

WPI

Assessing differentiation potential of C2C12 myoblastic cells on hydrogels, and development of stimulation device to induce contraction on regular and micropatterned C2C12 cells

Submitted By:

Adriana Martinez-Betancourt _____

Jeffrey Lessard _____

Kien Dao _____

Yow-Chyuan Yeh _____

Submitted to:

Professor Sakthikumar Ambady _____

Professor Raymond Page _____

April 26, 201

Abstract

We examined the differentiation characteristics of C2C12 mouse myoblasts on polyacrylamide hydrogels of varying stiffness' in comparison to widely used polystyrene tissue culture plates. The benchmarks we used included formation of myotube fibers, BrdU incorporation, myosin heavy chain expression, and fiber contraction. As a method of observing fiber contraction, we designed, built, and used an electrical stimulation device which allowed us to run a current through the cells after they had differentiated while viewing and recording the cells in real time. The data suggest that cells grown on hydrogels proliferate less but differentiate more similar to those *in vivo*.

Table of Contents

Abstract.....	2
Acknowledgements.....	5
Authorship Page.....	6
Table of Figures	7
Table of Tables	8
Table of Equations	9
Chapter 1: Introduction.....	10
Chapter 2: Literature Review.....	11
2.1 Tissue Engineering.....	11
2.2 Hydrogels.....	14
2.3 Electrical Stimulation.....	18
2.4 Micropatterning.....	21
Chapter 3: Project Strategy	25
3.1 Design.....	25
3.2 Initial Client Statement	26
3.3 Objectives, Constraints, and Functions.....	27
3.3.1 Feasible Objectives	29
3.3.2 Quantitative Analysis of Objectives.....	32
3.4 Revised Client Statement.....	39
3.5 Project Approach	39
Chapter 4: Alternative Designs.....	40
4.1 Needs Analysis.....	40
4.2 Previous Designs.....	40
4.2.1 Mechanical stretching device.....	40
4.2.2 Electrical stimulation: initial design.....	41
4.3 Feasibility Analysis.....	42
4.3.1 Mechanical Stimulation	42
4.3.2 Electrical Stimulation: initial design.....	42
4.4 Final Design Choice.....	43
4.5 Materials for Electrical Stimulator.....	44
4.6 Device Specifications.....	44

4.6.1. Stimulation procedure	Error! Bookmark not defined.
4.7 Design Validation	46
Chapter 5: Design Verification	46
5.1 Determination of Myoblast Differentiation	46
5.2 Differentiation of C2C12 cells on Polystyrene culture plates.....	48
5.3 Softwell™(Polyacrylamide hydrogels).....	51
5.4 Hydrogel Stiffness Testing.....	53
5.5 Micro-Patterning	55
Chapter 6: Discussion	56
Chapter 7: Final Design and Validation.....	58
7.1 C2C12 Mouse Myoblast cells	58
7.2 C2C12 culture and differentiation.....	59
7.2.1. Proliferation:	59
7.2.2 Differentiation:.....	59
7.3 Freezing C2C12 Cells	59
7.4 Immunocytochemistry	60
7.4.1 BrdU proliferation assay	60
7.4.2 Myosin Heavy Chain Expression analysis.....	60
7.5 Micro-Patterning	61
7.5.1. Coverslip method	61
7.5.2. Cell Printing Method.....	62
7.6 Testing Hydrogel Stiffness.....	63
7.7 Electrical Stimulation of C2C12 Multinucleated Fibers.....	64
Chapter 8: Future Work and Recommendations.....	64
Chapter 9: Conclusions	68
References.....	69
Appendices.....	73
Appendix A.....	73
Appendix B	84
Appendix C	87
Appendix D.....	90
Appendix E	93

Acknowledgements

We would like to thank the following people for all their help and guidance through the project:

Prof. Sakthikumar Ambady

Prof. Raymond Page

Justin Mih, Ph.D.

Satya S. Shivkumar, Ph.D

Heather Cirka

Lisa J. Wall

Chirantan Kanani

BME Department

WPI

Authorship Page

Section	Author	Editor
1. Introduction	J.L.	Y.Y./K.D.
2. Literature Review	A.M-B	Y.Y./K.D.
3. Project Strategy	A.M-B	Y.Y./K.D.
4. Alternative Designs	J.L.	Y.Y./K.D.
5. Design Verification	J.L./A.M-B	Y.Y./K.D.
6. Final Design & Validation	J.L/A.M-B	Y.Y./K.D.
7. Discussion	J.L	Y.Y./K.D.
8. Conclusion & Recommendations	J.L/A.M-B	Y.Y./K.D.

Table of Figures

Figure 1: Weighted Objective Tree.....	38
Figure 2: Flowchart of Project Approach	39
Figure 3: left) CAD drawing f removable tip. right) device schematic	45
Figure 4: C2C12 cell line in final stage of differentiation	48
Figure 5: Micro-Striation at Day 6 of Differentiation.....	49
Figure 6: BrdU/Hoechst stained Cells	49
Figure 7: Hoechst stained nuclei with fluorescent myosin heavy chains on day 9.....	50
Figure 8: Phase Contrast Images of C2C12 cells on hydrogels	51
Figure 9: Images of Most Successful Hydrogels Compared to the Polystyrene Control.....	52
Figure 10: Myosin Heavy-Chain Stain Comparison on Different Hydrogel Stiffnesses.....	53
Figure 11: Rheometer Testing.....	54
Figure 12: coverslip micropattern.....	55
Figure 13 Digilab’s commercial cell printer the Cell Jet	63
Figure 14: Screenshot sample of Code for Cell Printer	63
Figure 15: ESCC System Diagram	93
Figure 16: Microporous Alumina Membrane and Hole-Spotted PDMS Electrical Stimulation Device	93
Figure 17: Micropatterning on a Polyacrylamide gel (PAG).....	93
Figure 18: Micropatterning with Electrical Pulse Stimulation	94

Table of Tables

Table 1: Pairwise Comparison Table of main Objectives..... 33
Table 2: Pairwise Comparison of Sub-Objectives 1 34
Table 3: Pairwise Comparison of Sub-Objectives 2 34
Table 4: Pairwise Comparison of Sub-Objectives 3 35
Table 5: Pairwise Comparison of Sub-Objectives 4 35
Table 6: Pairwise Comparison of Sub-Objectives 5 36
Table 7: Pairwise Comparison of Sub-Objectives 6 36
Table 8: Stiffness Data Obtained from Samples 53

Table of Equations

Equation 1: Rubber Elasticity	15
Equation 2: Density of Crosslinking	17
Equation 3: Hydrogel Stiffness Equation.....	64

Chapter 1: Introduction

For this project, we investigated the effect which the use of polyacrylamide hydrogels on cellular gene expression and differentiation. To do this, we chose to use both regular methods of marking differentiation, namely phase contrast imaging and immunocytochemistry, and a functional assay for determining differentiation. The C2C12 mouse skeletal myoblast cell line was used in this project, due to the cells' differentiation characteristics and their ability to form contractile myotubes upon differentiation. Through literature review, it was found that the most effective functional assay which would be within the scope of the project would be electrical stimulation; however the cells could not be viewed in real time. Because of this, an electrical stimulation device designed, built, and implemented. This device was inexpensive, easy to use, and allowed us to view the cells in real time. This work is important to tissue engineering because through the design, the team developed a functional assay of skeletal muscle cells which is universal with all laboratory set ups, as well as being inexpensive and easy to sterilize.

Further, the team explored a method of testing the stiffness of the polyacrylamide hydrogels that can be routinely used in research labs. Such a method would eliminate the need for the more expensive atomic force microscopy. The method which was used for stiffness testing the hydrogels used a rotary rheometer on size 5 punches of a hydrogel, which was manufactured to be about 1 mm thick as opposed to the 100 μm thick hydrogel used in cell culture. This work is important to the tissue engineering field because the method the team developed can be used as adapted routine analysis in research labs and for high throughput quality control of hydrogel substrates by manufacturers of research grade substrates

Chapter 2: Literature Review

2.1 Tissue Engineering

The purpose of Tissue Engineering (TE) is to replace, repair, or regenerate tissues by signaling cells to carry out certain functions on a scaffold made from biocompatible materials. The main purpose is to control cell behavior to prompt specialized cell responses for structural and functional formations on a desired biomaterial (Luo and Shoichet, 2004). Tissue Engineering began in the late 1990s with the study of materials that could be combined and treated to withstand and reside in a semi-permanent manner inside of the body. This, soon became an enterprise that sought to attach the host's cells onto the material for better biocompatibility, tissue function, and overall healing (Varghese et al., 2005). The field of TE now focuses on using biomaterials as a temporary scaffold to give the cells the support until the tissue fully forms and performs the desired function on their own (Luo and Shoichet, 2004; Varghese et al., 2005; Tang et al., 2006; Hellman and Nerem, 2007).

Tissue Engineering relies heavily on previous work done in cell culture. Cell culture (CC) is a vital part of TE as host cell growth and differentiation depend on proper CC methods and experimentation. Much progress in TE methodology has been made in the last decade to realize the potential of TE across various platforms. Some of these platforms include Micro-Electro-Mechanical Systems (MEMS), cell printing, micropatterning on biomimetic materials, extracellular matrices (ECM), and 3-D scaffolds (Luo and Shoichet, 2004; Varghese et al., 2005; Tang et al., 2006; Hellman and Nerem, 2007; Li et al., 2008). However, before CC can be applied directly to TE, researchers use CC as a method of testing to see if a certain experimental method has the desired effect on the tissues and cells. The purpose of CC is to observe and analyze the behavior and characteristics of the cells that are being experimented on to understand

the reasons of the results so that they may later be applied to other applications in the future (Tang et al., 2006; Aldaye et al., 2010; Grainger and Putnam, 2011; Shepherd et al., 2011).

Muscle repair, or regeneration, is an important biological process that the body must be able to possess in order to maintain proper biological functions. The body does a capable job of replacing lost or damaged muscles up through approximately middle age. However, past middle age, the body's ability to maintain its muscle mass decreases every year. By the age of 80, humans have lost significant muscle mass (approx.. 40%), and have lost more than half of their ability to replace it on their own (Hansen et al., 2007). Myogenesis is a complex and regulated process that requires proliferation of myoblasts that have gone through the necessary morphological, biochemical, and molecular changes that result in myotubules that are multinucleated (Lee et al., 2009).

The purpose of this project was to understand the characteristics behind the differentiation of mouse C2C12 skeletal myoblasts to myotubes that make this particular cell line a good candidate to differentiate on a polyacrylamide hydrogel substrate and study the contractile properties of these cells when an electrical stimulation is applied *in vitro*. The C2C12 skeletal muscle line is a common, well-known *in-vitro* model that is used to observe differentiation of skeletal muscle cells and assessment of the contractile forces of the differentiated cells (Li et al., 2008). C2C12 cells are derived from the mouse skeletal muscle C2 cell line, and are used because these cells have similar characteristics to those of isolated human skeletal muscle cells (Luo and Shoichet, 2004; Gajsek et al., 2008; Hu et al., 2009; Lee et al., 2009). The myoblasts are known to differentiate and fuse into multinucleated myotubes in an *in-vitro* culture setting (Hansen et al., 2007). The cells also reach differentiation very quickly, a few days, after reaching a certain confluence and being switched to differentiation media. (Nishimura

et al., 2008). C2C12 *in vitro* cell culture is also a great model to exert physiological stresses to determine their relevant functions in the body (Kaji et al., 2010). The myotubes have the ability to contract in response to an electrical stimulation because the skeletal muscle has a calcium ion channel that allows Ca^{2+} inflow to trigger the contraction/relaxation cycle, and can do so to different voltages (Gutierrez-Martin et al., 2005). These known characteristics of the C2C12 are what make this cell line one of the best options to carry out the experiment.

The development of an electrical stimulation apparatus to test the differentiation of skeletal muscle cells requires a full understanding of mechanical signals of cells to be successful. Muscle cells interact with their surroundings through cytoskeletal connections with the extracellular matrix (ECM), which act as anchor points allowing them to cause traction. Morphology of the cell is dependent solely on its cytoskeleton, suggesting that morphology is intrinsically related to the cell's interaction with the ECM. Through analysis of differentiation and morphology on different substrate stiffness, the plan was to validate this theory. The team sought to explore the concept behind the production of hydrogels and how to control their mechanical properties, the interactions cells have with their substrate, and how the two relate to one another to influence the design of hydrogel substrates. This is important to cellular research as a whole because much of the success behind research is the elimination of variables. If researchers can grow tissues *in vitro* that can mimic *in vivo* behavior, then much more accurate representations of cellular processes can be attained. For tissue engineering to be successful, scaffolds and substrates should be tailored to the tissue at hand, and such fine control is attainable economically.

2.2 Hydrogels

The success of hydrogels for use in tissue engineering depends upon their mechanical and chemical properties. There are various methods that can be used to control the mechanical properties of gels, primarily, methods that control crosslinking, density, and biodegradable properties. Crosslinking density and degradation time are generally linked, which makes it very hard to separate these two properties. Studies have shown, however, that if a hydrogel possesses both hydrolytically labile units and multi-methacrylic groups it is capable of having its mechanical properties and degradation rate independent of each other (Cha, 2009). This suggests that it is possible to tailor hydrogels to accommodate the tissues being cultured on them.

A vital aspect of the use of hydrogels, in regards to TE, is as a substrate for cell culture. It is thus important to review the mechanical properties of hydrogels, particularly their rubber elasticity and viscoelasticity. Rubbers are able to instantaneously return to their original shape because their large free volume due to light crosslinking allows rapid rearrangement of polymer chains, which can be viewed in Equation 1. Hydrogels exhibit this property when they are swollen, with their mechanical behavior being dependent upon polymer network architecture. Viscoelasticity in a polymer results from the large size of polymer molecules, and in general hydrogels exhibit viscoelastic behavior and not simply elastic behavior. In viscoelastic materials, mechanical stress and strain leads to time dependence on the strain of the material as the segments of polymer chain move along one another in a sliding motion. Mechanical testing and analysis of hydrogels for these two properties include tensile testing for elastic, rubber-like behavior, and dynamic mechanical analysis for viscoelastic behavior (Anseth et al., 1996). (Anseth, 1996)

Equation 1: Rubber Elasticity

$$f = \left(\frac{dU}{dL}\right)_{T,V.} + T\left(\frac{df}{dT}\right)_{L,V.}$$

Where:

- ***f* is the retractile force in the elastomer due to a tensile force**
- **U is internal energy**
- **L is the length**
- **V is the volume**
- **T is the temperature in Kelvin**

The chemical properties of hydrogels and their influences on cells are often discussed when designing elements of tissue engineering. Certain cell factors such as trans membrane proteins, primarily integrins, transmit mechanical signals that act as anchors to the cytoskeleton by forming focal complexes, which is the source of fibrillar adhesions. Adhesive properties are dependent on the substrate's interaction with the cytoskeleton. Aside from adhesive properties, most cellular behavior is influenced by the substrate stiffness in which the cells alter their cytoskeleton; a cell's flattening behavior is in response to a substrate which is stiffer than its cytoskeleton (Brandl et al., 2007).

An important factor to this work was the differentiation characteristics of cells on polyacrylamide hydrogel substrate of different stiffness. Muscle cells akin to the C2C12 cell line has shown strong correlation between matrix compliance and cell responses similar to *in vivo* conditions (Brandl et al., 2007). In such situations, both biochemical and physical characteristics must be taken into consideration when designing ECM substrates, because much of the cell's activity depends upon cytoskeletal actions. The native tissues in which the cell originates should be used as a "morphological guideline" for how the cells should be grown, with regards to substrate stiffness, and how those cells should thus behave.

There are several methods available to determine tissue and hydrogel stiffness, including atomic force microscopy (AFM), magnetic resonance imaging, and fluorescent energy transfer. These methods are preferable to general material testing such as tensile/compressive testing, because cells do not only respond to substrate stiffness, but also to spatial variation (Brandl et al., 2007). The genetic makeup of a cell line can also be determined by mechanical analysis despite whether the cells are visually similar or not. Currently, the organization of cellular cytoskeletons is undergoing research, specifically changes in cell adhesion and morphology, and how changes in mechanical properties can indicate phenotype. So far, changes and anomalies in elasticity have been shown to indicate phenotypic events and disease when studying cancerous cell lines, which indicates that gene expression (differentiation) and mechanical properties of both the environment and the cells are linked (Cross et al., 2007). With the tissue stiffness in mind, along with chemical signals and markers, better hydrogels can be made for tissue engineering applications.

All cells respond to their surface substrate as much as any other stimuli. Most cells adhere to a surface once they've settled and given the correct conditions. Their adherence to their substrate, a cell's behavior is highly influenced by differences in elasticity of the substrate. Polyacrylamide gels are becoming commonly used in cell culture to mimic the elasticity of tissues. Furthermore, gels can mimic the mechanical environment of the ECM of cultured tissues. Therefore, an accurate measurement of stiffness and elasticity for these gels is crucial. Incorrect measurement could result in erratic conclusions while analyzing the effect of stiffness on cell behavior.. One way of measuring the stiffness and elasticity of these gels is through the use of a surface probe method like AFM. *In vitro* cells have an effective Young's modulus (E) of between 1-100 kPa. However, there are no reliable methods to estimate *in vitro* E values, and

therefore estimated values are used to extrapolate for analyzing in vitro experimental data (Engler et al., 2004). This is an important factor because even small differences in stiffness can affect how well cells adhere to the substrate and spread out, as stiffer substrates lead to more spreading by the cells.

As cells grow into their environment, their cytoskeleton deforms to their surroundings to a degree, and because of this, in vitro cell morphology is heavily influenced by substrate stiffness. As mentioned previously, cells exhibit a flatter morphology on stiffer substrates than on softer ones. However, they will preferentially migrate onto a harder surface when spreading. The response which the cells exhibit to substrate stiffness is cell type specific, meaning stiffness triggers gene expression depending on the cell type (Yeung et al., 2005). This means that the stiffness of the gel, and the gene expression of the cells cultured on it, can be controlled by adapting the density of crosslinking, which can be calculated by Equation 2.

Equation 2: Density of Crosslinking

$$G = \frac{nRT}{V}$$

Where:

- n is the number of crosslinks per volume
- V is the volume
- T is temperature
- R is the gas constant

A mechanical properties of tissue scaffold are critical to its success and functionality. In order to successfully seed cells they have to mimic the tissue which is being grown while maintaining a desired shape. Ideally, scaffolds should exhibit the mechanical functionality of synthetic substrates while having the bioactive capabilities of natural substrates, which makes

them not necessarily easy to manufacture (Almany and Seliktar, 2005). Research in determining the influence of substrate stiffness on cell morphology has included the extensive use of hydrogels, due to how easily their stiffness can be controlled through crosslinking density. Moreover, these substrates are transparent enough that allows imaging similar to regular tissue culture dishes. However, it is important not to only explore the morphology of muscle cells, but also their contractile properties, or the cell's ability to create a contraction, which is a major indication of cell differentiation (Bhana et al., 2010). It is important to be able to quantitatively assess if, and to what degree, muscle cells, like the C2C12 cell line, exhibit a twitch on different substrate stiffnesses. A study performed by Bhana, *et al* (Bhana et al., 2010) revealed that C2C12 cells showed spontaneous contractions after 5 days of differentiation on the softer substrates, but not on the stiffer substrates. They also showed that cells could be induced to exhibit sporadic clusters of contractions on the stiffer substrates non-synchronously. Ultimately, the authors point out that the cells' differentiation was regulated based on the stiffness of their respective substrates and they are more likely to exhibit *in vivo* differentiated phenotype when in an environment most closely resembling *in vivo* extracellular matrix.

2.3 Electrical Stimulation

Electrical stimulation can be used to excite muscle or nerve cells, allowing the development of *in vitro* models to study biochemical signaling, molecular change, and the stimulation of cellular functions. Muscle cell contractility has been produced *in vitro* via exogenous electrical stimulation. The excitation of muscle cells leads to various cellular responses such as triggering the activation of glucose uptake as well as glycogenolysis, the breakdown of glycogen long chains into glucose molecules (Derave et al., 2000). The work of Brevet, *et al* (Brevet et al., 1976) contributed to the early development of a device to stimulate a large number of cultured muscle cells and to maintain contraction over a controlled period of

time. Some of the limitations of this seminal work include the lack of consistent electric current to every well, the time consuming nature of delivering the electrical current to each well, and inflexible designs which make it difficult to replicate the experiment.

Applying an electric pulse *in vitro* to myocytes can generate a contraction of the cells. Periodic contraction of myotube fibers can be stimulated with an electric pulse between 0.5 Hz and 13 Hz (Aldaye et al., 2010). Tetanic contraction can be produced at frequencies higher than 5 Hz. Two-dimensional *in vitro* studies have shown that constant electrical stimulation, which mimics *in vivo* electrical signaling, can promote the maturation of primary muscle cultures in term of the expression of adult myosin heavy chain slow isoform (Flaibani et al., 2009). As for electrical stimulation in three-dimensional cultures, the cells proliferated at a higher rate, and the expression of myogenic genes was reduced (Flaibani et al., 2009).

Electrical pulse can also be used to investigate cellular functions with regards to myotube formation. A particular study analyzed the relationship between Ca^{2+} transients and sarcomere assembly in C2C12 myotubes. An electrical pulse stimulation was employed to manipulate the frequency of Ca^{2+} transient, which resulted in the myotubes showing remarkable contractile activity that was not initially exhibited (Fujita et al., 2007). This activity was concurrent with the establishment of sarcomere structure in the myotubes. There are still many unresolved questions associated with how tissue responses to electric impulse at molecular and cellular level, which in turn necessitate effective approach to deliver electrical impulse for vitro studies.

