2016

Flexible electronic substrates to deliver electromechanical stimuli to regenerative cardiac patches

Kalmykov, Anna

http://hdl.handle.net/2144/17089

Boston University
BOSTON UNIVERSITY
COLLEGE OF ENGINEERING

Thesis

FLEXIBLE ELECTRONIC SUBSTRATES TO DELIVER ELECTRO-MECHANICAL STIMULI TO REGENERATIVE CARDIAC PATCHES

by

ANNA KALMYKOV

B.S., University of Maryland, 2008

Submitted in partial fulfillment of the requirements for the degree of
Master of Science

2016
Approved by

First Reader
Joyce Y. Wong, Ph.D.
Professor of Biomedical Engineering
Professor of Materials Science and Engineering

Second Reader
Katherine Yanhang Zhang, Ph.D.
Associate Professor of Mechanical Engineering
Associate Professor of Biomedical Engineering

Third Reader
Robert H. Helm, M.D.
Assistant Professor of Medicine
Assistant Professor of Radiology
ACKNOWLEDGMENTS

I would like to thank Prof. Joyce Wong for giving me the privilege of working in her laboratory and her continuous support, mentorship, caring and advice. I am very grateful to Dr. Elaine Lee for her support of my work and guidance. I thank Dr. Vladimir Kleptsyn for his mentorship, guidance, and training. I thank Dr. Robert Helm and Dr. Katherine Zhang for their input and comments. I am grateful to all of the Wong Lab members, especially Dr. Joann Buczek-Thomas and Erin Roberts, for their help.
FLEXIBLE ELECTRONIC SUBSTRATES TO DELIVER ELECTRO-MECHANICAL STIMULI TO REGENERATIVE CARDIAC PATCHES

ANNA KALMYKOV

ABSTRACT

After myocardial infarction, the stressed environment may cause negative cardiac remodeling. An emerging treatment option, engineered cardiac patches can be mechanically conditioned to increase alignment or electrically stimulated to enable anisotropic conduction. While proper integration with native tissue may require both stimuli, very few studies have applied both simultaneously, and only to extracted tissues. To demonstrate feasibility, a rigid electrode prototype was constructed to incorporate electrical stimulation into a commercially available mechanical conditioning system. Electrodes were assembled to fit the system’s geometry, and parameters were optimized to mimic the human heart rate. Previously, a study used 5-Azacytidine (5-Aza) to differentiate mesenchymal stem cells (MSCs) toward cardiac lineage, which was used here for proof-of-concept testing. Unexpectedly, MSCs treated with 5-Aza and electrically stimulated showed a decrease in cardiac marker troponin and an increase in MSC surface marker gene expression. In this setup, current from rigid electrodes passes through the media; however, under physiologically relevant conditions, electrical signals should propagate directly through cardiomyocytes. Therefore, a method to apply electromechanical stimulation to individual cells was explored in a point source stimulation platform. Electroconductive adhesive (ECA), a composite of silver and polydimethylsiloxane, was used to fabricate flexible elastic microelectrode arrays that
provided positive and negative voltage sources to individual cells. Devices were not cytotoxic before applying an electric field; however, applied current caused electrolysis of media and cytotoxicity, even using current stimulation parameters lower than those in published studies. These findings suggest ECA electrochemical properties need more characterization and alternative materials for microelectrodes.
TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................................................................................ iv

ABSTRACT .......................................................................................................................... v

TABLE OF CONTENTS ..................................................................................................... viii

LIST OF TABLES ............................................................................................................... ix

LIST OF FIGURES ........................................................................................................... x

CHAPTER 1: Introduction and Background ................................................................. 1

1.1. A Need for Regenerative Heart Patch ................................................................. 1

1.2. Significance of Electrical and Mechanical Stimulation in Cardiac Engineering .... 1

1.3. Cardiac Patch Engineering ..................................................................................... 4

1.4. Project Aim and Rationale ..................................................................................... 4

CHAPTER 2: Electromechanical Stimulation Using Rigid Electrodes ....................... 6

2.1. Motivation and Experimental Design Rationale .................................................... 6

2.2. Materials and Methods .......................................................................................... 8

2.2.1. Rigid Electrode Prototyping and Optimization of Signal Parameters ............ 8

2.2.2. Proof-of-Concept Testing of Rigid Electrodes with Cells ............................... 10

2.2.2.1. Mouse Induced Pluripotent Stem Cells ..................................................... 10

2.2.2.2. Human Mesenchymal Stem Cells (MSCs) ............................................. 10

2.3. Results and Discussion for Proof-of-Concept Electrical Stimulation of MSCs ... 13
CHAPTER 3: Electromechanical Stimulation Using Flexible Electrodes for Point Source Stimulation

3.1. Motivation and Experimental Design Rationale .................................................. 18

3.2. Stretchable and Flexible Microelectrode Fabrication Technology ....................... 19
  3.2.1. Mask Design and Mask Writing .................................................................. 20
  3.2.2. Photolithography ...................................................................................... 22
  3.2.3. Polydimethylsiloxane (PDMS) Thin Films for Point Source Stimulation .. 24
  3.2.4. Elastic Conductive Composite Preparation and Processing ..................... 26

3.3. Cytotoxicity Testing and Proof-of-Concept Testing ............................................. 31
  3.3.1. Cytotoxicity Without Electric Field ......................................................... 32
  3.3.2. Cytotoxicity With Electric Field ............................................................. 33

CHAPTER 4: Conclusion and Future Directions .......................................................... 36

BIBLIOGRAPHY .................................................................................................... 40

CURRICULUM VITAE ............................................................................................ 43
LIST OF TABLES

Table 1. Fluorescence activated cell sorting (FACS) for mesenchymal stem cells (MSCs). MSC Surface Markers determined by FACS .......................................................... 11
## LIST OF FIGURES

| Figure 1 | Technical drawing of rigid electrode design | 6 |
| Figure 2 | Computer-aided design 3D model: rigid electrodes for electrical simulation | 7 |
| Figure 3 | PSPICE model of amplifier circuit and block diagram of the set up | 7 |
| Figure 4 | Change in gene expression of CD73 marker resulting from differentiation of MSCs with 5-Aza and electrical stimulation for 14 days | 14 |
| Figure 5 | Western blots for selected cardiac markers | 15 |
| Figure 6 | Change in protein expression of cardiac markers after differentiation and electrical stimulation using rigid electrodes | 15 |
| Figure 7 | A visual overview of photolithography process that outlines the fabrication steps for flexible elastic microelectrodes | 19 |
| Figure 8 | Concept of flexible point source device illustrated by the CAD models and by the photolithography mask designs | 21 |
| Figure 9 | Scanning electron microscopy images (SEM) of silicon wafers for PDMS molds | 22 |
| Figure 10 | Zygo profilometer depth of etching analysis: wafer for microelectrode mold | 23 |
| Figure 11 | Zygo profilometer depth of etching analysis: wafer for point contacts mold | 24 |
| Figure 12 | Zygo image of the wafer for point contacts mold and the resulting thin film PDMS structure | 25 |
| Figure 13 | New mask design and CAD model for device with smaller total area | 28 |
| Figure 14 | Light microscopy images of electromechanical stimulation devices | 29 |
| Figure 15 | Finite element analysis conducted using Ansys Simulation Software | 30 |
Figure 16. Cytotoxicity of devices without electric field after 7 days (live/dead assay).. 32
Figure 17. Current mirror circuit to control the current at a constant value ....................... 34
Chapter 1: Motivation and Significance

1.1. A Need for Regenerative Heart Patch

Cardiovascular disease is a leading cause of death, affecting 17.3 million people worldwide (1). Blocking blood flow in the coronary artery cuts off the oxygen supply to cardiac muscle, which results in necrosis of cardiomyocytes and myocardial infarction (MI). Post-MI tissue may remodel negatively, which can lead to wall thinning and stiffening, thus resulting in ventricular dilation that may lead to heart failure (2,3). To regenerate myocardial wall tissue, current methods involve injecting multipotent stem cells, mature myoblasts, or embryonic stem cell-derived cardiomyocytes (3). Drawbacks of these solutions include possibility of teratoma development (3) and formation of unorganized myofibers; the latter can also lead to creating defective conduction patterns that may cause turbulent cardiac electrical activity and result in arrhythmia (4,5).

