or dyssynchrony has been developed. This system was used to characterize the electrophysiological properties of those cardiomyocyte monolayers. Most mechanical strain experiments have studied protein production, DNA/RNA production, signal transduction pathways, and alignment.[11-17, 22] A limited number of studies have focused on the impact of strain on cardiomyocyte conduction properties.[22, 60] Similarly, a large proportion of electrically stimulated myocyte studies have focused on protein synthesis, DNA/RNA production, and cellular alignment.[23-27] While studies that research the impact of electrical stimulation on cardiomyocyte conduction properties are more common,[23, 26] they do not discuss arrhythmia susceptibility. This system has the ability to compare cardiomyocyte monolayers subjected to multiple types of stimulation during culture with control monolayers which has not been performed in previous studies. Additionally, this system has the ability to alter mechanical strain parameters including variable strain cycles and strain percentage. Furthermore, the characteristics of electrical field stimulation, such as pulse duration and stimulus amplitude, during culture can easily be altered. More importantly, the system has a controllable delay in which the timing of the electrical field stimulation with respect to the mechanical strain cycle can be varied. While previous studies limited their measurements to cardiomyocyte conduction properties, the susceptibility of monolayers to arrhythmias has also been measured in this study. The culmination of these added system features results in an enhanced capability to replicate cardiac

remodeling via multiple stimulation methods which are fundamental to cardiac

function.

Appendix A

The conditioning circuit diagram is located in Figure 19. This circuit was powered by a $\pm$ 15 V DC-DC converter (VESD1-S12-D15-SIP, V-Infinity). A low-noise dual operational amplifier (TL072, STMicroelectronics) was used to invert the analog signal, and in conjunction with a 100 kΩ trimming potentiometer, increased the gain of the signal and adjusted the DC offset. An optoisolator (H11L1, Motorola) was used to reduce signal noise by isolating the output timing pulse from the input waveform of the strain instrument. The selected optoisolator has an infrared-emitting diode (IRED) in combination with a Schmitt trigger output and is used to electrically isolate the timing signal. The optoisolator takes an input electrical signal and converts it to a beam of infrared light, where it crosses a gap and is detected by a photosensor, which then converts the signal back into an electrical output signal. The input and output of the optoisolator are identical; however, the output is unaffected by any electrical noise from the input signal since there is no electrical conduction through the gap between the IRED and photosensor. The Schmitt trigger is a comparator circuit which utilizes hysteresis. When the input signal is higher than a set threshold (6 V) then the output signal goes low, when the input signal crosses the same threshold again, the output goes high. Since the mechanical strain output waveform is trapezoidal, the Schmitt trigger transforms the signal into a square pulse. The output from the optoisolator – Schmitt trigger serves as the input to a Grass SD9 stimulator (Grass Technologies, RI). The square waveform triggers an electrical stimulus to be output by the Grass SD9 stimulator. The SD9 made it possible for the

duration and delay of the stimulation pulse, from the onset of mechanical strain to be

varied, which for these experiments was set to 4 ms and 1075 ms, respectively. The

polarity of the stimulation pulse output from the Grass SD9 stimulator was alternated
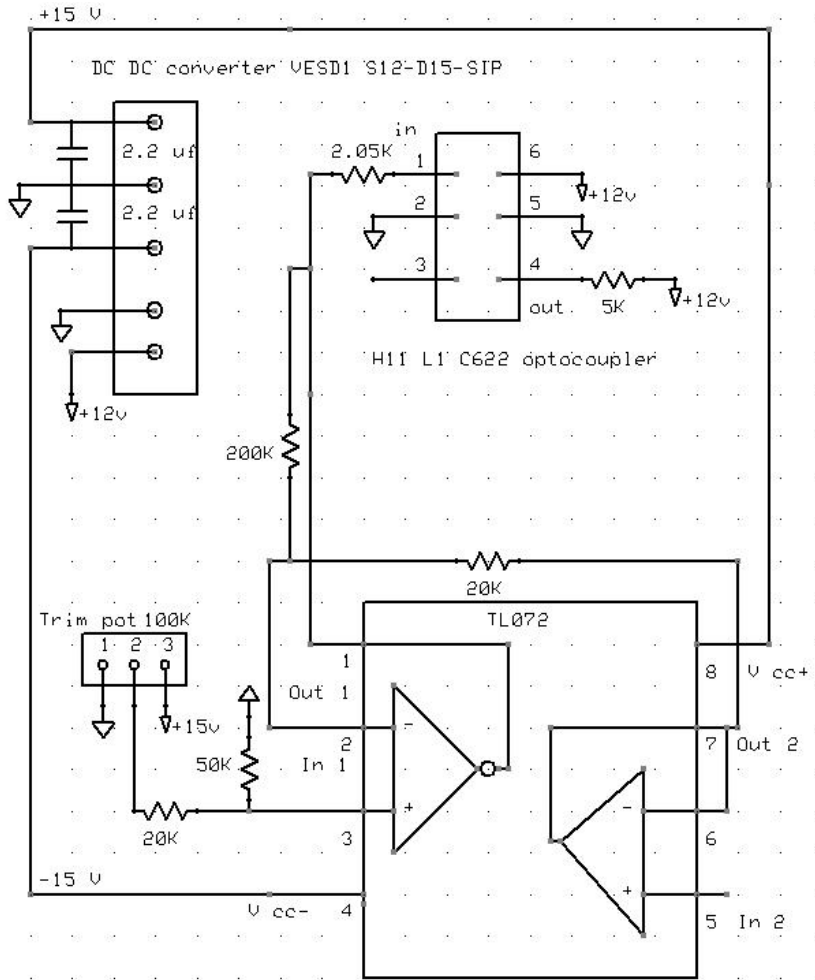
by the A385 stimulus isolator.

Fig. 19. Signal conditioning circuit schematic.

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