Electrical stimulation of tissue can be used in pharmaceutical application for treating tumor cells. Tumors have the ability to develop intrinsic pharmacoresistance mediated by drug extrusion mechanism, reducing the chemotherapeutic efficiency. There are studies that show

that low intensity and low frequency electrical stimulation by alternating current can interfere with tumor cell proliferation by affecting potassium channels (Janigro et al., 2006). The alternating current decreased multidrug resistance (MDRI) expression, and result in loss of drug extrusion ability of tumor cell as well as increased chemo-sensitivity. Electrical stimulation has the potential to enhance the efficacy of the currently available chemotherapeutic protocols.

Non-invasive electrical stimulus can be applied to a 3D collagen based scaffold to regulate cell adhesion and orientation of bone marrow-derived mesenchymal stem cells (MSCs) and fibroblast (Sun et al., 2006). Fibroblasts re-orient their direction perpendicular to current electrical stimulus and MSCs exhibit strong adhesion to 3D scaffold. In another study of electrical stimulation, the effects on cell orientation and alignment were studied using human adipose tissue-derived stem cells (hASCs). It was observed that when an electric field current stimulated the cells, the cells elongated and aligned perpendicular to the applied electric field (Derave et al., 2000). The ability to regulate the cell adhesion and orientation allows the development of a novel approach in controlling cell growth and differentiation.

It is important to develop a stable electrical stimulation system and measurement methodology in order to assess cell response in culture. The following model was designed and used in a study that placed a 6- or 24-well tissue culture plate between two ESCC connection cards that were attached to a transparent plastic support (Marotta et al., 2004). The electrodes carrying electrical impulses were made of platinum, which were sterilized with 70% ethanol prior immersing into media for stimulation. The cards were connected to an electrical stimulator by standard cables, which were able to generate different electrical impulses with variation of pulse duration, frequency and voltage. Appendix E below shows the diagram of aforementioned ESCC systems (Marotta et al., 2004).

Another device designed to stimulate a monolayer of C2C12 myotubes by electrical stimulation consists of a cell culture substrate made up of a microporous alumina membrane and a hole-spotted PDMS film (Kaji et al., 2010). The electrodes are placed above the cells and below the PDMS layers to generate electrical current. Myotubes that straddled the porous regions exhibited contractions when electrical impulse was applied. The myotube contraction in a wider area can also be triggered by applying the electrical impulse through the arrays of pores in PDMS films. The alumina membrane substrate was modified with an atelocollagen membrane to induced tissue-like stiffness. The study showed that Ca^{2+} transients induced by the electrical impulses increased the contractile activity of the cells. Appendix E illustrates the microporous alumina membrane and hole-spotted PDMS film electrical stimulation device (Kaji et al., 2010).

2.4 Micropatterning

Micro-patterning cell culture surfaces and 3D cell printing are two main methods to improving and changing the environment of cell culturing. Applying micro-patterns on surfaces has the potential to improve the differentiation of muscle cell culturing (Ilkhanizadeh et al., 2007). One of the most popular surfaces that are currently being used is a hydrogel in both 3D cell printing and micro-patterning (Melissinaki et al., 2011). Hydrogels have the properties of non-reactive and non-toxic control over the polymer properties, and are also biocompatible with most cells.

Micropatterning is fundamental for cellular biology, specifically for TE. One reliable method, that has shaped how most micropatterning is currently done, is photolithography. Photolithography is a method of using light to transfer a geometric pattern from a photomask to a light sensitive chemical to form a “stamp”. The stamp can then be filled with an ECM, which

cells can be embedded on, that will then be patterned on hydrogel surfaces with the desired pattern. The surface of the model illustrated in Appendix E was a polyacrylamide gel (PAG) that was treated with sulfo-SANPAH for collagen binding. The result was a high-degree of cell alignment, where the cells favored myotube differentiation and dystrophin expression (Ilkhanizadeh et al., 2007).

Micropatterning could also be combined with additional factors to help contraction and differentiation of myotubes, such as electrical pulse stimulation, which can be seen in Appendix E, cell traction force microscopy, and cyclic tensile strain system (Ilkhanizadeh et al., 2007; Ahmed et al., 2010; Nagamine et al., 2010). Controlling myotube contraction has the advantage of quantitative investigation of contraction-mediated metabolic alterations in myotube (Ilkhanizadeh et al., 2007). In a study by Nagamine *et al* , myotube contraction was generated by micropatterned embedded C2C12 myotubes in a fibrin gel pattern (Nagamine et al., 2010). The results showed that line patterns and contractile activities of myotubes supported by an elastic fibrin gel are maintained for a longer period of time than myotubes that are adhered on a conventional culture dish.

Micropatterning methods can control both cell alignment and strain direction. In a study done by Ahmed *et al*, different angles (0, 45, 90 degrees) of micropatterned fibronectin lines were placed on a PDMS substrate to attach C2C12 cells and form straight-line pattern (Ahmed et al., 2010). Cyclic tensile strain system (CTS) was applied on the PDMS substrate for four days, and analyze with extended focus image (EFI). The results showed that the C2C12 cells that were patterned on the 45-degree angle showed more sarcomere formation than regular C2C12 cell culture, or the other patterned angles.

Micropatterning is a method mainly used on two-dimensional cell culture surfaces to better improve cell differentiation, alignment, and other cell-to-cell functions. The aforementioned studies show that micropatterning on surfaces has significantly improved C2C12 differentiation. This method can also be combined with different applications to further on improve the quality of differentiation and cell expression.

Ink-jet printing on culture surfaces can be done on both two-and-three dimensional surfaces. Inkjet printers are a patterning method that is beneficial, and efficient, for the low-cost consumer market. There are two types of ink-jet printing: thermal printing and piezoelectric printing. In thermal printers, liquid is boiled by a pulse of energy at the surface of small heater, and the expanding bubble drives a drop of ink through the nozzle and onto the culture surface. In piezoelectric printers, an applied voltage pulse causes a glass tube, or a bending plate, to eject a droplet from the nozzle onto the desired surface. (Laura P. *et al*, 2003) The advantages of inkjet printers are that they are inexpensive, flexible to different samples, simple, desktop computer controlled (universal), and fast. (Luo and Shoichet, 2004)

Inkjet printing on cell culture surfaces has the ability to improve the environment of cell culture surfaces, and differentiation of cells. In a study done by *Shirin et al*, an inkjet printing method was used to print out macromolecules on hydrogels to steer neural stem cell differentiation (Ilkhanizadeh et al., 2007). The authors were able to print out biologically active macromolecules on poly-acrylamide-based hydrogels (HydroGel™) with seeded neural stem cell (NSC). As the result, inkjet printing method provide spatially well-controlled for NSC differentiation. More importantly, the differentiation efficiency of NSC was significantly improved (Calvert, 2007). Inkjet printing is not only accurate, but also able to print out a thin layer on cell culture surfaces. Drop on demand (DOD) devices are recently being used for

creation of ceramic pillar arrays, deposition of gold conductive track, deposition of optical microlens arrays, and deposition of polymers for microelectronics applications. (Luo *et al*, 2004) This method is a high-throughput, efficient, and fast way of printing out patterns on culture surfaces. Compared to micropatterning, inkjet printing is a cheaper way to pattern on cell culture surfaces. Even though it is not as exact, and small as micropatterning, there are no significant differences between the patterning on hydrogels.

Three-dimensional (3D) cell printing is commonly used for biological 3D scaffold creation. Three-dimensional printings have the advantage of cellular alignment, structure support, and cell growth guiding (Melissinaki *et al.*, 2011; Shepherd *et al.*, 2011). Hydrogels are commonly being used in 3D printing because it is biocompatible and non-adhesives to cells, allowing cell adhesion to be controlled. Planar substrates were patterned with adhesion signals that are similar to the actual space structure to guide cell attachment and function for controlling cell behavior and ultimately induce structural and functional tissue formation on surfaces (Shepherd *et al.*, 2011).

In study by Luo *et al*, hydrogels are being used as the matrices for guided axonal growth. (Luo *et al*, 2004) In their experiment, they have successfully demonstrated an example to immobilize biomolecules in selected volumes in a 3D hydrogel matrix using laser fabrication techniques and photochemistry (Shepherd *et al.*, 2011). As result, the adhesion channel had lead and improves the cell growth in hydrogel, which could further on use as cell regeneration guiding. Also in study by Aubin *et al*, authors have developed a simple and direct method to control cellular organization in 3D by cell encapsulated in hydrogel (Aubin *et al.*, 2010). Cell-laden 3D gelatin methacrylate (GelMA) was micro-patterned on PEG coated glass slides. The effect of cell alignment could be observed in four kinds of cell differentiation (HUVEC, C2C12,

CSP, Hep-G2). As the result, the micropatterned cells exhibited more line elongation structure formation and improved differentiation of the cells.

Chapter 3: Project Strategy

The ultimate goals of this project were to determine if a mouse myoblastic C2C12 cell line could differentiate on different elastic moduli, design and create a multi-well electrical stimulation device to electrically stimulate differentiated C2C12 myotubes, and develop a method to determine hydrogel stiffness in Softwell™ plates. The purpose of this chapter is to explain the step-by-step process this MQP group went to define and discuss the objectives for this project.

3.1 Design

The design process allows the designers to break down the project, into specific, individual parts to help determine the most cost-effective and optimal course of action that will meet all the needs and satisfy the client. This process is important when creating a method and/or design as it allows for logical and unbiased decisions to be made about the each detail. This section describes the steps the team took in the design process to revise the initial client statement, define project objectives, constraints, and functions, and prioritizes each goal and objective. The final purpose of this design process was to provide the tools for the team to determine how to differentiating C2C12 cells on different elastic moduli, create an electrical stimulation device for C2C12 myotubes, and develop a method to determine Softwell™ hydrogel stiffness.

Stakeholders of the project and its outcomes need to be identified before the design process can commence. The stakeholders for this project include the clients, the users of the Softwell™ plates, and the designers and users of the electrical stimulation device. The clients for

this project as a whole were Professors Sakthikumar Ambady and Raymond Page. These two were the ones who provided the group with the initial client statement that described the requests of the other client, and sponsor, Justin Mih, Ph.D., the need for either a mechanical or electrical stimulation device for differentiated myotubes, and what deliverables were expected from the designers at each project milestone. The other client, and sponsor, was Justin Mih, Ph.D., one of the founders of Matrigen™ of Worcester and the creator of Softwell™ plates. His client statement described the current techniques, materials, and methods of standard cell culture, the need for product verification, and the expected deliverables. The potential users of the electrical stimulation device are researchers, both students and Ph.D.'s, looking to stimulate muscle tissue at WPI and possibly in other labs across the United States. The potential users of the Softwell™ plates are students in a cell culture lab, graduate students, and researchers across the country. The design team included Adriana Martinez-Betancourt, Jeffrey Lessard, Kien Dao, and Yow-Chyuan Yeh, all of whom understood the needs and wants of all clients and users, and to create the necessary deliverables.

3.2 Initial Client Statement

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

“The behavior of cells and tissues such as growth and differentiation are depended on the relative softness or stiffness of the organ and the ECM surrounding the cells and tissues. It has been postulated that simulating the stiffness of specific tissues for *in vitro* cell culture can improve cell and tissue engineered products for clinical use. The mouse myoblast cell line C2C12 is a well-established cell line used extensively to study myogenic differentiation *in vitro*. Matrigen’s “Softwell™” is an alternative system to 3D cell culture wherein Softwell™ allows researchers to create an *in vitro* cell culture environment that simulates the stiffness of various tissues in the human

body. In order for the product to be commercialized extensively, it requires a reliable high throughput method to assess its stiffness.

The goal of this project is to develop a high throughput reliable method to determine stiffness of Softwell™ cell culture plates. Additionally, you will design a device to determine whether varying the stiffness of cell culture surface, application of mechano-transduction forces (stretch) and electrical stimulation or a combination of these three factors can improve myogenic differentiation of C2C12 cells.

Specific aims

- 1. Develop a high throughput reliable method to determine stiffness of Softwell™ cell culture plates.**
- 2. Determine optimal culture conditions/stiffness on Softwell™ culture plates or its “equivalent” for efficient differentiation of C2C12 mouse myoblast cells lines relative to current standards.**
- 3. Determine optimal electrical and/or mechano-transduction to improve differentiation of C2C12 mouse myoblast cells lines.**
- 4. Design and develop a device capable of delivering electrical and or mechanical (stretch) stimulus to C2C12 cells that allows real time monitoring of cellular changes during their differentiation process.**
- 5. Develop a quantitative assay/method to assess the relative efficiencies of each factor and their combination to effect myogenic differentiation of C2C12 cells.”**

As a design team, it is important to understand all parts of the client statement, which means the client statement must be broken down into a few statements that describe the objectives and constraints. This was accomplished by meeting with both Professor Ambady and Justin Mih, Ph.D. and asking questions to clarify exactly what the final deliverables were supposed to accomplish. After meeting with the clients, the design group met and discussed design ideas that incorporated the information obtained from the client meeting.

3.3 Objectives, Constraints, and Functions

Once the design goals are clarified, it's necessary to fully define the objectives and constraints. Afterwards, it is necessary to list some possible functions that will allow a product to

meet the defined objectives and constraints. The difference between objectives, constraints, and functions is that the objectives are the tasks the deliverables need to be able to perform to satisfy the client(s) and users, the constraints are the specific conditions the deliverables need to be able to meet in order to be considered functional, and functions are the means by which the objectives and constraints will be achieved.

Objectives:

1. Define C2C12 mouse myoblast myotube fiber differentiation
2. Determine optimal culture conditions on different stiffness on Softwell™ for efficient differentiation of C2C12 mouse myoblast cell lines
3. Develop quantitative assay and/or method to assess efficiencies of myogenic differentiation of C2C12 cells on Softwell™ plates
4. Determine optimal electrical and/or mechano-transduction to improve differentiation of C2C12 mouse myoblast cell lines
5. Design device capable of delivering specified electrical and/or mechanical stimuli to differentiated C2C12 myotube fibers
6. Develop a high-throughput and reliable method to determine stiffness of Softwell™ cell culture plates.

Constraints:

- C2C12 Mouse myoblast myotube fiber differentiation
 - Must meet the definition of differentiation
- Softwell™ Cell Culture Plates
 - Must meet relative current cell culture standards
- Electrical and/or Mechano-Transduction Device
 - Materials must not be cytotoxic
 - Materials must be easy to obtain
 - Finished device must not exceed \$20 to produce
 - Finished device must be sterilizable with resources available on-site (autoclave, UV light, alcohol bath, etc.)
 - Finished device must be able to fit under a microscope
 - Finished device must be able to allow for real-time monitoring of cellular changes during differentiation process
 - Finished device must have a method of controlling stimulation/stretch of cells
- Stiffness Testing
 - Cost-effective

- Easy
- Comparative to accepted stiffness testing standards.

Functions of the Each System:

- Softwell™ Cell Culture Plates
 - Able to seed cells that can adhere to the hydrogels that can proliferate and differentiate
 - Able to perform standard cell culture procedures
 - Able to maintain cell culture conditions
 - Able to visually monitor cells on each stiffness (no ocular disturbances)
- Electrical and/or Mechano-Transduction Device
 - Able to electrically stimulate and/or mechanically stretch the differentiated cells
 - Able to control electrical stimulation/mechanical stretch
 - Able to allow for real-time monitoring while stimulation/stretching occurs
 - Able to confirm differentiation of C2C12 mouse myotube fibers with either contraction via electrical stimulation or the ability to stretch and maintain structural integrity
- Stiffness Testing
 - Able to confirm hydrogel stiffness in a repeatable and reliable manner

3.3.1 Feasible Objectives

After creating a general and comprehensive list of objectives, constraints, and functions, the design team created a list of feasible objectives based on the meetings with the clients and the team's own discussions. Some objectives were removed, while others were combined. Each of the final objectives was broken into sub-objectives that needed to be fulfilled in order for the main objective to be able to be considered successful. The following list is the feasible objectives.

Objectives:

1. Define C2C12 mouse myoblast myotube fiber differentiation
 - a. Fusion of cells' cytoplasm
 - b. Multinucleated fibers
 - c. Myosin Detection using Myosin Heavy-Chain Staining
 - d. Lack of BrdU incorporation in C2C12 cells' nuclei
 - e. Striated Fibers
 - f. Spontaneous or stimulated contraction of myotube fibers

2. Assess ability of Softwell™ culture plates of different elastic moduli to differentiate myoblastic C2C12 cell line
 - a. Consistent Cell seeding density
 - b. Consistent protocols
 - c. Use standard cell practices and laboratory materials
 - d. Use previously described definition of C2C12 mouse myoblast myotube fiber differentiation
3. Develop parameters or a quantitative assay to correlate efficiency of differentiation status on Softwell™ plates compared to regular tissue culture plates
 - a. Polystyrene control for every experiment performed using Softwell™ plates with hydrogels of different elastic moduli
 - b. Start experiments at the same time
 - c. Use the same seeding cell density, media, incubator, protocols, and materials for the Softwell™ and regular polystyrene tissue culture plate
 - d. Compare spreading, proliferation, and differentiation of C2C12 differentiated fibers
 - e. Measure width of differentiated fibers
4. Determine optimal electrical stimulation for differentiated C2C12 mouse myoblast cell line
 - a. Define optimal electrical stimulation
 - b. Draw from literature reviews
5. Design and test a multi-well device capable of delivering specified electrical stimuli to differentiated C2C12 myotube fibers
 - a. Easy to use (integrated system with minimal components)
 - b. Easy to clean
 - c. Easy to sterilize
 - d. Easy to control
 - e. Must deliver electrical stimulation to several tissue culture-wells at once
 - f. Safe for humans and cells
6. Develop a high-throughput and reliable method to determine stiffness of Softwell™ cell culture plates.
 - a. Cost-effective
 - b. Following reliable and accepted standards

The differentiation of C2C12 myoblastic cell line needed to be defined so before the actual start of the project. The objectives regarding the ability of Softwell™ culture plates with hydrogels of different elastic moduli to differentiate the C2C12 cells, as well as the objective comparing the efficiency of the Softwell™ to the standard tissue culture plate directly depend on

this objective being met. The team determined that differentiation of the C2C12 cells met at least four of the following characteristics: fusion of the cells' cytoplasm, multinucleated fibers, myosin detection with immunocytochemistry of myosin heavy-chain, lack of BrdU incorporation in the nuclei, striated multinucleated fibers, and spontaneous or stimulated contraction of myotube fibers. These characteristics are all typical of differentiation, and are useful tools when determining if differentiation has occurred in a culture.

To complete the next two objectives, assessing the ability of Softwell™ culture plates to differentiate C2C12 cells and comparing the efficiency of differentiation of Softwell™ culture plates to regular tissue culture plates, the previous objective needed to be completed. The ability to be able to identify differentiated tissue is a huge factor when determining if this can be done on different substrate stiffness. In order to properly compare, and confirm, that differentiation is achieved and could be achieved again, everything needs to be kept consistent. The protocols and materials needed to be considered the “standard” for the lab, to ensure that differentiation could be achieved on the Softwell™ following the normal protocol for the lab. Monitoring cell spreading, proliferation, and the formation of fibers is also necessary when comparing the results of Softwell™ versus regular tissue culture plates.

The next two objectives are also dependent on one another. Determining the optimal electrical stimulation to apply to differentiated C2C12 directly applies to the design and testing of an electrical stimulation device. By determining the optimal stimulation to apply to differentiated C2C12 cells, it is possible to integrate this stimulation into the device to create a twitch/contraction of those differentiated fibers. Also, by defining the optimal stimulation, the extremes of electrical stimulation can also be identified, and prevention of permanently harming, or fully destroying, the cells can be avoided.

The final objective is developing a method to determine Softwell™ hydrogel stiffness. The stiffness of the Softwell™ culture plates needs to be confirmed so that users can be sure they are ordering the correct stiffness for their tissue culture to obtain the best results. For a company like Matrigen™, whose goal is to mass produce these tissue culture plates, a quick and reliable method is most desirable. By using a widely accepted method by the scientific community to test the stiffness, the integrity and validation of the hydrogels can be supported, and any skeptics can be placated.

3.3.2 Quantitative Analysis of Objectives

When developing a method and designing a device, it is necessary to ensure that the method/device performs the specified functions safely, in a cost and time efficient manner, and ultimately satisfies the client's needs. It is important to remember that there is a possibility that not all the objectives can be met with the best solution, as the solution might conflict or not require with another objective. Therefore, in order to produce the best possible product and method, it was necessary to weigh and prioritize the objectives.

In order to prioritize the objectives, pairwise comparison charts were created. The main objectives were compared against each other first, and then the sub-objectives were compared against each other for each main objective. Two objectives were compared at the same time. The objective determined to be more important received a score of 1 while the objective determined to be less important receives a zero. If both the objectives were determined to be of the same importance, a score of 0.5 is given to each of the objectives. In order to normalize the score, each aggregated score had 1 added to the aggregated score to ensure a non-zero value for any of the objectives. Finally, each score was weighted to allow for a quick understanding of which

objectives were found to be most important, and by what percentage. The following tables show the pairwise comparisons for the main objectives, and their sub-objectives.

Table 1: Pairwise Comparison Table of main Objectives

Main Objectives	1	2	3	4	5	6	Aggregated Score	Normalized Score	Weight (%)
1. Define C2C12 Differentiation		0.5	0.5	0.5	1	1	3.5	4.5	20
2. Softwell™ to differentiation of C2C12 cells	0.5		0.5	0.5	0.5	1	3	4	18.5
3. Comparing Softwell™ to regular TC plates	0.5	0.5		0.5	0.5	1	3	4	18.5
4. Determine Optimal Electrical Stimulation	0.5	0.5	0.5		0.5	0.5	2.5	3.5	16
5. Design and Test Electrical Stimulation Device	1	0.5	0.5	0.5		1	3.5	4.5	20
6. Develop method to determine Softwell Stiffness	0	0	0	0.5	0		0.5	1.5	7

The pairwise comparison chart above shows that all of the objectives are important to the project, however some of the objectives are considered more important to the project than the others. The two most important objectives, according to the team and stakeholders, were first defining C2C12 differentiation, and designing and testing an electrical stimulation device. Following those two objectives in importance are being able to assess how cells differentiate on Softwell™ hydrogels with different elastic moduli, comparing C2C12 cell differentiation on Softwell™ versus regular tissue culture plate, determining optimal electrical stimulation to apply to differentiated C2C12 fibers for contraction, and finally, developing a method to determine Softwell™ stiffness.