Therefore, for functional myocardial tissue regeneration, cardiomyocytes should be aligned to allow for proper conduction patterns. To achieve such, constructs may require electrical and mechanical conditioning in vitro prior to implantation to allow proper integration with the native tissue.

1.2 Significance of Electrical and Mechanical Stimulation in Cardiac Engineering

Electrical stimulation can induce cytoskeletal rearrangement, migration, proliferation, alignment, differentiation, and maturation of committed lineages (6). In mice, electrical stimulation promoted cardiac lineage commitment of embryonic stem cells, as well as increased functionality and contractility of engineered cardiac tissues in vitro. In addition
to electrical stimulation, mechanical conditioning is essential to developing and remodeling myocardium, as cardiac tissue is constantly subjected to dynamic environments *in vivo* (3). Mechanical stimulation has previously been shown in the literature to affect differentiation and proliferation (7). By stimulating stretch-activated channels, mechanical cues also lead to cytoskeletal rearrangement. The membranes attached to extracellular matrix (ECM) remodel, affecting membrane-bound integrins that, in turn, transmit mechanical cues into the cell and trigger the metabolic switch from proliferation to differentiation (7).

Electrical and mechanical stimuli are strongly coupled in cardiac tissue, and are critical for development of proper contraction (8). The goal of this work is to develop a flexible electronic circuit that can simultaneously deliver electrical and mechanical stimuli, which can be used to accelerate differentiation and commitment of stem cells or progenitor cells to cardiac lineage.

Several previous studies have used various bioreactor systems to provide simultaneous electrical and mechanical stimulation to extracted tissues (8-10). However, these systems relied on conductive characteristics of a whole tissue and allowed for stimulation at the macroscale, thus only approximating the cellular microenvironments *in vivo* (11). Such bioreactor systems were largely employed to stimulate embryonic, neonatal, or developed tissues (10). During bioreactor stimulations, keeping the tissue in a stable position between the electrodes is essential for the electric field to be maximally effective (12). Although plausible for such a construct in a stationary system, it may be challenging in the dynamic environment of Flexcell, a commercially available cell culture
platform that can apply cyclic strain to cells and tissues. Moreover, these systems rely on stimulation by electric field in the culture media, where current passes through the media. However, under physiologically relevant conditions, electrical signals should propagate directly through the cells.

Some researchers have used microelectrode arrangements for direct cardiac cell stimulation in lab-on-a-chip systems. Tandon and colleagues previously used indium tin oxide to create micropatterns that mimicked microscale environments. This stimulation system involved alternating positive and negative electrodes evenly spaced 200 μm apart (12). Electrical stimulation of neonatal rat cardiac cells using this system showed increase in proliferation, elongation, alignment, and expression of connexin-43 gap junctions. However, the materials used in these electrodes cannot be stretched, therefore restricting this set up to electrical stimulation only. A recent study by Pavesi and colleagues constructed a microfluidic device that combined mechanical and electrical stimuli (11). In this study, the linear electrodes providing stimulation to human mesenchymal stem cells (hMSC) were located 1.2 mm apart. Although this system aims to better mimic microenvironments, it is unable to provide individual cells with a direct voltage potential, as cells dimensions range from 10 μm to 100 μm. Delivering stimulation to individual cells via microelectrodes provides precise spatial control of the electric field and ensures that the electric field is passed directly through cells (12). Direct contact of microelectrodes to cells adds physiological relevance to the system. Previous studies showed that point source electrical stimulation alone was able to induce cardiac lineage commitment of murine embryonic stem cells (29).
1.3 Cardiac Patch Engineering

The long-term goal of this work is to generate patient-specific tissues from stem cells in a scaffold-free environment. In a recent publication, we proposed a novel method for grafting thermoresponsive copolymer poly(N-isopropylacrylamide-co-acrylic acid), p(NIPAAm-co-AAc) onto Flexcell Uniflex-Amino plates, which allow for uniaxial strain and are coated with amines on the surface (13). This modification to the platform allowed for controlled detachment and subsequent successful transfer of engineered cell sheets (13) that can be used to assemble preconditioned cardiac patches layer-by-layer. The current work proposes a novel electrical stimulation system coupled to the Flexcell mechanical conditioning system to be used with the thermo-responsive polymer modification for cell sheet engineering.

1.4 Project Aim and Rationale

The project aims to incorporate electrical stimulation into the commercially available Flexcell Tension System, which allows cyclic uniaxial strain to be applied to cells. Devices that combine both electrical and mechanical stimulation will be designed, built, and tested with cells. This device is a tool that can be used to understand the influence of electrical and mechanical stimuli on lineage commitment and maturation of cardiac progenitor cells.

Current stem cell-based methods of myocardial regeneration do not provide cues to mimic the hierarchical structure of the heart. Polymer-based scaffolds that incorporate structural cues may elicit inflammatory responses (5). Because electrical and mechanical
stimuli are highly coupled in cardiac tissue, pre-conditioning with both electrical and mechanical stimuli may increase myofiber alignment, improve electrical conductivity, and induce ECM secretion that mimics native tissue biomechanics.

Previously described methods provided macroscale stimulation and were unable to mimic native microenvironments. The electrode micropatterns proposed in this work are based on cardiac cell dimensions, and allow electromechanical stimulation to each cell in developing tissue. Such customized electrical stimulation mimics the tissue development as it ensures that currents go through the cells instead of over the cells through the media (12). Moreover, developing cardiac tissue lacks sufficient connexin-43 gap junctions to allow for signal propagation, making individual cell stimulation more attractive. The novel electrode design proposed in this work may overcome the current limitations of electrical stimulation. Additionally, this tool can be incorporated into a commercially available and validated mechanical conditioning culture system, circumventing the need to build a complex control system. Furthermore, this system has potential use in cell sheet engineering, which avoids the need for scaffolds and consequently the undesired inflammatory response.
Chapter 2: Electromechanical Stimulation Using Rigid Electrodes

2.1. Motivation and Experimental Design Rationale

Electrical stimulation parameters were selected to mimic the pacing of the human heart, which can be modeled as a pulse with a frequency of 60 beats per minute. A prototype consisting of a pair of rigid electrodes, a pulse generator, and an amplifier circuit was built. Electrodes must be non-toxic, easily sterilizable, and able to provide consistent and measurable currents while maintaining cell viability and proliferation. Because the electrodes were intended to work in conjunction with the Flexcell system, electrode geometry was based on the dimensions of a Flexcell plate (Figure 1). Materials for the rigid electrodes must be highly conductive, chemically inert, biocompatible, and easily manipulated to achieve the desired geometry. The electrode body was insulated in polydimethylsiloxane (PDMS), and only areas directly opposing the cell layer were exposed, as pictured in computer-aided design (CAD) models in Figure 2. Such constructs were designed to generate a constant electric field.