Table 2: Pairwise Comparison of Sub-Objectives 1

Main Objectives	1	2	3	4	5	6	Aggregated Score	Normalized Score	Weight (%)
1.Fusion of Cells' Cytoplasm		0.5	0.5	0.5	0.5	0.5	2.5	3.5	16.6
2.Multinucleated Fibers	0.5		0.5	0.5	0.5	0.5	2.5	3.5	16.6
3.Myosin Detection	0.5	0.5		0.5	0.5	0.5	2.5	3.5	16.6
4.Lack of BrdU Incorporation	0.5	0.5	0.5		0.5	0.5	2.5	3.5	16.6
5.Striated Fibers	0.5	0.5	0.5	0.5		0.5	2.5	3.5	16.6
6.Fiber contraction	0.5	0.5	0.5	0.5	0.5		2.5	3.5	16.6

Within the first objective, it was determined that every sub-objective was equally important. The parameters that the team and Stakeholders determined defined differentiation are all equally useful and necessary when determining differentiation of C2C12 cells. It was also determined that 4 out of the 6 parameters must be met, as some of the parameters might not be met due to human error, but differentiation would still have occurred. Due to their equal importance, it is not necessary for all sub-objectives to be met.

Table 3: Pairwise Comparison of Sub-Objectives 2

Main Objectives	1	2	3	4	Aggregated Score	Normalized Score	Weight (%)
1.Consistent Cell Seeding Density		1	0.5	0.5	2	3	30
2.Consistent Protocols	0		0.5	0.5	1	2	20
3.Use Standard Cell Procedures	0.5	0.5		0.5	1.5	2.5	25
4.Using Definition of C2C12 Differentiation	0.5	0.5	0.5		1.5	2.5	25

Within the second objective, it was determined that the most important sub-objective was keeping the cell seeding density consistent throughout experiments. This was shortly followed by using standard lab cell-culture procedures and using the team's definition of C2C12 mouse myoblast differentiation. The least important sub-objective was determined to be keeping the

protocols consistent, as the Softwell™ may require some modifications in regards to protocols to achieve the necessary results.

Table 4: Pairwise Comparison of Sub-Objectives 3

Main Objectives	1	2	3	4	5	Aggregated Score	Normalized Score	Weight (%)
1.Polystyrene Control		0.5	0.5	0.5	1	2.5	3.5	23.3
2.Start Experiments at the Same Time	0.5		0.5	0.5	0.5	2	3	20
3.Keeping everything Consistent (Seeding Densities, Media, etc.)	0.5	0.5		0.5	1	2.5	3.5	23.3
4.Comparing Spreading, Proliferation , and Differentiation	0.5	0.5	0		0.5	1.5	2.5	16.6
5.Measuring Width of C2C12 Fibers	0	0.5	0.5	0.5		1.5	2.5	16.6

Within the third objective, it was determined that having a polystyrene control for each Softwell™ plate with hydrogels of different elastic moduli and keeping everything consistent were the most important sub-objectives. These sub-objectives were closely followed by starting the experiments of differentiating C2C12 on Softwell™ plates and polystyrene controls at the same time. The last two important objectives were comparing the spreading, proliferation, and differentiation of the fibers on the Softwell™ plates and the polystyrene plate, and measuring the width of the C2C12 fibers.

Table 5: Pairwise Comparison of Sub-Objectives 4

Main Objectives	1	2	Aggregated Score	Normalized Score	Weight (%)
1.Define Optimal Electrical Stimulation		0.5	0.5	1.5	50
2.Draw from Literature Review	0.5		0.5	1.5	50

Within the fourth objective, it was determined that both of the sub-objectives were equally important. In fact, the first sub-objective can only be determined by the second sub-

objective. This particular objective requires full completion for the success of the fifth objective, and the success/functionality of the electrical stimulation device.

Table 6: Pairwise Comparison of Sub-Objectives 5

Main Objectives	1	2	3	4	5	6	Aggregated Score	Normalized Score	Weight (%)
1.Easy to Use		1	0.5	0.5	0.5	0	2.5	3.5	17
2.Easy to Clean	0		0.5	1	0	0	1.5	2.5	12
3.Easy to Sterilize	0.5	0.5		1	0.5	0.5	3	4	19.5
4.Easy to Control	0.5	0	0		0	0	0.5	1.5	7
5.Must deliver electrical stimulation to all tissue culture-wells at once	0.5	1	0.5	1		0.5	3.5	4.5	22
6.Safe for Humans and Cells	0.5	1	0.5	1	0.5		3.5	4.5	22

Within the fifth objective, it was determined that the two most important sub-objectives were that the device must deliver electrical stimulation all tissue-culture wells at once, and it must be safe for both humans and the cells being stimulated. Following these two objectives, the device must be easy to sterilize, easy to use, easy to clean, and finally, easy to control. This is the order in which we will prioritize the electrical stimulation device.

Table 7: Pairwise Comparison of Sub-Objectives 6

Main Objectives	1	2	Aggregated Score	Normalized Score	Weight (%)
1.Cost-Effective		0.5	0.5	1.5	50
2.Following reliable and acceptable standards	0.5		0.5	1.5	50

Within the sixth, and last, objective, it was determined that both of the sub-objectives were equally important. The method chosen to determine substrate stiffness must be cost effective, either worth it to purchase the materials necessary to determine the stiffness, or inexpensive enough to have the process done by a third party. This method must also be

considered reliable and readily accepted as a method to determine substrate stiffness by a majority of the scientific community so that the results of the testing can be fully supported and not doubted.

After the seven pairwise comparison tables were completed, the team was able to understand which objectives, and sub-objectives, had more weight in regards to the project, and thus required more focus for the development and success of the project deliverables. To summarize the full results of all the pairwise comparison tables, a weighted objectives tree was created, as seen in Figure 1. Within the objectives tree, each branch has two sets of numbers: the first number is the weight that particular branch holds in relation to that Objective, and the second number is the weight that branch holds in relation to the tree as a whole.

Project	Objective	Weighted Objective
Defien C2C12 Differentiation 20% : 20%	Fusion of Cells' Cytoplasm	16.6% : 3.32%
	Multinucleated Fibers	16.6% : 3.32%
	Myosin Detection	16.6% : 3.32%
	Lack of BrdU Incorporation	16.6% : 3.32%
	Striateed Fibers	16.6% : 3.32%
	Contraction of Fibers	16.6% : 3.32%
Ability of Softwell™ to Differentiate C2C12 Cells on Different Stiffness 18.5% : 18.5%	Consistent Cell Seeding	30% : 5.5%
	Consistent Protocols	20% : 3.7%
	Standard Laboratory Practices and Materials	25% : 4.6%
	Previously Described Definition of C2C12 Differentiation	25% : 4.6%
Compare differentiation on Softwell™ to regular TCP 18.5 % : 18.5%	Polystyrene Control	23.3% : 4.3%
	Start Experimetns at the Same Time	20% : 3.7%
	Consistency (cell seeding density, media, etc.)	23.3% : 4.3%
	Compare Spreading, proliferation, and differentiation	16.6% : 3.1%
	measure width of differentiated Fibers	16.6% : 3.1%
Determine Optimal Electrical Stimulation for Contraction 16% : 16%	Define Optimal Electrical Stimulation	50% : 8%
	Draw From Literature Reviews	50% : 8%
Design/Test Electrical Stimulation Device 20% : 20%	easy to use	17% : 3.4%
	easy to clean	12% : 2.4%
	easy to sterilize	19.5% : 3.9%
	easy to control	7% : 1.4%
	deliver electrical stimulation to several tissue culture-wells at once	22% : 4.4%
	safe for humans and cells	22% : 4.4%
Develop Method to Determine Stiffness of Softwell™ 7% : 7%	cost effective	50% : 3.5%
	following reliable and accepted scientific standards	50% : 3.4%

Figure 1: Weighted Objective Tree

3.4 Revised Client Statement

After meeting with the clients and users, the brainstorming session, and conducting analysis of the objectives using the pairwise comparison tables, the original client statement was revised to the following:

1. **Assess the ability of Softwell™ culture plates of different elastic moduli to differentiate mouse myoblastic C2C12 cell lines**
2. **Develop parameters or quantitative assays to correlate efficiency of differentiation status of C2C12 cells on Softwell™ and regular tissue culture plates**
3. **Design and develop a multi-well electrical stimulation device to induce contractions in differentiated C2C12 cells**
4. **Develop a reliable method to determine stiffness of Softwell™ culture plates**

3.5 Project Approach

The group developed a three-step approach to represent the process by which the hydrogels stiffness were tested, the cells were differentiated on Softwell™ culture plates and compared to regular tissue culture plates, and then electrically stimulating the differentiated cells with the device. This process is represented in the Figure 2.



Figure 2: Flowchart of Project Approach

Chapter 4: Alternative Designs

4.1 Needs Analysis

The modulation of C2C12 gene expression has been investigated by previous MQP teams, such as the design which used a “dog-bone” shape to produce tissues which had inherent anchor points from which they could be stretched. Prof. Rolle’s previous MQP using tissue rings to examine the effect of mechanical stimuli was also an inspiration for the initial mindset into design concepts. Because the team’s primary goal was a *functional* assay of the cells once they had differentiated, the team needed to develop a reproducible device which would trigger the contraction of the myosin fibers to establish how well the cells functioned on the polyacrylamide hydrogels in comparison to those grown on regular polystyrene plates.

Initially, the team did not consider electrical stimulation too thoroughly. The team only learned of it fully after literature review on the subject of cell stimulus response *in vitro*. The team had noticed in previous work with C2C12 cells that after they had fully differentiated they had a tendency to spontaneously twitch on their own. The hypothesis was that these twitches were due to minute mechanical stimuli acting upon the plate which the team could not observe visually. Because of this, the team pursued mechanical stimulation initially. Once the non-feasibility of mechanical stimulation became apparent, the team chose to focus on electrical stimulation.

4.2 Previous Designs

4.2.1 Mechanical stretching device

When developing a functional assay, the team initially considered a mechanical transducer to stretch the cells grown on an elastic polymer bottom. Works such as this had been done before to induce a cyclic contraction of cardiac tissue, whose cells have a natural heartbeat. The team

considered the design of a square tissue culture well produced by a previous MQP by Prof. Raymond Page as an outline for a new mechanical stimulation platform. The concept which was considered was a thinner rectangle with the same polymer bottom and a PDMS housing, which would provide a mechanical strain similar to that which the C2C12 cells would experience *in vivo*.

4.2.2 Electrical stimulation: initial design

Upon realization that mechanical stimulation was not feasible within the budget and time frame, the use of an electrical current to create a simulated nerve action potential was investigated. The team initially chose to integrate electrodes onto the base of a 6 well plate, which would then have cells seeded onto the bottom of it. Materials for this design were much easier to access, since the necessary wire and components could be obtained from teaching labs at WPI. Standard wire would run into the plate bottom on one side and essentially use the cells and media as a resistor. Since all the parts were inert, sterility would be easily achieved through a 70% ethanol bath or UV sterilization. For this design, current 6-well plates could easily be adapted by incorporating electrical components into their design. This meant that the team could use the Softwell™ plates provided by the project sponsor by adapting them using wire components and then sterilize them using UV light. This meant hydrogels did not have to be produced for experiments, something which was a major advantage to this design and methodology.

4.3 Feasibility Analysis

4.3.1 Mechanical Stimulation

The team immediately had problems with this design's usefulness as a functional assay for cells grown on a hydrogel substrate. The Softwell™ plates use a proprietary method to attach the gel to the bottom of the culture plate. This along with the frailty of the gel itself made removing the gel from the plates provided by the project's sponsor impossible. The team would have had to produce hydrogels by hand. The method of producing polyacrylamide hydrogels is possible in a laboratory setting with minimal experience; however these gels have to be:

- Sterile
- ~100-200 μm in thickness
- Able to attach to a ligand
- Easily placed in the bottom of a culture plate

With these requirements of the gel, producing gels by hand would have been inefficient and time consuming. There was also questionable hydrogel integrity. Any gels which the team produced were extremely fragile. About half of the gels would disintegrate during production, others would tear when they were removed, and others would break during regular handling. Overall, an estimated 10% to 20% of hydrogels produced were actually usable in this application. Finally, the team encountered an issue with how it would be built. Materials for the device would have to largely be fabricated by hand and would be outside the project budget. Given these limitations, the team decided that mechanical stimulation was not a viable option.

4.3.2 Electrical Stimulation: initial design

Issues with this design arose with replication of experiments. Because the *base* of the culture plate was used, the stimulation plates were one-time-use. Experiments using different

wire in media found that when a current was applied, certain wire metals rapidly oxidized. This rapid oxidation sometimes drastically lowered to pH of the media, and could have caused cytotoxicity. To avoid this, better metals would have to be used at the media interface to avoid rapid corrosion; however, due to their cost these metals would not be expendable given the available budget. Further, it was difficult to build a parallel circuit in the confined space of the 6 well plates. Having the wells set up as linear resistors would not be acceptable because the voltage drop across each well compromised the consistency and reproducibility of data. With further effort, a bottom which placed the wells in a parallel circuit could have been developed; however, by this time the team had realized that placing the electrodes on a removable and reusable cover was a better option. Therefore, development of this design ceased.

4.4 Final Design Choice

For the final design the team chose to use a top-insertion method to insert electrodes into the media such that a current would run across the cells on the plate. This was chosen in lieu of the bottom electrodes because it would be reusable and easy to sterilize. The design would act as the cover to a plate which already had cells grown on it, and could be used on multiple wells; meaning only one stimulator had to be produced. A reusable stimulation device, as opposed to a disposable one, allowed the team to invest better materials into the design to improve its characteristics. The circuitry was laid out on the top of the plate cover, with only the electrode tips sticking through the top into the media. With a smaller surface area facing the cells in media, this method would be easier to sterilize. Since the circuitry was on the outside of the plate cover, it would be easier for the team to design the wells as resistors in parallel. Further, the team chose to institute banana plugs as a contact to the power source. The team also decided on implementing an on/off switch for each of the two halves of a 6 well plate, in order to have one

side stimulated while the other side acted as a control. In case the tips had to be replaced due to wear, a threaded attachment was used as the interface between the tip and the circuit.

4.5 Materials for Electrical Stimulator

The team used available 6-well tissue culture plate covers and stock electrical material to fabricate the device. These would meet the objective of allowing live observation. As a source of current, the team used an available BioPac pulse generator to apply a voltage to the circuit through banana plug inputs on the side. The use of platinum lead to a slightly higher cost, however since the device is less reusable the cost was made up by a long usage life. Components were adhered to the cover by using a glue gun. For the circuitry, standard copper wire was used. A threaded conduit was glued onto specified points for each of the wells. A corresponding threaded conduit was attached to each of the platinum wire tips so that they could be screwed into place into the circuit. Plastic sheathing was housed around the platinum tip allowing only the very end of the tip to contact the medium. This sheathing was implemented to maintain the shape of the platinum wire.

4.6 Device Specifications

The device which was decided upon was the addressed all the design objectives. All materials except for the platinum wire did not have to be ordered, they were available from stock material on campus. Because of the universal banana plugs, the design meets the requirement of being easy to use. The plate-cover design and reliable electrode tips allow for the design to be reusable and biocompatible. Finally, the clear top allows for live observation. In each well, the holes are 3 cm apart in each well. On each side of the plate, different color insulation is used for the wire circuit to indicate positive and negative charge (Figure 3). The device is compatible with any laboratory set up, however for our device we

used an Agilent 33220A pulse generator. The device was sterilized by spraying 70% ethanol and placing it under the UV light inside a culture hood for 20 minutes. After sterilization was completed, the device was placed onto a standard 6 well-plate which contained cell cultures which were to undergo stimulation. The device and the plate were brought to the microscope for live observation during the stimulation.

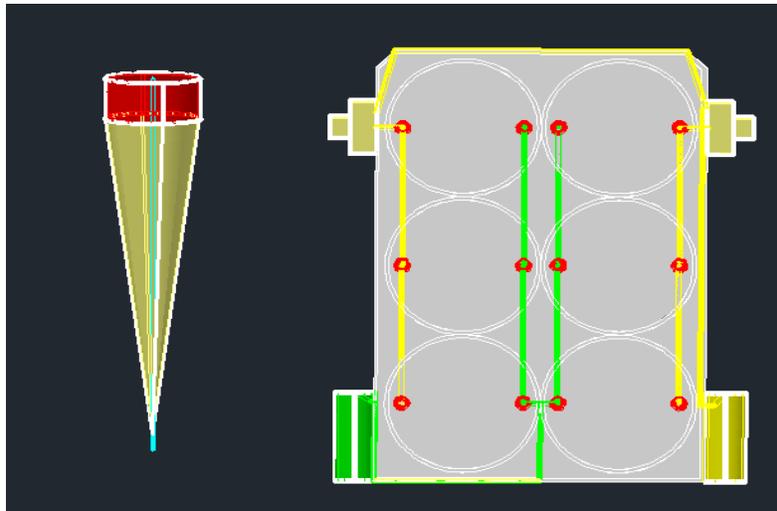


Figure 3: CAD drawing of removable tip (left). device schematic (right)

4.6.1. Stimulation procedure

1. The pulse generator was connected to the device through the banana plugs, with the positive and negative leads connected to their respective plugs. The pulse setting was specified.
 - The appropriate setting was varied between (1-5 hz, 10ms-100ms, 1 – 10 V)
2. Images and live videos were recorded under a microscope using Camtasia Studio 7.
3. The device was cleaned after use as described before, and stored.

4.7 Design Validation

The choice of platinum wire was made after initial testing of the device showed that upon running a current through regular copper and silver coated wires two things occurred: the metal wires rapidly oxidized, within seconds, causing bubbles to form from the wire interface, and the pH of the media also decreased rapidly, as seen by the changing color of the media. Platinum was found to be the least reactive of possible wire metals due to its high oxidation resistance. Because of this the team concluded that it would be the longest lasting and most biocompatible material for this application. Plastic sheathing was placed around the platinum leads to prevent them from bending. This sheathing was attached by a threaded screw-like mechanism for ease of replacement of the platinum wires, as the leads have the shortest life span of any part of the design. A switch was included for each row of wells so that one row could be used as a control for an experiment. A glue gun was used to adhere the parts together for ease of manufacture and so that any crevices could also be sealed using this method.

Chapter 5: Design Verification

5.1 Determination of Myoblast Differentiation

To determine the level to which the myoblasts had differentiated into myotubules, the following criteria were used.

- The myoblasts must show fusion of the cytoplasm.
- Once fused, the nuclei must align along one axis. This becomes the midpoint for the muscle fiber.
- The fused myoblast must form large fibers, generally aligned parallel along one axis.

- The large fibers must contract, either spontaneously or when induced by an electrical charge.
- The nuclei of the cells must cease incorporating Bromodeoxyuridine (BrdU) into DNA strands in the presence of BrdU labeled media.
- The fused myoblasts must express myosin heavy chain proteins in the presence of fluorescent myosin-antibodies.

Each of the aforementioned criteria is typical of C2C12 myoblast differentiation, and is considered the number of criteria which the cells met as a determination of their level of differentiation. Establishing methods to determine how well the cells met these criteria was essential to each experiment. Cells were viewed and images were recorded using an Axiovert microscope at 10x, 20x and 40x magnifications. Fusion of the cytoplasm was determined through visual examination of the cells using phase contrast microscopy and a immunocytochemical analysis to determine the expression of skeletal muscle specific protein, namely the myosin heavy-chain. Nuclear alignment was determined by a) visual examination the cells, and b) BrdU fluorescent marking using anti-BrdU antibody and AlexaFluor 488 anti-mouse antibody, and through Hoechst staining the cells.

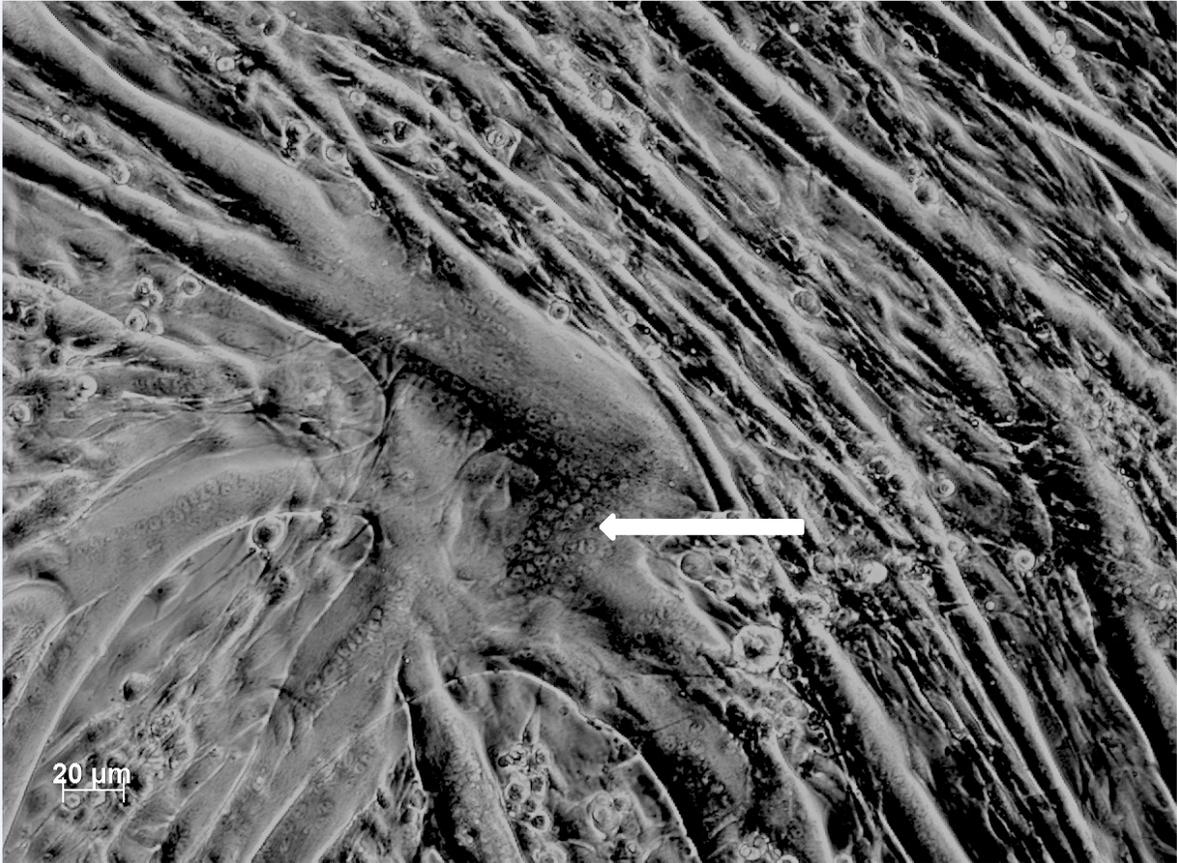


Figure 4: C2C12 cell line in final stage of differentiation

Nuclei can be clearly seen along the center axis of the fiber. Taken with phase-contrast microscopy at a 20X magnification.

5.2 Differentiation of C2C12 cells on Polystyrene culture plates

The differentiation assays performed on the polystyrene plates showed a typical timeline of about 9-10 days. At about 70% confluency the cells were considered to be at day 0 of differentiation. At day 2 the cells start the process of fusion on a small, localized scale. By day 3, locally fused cells align along an axis, which is the beginning of a muscle fiber formation. Fusion continues through day 4 and 5 creating large fiber formations grouped together. At day 6 the fibers are generally fully formed and are aligned along an axis, appearing striated in nature

(Figures 4 and 5). In the absence of micropatterning they can't form full striations; however they do exhibit similar behavior in localized formations.

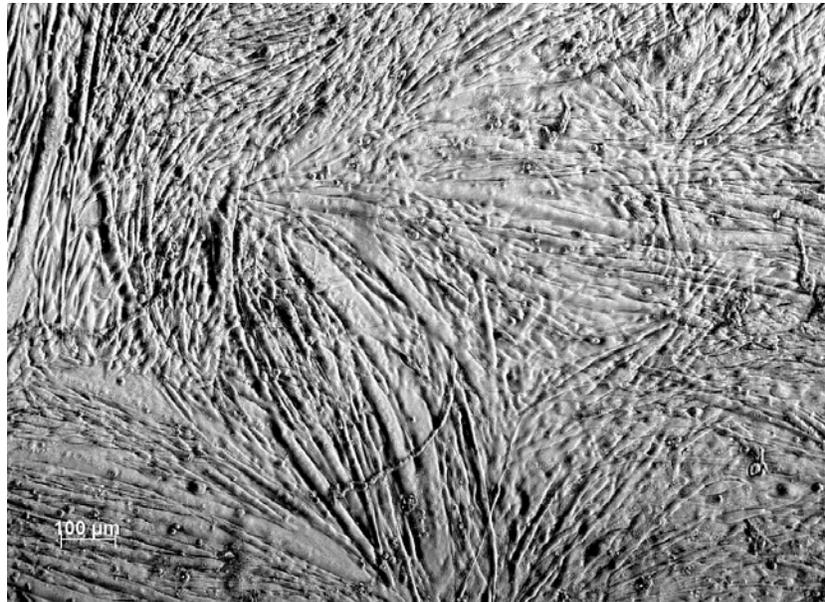


Figure 5: Micro-Striation at Day 6 of Differentiation

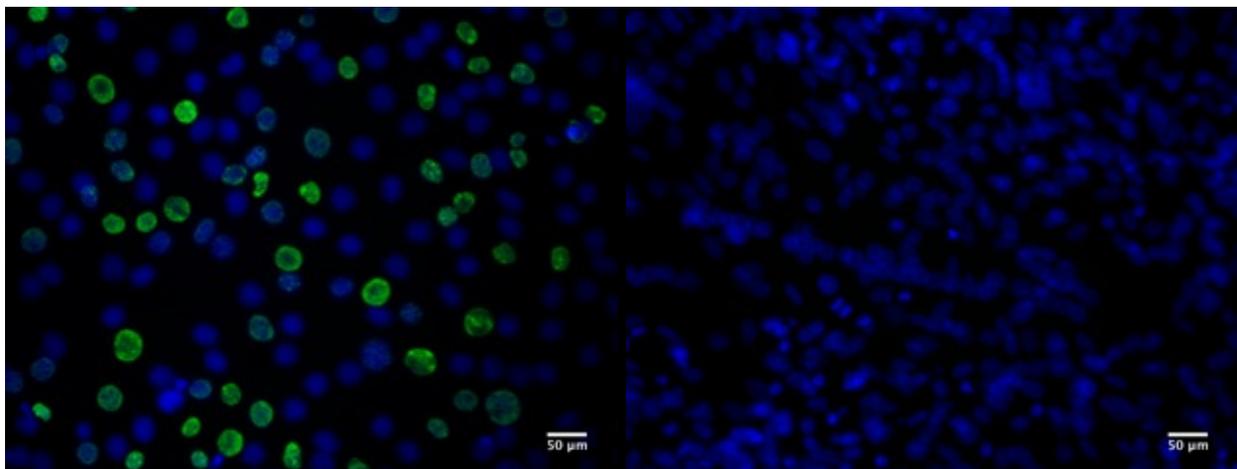


Figure 6: BrdU/Hoechst stained Cells

Left: day 2 of differentiation. Right: day 9 of differentiation. Notice the linear alignment of nuclei in the middle of the visual field in the right hand image at day 9, when the fibers exhibit optimal contraction

After day 6, the cells do not show any substantial growth in fibers. At this point we can clearly observe the nuclei of fused cells aligning in the middle of the fiber end-to-end. From days 6-9, the fibers stop fusion of the cytoplasm and expression of myosin chains increases. By day 9, MHC protein production is sufficient enough such that the when electrically stimulated they can induce a contraction, or “twitch”. From the fluorescent microscope images, one can see that the fibers by day 7 are expressing myosin heavy chains. It can also be seen from the fluorescent images that the cells have declined the incorporation of BrdU in their nuclei by day 6. By day 9, there is no incorporation of BrdU (Figure 7). From the images and the contractions induced by the device, the evidence strongly suggests that these cells grown on polystyrene plates differentiate well in 9-10 days, forming bundles of fibers containing contractile myosin heavy chains.

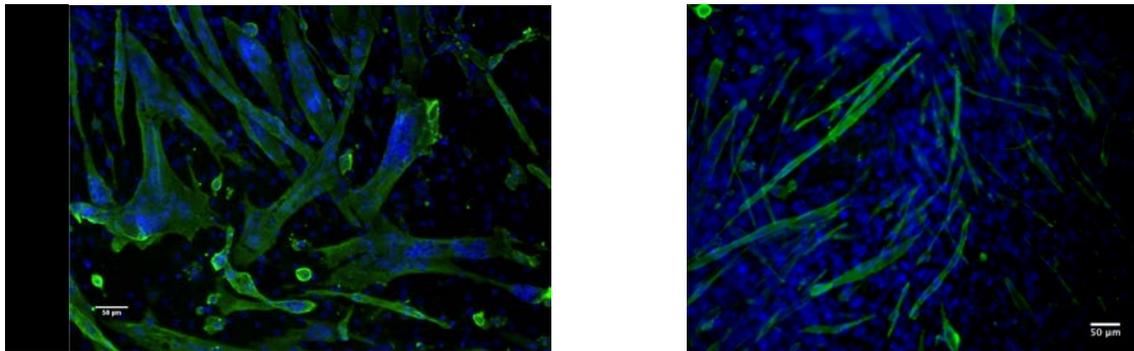


Figure 7: Hoechst stained nuclei with fluorescent myosin heavy chains on day 9

Left: polystyrene; Right: 0.5 kPa hydrogel. Notice the nuclei are closer together on polystyrene, suggesting the cells fuse more completely on flatter surfaces due to increased cell-to-cell contact

5.3 Softwell™ (Polyacrylamide hydrogels)

The Softwell™ plates showed caused a completely different morphology during the experiments. Cells plated on the hydrogels spread out much less than on the polystyrene plates. On each of the varying stiffnesses, the propensity for the cells to spread out over the surface area was proportional to the stiffness of the substrate. Because of this, cells on softer substrates had much less cell to cell contact.

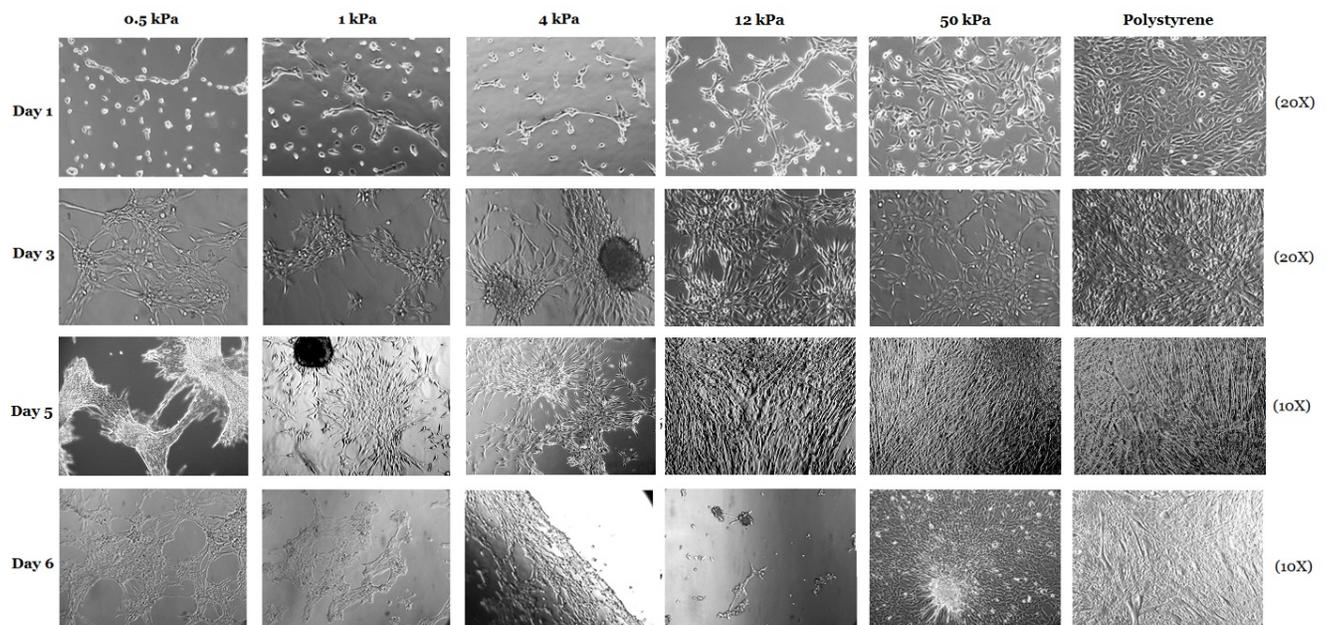


Figure 8: Phase Contrast Images of C2C12 cells on hydrogels

A Rat-Tail Collagen I extracellular matrix was used on all the gels above

C2C12 cells plated on softer hydrogels exhibited less propensity to proliferate compared to the standard polystyrene surface. Because of the reduction in cell density, differentiation occurred less efficiently on softer hydrogels than the stiffer ones. Fewer cells fibers were formed by the fusion of cells in all of the wells. However fibers formed on softwells exhibited a three dimensional morphology (Figure 8). ICC analysis on myofibers formed on softwells indicate that myosin expression of fibers on 0.5 and 1 kPa stiffness was comparable to polystyrene.

Intermediate softness (4 kPa, 12 kPa, and 50 kPa) exhibited neither appreciable myofiber formation nor extensive myosin protein expression (Figure 8). BrdU incorporation showed no discernible difference between the polystyrene control and the hydrogels at any stiffness. The 4 kPa and 12 kPa hydrogels during the experiments had a tendency to lift off the bottom of the plate around day 5-7, particularly when electrical stimulation was attempted. This suggested that collagen coated softwells were unable to support the contractile forces of myofibers. Fibers produced in the 0.5 kPa and 1 kPa did not induce a twitch at day 9 and 10 (Figures 9 and 10).

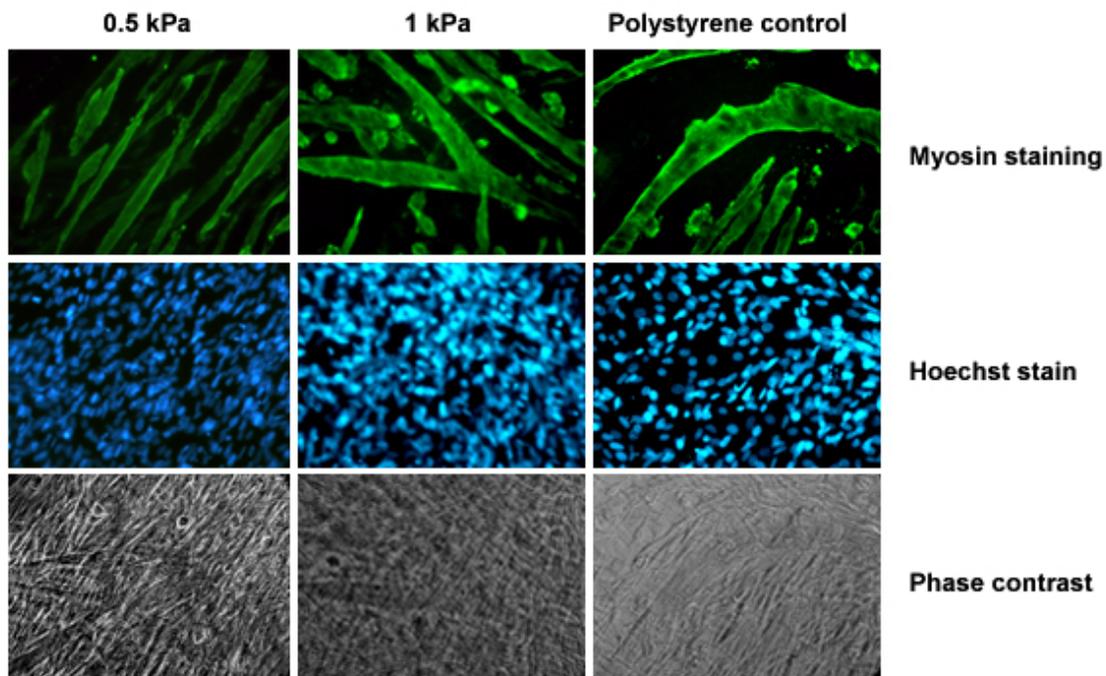


Figure 9: Images of Most Successful Hydrogels Compared to the Polystyrene Control

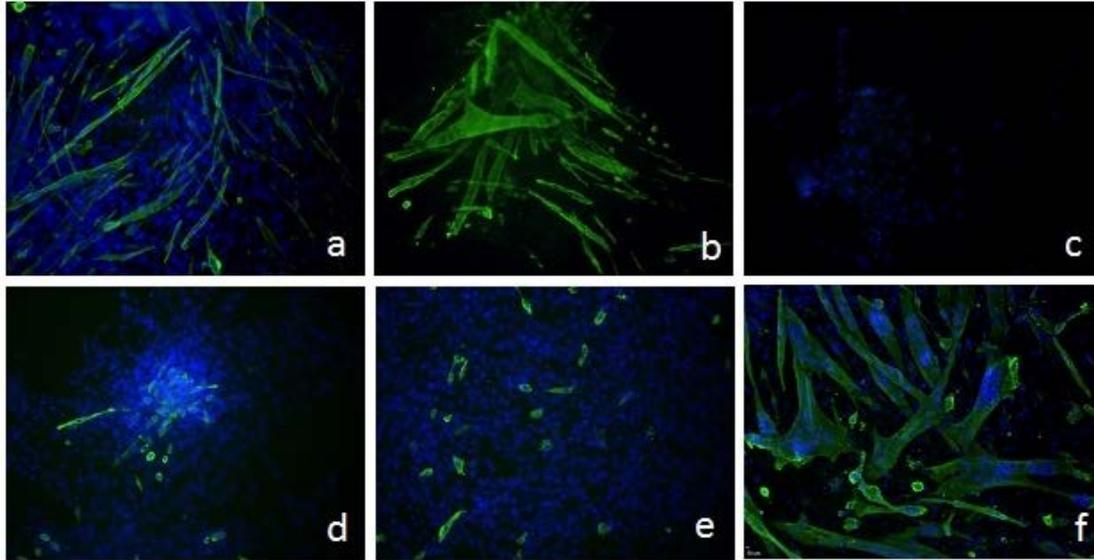


Figure 10: Myosin Heavy-Chain Stain Comparison on Different Hydrogel Stiffness

(a) 0.6 kPa, (b) 1.2 kPa, (c) 4 kPa, (d) 12 kPa, (e) 50 kPa, and (f) Polystyrene. Wells were anti-body stained for MHC and counterstained with Hoechst for cell nuclei. Collagen I was used as a ligand. 20X magnification.

5.4 Hydrogel Stiffness Testing

Sample	inert_g	inert_s	Jsample_c	rmachine	Rsample	G'rheo	true G'	true G' (kPa)	expected G'	
3.1	3.98E+13	31733338	1036.6547	2244.2057	4.5	130.4	163648532	163648.5324	50 kPa	
3.1	3.48E+13	1.398E+09	411605.41	2170.2356	9.2444	135.6	3376964	3376.964036		
3.1	5.95E+13	1.403E+09	366315.44	2481.0599	3.2054	135.6	5750395.7	5750.395692		
3.3	3.61E+13	592259719	14397.228	2189.6046	3.1053	184.7	11254265	11254.26469		
3.3	3.81E+13	59350419	25004.132	2218.8629	0.022855	185.3	118815507	118815.5069		
3.3	3.94E+13	6.9196E+08	4.4000E+04	2.2376E+04	1.0567E+01	1.8560E+02	10556208	10556.20787		
2.1	4.72E+13	1.853E+09	125711.29	2341.9271	11.1489	8.526	217345.55	217.3455452		4 kPa
2.1	3.59E+13	676338025	66444.903	2187.3041	25.5422	9.195	488567.12	488.567123		
2.1	3.19E+13	4.675E+09	2171068.6	2123.7819	31.8882	9.432	64439.644	64.43964354		
2.2	4.54E+13	532856423	63952.285	2319.1992	18.0578	7.683	654896.26	654.8962614		
2.2	3.38E+13	84420739	2937.3345	2154.6424	13.4753	8.675	3477128.9	3477.128947		
2.2	3.35E+13	73667632	23073.251	2148.9347	11.9813	8.773	3987162.4	3987.162413		
2.3	2.3E+13	262459579	37687.399	1957.4564	2.8545	11.18	981855.16	981.8551586		
2.3	3.69E+13	12478400	6012.4148	2201.7057	1.4942	12.4	36660594	36660.59358		
2.3	6.08E+13	17529407	831.6186	2494.4967	1.4925	12.65	43868691	43868.69104		
1.1	4.56E+13	1.201E+10	3652490.9	2321.8489	32.6904	0.4522	1717.6438	1.717643797	1 kPa	
1.1	2.94E+13	894187812	270839.89	2080.5491	37.5299	0.4689	15426.344	15.42634375		
1.1	5.88E+13	1.265E+10	11726991	2474.1384	36.6903	0.4762	2215.3436	2.215343634		
1.2	3.34E+13	84151527	25040.128	2147.9034	13.475	0.394	156456.23	156.4562302		
1.2	4.6E+13	226456337	192262.8	2326.6842	1.8214	0.3285	66742.165	66.74216497		
1.2	4.8E+13	2.523E+10	31246766	2351.7946	34.1774	0.3555	676.83474	0.676834736		

Table 8: Stiffness Data Obtained from Samples

Sample 3.2 (i.e: the second sample of the 50 kPa gel) did not return any stiffness data from the "point_collection" program. Therefore, it is absent from the table

Testing the stiffness of polyacrylamide hydrogel samples using a rheometer yielded inconsistent results (Table 8). This is attributed to several factors. Prior to the stiffness testing, the samples had dried out due to the loose packaging. The Softwell™ plates are sealed air-tight and often vacuum sealed. Moreover, in order to measure small samples of hydrogel materials, rheometer was calibrated and used differently. The large variations in the observed stiffness readings using the current settings indicate that fine tuning of the rheometer settings would be necessary for more consistent results. The team hypothesized that the stiffening of the gels led to the data from Table 1 being so much higher than the defined stiffness to which they were manufactured. However, it can be seen from the data that the stiffer gels are still measured to be stiffer than those defined to be softer. Further, when the expected G' is lower, the true G' values are closer to the defined stiffness (Figure 11). This could imply that the stiffer gels dried out more rapidly



Figure 11: Rheometer Testing

Image of hydrogel punch as taken from the rheometer (Left). Adjusted image with punch area colored red (Right). Using MatLab code “point collection”, five points were defined along the curved edge of the rheometer base, and four points on the outside of the red area were defined such that the red area could be analyzed by the program.