Figure 1. Drawing of Flexcell with rigid electrodes. A. Top view B. Side view of the system (front) C. Side view of the system (side). Dimensions are in inches.
Figure 2. Computer-aided design 3D model: rigid electrodes for electrical simulation.

A. A rigid copper electrode system. B. Copper electrodes insulated with PDMS. C. Electromechanical stimulation device in Flexcell well.

An amplifier circuit was assembled to amplify the signal from a function generator (Figure 3). Signal parameters were optimized to mimic the pacing of the human heart.

Figure 3. A. An amplifier circuit diagram (PSPICE). B. Block diagram of the set up.
Rigid electrodes provide a linear electrical potential in the media spanning the mechanical stimulation area. Such linear electrical potential was designed according to the simulation parameters from previous research by Tandon and colleagues in their work to optimize electrical stimulation parameters for cardiac engineering (9).

2.2. Materials and Methods

2.2.1. Rigid electrode prototyping and optimization of signal parameters

An early prototype used copper for the core of the electrodes because of its high conductivity, accessibility, and ease of manipulation. Because copper easily oxidizes and thus can decrease biocompatibility, the electrodes were sputtered with 300–400 nm gold layers. The original electrode design intended for each electrode to fit tightly with the PDMS step of the Flexcell well, as shown in Figure 2. Copper foil of 3 different thicknesses (50 µm, 75 µm, 125 µm) was tested. Electrodes were connected using gold wires to an amplifier circuit and a function generator outputting a square waveform (1 Hz frequency, 50% duty cycle, 2-5 Vpp, 1-2.5 VDC offset).

Copper electrode surfaces were cleaned by submerging in 6 M hydrochloric acid (HCl) for 5 minutes, rinsing with sterile deionized water, and drying under compressed nitrogen gas. The copper surface was sputtered with gold under vacuum for 3 min at 100 mA current, resulting in a 300–400 nm thick layer of gold on copper. PDMS was spin-coated onto each electrode at 3000 rpm, 30 s, to produce a thin insulating coat of PDMS with controlled thickness, and cured overnight at 80°C. Electrodes were sterilized under
ultraviolet (UV) light overnight or autoclaved to determine the material robustness after sterilization.

Square monophasic pulses applied to the electrodes caused electrolysis of water in the media and created copper oxide buildup on one of the electrodes. Moreover, electrolysis triggered a significant pH change that the buffering capacity of the media was not able to oppose.

To further shield the copper electrodes and increase biocompatibility, a second prototype of the electrodes was constructed with titanium, silver, and gold. Thin layers of each metal were deposited onto copper electrodes using direct current/radio frequency (DC/RF) Magnetron Sputter (Discovery 18, Denton Vacuum) to achieve a thicker layer of inert materials. The target thicknesses were set to 100 nm titanium for increased surface adhesion, 10 μm of silver to increase total thickness, and 0.5 μm gold layers to increase biocompatibility with cells. The electrodes were sterilized by autoclaving and used to stimulate bovine vascular smooth muscle cells (BVMSCs).

Signal parameters were optimized to reduce total current in the system and thus decrease electrolysis of cell culture media. The duty cycle of the square waveform was reduced to a pulse waveform with width of 2 ms; the height of the signal was reduced to 2 Vpp with +1 V DC offset. Frequency of 1 Hz was maintained to mimic heart rate at 60 beats per minute. An oscilloscope was connected across a resistor in series with the stimulation electrodes and used to monitor the signal in the circuit. The measured and displayed voltages were used to calculate the currents in each device.
The gold-sputtered electrodes continued to electrolyze the media. Therefore, in search for more inert materials, the third prototype used platinum wire for electrodes. To keep the desired geometry and fit within Flexcell wells, PDMS molds with the same dimensions as copper electrodes were used to anchor the wire and create a set of parallel electrodes as previously designed.

2.2.2 Proof-of-Concept Testing of Rigid Electrodes with Cells

2.2.2.1 Mouse Induced Pluripotent Stem Cells

Mouse induced pluripotent stem cells (iPSCs) were a generous gift from Dr. Darrell Kotton and George Kwong. Two wells seeded with mouse iPSCs were electrically stimulated for 1 week using the rigid electrode prototype with platinum wire with 2 ms pulses at 1 Hz, 2 Vpp, +1 V DC offset in complete serum-free differentiation medium (cSFDM) without addition of growth factors. Two wells cultured under the same conditions without electrical stimulation were used as controls. However, data from this study are inconclusive (data not shown). We recommend that experiments be repeated, and ribonucleic acid (RNA) be collected for real time polymerase chain reaction (qPCR).

2.2.2.2 Human Mesenchymal Stem Cells

Human mesenchymal stem cells (hMSCs) were collected from placental tissue and analyzed for MSC surface markers using fluorescence-activated cell sorting (FACS) (see Table 1).
Table 1. Fluorescence activated cell sorting (FACS) for mesenchymal stem cells (MSCs). MSC Surface Markers determined by FACS.

<table>
<thead>
<tr>
<th>Markers</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>VioGreen</td>
<td>99</td>
</tr>
<tr>
<td>PerCp</td>
<td>92</td>
</tr>
<tr>
<td>APC</td>
<td>12</td>
</tr>
<tr>
<td>PE</td>
<td>85</td>
</tr>
<tr>
<td>PE</td>
<td>88</td>
</tr>
<tr>
<td>FITC</td>
<td>72</td>
</tr>
<tr>
<td>PEVio770</td>
<td>88</td>
</tr>
</tbody>
</table>

A Flexcell plate was seeded with MSCs at $5 \times 10^5$ cells/cm$^2$ and cultured using low glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, HyClone), 1% Antibiotic/Antimycotic (ABAM). After reaching confluency, 4 out of 6 wells were differentiated into cardiomyocytes by supplying the media with 10 μM 5-Azacytidine (5-Aza) for 24 hours as previously described (15).

Cells were switched to post-differentiation media (low glucose DMEM, 10% FBS, 1% ABAM, 2 mM L-Glutamine (Invitrogen), recombinant human fibroblast growth factor (rhFGF, 10 ng/ml, R&D Systems)) and stimulated with 2 Vpp, 2 ms pulse signal with +1 V DC offset for 2 weeks. Undifferentiated cells (N=2) and differentiated but unstimulated cells (N=3) were used as controls. Media was exchanged every 48 hours. Following stimulation, RNA and protein were collected to analyze changes in gene
expression of MSC and cardiac markers.

To determine gene expression changes in differentiation with 5-Aza or in conjunction with electrical stimulation, qPCR was performed to analyze the expression of MSC marker CD 73. Samples were lysed, and RNA and protein were collected using RNeasy Plus Mini Kit (Qiagen) per standard manufacturer’s protocol. RNA (1 μg) was reverse-transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Analysis of gene expression was performed using TaqMan assays using human CD73 (Assay ID Hs00159686_m1, Applied Biosystems) on an ABI 7300 Real Time PCR system. Beta actin was used as an endogenous control, and gene expression was calculated using the ΔΔCt method.