5.5 Micro-Patterning

Micropatterning of C2C12 was attempted using two different methods, viz, cell printing using a Digilab cell printer and coverslip method. The 350 micron size nozzle of the printer head that was used was too large for the micropattern size required for patterning C2C12 cells. The coverslip method yielded relatively larger pattern of approximately 1600 microns (Figure 12). It is therefore imperative to use nozzle size of the right size that are less than 50 microns. In order for a usable fiber to be patterned, the necessary size cell printing needle width would be 30-50 μm .

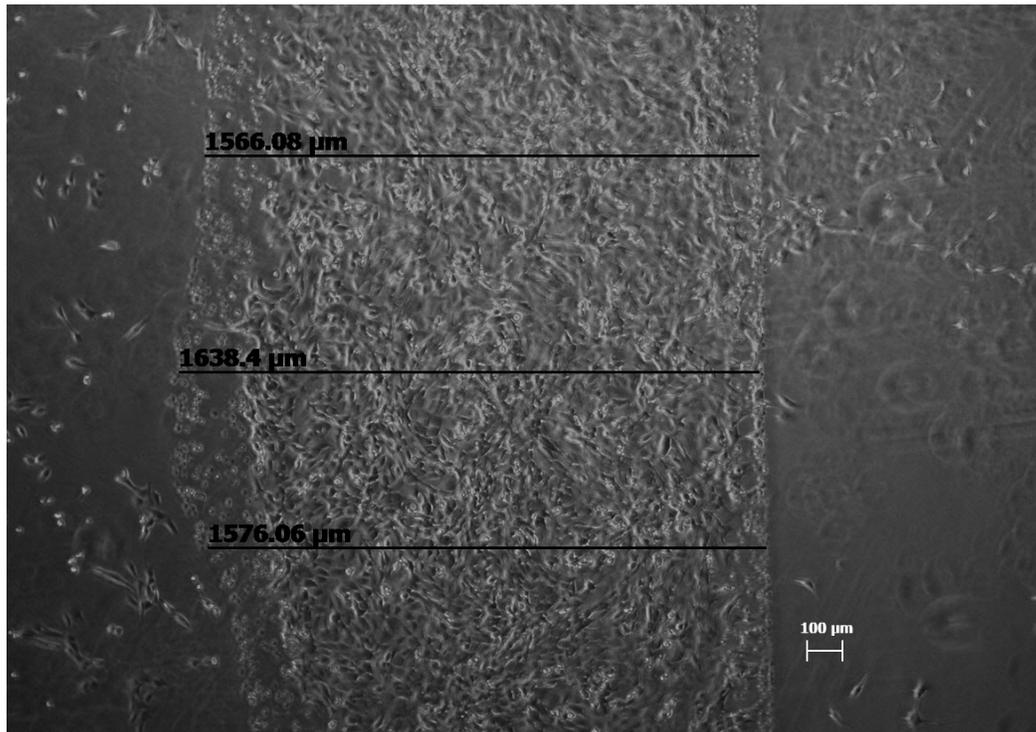


Figure 12: coverslip micropattern

Cell/collagen mixture was left to gel for 20 min. 4 million cell/mL solution used.

Chapter 6: Discussion

We observed that the C2C12 differentiated to aligned multinucleated myofibers both on polystyrene and Softwell™ plates with similar efficiencies. The myoblasts did not differentiate as efficiently on the Softwell™ plates as they did on the polystyrene plates. We hypothesize that this is because of a cell's tendency to spread over a larger surface area and be generally more motile on stiffer substrates. During observations, the fibers which were produced on the Softwell™ plates three dimensional morphology, and appeared more pronounced than on the polystyrene plates. In each of the wells, they exhibited a clearly multi-layered morphology, however only the 0.5 kPa and 1 kPa hydrogels produced fibers which could exhibit contraction. A possible hypothesis is that the fibers would be stronger on the softer substrate than they would on the polystyrene plates. However this was not testable. The data suggests that the hydrogels produce fewer differentiated fibers.

In this series of experiments, the team was unable to incorporate micro-patterning. This was due to a lack of available materials to produce *viable* micro-patterned designs, specifically patterns of the correct width. In order for us to generate myotubes of a specific pattern, a cell printing needle of width 30-50 μm would be needed. Micropatterns prepared using 350 μm exceeded the optimal width required for aligning C2C12 cells. Cells on the “micro-patterned” surfaces exhibited no discernible difference from those which were grown on regular plates. With the correct cell-printing tip, this could have been more successful. As for the stiffness testing, it is important to use fresh samples as well as find a way to calibrate the rheometer to test smaller samples.

The team's development of a functional assay of myoblast differentiation proved successful. The team was able to successfully design, build, and implement an electrical

stimulator, and was able to induce a twitch. The team was able to control these twitches using different frequencies. Cells seeded on the hydrogels lifted off the plate either before the cells could be stimulated them or during stimulation. Because of this, the team was unable to observe the differences in contraction between the two substrates. Further work should investigate a possible method of maintaining cell attachment, especially during contraction. Despite this, the team's development of a functional assay of differentiation was successful.

Limitations of the data include possible human error in protocols. For cell seeding density, errors in calculation along with possible inaccuracy in laboratory equipment could have led to mild inconsistency in cell behavior. For the stiffness testing, much inaccuracy was found in the results. The results were found to be inconclusive, as no accuracy could be discerned. The testing did show that the method described *can* find stiffness data. However, more thorough investigation of this method is required to increase accuracy. From the electrical stimulation data, a major limitation is the lack of quantitative data provided from videos taken. A possible solution could be quantum dot displacement to measure strength of contraction.

In terms of economics, the final design would directly affect the biological research community the most. Developing complex procedures, such as a functional assay of a cell's differentiation and gene expression, is sometimes out of the range of research laboratories. Since most research is based on the funding of individual or corporate grants, an easily accessible method of functionally assessing cell stimulation would provide smaller research laboratories more resources. Without any commercially available or commercially viable platforms available, researchers would have to improvise methods of stimulating muscle cells. By providing a commercially available product, those procedures would be operable in a more consistent and stable format.

As for environmental or societal impact, the design would have little. Materials used in the design are recyclable and readily available. Further, manufacture would require little resources, keeping negative impacts on the environment to a minimum. The societal impact would be largely indirect, only affecting those who benefit from cell culture research. A possible impact could be improved economics of drug development, as inexpensive protocols could possibly translate to less expensive pharmacotherapies. This could have possible political effects, since healthcare costs are a major concern for millions of Americans and an aging population.

The design which the group produced does not have any major ethical concerns. It does not pose any health and safety concerns in itself, although a possible concern would be the use of AC electric current with the device. An issue such as this could be resolved by issuing a safety warning with the final product imploring users to maintain safety when using electric devices. As stated before, the final design is easy to manufacture and mass market. A possible side effect in terms of sustainability would be the use of platinum as an electrode material. An investigation into this could consider the cost/benefit analysis of different metals, such as gold, copper, and stainless steel. Otherwise, the device is completely recyclable and poses little to no toxicity risk.

Chapter 7: Final Design and Validation

7.1 C2C12 Mouse Myoblast cells

The cells used for this project were from a C2C12 mouse skeletal myoblastic cell line. This cell line was chosen because it shows excellent differentiation *in vitro* and proliferates in the presence of Fetal Bovine Serum (FBS). The cells are also resilient, which was necessary for certain protocols and imperfect conditions during the project. The cells were obtained from a Worcester Polytechnic Institute frozen stock. The cells were grown and differentiated in an

incubator at 37°C with 5% CO₂, and for long term storage, the cells were frozen and stored at -80°C.

7.2 C2C12 culture and differentiation.

7.2.1. Proliferation:

For regular proliferation and expansion of C2C12 cells, they were grown in 100 mm polystyrene culture plates in culture media comprised of Dulbecco's Modification of Eagle's Media (DMEM, Mediatech) supplemented with 10% FBS (PAA laboratories), 2mM glutamax (Invitrogen) and 1% Penicillin-Streptomycin (Lonza) in a humidified incubator at 37°C with 5% CO₂.

7.2.2 Differentiation:

Differentiation media comprised of DMEM supplemented with 2% adult donor horse serum (PAA laboratories), 2mM glutamax, 1% Penicillin-Streptomycin and 1X insulin, transferrin, selenium supplement (ITS, Invitrogen). Different cell numbers were seeded per well to determine the optimal density for differentiating C2C12 cells. We empirically determined that 1.1×10^5 cells and 4.5×10^5 cells per well in 24-well and 6-well plates respectively provided sufficient confluency for experiments. This cell density was used for all differentiation experiments. Cells were seeded in proliferation media for approximately 2-6 hours to allow the cells to attach. Cells were then switched to differentiation media to start the differentiation process; and cultured over a period of 6 to 9 days to allow complete differentiation.

7.3 Freezing C2C12 Cells

In order to ensure a steady supply of early passage cells and to ensure that the same population of cells was used across all experiments and to keep the seeding densities constant, C2C12 cells were expanded in large quantities in the initial phase of the project. Cells were subcultured at about 70% confluency. All cells were frozen at the same time at specific densities.

Cells were frozen in freezing media containing DMEM, 10% FBS and 10% DMSO (Mediatech). The freezing densities were determined by the total numbers of cells required to seed all wells in either 24-wells or 6- well plates as mentioned earlier.

7.4 Immunocytochemistry

7.4.1 BrdU proliferation assay

The differentiation status of C2C12 cells was determined by assessing the ability of cells to uptake the nucleotide analog 5-Bromo-2'-deoxyuridine (BrdU). BrdU staining solution (Invitrogen) was added to cell culture medium to a final dilution of 1:100 and cultured for a further 12 hour period to allow BrdU incorporation into the nuclei of proliferating cells. After the 12 hour incubation period, cells were fixed in ice-cold methanol, rinsed 2X with DPBS containing Ca⁺⁺ and Mg⁺⁺ (Mediatech). Standard ICC protocol was followed for immunocytochemistry. Briefly, fixed C2C12 cells were treated with 1.5N HCl, rinsed 3X with DPBS for 5 minutes each. Anti BrdU antibody (DSHB, University of Iowa) diluted 1:100 in DPBS/0.05% Tween-20 (Invitrogen) was added and incubated for 30 minutes at room temperature, aspirated, and rinsed 3X with DPBS for 5 minutes each. This was followed by 30 minute incubation with Alexafluor-488 conjugated secondary antibody (Invitrogen), diluted 1:500 in DPBS/0.05% Tween-20. Cells were rinsed, stained with 0.5 µg/ml Hoechst 33342 (Calbiochem) for 10 minutes, rinsed and stored in DPBS until imaging. Imaging was performed using a Zeiss Axiovert inverted fluorescent microscope and images acquired using appropriate filters and analyzed using the Axiovision software (Zeiss).

7.4.2 Myosin Heavy Chain Expression analysis

Differentiated C2C12 cells were fixed at different time points in ice-cold methanol, rinsed twice with DPBS containing Ca⁺⁺ and Mg⁺⁺ (Mediatech). Cells were blocked with 3% FBS for

30 minutes at room temperature. A mouse anti-chicken myosin heavy chain (DSHB, University of Iowa), diluted 1:500 in DPBS/0.05% Tween-20 (Invitrogen), was added and incubated for 30 minutes at room temperature, aspirated, rinsed 3X with DPBS for 5 minutes each. This was followed by 30 minute treatment of Alexafluor-488 conjugated secondary antibody (Invitrogen), diluted 1:500 in DPBS/0.05% Tween-20. Cells were rinsed, stained with 0.5 µg/ml Hoechst 33342 (Calbiochem) for 10 minutes, rinsed and stored in DPBS until imaging. Imaging was performed using a Zeiss Axiovert inverted fluorescent microscope, and images acquired using appropriate filters and analyzed using the Axiovision software (Zeiss).

7.5 Micro-Patterning

7.5.1. Coverslip method

C2C12 cells were cultured in DMEM/10% FBS/2mM glutamax/1% Pennstrep until about 70% confluency. Cells were trypsinized using 0.05% Trypsin-EDTA. Specific cell numbers were pelleted at 200G for 10 minutes, supernatant removed and cell pellets resuspended in 250 µl of 0.5% PureCol® EZ Gel bovine type I collagen solution (Advanced Biomatrix) to a final concentration of 4 or 8 million cells per ml by repeated pipetting. Collagen/C2C12 mixture was stored on ice until use. Coverslips (22 x 22mm, 0.25 mm thick) were sterilized by rinsing in 70% ethanol followed by DPBS rinse. Sterilized coverslip was dipped in collagen/C2C12 mixture and stamped in 6-well plates to make a linear pattern. The 6-well plate was incubated for a period ranging from 10 to 60 minutes in 10 minute increments at 37°C after patterning to allow the collagen to gel completely. It was determined that 10 to 20 minute incubation was optimal for complete gelling of collagen. Incubations over 20 minutes resulted in drying of the collagen and subsequent cell death. Fresh complete medium was added to the plates after incubation and cultured for 3 days before initiating differentiation. Patterns were imaged the one day after

patterning using Zeiss Axiovert microscope and image analyzed using Axiovision software.

Thickness of the micropatterns was determined by averaging measuring taken at three points along the patterns spanning the borders created by collagen patterning (Figure 8).

7.5.2. Cell Printing Method

The precursor (prototype) of Digilab's commercial cell printer (Figure 13), the Cell Jet, was employed for C2C12 micropatterning on standard 6 well plates. The micropattern machine allows the aspiration of any liquid form via a tip and dispersion of the liquid onto surface of tissue culture well in specific pattern, speed and volume. The tip that was used in this experiment has a diameter of 350 μm . The nozzle was sterilized by dipping in 70% ethanol and rinsing in DPBS. The operation of this device was controlled using the AxSys MFC software, controlled by a computer connected to the device. We developed a code for this project (Figure 14) to instruct the machine to aspirate the collagen and cell mixture from a well (300 μl) of a 96 well-plate, and disperse the mixture onto the surface of the wells in 6-well plate. The machine dispersed the liquid in straight lines (1 cm), at the end of each operation, 3 lines of collagen and cell mixture in each well of a 6 well-plates would be produced, and 18 patterned line in total. Each well is varied by the volume of dispersion, which is ranged from 3 $\mu\text{l}/\text{cm}$ and decreased by 0.5 $\mu\text{l}/\text{cm}$ in each consecutive well.



Figure 13 Digilab's commercial cell printer the Cell Jet

```

6well_all.ad - Cartesian AxSys
File Edit Insert View Run Tools Help
MAIN PROGRAM
( ) Call start up
( ) Comment \\ Program to determine cell number consistency after printing with cell printer
( ) Comment \\ Dispense cell suspension in wells Pre-filled with media
( ) Pause Operator determined delay
( ) Call Aspirate 250 uL_cell_suspension_from Front holder Plate B1
( ) Move X = None, Y = None, Z = 20.000mm Absolute
( ) Loop 10 x (Call Pre_Disp_just_above_source_well)
( ) Move X = None, Y = None, Z = 0.000mm Absolute
( ) Call Z safe
( ) Call 1_1 6well
( ) Call 1_2 6well
( ) Call 1_3 6well
( ) Call 2_1 6well
( ) Call 2_2 6well
( ) Call 2_3 6well
( ) Comment \\ Clean tips
( ) Call Z safe
( ) Syringe Speed InvalidMP(1)
( ) Call clean tips
( ) Move X = Home, Y = Home, Z = Home
( ) End
  
```

Figure 14: Screenshot sample of Code for Cell Printer

7.6 Testing Hydrogel Stiffness

To test the stiffness of the Softwell™ hydrogels, 1mm thick punches obtained from hydrogels with stiffness ranging from 0.5 to 50 kPa were tested using a TA Instruments AR-G2 rotary rheometer. Gap height was measured by lowering geometry until a low (~3-5 N) normal force was seen. Data was collected using TA Advantage software. G'_{rheo} values were found at

0.01% strain. Images were taken after each test. These images were then adjusted in Adobe Photoshop to mark the hydrogel area red and analyzed using several MatLab codes which found the moment of inertia and the area for each image. The MatLab code “point_collection” found the $inert_s$ and $inert_g$ of the image, which correspond to the J_{rheo} and J_s values in the following equation:

Equation 3: Hydrogel Stiffness Equation

$$G_s^* = \frac{J_{rheo}}{J_s} G_{rheo}^* = b G_{rheo}^*$$

The ratio of J_{rheo}/J_s is a corrective factor to the measured stiffness, G_{rheo}^* . This corrective factor, b , adjusts the measured stiffness to find the true hydrogel stiffness, G_s^* .

7.7 Electrical Stimulation of C2C12 Multinucleated Fibers

C2C12 cells were seeded in 6-well plates and differentiated following standard protocols. Cells were stimulated by the electrical stimulation device designed and built for this project. The device, integrated into the cover of a 6-well plate, has electrical leads that enter the wells and can be controlled by an outside electrical source (Agilent 33220 A). The device can stimulate the cells for at least an hour or longer, at frequencies of 1-10 Hz, pulse durations of 10-200ms, and amplitudes between 1-50V. Each side of the plate can be controlled independently of each other and simultaneous electrical stimulation experiments can be run.

Chapter 8: Future Work and Recommendations

The effects that an *in vitro* tissue culture’s substrate stiffness has on its cell growth and morphology were the central focus of this project. Primarily, what the team examined was the extent to which gene expression, and subsequently differentiation, was affected by placing cells

in an environment with *in vivo* mechanical properties. In terms of gene expression, the production of myosin in skeletal muscle myoblasts was used as an indicator of gene expression as well as differentiation. Fluorescence imaging has already been widely used as a method of determining myosin expression visually; however the team also wanted to use a functional assay as a determinant of myosin expression.

When discussing the differences between the Softwells™ and the polystyrene plates, “gene expression” and “differentiation” must be clearly defined, including how they are separate yet related. “Differentiation” defined as the process the myoblasts take from a proliferating state to the formation of myotubes (the contractile unit of skeletal muscle). This is specifically talking about the fusion of the cytoplasm, the alignment of nuclei along a single axis, and the formation of a fiber along that axis. Once the fiber is formed, the nuclei express the gene for myosin. “Gene expression” is defined as the production of proteins which comes along with differentiation; in the case of C2C12 cells, the production of myosin heavy chains. The focus of the project was the effect on differentiation; gene expression was essentially used as a marker of differentiation.

By using both fluorescence and electrical stimulation to examine myosin expression, the team was not only able to see how efficiently the cells differentiated, but were able to examine the force of contraction from gene expression. Fluorescence imaging allowed us to examine how widely myosin heavy chains were produced by the cells, with a higher density of fibers translating to more efficient differentiation. Results show that cells grown on polystyrene plates differentiated more efficiently. Examination of contraction strength was primarily qualitative; in order to achieve quantitative data on the strength of contraction, displacement measurements would have to be made during contraction. This could be achieved through the use of quantum

dot fluorescence on real-time contracting cells. What was achieved in this project was validation that electrical stimulation can be used as a method of assessing the *function* of muscle cells once they differentiate and express myosin. To examine this the team had to develop a device which allowed us to view the cells in real-time.

In the results, there was a trade-off between the Softwell™ plates and the polystyrene plates. On the polystyrene plates, the cells were able to proliferate much more effectively than on the Softwells™, causing them to differentiate more efficiently. It can be hypothesized that this is because the cells were able to spread out over a larger area with a polystyrene substrate, meaning that they had more cell-to-cell contact. This can be seen this in both the phase contrast images and in the fluorescence images; the regular polystyrene plates simply produced more myosin fibers. Although differentiation was less efficient in the Softwells™ than on regular polystyrene, the fibers which resulted were more similar to that which would be seen *in vivo*. The tissues produced had a multi-layered morphology and therefore produced fibers with a three dimensional morphology, whereas the regular polystyrene plates had a flat morphology. For the team's experiments, the team used the same cell-seeding density for both the polystyrene plates as the Softwells™ in order to maintain consistency, as well as in the interest of time. Otherwise, observing a correlation in efficiency would be inconclusive. To achieve the same density of fiber formation, a higher cell seeding density would be required.

As mentioned, we used the same seeding density for the wells. Before the team began the differentiation experiments using 24-well Softwells™, trial and error was used to find the cell seeding density which would obtain 70% confluency as soon as the cells adhere. In the 24-wells, this was 110,000 cells per well. Normally, the cells are seeded at about 30% and allowed to grow to 70%-100% confluency, which generally takes about 4-5 days. By seeding at confluency,

the cells weren't necessarily allowed to fully adjust to their new environment. Because of this, some cells took longer to fully exit the cell cycle. For this reason, BrdU incorporation was seen up to and including day 6 of differentiation in the results. Had the cells been allowed to grow from a smaller density, the team most likely would not have seen this behavior. However, in the interest of consistency, the team chose to seed at confluency, accepting that many of the cells would not exit the cell cycle as quickly as they would normally.

During the project, the team was also tasked with determining a reliable method of testing the stiffness of the Softwell™ hydrogel, essentially as a quality control. The goal was to find a cost-effective method to determine the stiffness of hydrogel substrates. To keep it cost-effective, a method of quality control which lowers production costs is also necessary. Currently, the gold standard of hydrogel stiffness testing is atomic force microscopy (AFM), an expensive procedure for a manufacturer to undertake. Proper calibration of the rheometer to measure the stiffness of smaller hydrogel samples will be much more cost-effective and affordable for research labs as well as hydrogel manufacturers. A thorough investigation of substrate stiffness on tissue cultures, such as the work done in this project, gives insight as to the behavior and gene expression of cells in environments with different mechanical properties. Here the team directly compared the differentiation characteristics between the two types of surfaces in terms of cell function. For this reason, a simple functional model was used: whether or not contraction was achieved. To what degree it was achieved would require more quantitative work. This is a relatively simple model; more complex models such as glandular cells would require a more complex functional assay. From the results of this research, further research should be done concerning the function of more complex cells in *in vivo* mechanical environments. The team's research serves to elucidate some of the effects which the substrate stiffness has on the

functionality of cells, which perhaps will be advanced further through other projects and publications.

Chapter 9: Conclusions

The final design succeeded in its purpose as a functional assay of differentiation. Through its implementation, the team was able to observe function of cells in the final stages of differentiation on polystyrene plates. No contraction of C2C12 cells was observed on Softwell™ plates because the gels either lifted off of the plate or did not exhibit a contractions at all. Through the use of widely used markers of cell differentiation, such as immunocytochemistry, the results of the functional analysis was validated. Cells which exhibited a twitch had more widespread differentiation and more fibers, as evidenced by myosin fluorescence imaging. As a method of functionally determining differentiation, the device is validated by previous techniques. The Softwell™ plates were shown to induce cells to less widespread differentiation than the polystyrene. Fibers were, however, morphologically similar to *in vivo* tissue due to their three-dimensional shape.

References

- Aldaye, Faisal. Senapedis, William. Silver, Pamela. Way, Jeffery.” A Structurally Tunable DNA-Based Extracellular Matrix”. *Journal of the American Chemical Society*. **132**, 14727-14729 (2010)
- Almany, Liora. Seliktar, Dror. “Biosynthetic hydrogel scaffolds made from fibrinogen and polyethylene glycol for 3D cell cultures”. *Biomaterials*, Vol. 26 (2005). 2467-2477.
- Anseth, Kristi S. Bowman, Christopher N. Brannon-Peppas, Lisa. “Mechanical properties of hydrogels and their experimental determination – Review”. *Biomaterials* Vol. 17 (1996). 1647-1657.
- Bhana, Bashir. Iyer, Rohin K. Chen, Wen Li Kelly. Zhao, Ruogang. Sider, Krista L. Likhitanichkul, Morakot. Simmons, Craig A. Radsisc, Milica. “Influence of Substrate Stiffness on the Phenotype of Heart Cells”. *Biotechnology and Bioengineering*, Vol. 105, No. 6 (April 2010)
- Brandl, Ferdinand, et al. “Rational design of hydrogels for tissue engineering: Impact of physical factors on cell behavior”. *Biomaterials* vol 28(2007); 134-146.
- Brevet, A., E. Pinto, J. Peacock, and F.E. Stockdale. 1976. Myosin synthesis increased by electrical stimulation of skeletal muscle cell cultures. *Science* 193:1152-1154.
- Calvert, Paul. “Printing Cells”. *Science*. Vol 318. 2007, Oct. 11.
- Cirka, Heather. Billiar, Kristen. “Eccentric rheometry for viscoelastic characterization of small, soft, anisotropic, and irregularly shaped biopolymer gels and tissue biopsies”. DOI 10.1007/s10439-012-0532-5
- Cha, Chaenyung, et al. “Biodegradable Polymer Crosslinker: Independent Control of Stiffness, Toughness, and Hydrogel Degradation Rate”. *Advanced Functional Materials* vol 19 (2009); 3056-3062.
- Cross, Sarah E. Jin, Yu-Sheng. Rao, Jianyu. Gimzewski, James K. “Nanomechanical analysis of cells from cancer patients”. *Nature*, Vol. 2 (Dec 2007).
- Derave, W., H. Ai, J. Ihlemann, L.A. Wit-ters, S. Kristiansen, E.A. Richter, and T. Ploug. 2000. Dissociation of AMP-activated protein kinase activation and glucose transport in contracting slow-twitch muscle. *Diabetes* 49:1281-1287.
- Engler, Adam J. Richert, Ludovic. Wong, Joyce Y. Picart, Catherine. Discher, Dennis E. “Surface probe measurements of the elasticity of sectioned tissue, thin gels and polyelectrolyte multilayer films: Correlations between substrate stiffness and cell adhesion.” *Surface Science*, Vol. 570 (2004) 142-154.
- Flaibani, M. Boldrin, L. Cimetta, E. Piccoli, M. De Coppi, P. Elvassore, N. “Muscle differentiation and myotubes alignment is influenced by micropatterned surfaces and exogenous electrical stimulation”. *Tissue Eng Part A*, **15** (2009). 2447–2457.

Fujita, Hideaki. Nedachi, Taku. Kanzaki, Makoto. "Accelerated de novo sarcomere assembly by electric pulse stimulation in C2C12 myotubes". *Experimental Cell Research*. Volume 313, Issue 9 (2007). 1853-1865

Gajsek, Nina. Marko Jevsek, Tomaz Mars, Katarina Mis, Sergej Pirkmajer, Janez Breclj, and Zoran Grubic. Synaptogenetic Mechanisms Controlling Postsynaptic Differentiation of the Neuromuscular Junction are Nerve-Dependent in Human and Nerve-Independent in Mouse C2C12 Muscle Cultures. *Chemico-Biological Interactions*. **175**. 50-57 (2008).

Grainger, Stephanie and Putnam, Andrew. "Assessing the Permeability of Engineered Capillary Networks in a 3D Culture". *PLoS One*. **6**, 1-9 (2011).

Guillotin B., Guillemot F. Cell patterning technologies for organotypic tissue fabrication. *Trends in Biotechnology*, 2011, Vol 29, No. 4

Gutierrez-Martin, Yolanda. Martin-Romero, Francisco Javier. Henao, Fernando." Store-Operated Calcium Entry in Differentiated C2C12 Skeletal Muscle Cells". *Biochimica et Biophysica Acta*. **1711**, 33-40 (2005).

Hansen, Jason. Klass, Markus. Harris, Craig. Csete, Marie. "A Reducing Redox Environment Promotes C2C12 Myogenesis: Implications for Regeneration in Aged Muscle". *Cell Biology International*. **31**, 546-553 (2007)

Hellman, Kiki B. Nerem, Robert M. Editorial: Advancing Tissue Engineering and Regenerative Medicine. **13**, 2823-2824 (2007).

Hug A., Jason W., Che B., Hojae B., Alisha L., Donald M., Payam A., Ali K., Directed 3D cell alignment and elongation in microengineered hydrogels. *Biomaterials*. **30**. 2011. 1-11.

Janigro, D. Perju, C. Fazio, V. Hallene, K. Dini, G. Agarwal, M.K. Cucullo, L. "Alternating current electrical stimulation enhanced chemotherapy: a novel strategy to bypass multidrug resistance in tumor cells". *BMC Cancer*, **17** 6: 72

Jennifer N., Sara T., Robert F., Martha U., Jennifer A., Ralph N. "3D Microperiodic hydrogel scaffolds for Robust neuronal cultures".

Kaji, H., Ishibashi, T., Nagamine, K., Kanzaki, M., & Nishizawa, M. (2010). "Electrically induced contraction of C2C12 myotubes cultured on a porous membrane-based substrate with muscle tissue-like stiffness". *Biomaterials*, **31**(27), 6981-6986.

Kaji, Kirokazu. Ishibashi, Takeshi. Nagamine, Kuniaki. Kanzaki, Makoto. Nishizawa, Matsuhiko. "Electrically Induced Contraction of C2C12 Myotubes Cultured on a Porous Membrane-Based Substrate with Muscle Tissue-Like Stiffness". *Biomaterials*. **31**, 6981-6986 (2010)

Kondo, Hideo and Yamasaki, Kenichi. "Movement of Cultured Myotube with Electrical Stimulation". Department of Biomedical Engineering, Research Institute of National Cardiovascular Center, Suita, Japan.

- Kuniaki N., Takeaki K., Takeshi I., Hirokazu K., Makoto K., Matsuhiko. Micropatterning contractile C2C12 myotubes embedded in a fibrin gel. Wiley InterScience 2009. Dec. 11
- Laura P., W. Cris W., Thomas B., Characterization of Patterned self-assembled monolayers and protein arrays generated by the ink-jet method. *Langmuir*. Vol 19 . 2003. 1462-1466
- Lee, Ju-Hye. Tachibana, Hirofumi. Morinaga, Yoshiko. Fujimura, Yoshinori. Yamada, Koji. "Modulation of Proliferation and Differentiation of C2C12 Skeletal Muscle Cells by Fatty Acids". *Life Sciences*. **84**, 415-420 (2009)
- Li, Bin. Lin, Michael. Tang, Ying. Wang, Bing. Wang, James. "A Novel Functional Assessment of the Differentiation of Micropatterned Muscle Cells". *J Biomech*. **41**, 3349-3353 (2008).
- Luo, Ying and Shoichet, Molly. "A photolabile hydrogel for guided three-dimensional cell growth and migration". *Nature Publishing Group*. **3**, 249-253 (2004).
- Luo Y., Schoichet M. A photolabile hydrogel for guided three-dimensional cell growth and migration. 21 March 2004; doi:10.1038/nmat1092
- Lo, C-M. Wang, H-B. Dembo, M. Wang, Y. "Cell Movement is Guided by the Rigidity of the Substrate". *Biophysical Journal* **79**. 2000, 144-152.
- Marotta, Mario. Bragós, Ramón. Gómez-Foix, Anna M. "Design and performance of an electrical stimulator for long-term contraction of cultured muscle cell". *BioTechniques*. 2004. 36:68-73
- Masuhiko Nishimura, Takeshi Nikawa, Yuichi Kawano, Mitsuo Nakayama, and Muneharu Ikeda. Effects of Dimethyl Sulfoxide and Dexamethasone on mRNA expression of housekeeping genes in cultures of C2C12 Myotubes. *Biochemical and Biophysical Research Communications*. **367**, 603-608 (2008).
- Melissinaki, V. Gill, A A. Ortega, I. Vamvakaki, M. Randella, A. Haycock, J W. Fotakis, C. Farsari, M. Claeysens, F. "Direct laser writing of 3D scaffold for neural tissue engineering application". *Biofabrication* **3**. 2011.
- Ni, Ming. Tong, Wen Hao. Choudhury, Deepak. Abdul Rahim, Nur Aida. Iliescu, Ciprian. Yu, Hanry. "Cell Culture on MEMS Platforms: A Review". *International Journal of Molecular Sciences*. **10**, 5411-5441 (2009).
- Shepherd J., Parker S., Shepherd R., Gillette M., Lewis J., Nuzzo R. 3D Microperiodic Hydrogel Scaffolds for Robust Neuronal Culture. *Adv. Funct. Mater*. 2011, 21, 47-54
- Shepherd, Jennifer Hanson. Parker, Sarah. Shepherd, Robert. Gillette, Martha. Lewis, Jannifer. Nuzzo, Ralph. "3D Microperiodic Hydrogel Scaffolds for Robust Neuronal Cultures". *Advanced Functional Materials*. **21**, 47-54 (2011)
- Shirin I., Ana I., Ola H., Inkjet printing of macromolecules on hydrogels to steer neural stem cell differentiation. *Biomaterials* **28**. 2007. 3936-3943

Sun, Shan. Titushkin, Igor. Cho, Michael. "Regulation of mesenchymal stem cell adhesion and orientation in 3D collagen scaffold by electrical stimulus". *Bioelectrochemistry*, Volume 69, Issue 2, October 2006, Pages 133-141

Tang, Zhiyong. Wang, Ying. Podsiadlo, Paul. Kotov, Nicholas A. "Biomedical Applications of Layer-by Layer Assembly: from Biomimetics to Tissue Engineering". *Advanced Materials*. **18**, 3202-3224 (2006).

Varghese, David. Deshpande, Malay. Xu, Tao. Kesari, Priya. Ohri, Sunil. Boland, Thomas. "Advances in Tissue Engineering: Cell Printing". *The Journal of Thoracic and Cardiovascular Surgery*. **129**, 470-472 (2005).

von Keller, A., Coster, H. G. L., Schnabl, H., & Mahaworasilpa, T. L. (1997). Influence of electrical treatment and cell fusion on cell proliferation capacity of sunflower protoplasts in very low density culture. *Plant Science*, *126*(1), 79-86.

Wylie W., Tobias W., Alexandra M., Kirstina B., Borja A., Martin M., Joachim P., Taher A., Jurgen G., Ralf K. "Myoblast morphology and organization on biochemically micro-patterned hydrogel coatings under cyclic mechanical stain". *Biomaterials* 31. 2010. 250-258

Yeung, Tony. Georges, Penelope C. Flanagan, Lisa A. Marg, Beatrice. Ortiz, Miguelina. Funaki, Makoto. Zahir, Nastaran. Ming, Wenyu. Weaver, Valerie. Janmay, Paul A. "Effects of Substrate Stiffness on Cell Morphology, Cytoskeletal Structure, and adhesion". *Cell Motility and the Cytoskeleton*, Vol. 60 (2005). 24-34.

Zhen-Ming Hu, Sean Peel, Stephen Ho, George Sandor, and Cameron Clokie. Induction of Bone Matrix Protein Expression by Native Bone Matrix Proteins in C2C12 Culture. *Biomedical and Environmental Sciences*. **22**, 164-169 (2009).

Appendices

Appendix A

MatLab code:

- **point_collection** has user define boundary points
- **process_image** uses boundary points to (in order)
 - o **1st**: find moment of inertia
 - o **2nd**: generate black and white contrast image
 - o **3rd**: find integral of red-colored area
- Data from **process_image** is used to find variables
 - o inert_s
 - o inert_g
 - o Jsample_c
 - o rmachine
 - o Rsample
- Output function in **point_collection** plots values found above into an Excel file named “outputs.xlsx”

“circfit.m”

%use with Matlab 2009b

```
function [xc,yc,R,a] = circfit(x,y)
%
% [xc yx R] = circfit(x,y)
%
% fits a circle in x,y plane in a more accurate
% (less prone to ill condition )
% procedure than circfit2 but using more memory
% x,y are column vector where (x(i),y(i)) is a measured point
%
% result is center point (yc,xc) and radius R
% an optional output is the vector of coeficient a
% describing the circle's equation
%
%  $x^2+y^2+a(1)*x+a(2)*y+a(3)=0$ 
%
% By: Izhak bucher 25/oct /1991,
x=x(:); y=y(:);
a=[x y ones(size(x))]\[-(x.^2+y.^2)];
xc = -.5*a(1);
yc = -.5*a(2);
R = sqrt((a(1)^2+a(2)^2)/4-a(3));
```

“imageanalysis.m”

% Use with Matlab 2009b

```
PIC2 = imread('IMG_5993.JPG');
figure
imshow(PIC2)
I=PIC2(:,:,1)-PIC2(:,:,2);
figure
imshow(I)
level = graythresh(I);
bw = im2bw(I,level);
figure
imshow(bw)
s=regionprops(bw, 'centroid');
centroids=cat(1, s.Centroid);
imtool(bw)
hold(imgca,'on')
plot(imgca,centroids(:,1),centroids(:,2),'r*')
hold(imgca, 'off')
xcentroid=s.Centroid(1,1);
ycentroid=s.Centroid(1,2);

%Calculating the inertia of the sample if it was about its center
Z=zeros(2048,3072); %creates a zero matrix the size of the picture
nR=size(bw,1);
nC=size(bw,2);

for i=1:nR;
    for j=1:nC;
        if bw(i,j)== 1; %if BW is white (ie has a value of one)
            Z(i,j)=(sqrt(((j-xcentroid).^2 + (i-ycentroid).^2));
        else
            Z(i,j)=0; %if bw is zero, it does not contribute (ie, it is not part of the sample
        end
    end
end
inertia_c= 0;
for j=1087:2195;
    for i=523:1167;
        inertia_c= inertia_c + (Z(i,j).^2).*bw(i,j);
    end
end
Jsample_c=inertia_c;
```

hold on

```
th = linspace(0,2*pi,20);  
R = 1.1111111;  
sigma = R/10;  
x = [2235 2311 2375 3395 2311 2243 2295];  
y = [487 671 915 1139 1863 519 1183];
```

```
plot(x,y,'o'), title('measured points')  
[xc,yc,Re,a] = circfit(x,y);  
xe = Re*cos(th)+xc; ye = Re*sin(th)+yc;
```

```
plot(x,y,'o',[xe;xe(1)],[ye;ye(1)],'-.',R*cos(th),R*sin(th)),  
title('fitted circle')  
text(xc-R*0.9,yc,sprintf('center (%g , %g ); R=%g',xc,yc,Re))  
xlabel x, ylabel y
```

```
axis equal  
plot(xc,yc,'r*')  
nR=size(bw,1);  
nC=size(bw,2);  
rC=xc %rC and rR stands for reference column and reference row respectively  
rR=yc  
D=zeros(2048,3072);  
for i=523:1167  
    for j=1087:2195;  
        if bw(i,j)== 1;  
            D(i,j)=(sqrt(((i-rR)).^2 + (j-rC).^2));  
        else  
            D(i,j)=NaN;  
        end  
    end  
end  
D(D==0)=NaN;
```

```
Rc2Redge= max(D(:))  
R1 = min(D(:))  
Rsample=0.5*(Rc2Redge-R1);
```

```
R=zeros(2048,3072);  
for i=1:nR;  
    for j=1:nC;
```

```

    if bw(i,j)== 1;
        R(i,j)=(sqrt(((j-rC).^2 + (i-rR).^2));
    else
        R(i,j)=0;
    end
end
end
inertia= 0;
for j=1087:2195;
    for i=523:1167;
        inertia= inertia+ (R(i,j).^2).*bw(i,j);
    end
end
Jsample=inertia;

rmachine=Re;
Jrheo= 3.14*0.5*(rmachine)^4

```

```

%torque as a function of Rsample and Rc2Redge= 0.5* rs^4 * [1
% %+2*(R/rs-1)^2)
%
%
Inertia = 0.5*pi*(Rc2Redge)^4*(Rsample/Rc2Redge)^4*(1+2*(((Rc2Redge/Rsample)-1)^2))

```

“process_image.m”

% Use with Matlab 2009b

```

function [inert_s inert_g Jsample_c rmachine Rsample] = process_image(image, x1x2, y1y2,
rgx, rgy)
% Process image file
% image:   RGB image from 8-bit tif file
% x1x2:   Limits of integration in the x-direction (in pixels)
% y1y2:   Limits of integration in the y-direction (in pixels)
% rgx:    Vector containing x-coordinates of radius of geometry
% rgy:    Vector containing y-coordinates of radius of geometry
% inert_s: Inertia of sample (in pixels)
% inert_g: Inertia of geometry (in pixels)

I = image(:, :, 1) - image(:, :, 2);
level = graythresh(I);
bw = im2bw(I, level);
th = linspace(0, 2*pi, 20)';
R = 1.1111111;
sigma = R/10;

```

```

[xc, yc, Re, a] = circfit(rgx, rgy);
xe = Re * cos(th) + xc;
ye = Re * sin(th) + yc;

nR = size(bw, 1);
nC = size(bw, 2);
rC = xc; %rC and rR stands for reference column and reference row repectively
rR = yc;
D = zeros(nR, nC);
for i = y1y2(1):y1y2(2);
    for j = x1x2(1):x1x2(2);
        if bw(i, j) == 1;
            D(i, j) = (sqrt(((i-rR)).^2 + (j-rC).^2));
        else
            D(i, j) = NaN;
        end
    end
end
D(D==0) = NaN;

Rc2Redge = max(D(:));
R1 = min(D(:));
Rsample = 0.5*(Rc2Redge - R1);
Dsample= Rc2Redge-R1;

R = zeros(nR, nC);
for i = 1:nR;
    for j = 1:nC;
        if bw(i, j)== 1;
            R(i, j)=(sqrt(((j-rC)).^2 + (i-rR).^2));
        else
            R(i, j) = 0;
        end
    end
end
inertia = 0;
for j = x1x2(1):x1x2(2);
    for i = y1y2(1):y1y2(2);
        inertia = inertia + (R(i, j).^2).*bw(i, j);
    end
end
ansDD = inertia;

rmachine = Re;
bmachine = 3.14*0.5*(rmachine)^4;

```

```
Inertia = 0.5*pi*(Rc2Redge)^4*(Rsample/Rc2Redge)^4*(1+2*(((Rc2Redge/Rsample)-1)^2));
```

```
inert_s = ansDD;  
inert_g = bmachine;
```

```
s=regionprops(bw, 'centroid');  
centroids=cat(1, s.Centroid);  
imtool(bw)  
hold(imgca,'on')  
plot(imgca,centroids(:,1),centroids(:,2),'r*')  
hold(imgca, 'off')  
xcentroid=s.Centroid(1,1);  
ycentroid=s.Centroid(1,2);
```

```
%Calculating the inertia of the sample if it was about its center  
Z=zeros(2048,3072); %creates a zero matrix the size of the picture  
nR=size(bw,1);  
nC=size(bw,2);
```

```
for i=y1y2(1):y1y2(2);  
    for j=x1x2(1):x1x2(2);  
        if bw(i,j)== 1; %if BW is white (ie has a value of one)  
            Z(i,j)=(sqrt(((j-xcentroid).^2 + (i-ycentroid).^2));  
        else  
            Z(i,j)=0; %if bw is zero, it does not contribute (ie, it is not part of the sample)  
        end  
    end  
end  
inertia_c= 0;  
for j=x1x2(1):x1x2(2);  
    for i=y1y2(1):y1y2(2);  
        inertia_c= inertia_c + (Z(i,j).^2).*bw(i,j);  
    end  
end  
Jsample_c=inertia_c;
```

```
end
```

```
“MAIN_run_all_data.m”
```

```
% Use Matlab 2009b  
% Import data
```

```
close all;  
clear all;  
clc;
```

```

% File list
files = {
    'C6342.TIF'
%     'C6341.Tif'
%     'C6340.Tif'
%     'C6339.TIF'
%     'C6322.TIF'
%     'C6321.TIF'
%     'C6320.TIF'
%     'C6319.TIF'
%     'C6310.TIF'
%     'C6309.TIF'
%     'C6308.TIF'
%     'C6307.TIF'

};

total = length(files) + 1;
count = 0;
h = waitbar(count/total, 'Processing...');

% Get parameters from excel file
[~, ~, params] = xlsread('Torque at center.xlsx');
i = 1;
for k = 2:length(params(:, 1))
    x1x2(i, :) = str2num(char(params(k, 2)));
    y1y2(i, :) = str2num(char(params(k, 3)));
    rgx(i, :) = str2num(char(params(k, 4)));
    rgy(i, :) = str2num(char(params(k, 5)));
    i = i + 1;
end

% Process image
for j = 1:length(files);
    waitbar(count/total, h);
    image = imread(char(files(j)));
    [inert_s(1, j) inert_g(1, j)] = process_image(image, x1x2(j, :), y1y2(j, :), rgx(j, :), rgy(j, :));
    count = count + 1;
end

waitbar(count/total, h, 'Saving...');

% Write to excel

```

```
headings = {'File' 'inert_g' 'inert_s'};
xlswrite('results.xlsx', headings);
xlswrite('results.xlsx', files, 1, 'A2');
xlswrite('results.xlsx', inert_g, 1, 'B2');
xlswrite('results.xlsx', inert_s, 1, 'C2');

close(h);
```

“point_collection.m”

```
% Use Matlab 2009b
% Import data
```

```
close all;
clear all;
clc;
```

```
% File list
```

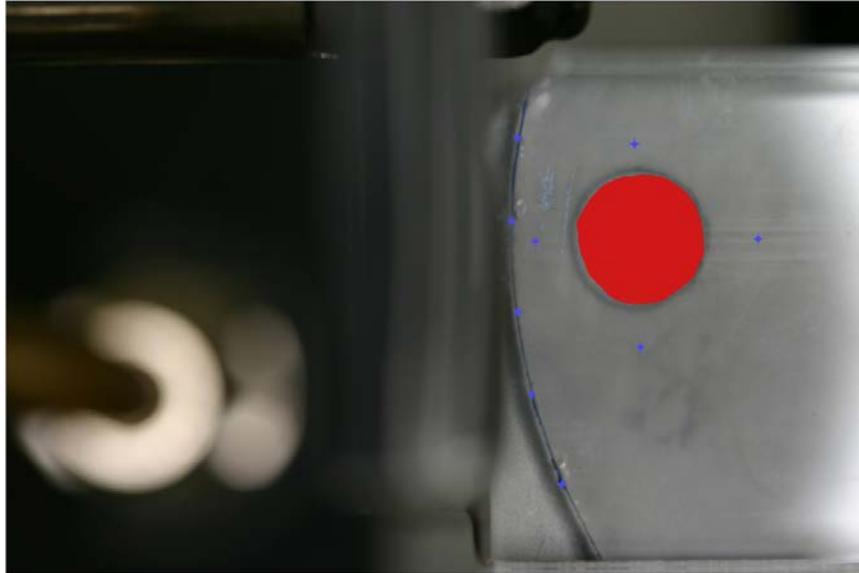
```
files = {
'C7814.tif'
'C7817.tif'
'C7818.TIF'
'C7820.tif'
'C7821.tif'
'C7823.tif'
'C7824.tif'
'C7826.tif'
'C7827.tif'
'C7829.tif'
'C7830.tif'
'C7831.tif'
'C7832.tif'
'C7834.tif'
'C7835.tif'
'C7836.tif'
'C7837.tif'
'C7838.tif'
'C7839.tif'
'C7840.tif'
'C7841.tif'
'C7842.tif'
'C7843.tif'
}
```

% listed above are file names of the images saved in the same file as the
% code. the names correspond to the trial number of the rheometer.