To quantify protein expression changes in differentiation with 5-Aza and electrical stimulation, Western blot (WB) analysis was performed against cardiac markers. The protein fraction separated using Qiagen kit was precipitated from a protein column using 200 proof ethanol, resuspended in 50 mM LDS buffer with β-mercaptoethanol, and denatured at 99°C for 5 min. The protein was separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) using 4-12% Criterion gels (BioRad) at 100 V for 90 min. Protein was transferred from gel to polyvinylidene difluoride (PVDF) membrane using transfer buffer (25 mM Tris, 190 mM glycine with 0.25% SDS, 20% methanol, pH 8.3) for overnight transfer at 22 V, 4°C. Blots were blocked in 5% nonfat milk in Tris-Buffered Saline with Tween (TBST) for 1 hr at room temperature before probing with primary antibodies for cardiac markers connexin-43 (Abcam ab11370; 1:6000 dilution), cardiac Troponin I (Abcam ab47003; 1:4000),
homeobox protein Nkx2.5 (Abcam ab91196; 1:4000), α-actinin (Abcam ab68167; 1:5000). Beta actin (Sigma Aldrich A5441; 1:5000) was used as housekeeping gene. All antibodies were diluted in TBST. Blots were incubated with primary antibody solutions overnight at 4°C and washed 3 times with TBST. Blots were incubated in the corresponding secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, Jackson Immuno Research at 1:10,000 dilution) for 1hr at room temperature. Blots were developed using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific P/N 34080). Blots were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) between probes. WB images were analyzed using Image J software, and peak areas were normalized to protein expression of β-actin. Each data set was compared categorically using standard t-test (assuming equal variances). Values with difference of p<0.05 were considered statistically significant.

2.3 Results and Discussion for Proof-of-Concept Electrical Stimulation of MSCs
MSCs were previously noted as an abundant stem cell source and potential candidates for myocardial regeneration (18). After engraftment of MSCs onto the myocardium, they demonstrated increase in expression of cardiac and vascular markers (troponin T and αSMA). These cells also exhibited greater expression of connexins, as they formed intracellular junctions with surrounding cardiomyocytes (20).

MSCs collected from placenta tested positive for MSC markers using FACS (Table 1). Because this MSC population was highly positive for CD 73 and CD105 markers, these markers were selected as targets for gene expression analysis by qPCR.
The fold change in CD73 for undifferentiated, differentiated, as well as stimulated and differentiated cultures is presented in Figure 4.

**Figure 4.** Change in gene expression of CD73 marker resulting from differentiation of MSCs with 5-Aza and electrical stimulation for 14 days.

Changes in expression of CD73 were not statistically significant due to a large variation in undifferentiated samples. Samples differentiated with 5-Aza alone showed no change in expression of CD73, but samples differentiated with 5-Aza and electrically stimulated showed a slight increase in CD73 expression. These results contradicted the proposed hypothesis that predicted MSC surface marker expression would decrease as MSCs differentiated toward cardiac lineages, and therefore reduce CD 73 expression.

Western Blotting was used to quantify protein expression for connexin-43, Troponin I, and Nrk2.5 (Figure 5). Sarcomeric α-actinin antibody did not show binding to samples (data not shown).
Figure 5. Western blots for selected cardiac markers. Lanes 1, 2: undifferentiated samples, lane 3: differentiated and electrically stimulated sample, and lanes 4-6: differentiated samples.

Figure 6. Change in protein expression of cardiac markers after differentiation and electrical stimulation using rigid electrodes. Error bars represent ±1 standard deviation.

Although changes in expression of connexin 43 and Nkx2.5 were not statistically significant, cardiac troponin protein expression showed a significant decrease between undifferentiated and differentiated cells (P<0.05) and between differentiated and
electrically stimulated cells (P<0.05) (Figure 6). Troponins are late-stage cardiomyogenic markers, as well as indicators of cardiac ischemia (18, 19). Increase in troponins cTnT and cTnI were noted as markers of cardiac damage and myocardial necrosis (19). Troponin subunits T, C, and I combine into a complex that regulated contraction of cardiac muscle (22). The higher expression of troponin in undifferentiated MSCs is surprising; however, researchers have previously identified spontaneous exhibition of cardiac phenotypes in human MSCs in vitro (21). Alternative explanations of potential cross reactivity of the antibodies resulting in non-specific binding or insufficient blot stripping were hypothesized. To evaluate this proposition, blots probed with troponin were stripped twice and probed for cross reactivity with secondary antibodies. Both showed no signal, indicating that blots were sufficiently stripped between probes.

Another factor in this analysis involved uneven sample loading. Undifferentiated controls were more heavily loaded compared to differentiated samples, which affected the peaks detected in Image J. Chemiluminescence detection in overloaded samples becomes non-linear and it may saturate at high concentrations, altering the results.

5-Aza has been previously used to drive differentiation toward a cardiac lineage (15); however, previous studies have noted that in some cases cell death occurred after treatment with 5-Aza. In this study, greater cell death was also observed in samples treated with 5-Aza compared to the undifferentiated controls, which resulted in lower cell density and thus less protein harvested. 5-Aza is a demethylating agent, treatment with which leads to unmasking methylated promoters and expressing genes that were previously silenced. Its greatest disadvantage involves lack of specificity; it does not
target any particular genes, including those that may induce cardiac lineage commitment (18). Such non-specific demethylation may result in disruption of essential gene expression, subsequent metabolic changes, and cell death. Additionally, cell death may have contributed to the decrease in expression of connexin (Figure 6), as 5-Aza may have disrupted cell-cell connections (18).

The unexpected presence of cardiac markers in the unstimulated and undifferentiated control samples may possibly be attributed to inhomogeneity of the initial MSC population. MSCs collected from different patients may exhibit greater variability in differentiation because their initial states are largely varied. Future studies should pre-sort harvested cells based on the desired MSC surface markers. Alternatively, MSCs should be reprogrammed to iPSCs, followed by directed differentiation, and pre-sorted based on markers characterizing cardiac lineage.

The rigid electrode design stimulated MSCs by a voltage potential gradient in the culture media above the cells. A design to deliver point source currents directly to cells may improve differentiation toward the cardiac lineage, as signal that propagates directly through the cells may allow for electrical stimulation of each individual cell.
Chapter 3: Electromechanical Stimulation Using Flexible Electrodes for Point Source Stimulation

3.1 Motivation and Experimental Design Rationale

Electrical stimuli during tissue development may lead to cardiac lineage commitment by establishing structure and function to forming myocardium, which can manifest as striations and cell-to-cell coupling through gap junctions (28). Developing tissues lack extensive connexin-43 gap junctions that are responsible for electric signal propagation. Stimulating disjoined cells via electric field applied to the media may not be effective, as it neither provides for homogeneous stimulation nor mimics cellular microenvironments.

Previous research implemented point source stimulation systems for murine embryoid bodies to provide direct contact of electrodes with cells. Due to the direct contact, these systems were able to add physiological relevance to electrical stimulation (29). Because such direct current injection mimics native conditions more closely, the study showed this type of stimulation alone to be effective for directing cardiac lineage commitment in murine embryonic stem cells (28). To ensure that the same potentials are applied to each cell and current passes through cells instead of over cells, a point source stimulation system was designed to directly stimulate each cell in the developing tissue with the same electrical potential. The point source stimulation system in this study was designed to be compatible with the Flexcell mechanical conditioning platform.