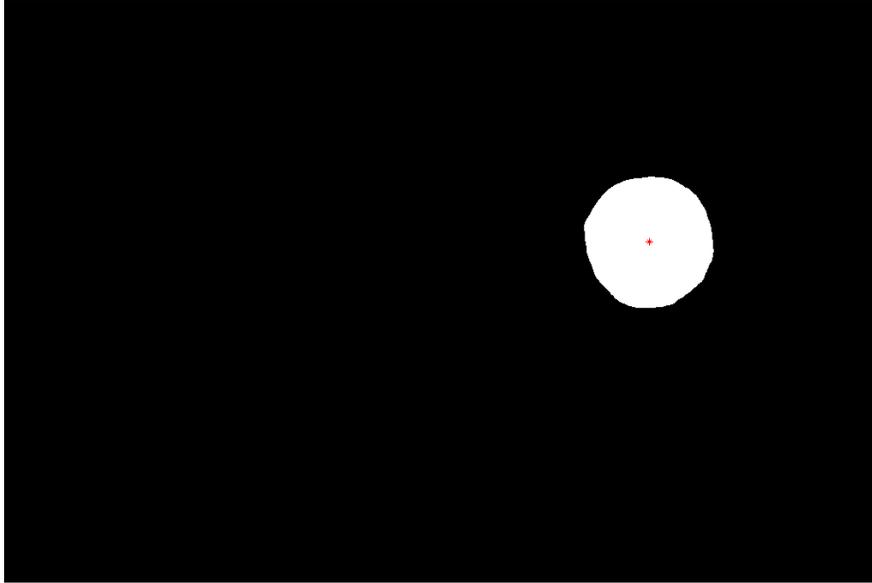
```
};  
% gets number of images by taking length of first column in excel  
% starts loop to run through all the images  
  
circpoints=zeros(2,5,length(files));  
edgepoints=zeros(2,4,length(files));  
for f=1:length(files)  
    close all;  
    imfix=imread((char(files(f))));  
    imfix=imfix(:,:,1:3);  
    imshow(imfix)  
    for i=1:5  
        h1=impoint;  
        h=wait(h1);  
        circpoints(:,i,f)=h;  
    end  
  
    for i = 1:4  
        h1=impoint;  
        h=wait(h1);  
        edgepoints(:,i,f)=h;  
    end  
end  
edgepoints = round(edgepoints);  
circpoints = round(circpoints);  
for f =1:length(files)  
    im=imread(char(files(f)));  
    im=im(:,:,1:3);  
    [inert_s(f) inert_g(f) Jsample_c(f) rmachine(f) Rsample(f)]=process_image(im,  
edgepoints(1,1:2,f),...  
    edgepoints(2,3:4,f), circpoints(1,:,f), circpoints(2,:,f));  
    f  
end  
  
outputs=cell(length(files)+1, 6);  
  
headings = {'File' 'inert_g' 'inert_s' 'Jsample_c' 'rmachine' 'Rsample'};  
  
outputs(1,:) = headings;  
outputs(2:end,1) = files;  
outputs(2:end,2) = cellstr(num2str(inert_g'));
```

```
outputs(2:end,3) = cellstr(num2str(inert_s'));
outputs(2:end,4) = cellstr(num2str(Jsample_c'));
outputs(2:end,5) = cellstr(num2str(rmachine'));
outputs(2:end,6) = cellstr(num2str(Rsample'));
```

```
xlswrite('outputs.xlsx', outputs);
```



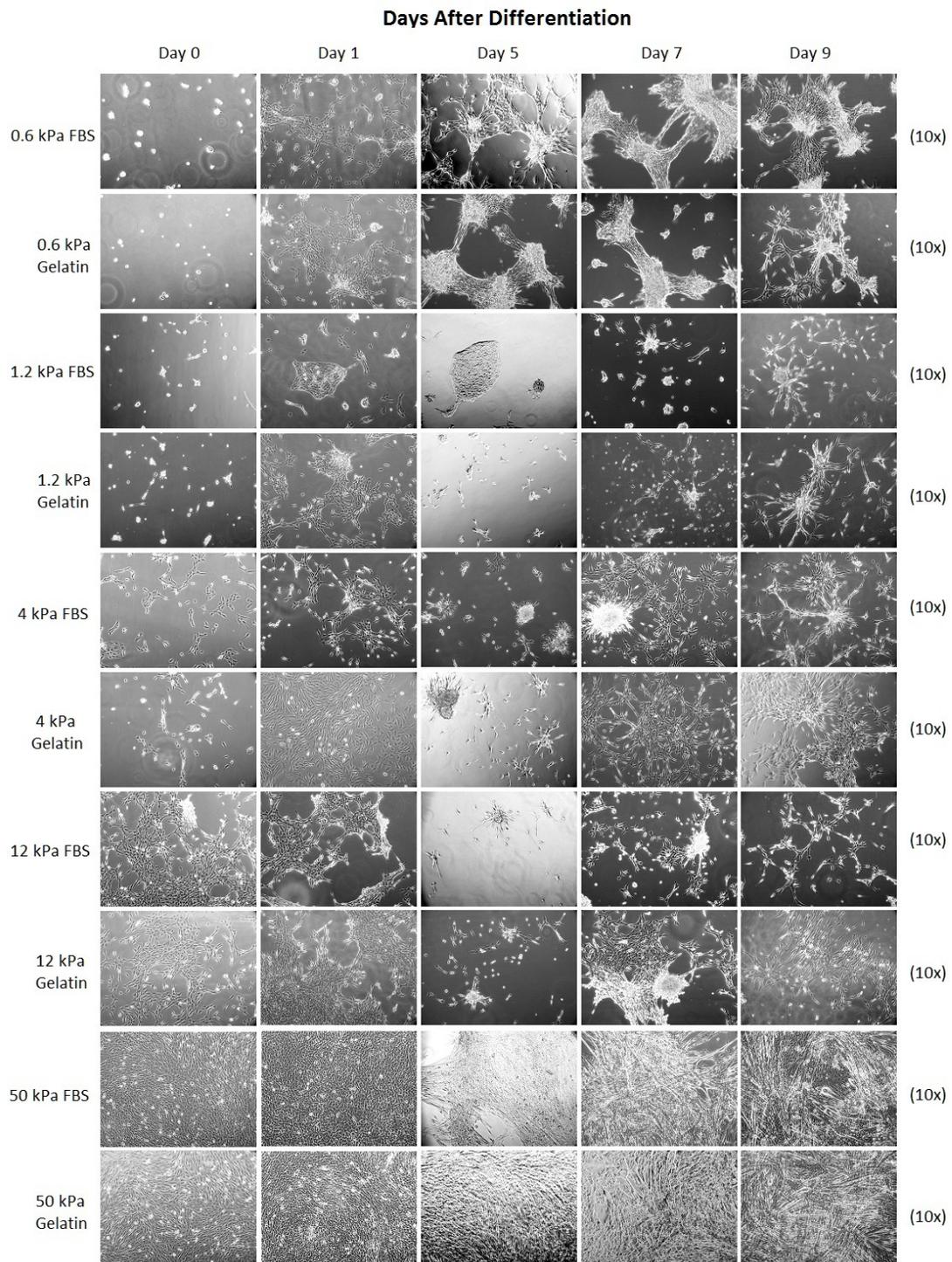
- Images of the hydrogel were marked red along the entire area of the hydrogel using Adobe PhotoShop
- Five points are defined along the curvature of the rheometer to the left of the hydrogel
- Four points are defined on the outside of the red area in the following order
 - o Top
 - o Bottom
 - o Left
 - o Right



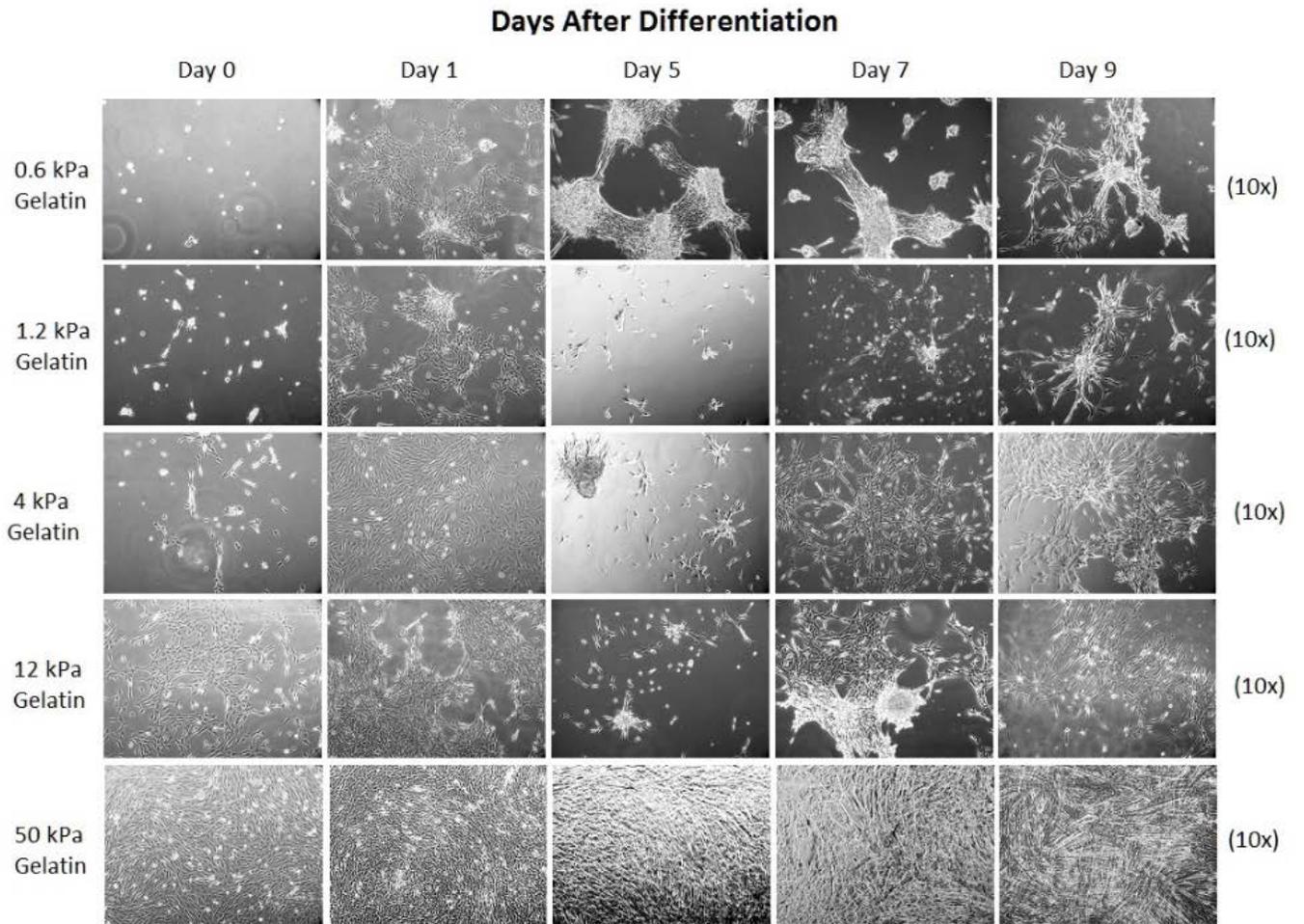
- What is produced is a Black/White contrast of the hydrogel which is used to
 - o Measure the area of the gel by taking its integral
 - o Find the moment of inertia (centroid) of the gel

Appendix B

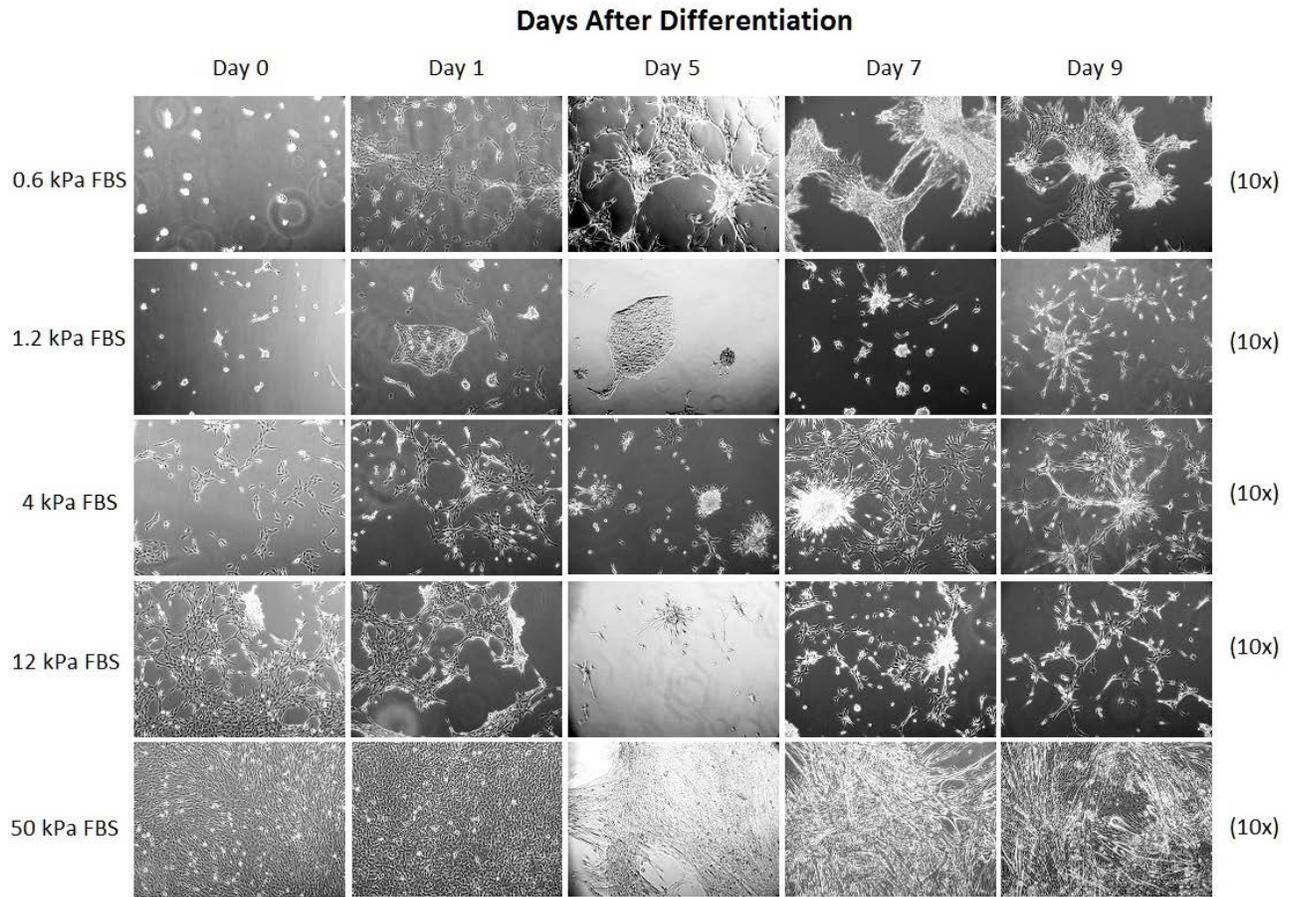
Comparison of Extracellular Matrix testing



Exploded view: Gelatin Extracellular Matrix

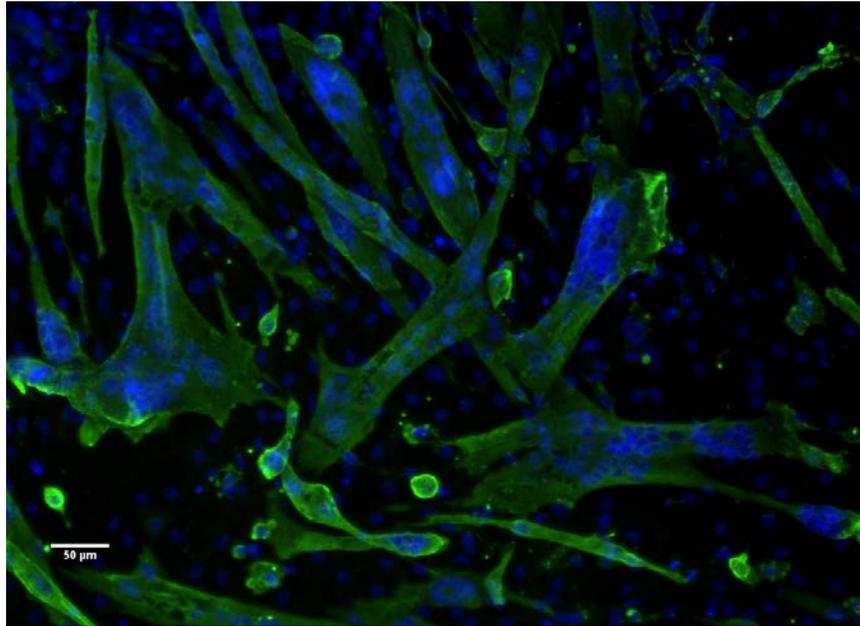


Exploded view: Fetal Bovine Serum Extracellular Matrix

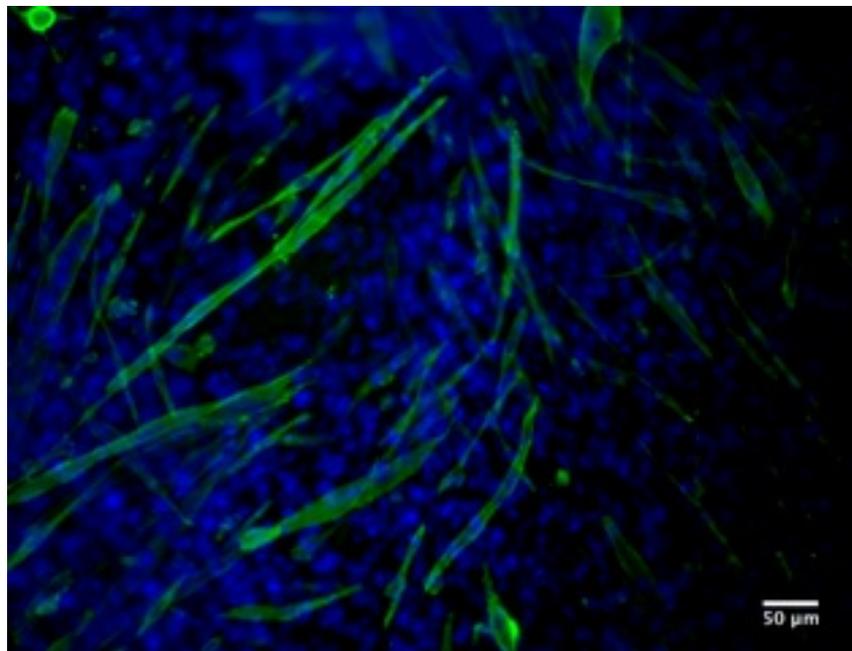


Appendix C

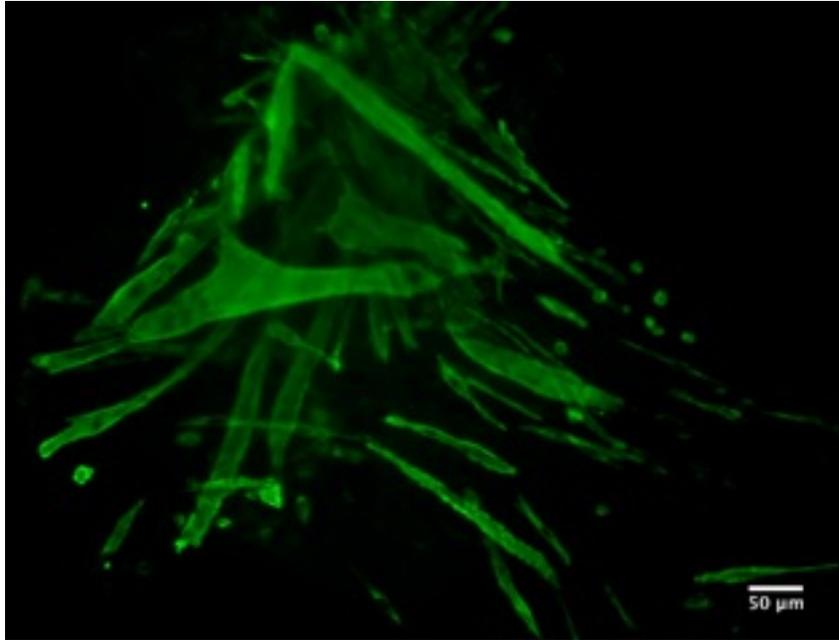
Myosin Heavy Chain fluorescence.