The point source stimulation system will be compared to the rigid electrode system to determine whether stimulating individual cells will accelerate differentiation at
higher efficiency than an applied uniform electric field in culture media. Moreover, stimulation with precise spatial control over a limited area may help elucidate the significance of physically coupling electrical and mechanically active substrata for cellular differentiation. To combine point source stimulation with uniaxial strain, a flexible and elastic microelectronic circuit was designed and manufactured using standard soft photolithography techniques.

3.2. Stretchable and Flexible Microelectrode Fabrication Technology

Figure 7. A visual overview of photolithography process that outlines the fabrication steps for flexible elastic microelectrodes. Si- silicon wafer, ECA- electroconductive adhesive, PDMS – polydimethylsiloxane, P-photoresist (15).
3.2.1 Mask Design and Mask Writing

Masks were designed using computer-aided design software Draftsight (Figure 8 B, C). The first mask was designed to outline electrodes for positive and negative voltage sources (Figure 8B). Two interpenetrating comb-like structures were uniformly spaced to create alternating voltage sources, which were connected on each side to contact pads (Figure 8). Previous research showed that myofibrils will align in the direction of electrical field lines (9) and perpendicular to the vector of cyclic strain. Hence the direction of the electrodes was perpendicular to the direction of cyclic strain, applied as illustrated by force vectors on Figure 8 C.

A second mask was designed to provide openings for points of charge directly above the microelectrode grooves to each individual cell. The contacts were designed as squares with periodicity matching that of the electrode mask design (Figure 8). The layers were stacked to construct the flexible stimulation device with point source contacts.

Dimensions of cardiac progenitor cells (CPCs) and mature cardiomyocytes dictated the design input of the device geometry. For the grooves to create the microelectrodes and the spacing between the electrodes, various widths were selected to match cellular dimensions (varying from 20 μm to 100 μm) and allow cells to align between the electrodes. The stimulation array was designed to fit the stretching area (24 mm x 15 mm) of the Flexcell system that provides for uniaxial strain mechanical conditioning. Iron oxide masks were created using a laser lithography system for mask writing (Heidelberg Instruments DWL66). Masks were developed in MF-319 (TMAH), and photoresist was removed using acetone.
Figure 8. Concept of flexible point source device illustrated by the CAD models (A,B, C) and by the photolithography mask designs (C,D). A, B. CAD Model of device PDMS device in grey, microelectrodes in gold. C. Arrows indicate the direction of applied uniaxial strain, perpendicular to electric field. D. Mask for microelectrodes E. Mask designed for point source contacts.
3.2.2 Photolithography

Standard photolithography procedures were used to fabricate the patterns on silicon wafers for PDMS molds that outline electrodes. Targeted thickness (6-7 μm) was achieved by spin coating AZ4620 (AZ innovation in Semiconductor Chemistry) photoresist on a 4-inch silicon wafer at 4000 rpm, pre-baking at 60°C for 15 min, exposing under UV using Karl Suss MA6 Mask Aligner for 30 s, post-exposure baking at 90°C for 15 min, and developing with AZ® 826 MIF (2.38% Tetra Methyl ammonium hydroxide in H₂O₂). Deep reactive ion etching (DRIE) was used for anisotropic etching to create high aspect grooves with width-to-height ratios that range 2:1, 1:1, and 1:2, and resulting etch depths of 20 μm to 100 μm. Piranha solution (a mixture of phosphoric acid H₃SO₄ and peroxide H₂O₂) was used to remove photoresist Surfaces were passivated with silane under vacuum overnight. The wafers with etched surface topography served as molds for PDMS structures that comprised the device: a PDMS base that outlined microelectrodes and a strainer-like thin film to allow for point sources.

\[\text{A.} \quad \text{B.}\]

**Figure 9.** Scanning electron microscopy (SEM) images of wafers with varied microelectrode geometries and spacing were produced using Zeiss Field Emission Scanning Electron Microscope.
Scanning electron microscopy (SEM) was used to evaluate the wafers for potential defects and ensure the proper geometry and structural integrity (Figure 9). ZYGO NewView 6300 optical profilometer was used to confirm etching depths on the silicon wafers (Figures 10 and 11).

**Figure 10.** Zygo profilometer depth of etching analysis: wafer for microelectrode mold, with measured etch depth of 59 μm. A. Top view of wafer with microtrenches. B. Side view of the wafer with microtrenches.

**Figure 11.** Zygo profilometer depth of etching analysis: wafer for point contacts mold, with measured etch depth of 15.5 μm. A. Top view of wafer for point-contacts. B. Side view of the wafer for point-contacts.
3.2.3 PDMS Thin Films for Point Source Stimulation

To allow for direct point contacts to cells and to minimize exposure to the silver composite used for creating electrodes, the second mask will be used to make a layer of thin PDMS with a strainer-like pattern.

A thin film PDMS screen technology was developed to create a film of PDMS sufficiently thin enough to maintain contact between cells and microelectrodes. Several wafer etch depths were tested for the thin film PDMS layer (20 μm, 40 μm, 50 μm, and 90 μm). The high viscosity of PDMS sometimes prevented holes from fully forming openings in the films for the strainer-like pattern. Moreover, thin films were fragile and difficult to remove from the wafers without damage.

To increase the robustness of PDMS thin films, the ratio of PDMS base to curing agent was increased to 10:2 and cast on mold wafers of increasing etch depth (30 μm, 40 μm, 50 μm, and 90 μm). However, thin films were still fragile and damaged upon detachment. For controlled and uniform thin film detachment, Microposit photoresist S1813 (MicroChem) was spin coated onto the wafer at 2000 rpm for 1 min. PDMS was diluted with toluene (40:60) to reduce viscosity and allow for uniform thickness. To form robust thin films with openings, an optimal etch depth was determined to be at 30–40 μm. The films were post baked at 80°C, 2 hrs and submerged in acetone. Upon gentle shear, photoresist dissolved, releasing the films. Methanol was used to keep the films flat for alignment with microelectrodes. Zygo images of the wafers and the films on Figure 12.
Figure 12. The wafer for point contacts mold and the resulting thin film PDMS structure.

Etch depth was measured at 30μm A. Top view of wafer. B. Side view of the wafer.

PDMS film with through holes form strainer like structures. C. Top view D. Side view.

As the presence of holes could not be visually assessed by light microscopy for the total area of the film, a test was designed to ensure the presence of openings in the thin films. The films were placed on one side of PVDF membranes that were pre-treated with 10% nonfat milk in TBST. As methanol activates PVDF, methanol was used to wet the opposite side of the membrane. Ponceau S (Sigma, P7170) was applied drop-wise to the PDMS side of the PVDF membrane. Ponceau S collected in large drops on hydrophobic PDMS in the absence of holes, but diffused onto the opposite side and stained milk proteins on PVDF membrane red when holes were present. PVDF membranes were also used to transfer and align PDMS thin films with the microelectrodes. Wetting the PVDF
membrane with methanol released the thin film onto the device’s surface, providing for precise alignment with microelectrodes.

3.2.4 Elastic Conductive Composite Preparation and Processing

Electroconductive adhesive (ECA), a composite of silver in PDMS, was selected as a flexible, elastic, and conductive material. ECA can be screen-printed as previously described (14) into the device’s grooves to create the electrodes. Initial silver–PDMS composites were fabricated using 66.7%-80% v/v of 4 nm silver nanoparticle solution in toluene. The solution was mixed on a stir plate, homogenized by ultra-sonication (60 min, Branson 1510 Ultrasonicator), degassed, and cured. The resulting ECA was elastic but nonconductive.

Previous research showed less agglomeration and increased conductivity when silver flakes were substituted for nanoparticles (14). Therefore, consequent ECA used silver flakes and were mixed at 80% w/w silver rather than by volume. Because heat produced by ultrasonication may have caused premature curing of silver clusters separated by insulating PDMS, the curing agent was added after sonication. The solution was screen-printed into grooves and cured at 150°C, the temperature at which ECA transitions from insulating to conductive. However, the resistivity between the devices was inconsistent, as some grooves were insufficiently filled with silver. Additionally, some grooves were overfilled with excess of ECA on top of the grooves, producing short circuits.
To optimize the amount of silver deposited in the grooves, a greater etch depth was tested to increase volume for silver deposition. A 2:1 aspect ratio of groove width to depth was tested with etching depths of 25 μm, 30 μm, 50 μm, and 100 μm. However, an aspect ratio of greater than 1:1 caused the PDMS mold to bend and expel the ECA when force was applied during the screen-printing process. A 1:1 aspect ratio with 50 μm grooves with 50 μm depths was determined to be optimal for screen-printing conductive microelectrodes while maintaining elasticity for mechanical conditioning.

Decreasing the viscosity of the ECA composite was used to address screen printing limitations. ECA diluted with toluene increased the homogeneity of the solution during sonication and improved yields of screen-printed devices. Because of its volatility, toluene evaporated during the post bake, thus having no effect on the total percent composition of the ECA. Since increased (> 30% v/w) toluene dilutions resulted in excess PDMS swelling and deformation of the device, concentration of toluene in ECA was optimized at 25-30% v/w. Unevaporated toluene caused ECA to become porous and therefore non-conductive, so structures were baked under vacuum at temperatures of 150–160°C, which degassed the PDMS and decreased its porosity.

Thin 400 μm silver wires were used to connect the microelectrodes to the rest of the circuitry. However, the contact pads were not filled completely with ECA during the screen-printing process, making the contact challenging. When excess ECA was added to mitigate lack of conductivity, the presence of unevaporated toluene caused swelling of PDMS and subsequent short circuits. These short circuits and other defects were repaired manually under a light microscope prior to final post-bake and curing. Future prototypes
can address this design flaw by redesigning the masks with larger contact pad areas and increasing the distance between the side contact and the opposite microelectrode. The new mask design is illustrated on Figure 13.

**Figure 13.** A new mask design (A) and CAD model (B) covers a smaller area to avoid probability of defects. The design features enlarged contact pads and increased distance between the side contact and the opposite microelectrode to mitigate short circuits.

Device layers were assembled and sealed with PDMS, to form elastic and flexible microelectrode stimulation devices (Figure 14). After sterilization by autoclaving, the ECA maintained conductivity. To test for ECA leaching, the devices were exposed to
DMEM (low glucose) for 1 week. No pH change or leaching of the ECA into the solution was detected.

Figure 14. Microscopy images of devices. A. Basal part with flexible microelectrodes. B. Total assembled device with thin film structure aligned with microelectrodes.

To predict performance under uniaxial strain, CAD models of the devices were generated. The models were imported into Ansys Simulation Software 14.0 and treated as solids with a Poisson ratio of 0.498. As the Young’s modulus of PDMS varies between 360 kPa and 870 kPa, both values were used to calculate the minimum and maximum forces necessary to generate 10% displacement (Figure 15 D).

Since the model is linear, strains were symmetrical. Maximal local strain of ~14% was detected where the contact pads connect to the microelectrodes (Figure 15 C). Numerical solution at Young’s modulus value of 360 kPa identified that 0.12189 N force needs to be applied at the flat surface to gain 10% deformation, while at 870 kPa, the force of 0.29456 N is needed. Because this model is linear, the reaction force scaled linearly.
Figure 15. The finite element analysis conducted using Ansys Simulation Software 14.0 for solid with Poisson ratio of 0.498 and Young’s modulus of 360 kPa and 870 kPa. A. CAD model with microelectrode geometry. B. Forces applied to end surfaces, modeled to remain flat, with no restriction placed on the in-plane motion. C. Fine mesh was generated as illustrated with minimum edge length of 5x10⁻² mm. D. The load applied at the end surface resulting in 10% elongation. Equivalent elastic strain in the model using 380 kPa Young’s modulus and 780 kPa Young’s modulus presented in E and F respectively.
These data were used to predict the necessary force to achieve desired deformation while avoiding yield strength of the device assembly. To create PDMS molds with a desired thickness (0.5–1mm), PDMS was spin-coated at 2000 rpm for 30 s and post-baked at 80°C for 10 min three times. PDMS-coated wafers were post-baked at 80°C for 2 hrs. The PDMS molds were screen-printed with ECA, post baked, PDMS thin films were aligned with the ECA microelectrodes and two layers were joined by PDMS.

3.3. Cytotoxicity Testing and Proof-of-Concept Testing

ECA from previous studies (14) was designed to form flexible, elastic electronics, and the cytotoxicity of this material was not extensively described. Previous work by Oropeza-Ramos and colleagues described composites of silver and carbon in electrodes that were used for electrical stimulation of chick embryo heart cells (17). However, the sizes of these electrodes did not provide for stimulation of individual cells. The cytotoxicity study was designed to examine ECA biocompatibility with and without an applied electric field. The thin strainer-like top film was designed to minimize cell exposure to the silver composite, and cytotoxicity studies with and without the top film evaluated its necessity. A subsequent study evaluated potential formation of oxidative products of the silver composite due to electrolysis of the media. Because cardiomyocytes are non-proliferative, bovine vascular smooth muscle cells (BVSMCs) were used to test cytotoxicity. Moreover, BVSMCs are robust and inexpensive to maintain. As BVSMCs are muscle cells, their response to electrical stimulation can be evaluated on flexible substrata.
3.3.1. Cytotoxicity without electric field

Devices (N = 12) were assembled and autoclaved. To enhance cell attachment to the devices, flat PDMS stamps were sterilized and plasma-treated (1200 mTorr, 30 s, medium intensity), coated with 100μL of 75% fibronectin (FN)/ 25% glycerol solution (final concentration 0.75mg/mL), and used to stamp device surfaces. Each device was seeded with BVSMCs at 1x10^6 cells. Cells were cultured for one week and analyzed using live/dead assay (Life Technologies) per standard manufacturer’s protocol. Cells were viable after one week, and dead cells mostly concentrated in the holes for contacts that directly interfaced the cells with the silver composite (Figure 16).

**Figure 16.** Cytotoxicity of devices without electric field after 7 days (Live/dead assay). Live cells are shown in green, dead cells are in red. **A.** 20x image **B.** 5x images. **C, D.** Cytotoxicity to BVSMCs on device without thin film layer after one week. **C.** 20x image **D.** 5x image.
The cytotoxicity study was repeated without the top layer thin film (N = 6) to determine cell viability upon direct contact with the silver composite microelectrodes. After 7 days, cells remained viable and showed alignment along the microelectrodes due to topographical cues of the devices (Figure 16).