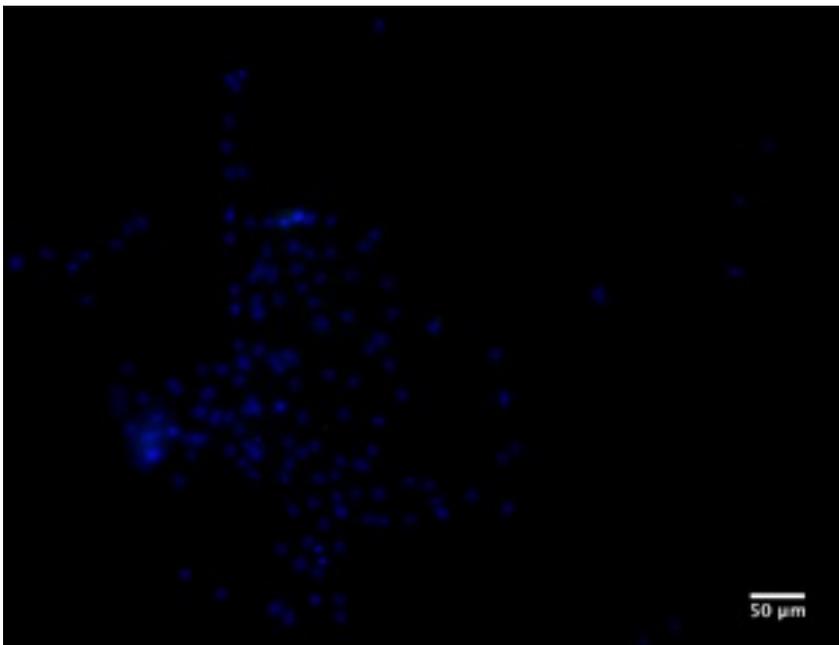
Day 9 of differentiation on polystyrene. Myosin anti-body & Hoechst nuclei stain. 20X magnification.



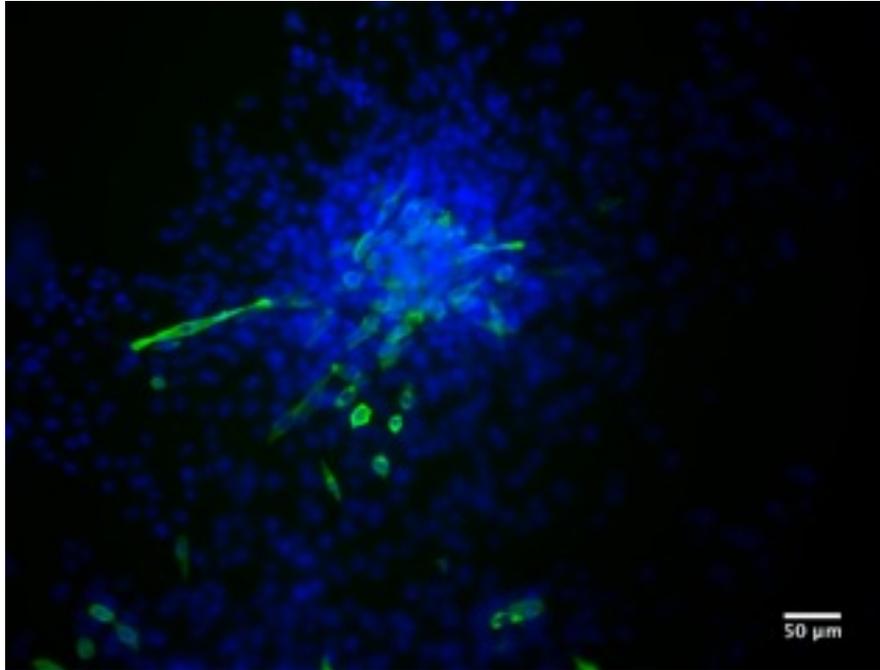
Day 9 of differentiation on 0.5 kPa hydrogel. Myosin anti-body & Hoechst nuclei stain. 20X magnification.



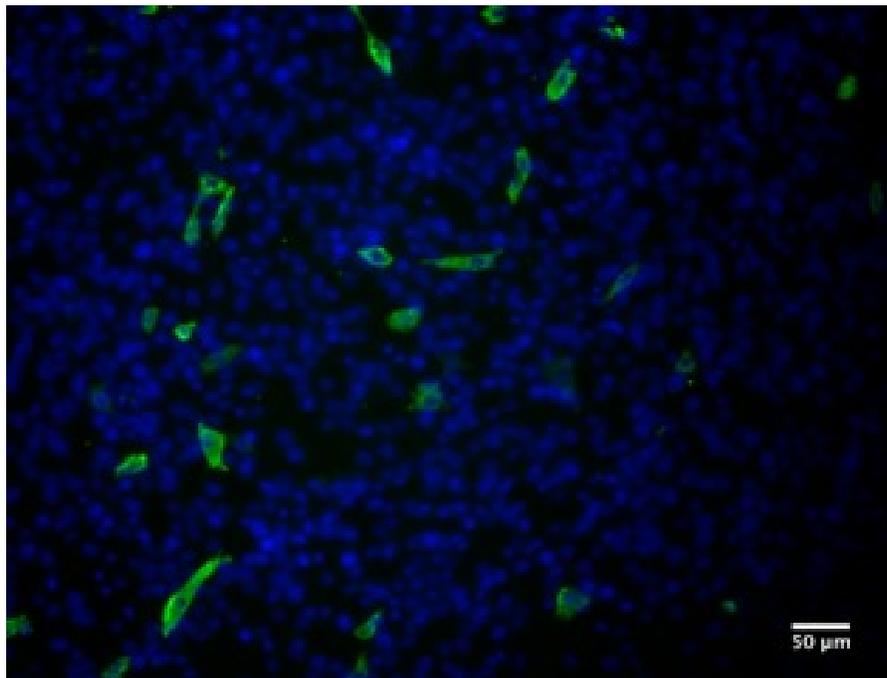
Day 9 of differentiation on 1 kPa hydrogel. Myosin anti-body & Hoechst nuclei stain. 20X magnification.



Day 9 of differentiation on 4 kPa hydrogel. Myosin anti-body & Hoechst nuclei stain. 20X magnification. No myosin fluorescence was observed in this sample. This could be due to one of many reasons, possibly error.



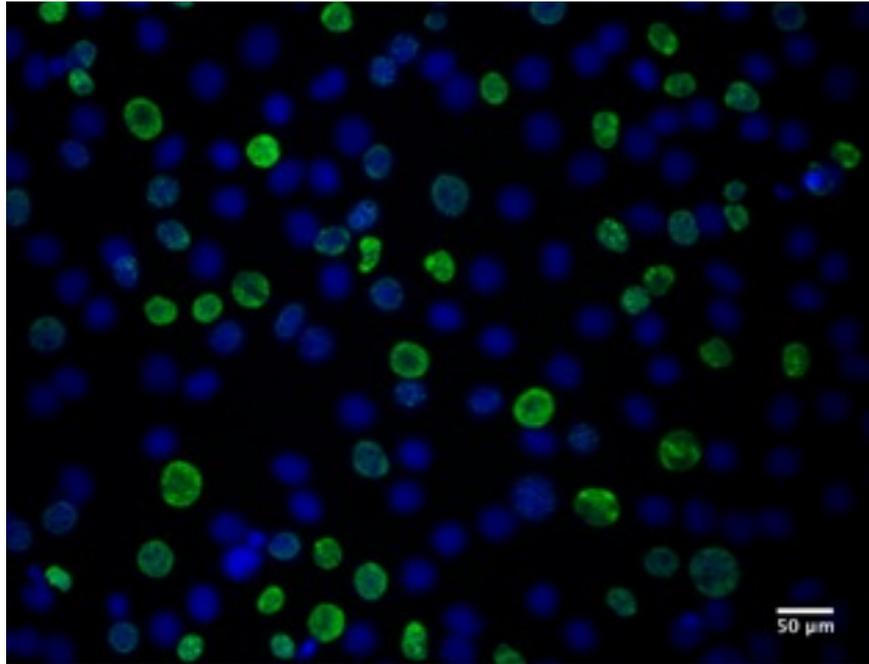
Day 9 of differentiation on 12 kPa hydrogel. Myosin anti-body & Hoechst nuclei stain. 20X magnification.



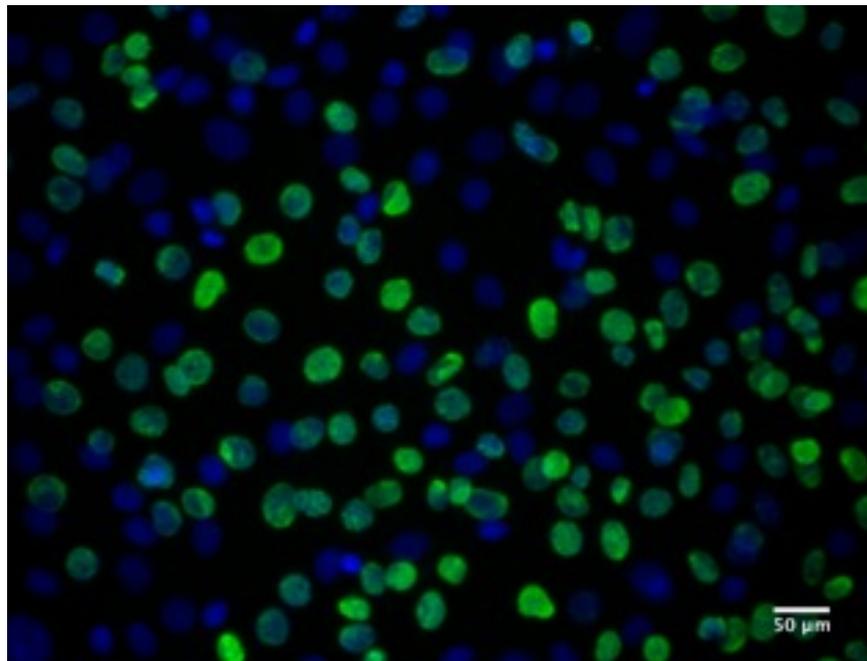
Day 9 of differentiation on 50 kPa hydrogel. Myosin anti-body & Hoechst nuclei stain. 20X magnification.

Appendix D

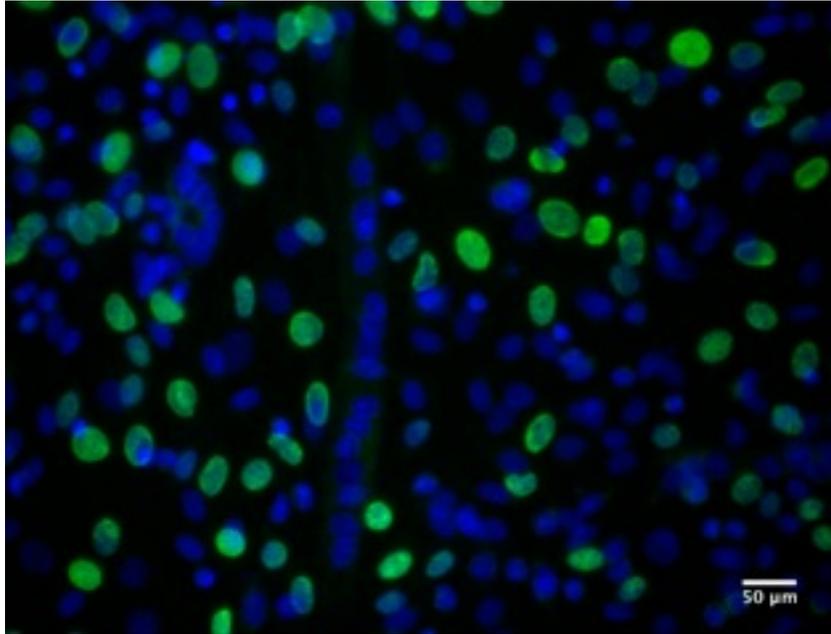
Bromodeoxyuridine (BrdU) incorporation experiment on polystyrene.



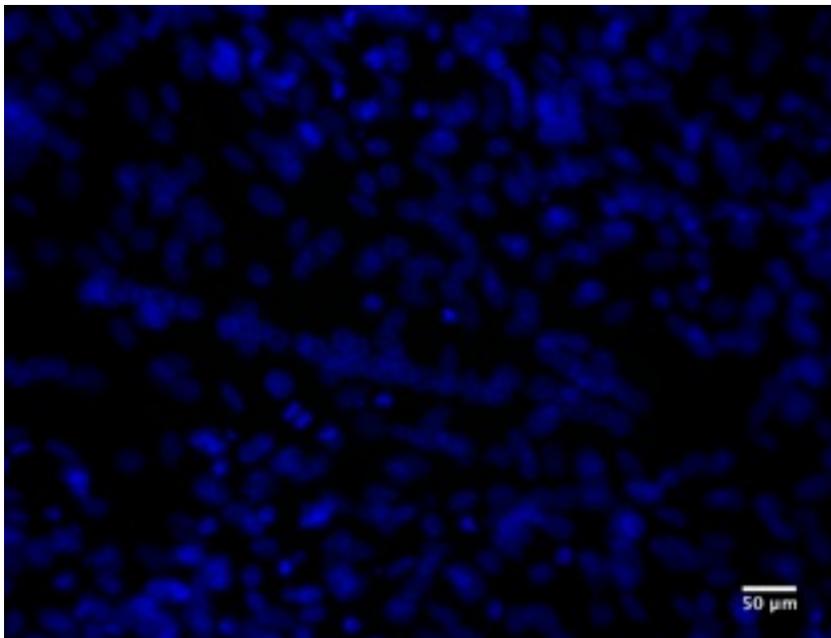
Day 2 of differentiation. BrdU anti-body and Hoechst stain. 20X magnification.



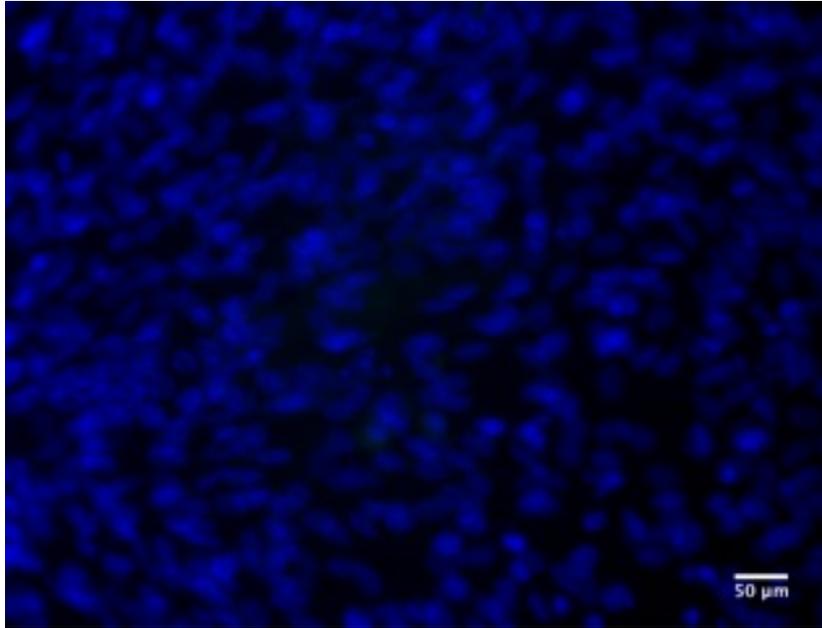
Day 3 of differentiation. BrdU anti-body and Hoechst stain. 20X magnification.



Day 6 of differentiation. BrdU anti-body and Hoechst stain. 20X magnification.



Day 9 of differentiation. BrdU anti-body and Hoechst stain. 20X magnification.



Day 9 with cells left in proliferative media. BrdU anti-body and Hoechst stain. 20X magnification. Cells have stopped incorporating BrdU, however fibers are not as pronounced or widespread when observing Hoechst stained nuclei. There is, however, some nuclear alignment observed.

Background figures

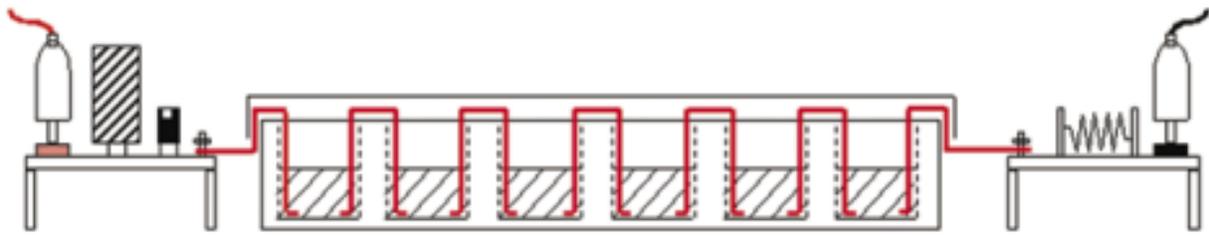


Figure 15: ESCC System Diagram

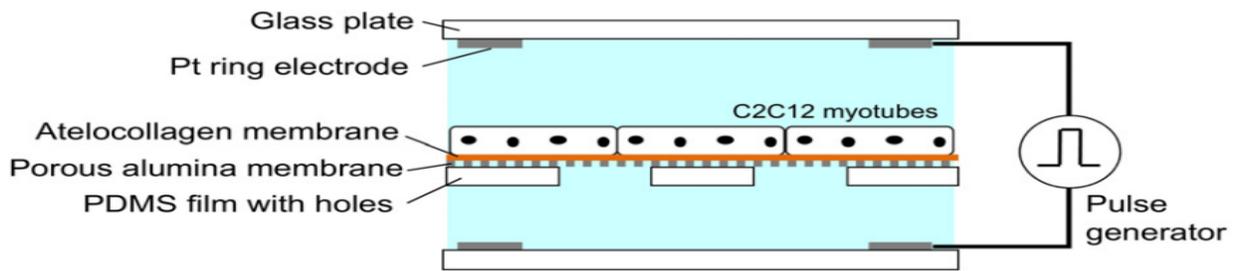


Figure 16: Microporous Alumina Membrane and Hole-Spotted PDMS Electrical Stimulation Device

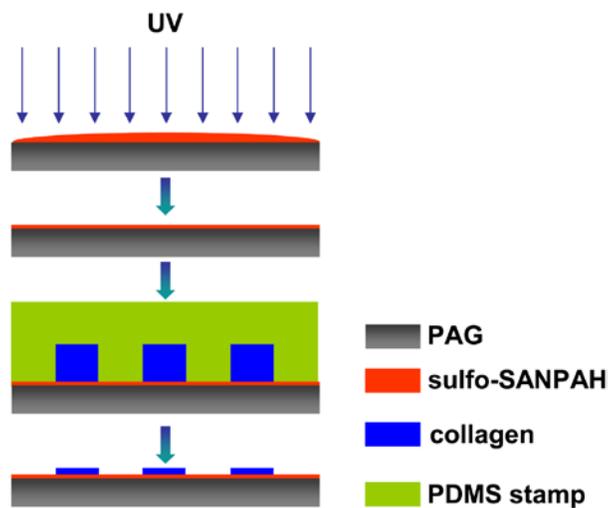


Figure 17: Micropatterning on a Polyacrylamide gel (PAG)

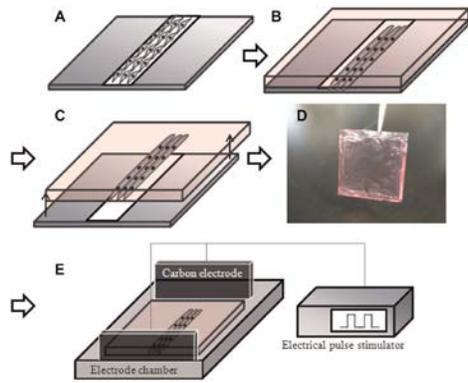


Figure 18: Micropatterning with Electrical Pulse Stimulation