3.3.2. Cytotoxicity with electric field

Cytotoxicity studies with applied electric field were conducted without the top film, as cells remained viable without the top film in the first set of cytotoxicity studies and thus allowed testing with the cell-composite interface maximized. For this study, devices with 50 μm electrodes spaced 100 μm apart were used. Autoclaved devices were plasma-treated for 30 s at 1200 mTorr, and 50 μl FN (1 mg/mL) was added to the seeding surface. Devices with physisorbed FN were dried in the laminar flow hood under ultraviolet light for 1 hr. Devices (N = 3) were placed in a 6-well plate and connected to the amplifier circuit and pulse generator; devices (N = 3) with no applied electric field served as controls. BVSMCs were seeded at 1x10⁶ cells per well and cultured for 2 days prior to stimulation. Cells were subjected to 2 ms pulses of 2 Vpp (1 VDC offset) at 1 Hz for 7 days.

Live/dead assay indicated local cell death around the electrodes after stimulation. Resistance largely varied across microelectrodes, with average resistance normalized per distance at 56.4±12.5Ω per 1 mm. Because devices were connected in parallel, high and variable currents may have caused this localized cell death. A current mirror circuit was
designed to control the current at a constant value, regardless of the variable in resistance (Figure 17).

![Current mirror circuit](image)

**Figure 17.** Current mirror circuit to control the current at a constant value. Devices were connected in series, and bipolar junction transistors (BJTs) were used to control current at a maximum value of 2 mA.

Previous work measured 4 mA currents in a stimulation bioreactor patterned with indium tin oxide (ITO), where the distance between the positive and the negative electrodes was 200 μm (12). Thus, electrical stimulation was repeated with cells stimulated at 2 mA constant controlled current for 10 hrs. Despite the reduced and constant current, the cell death persisted.

Moreover, cell death was localized to the stimulation area of the device spanned by microelectrodes; cells surrounding the stimulation area remained viable. ECA toxicity due to leaching of silver ions under electrical field was investigated as another cause of cell death. Conditioned media was collected post-stimulation and added to confluent layer of BVSMCs on a tissue-culture polystyrene dish. The conditioned media did not have a dramatic negative effect on cell growth and viability.
Therefore, the reactivity of electrodes with culture media under electric field was investigated. The voltage was increased, and the electrodes and media changed color, indicating a change in pH and chemical reaction on the surface of the electrodes. Moreover, gas bubbles were observed and identified by smell as chlorine gas and hypochlorides, which indicate that the media was electrolyzing as reactive radicals from electrolytes in the solution combined with the electrodes to form toxic species. Moreover, hypochlorides form hypochlorous acid in water (23), contributing to the pH change. Therefore, electrolysis at the electrode surface was identified to be the cause of toxicity.

Generally, electrochemical experiments involve electrodes from noble metals such as platinum and gold; thus, as the electrochemical window of silver is small, a deviation outside of this window will result in a chemical composition change in electrodes and result in electrolysis. Therefore, coating silver with a less reactive material may increase the electrochemical window and alleviate electrolysis. The microelectrode surfaces for the devices were modified by sputtering 10 Å titanium and 100 Å gold onto devices with uncured ECA. Devices were post-baked at 150°C, allowing the gold layer to adhere to the microelectrodes. Because PDMS is not readily metallized, a razor blade was used to rapidly remove the gold layer between the electrodes, and remaining flakes were lifted using tape. The resulting devices contained gold-coated microelectrodes, which remained conductive at 10% stretch. However, 3 hours post-stimulation, the electrodes darkened upon visual inspection, the media had a pH change, and hypochlorite smell was observed. Under a light microscope, the gold layer had localized cracking, allowing silver to electrolyze the media.
Chapter 4: Conclusion and Future Directions

This work presents a way to combine electrical and mechanical stimulation for preconditioning cellular contracts in vitro. This platform explored both rigid and flexible elastic electrodes to be coupled with Flexcell, a system that can provide uniaxial strain to cells. A point source electrical stimulation device was designed and fabricated that featured flexible and elastic circuitry to stimulate individual cells in developing tissues. Composites of silver and PDMS were used to form microelectrodes; however they became cytotoxic under an applied electric field due to electrolysis of culture media. Optimization of signal parameters and reducing currents did not mitigate the electrolysis.

Because electrolysis persisted despite reduced applied current, the electrochemistry of ECA should be investigated further. To appropriately use ECA in electrical stimulation of physiological systems, it is essential to use this flexible electronics material in an electrolyte-rich solution such as DMEM. To study the electrochemical window of ECA, a three-electrode system involving working, auxiliary, and reference electrodes (e.g. silver/silver chloride electrode) may be used with a potentiostat to control and amplify the signal measured with respect to the reference electrode. To establish the electrochemical window for ECA, a cyclic voltammetry experiment with a gradual triangular waveform and cathodic potential can be applied to the working electrode. The measured current at the working electrode can then be plotted against applied voltage to determine its point of zero charge (PZC). The electrochemical window of pure silver is very small (silver dissolution occurring at 3.7 V Li/Li+), and the PZC of silver chloride is 4; silver iodine, 5.6; and silver sulfide, 10.2 (24). Staying in this
small electrochemical operational window allows several molecular layers in the solution to be charged by capacitance without causing the current to propagate 50-100 μm to the next electrode.

To form a sufficient electric field, future iterations of the device may require microelectrode materials with a greater electrochemical operational window. Carbon powder, such as small particle size high-structure carbon black, can be uniformly mixed to increase conductivity while providing a greater electrochemical window. High-structure carbon black had been previously used to achieve up to $10^8 \ \Omega$/cm resistivity at 50–60% w/v ratio. Conductive polymer composites, such as carbon black filled ethylene-propylene diene monomer (EPDM), may also provide suitable alternatives (25). Other highly conductive biocompatible carbon structures that also allow for flexibility include carbon nanotubes and graphene (26).

In addition to replacing the materials that may cause electrolysis, a circuit with a low noise operational amplifier could be used to both supply and measure microampere currents. This current source needs to be voltage controlled, as non-linearity may lead to unbalanced waveforms that can drift over time and exacerbate charge build up on the electrode’s surface (27). Supplied voltages and currents must be measured to ensure that both parameters stay within safe and physiologically relevant ranges.

Another solution can involve alteration of the waveform. Previous studies explored biphasic waveforms of 10 ms duration, where the load was actively grounded between the pulses to clear the residual charge on the electrodes (27, 28). These systems are charge balanced and were previously noted to decrease electrochemical reactions
(29). Furthermore such biphasic signals are more biomimetic, as they closely approximate action potentials of the polarization and depolarization of cellular membranes. For the point source stimulation system, further reducing currents to magnitudes similar to transmembrane currents (10–60 μA) may be necessary.

Finally, a system that removes toxic compounds from electrolysis may decrease cytotoxicity from the devices under applied electric field, such as a circulation system with continuous media feed and removal. Moreover, to effectively remove toxic species, this type of circulation needs to take place directly at the microelectrode interface. Cells are subject to some shear stress under cyclic mechanical conditioning; thus, the flow direction should be parallel to microelectrodes and parallel to the direction of strain.

Because circulation on the macro scale may add shear and negatively affect cells, a microfluidic solution may be more applicable. However, a single inlet and a single outlet would create large variabilities in velocity profiles and may add to turbulence and shear. To avoid these problems, a single inlet may be split multiple times and branched to achieve constant fluid flow to individual microchannels at each of the microelectrodes. Shear stress depends on volumetric flow, which in turn, is a function of channel dimensions and fluid viscosity (30). These parameters can be modeled numerically and used in estimating shear and velocity profiles.

In conclusion, this work presents a novel method for electrical stimulation that may be coupled with a commercially available mechanical stimulation platform for uniaxial strain. This work explored flexible and elastic microelectronics in a novel application. Stimulating cells at the microscale by point sources has the potential to
provide for more physiologically relevant cues to the cells in developing tissue. In this work, composites of silver and PDMS were used to create flexible electronics for point source stimulation. Although the devices were not inherently cytotoxic, applying current to the device caused electrolysis and cell death. Future work may require better characterization of ECA electrochemical properties, signal optimization, or alternative materials to reduce electrolysis.
BIBLIOGRAPHY


in concert improved the assembly of engineered cardiac tissue. *Journal of Tissue Engineering and Regenerative Medicine, 6*(10), pp.e12-e23.


CURRICULUM VITAE

Anna Kalmykov
YOB: 1985

8 Royce Road Apartment 1, Allston, MA 02134

EDUCATION

**Boston University**, Boston, MA
Masters in Science, Biomedical Engineering, expected 2016 (GPA 3.91/4.0)

**University of Maryland**, College Park, MD
Bachelors of Science, Biochemistry, 2008 (GPA 3.4/4.0)

EMPLOYMENT

**Boston University**, Biomedical Engineering Department, Boston, MA
April 2015-Present

*Research Assistant for Dr. Joyce Wong, Biomimetic Materials Engineering Laboratory*
- Designed electrical stimulation system for cardiac patch engineering.
  - Coupled electrical stimulation system to uniaxial cyclic strain cell culture platform using fabricated flexible electrodes with electrically conductive composites
  - Used platform to determine effects on human cardiomyocyte progenitor and induced pluripotent stem cells.
- Mechanically conditioned vascular smooth muscle cell sheets, modulating anisotropy via protein micropatterning.
  - Tested tensile strength, quantified cell alignment, and examined contractile protein expression change.
- Utilized temperature-responsive polymer for cell sheet detachment, transfer, and stacking.

**Boston University**, Mechanical Engineering Department, Boston, MA
September 2015-Present

*Teaching Assistant for Introduction to Engineering Design*
- Assisted students with circuit and control loops design, and computer-aided design of mechanical components.
- Provided feedback to teachers on student assignments.
**Boston University**, Electrical/Computer Engineering Department, Boston, MA  
*Electronics Laboratory Assistant*  
- Assisted students with laboratory and project assignments.  
- Performed basic equipment repair and maintenance.

**Genocea Biosciences**, Cambridge MA  
June 2014-September 2014  
*Process Development Summer Intern*  
- Optimized vaccine production using insect culture upstream process development.  
- Developed and performed Analytical Development Assays (WB, SDS-PAGE, ELISA).  
- Sourced and installed equipment for expansion of capabilities.

**Paragon Bioservices**, Baltimore, MD  
February 2009-January 2014  
*Business Development Associate*  
*Research Associate Scientist*  
- Nurtured client relationships and generated proposals for pre-clinical and GMP manufacturing projects  
- Built new partnerships by representing at meetings and conferences  
- Conducted process development projects for industrial and academic clients.  
  - Optimized production of bacterial recombinant proteins using expression analysis, transformation, best expression analysis, and scale-up process development (*E. coli, Pseudomonas sp.*).  
  - Produced recombinant proteins in mammalian cell culture via chemical transfection, electroporation.  
  - Developed vaccines through virus-like particles produced in mammalian cells on microcarriers.  
  - Utilized yeast expression systems for recombinant protein production (*Pichia, Saccharomyces*).  
  - Developed bacteriophage production process for food safety industry (*E.coli, Salmonella sp.*).  
  - Conducted protein expression in insect cell culture on a small and large scale (*T.ni, SF21, SF9*).  
- Managed microbial upstream laboratory, trained and supervised laboratory technicians, scheduled projects.
Bioprocess Scale-Up Facility, University of Maryland, College Park, MD
June 2007–January 2009

*Senior Laboratory Technician*
- Trained and supervised student technicians.
- Conducted fermentation projects for industrial and academic clients
  - Prokaryotic scale-up using pilot scale bioreactors (2.5L-250L).
  - Downstream and upstream processing of products.
- Performed product analysis and separations through continuous centrifugation, gel electrophoresis, tangential flow ultra filtration, and column chromatography.
- Assisted in teaching educational workshops for biotech companies, undergraduates, government agencies.

University of Maryland, Biochemistry Department, College Park, MD
September 2006-May 2007

*Research Internship*
- Studied biotin carboxylase-biotin carboxylase carrier protein complex.
- Purified the complex using column chromatography, analyzed its size and conformation by ultracentrifugation.
- Studied preliminary binding kinetics by stopped flow.

Johns Hopkins University, Bloomberg School of Public Health, Baltimore, MD
June 2002-May 2004

*Research Internship*
- Conducted microbiology and molecular biology research with environmental focus.
  - Utilized 16S rRNA sequencing to identify bacteria present in soil in Aberdeen Proving Ground, MD.
  - Analyzed species present for bioremediation abilities of depleted uranium.
- Developed a method of rapid identification of *Sphingomonas wittichii* used in environmental cleanup.
  - Identified the dioxin deoxygenase enzyme using whole proteome digest of crude cell extracts by matrix assisted laser desorption/ionization time of flight mass spectrometry.
  - Utilized transforming dioxins ability of *S. wittichii* in bioremediation of contaminated soils.

**PROJECTS**

Identification of Novel Transcription Factor Binding Sites using ChIP-Seq
(Molecular Bioengineering Course)
Minimally Invasive Catheter Deployed Pericardium Sensor Design (Advanced Biomedical Design and Development)
- Measured depth of ablation to prevent esophageal fistulas during electrophysiological arrhythmia treatments

**SKILLS**

Fermenters, centrifuges, bioreactors, microfluidizer processor, TFF, ViCell and CEDEX cell viability analyzers, NOVA Flex (nutrient, metabolite, gas analyzer), electroporation, ultracentrifuges, microscopes, aseptic technique, Matrix Assistant Laser Desorption Ionization Voyager Mass Spectrometer, agarose gels, denaturant gradient gel electrophoresis, SDS-PAGE, western blot, ELISA, LiCor Odyssey Infrared and BioRad ChemiDoc Imaging Systems, spectrophotometers, Nanodrop, AKTA prime, protein purification, PCR, ChIP-Seq, FlexCell, Instron, MATLAB.

**PUBLICATIONS**


Lee EL, Bendre HH, Robinson MK, Kalmykov A, Wong JY. Controlling cellular orientation and modulating contractile protein expression to create anisotropic vascular smooth muscle cell sheets. (in review)

Lee EL, Backman DB, Bendre HH, Robinson MK, Kalmykov A, Wong JY. Effects of modulating anisotropy and mechanical conditioning on mechanical properties of cell sheets. (in preparation)

**PRESENTATIONS**


**HONORS AND AWARDS**

Boston University Masters in Science Merit Scholarship, Boston University, Boston, MA Incentive Awards Scholarship, a full scholarship, University of Maryland, College Park.