UNIVERSITY OF CALIFORNIA,
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Development of 2D and 3D In Vitro Models of Human Skeletal Muscle

THESIS

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MASTER OF SCIENCE

In Biomedical Engineering

by

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Associate Professor Anna Grosberg, Chair
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ABSTRACT OF THE THESIS

Development of 2D and 3D in vitro Models of Skeletal Muscle

By

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Master of Science in Biomedical Engineering

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Associate Professor Anna Grosberg, Chair

The development of 2D and 3D in vitro models of human skeletal muscle would provide the ability to examine the structural organization and function of muscle tissue for use in predictive drug screening and disease modeling. Application of such models may offer insight into the initiation mechanisms of genetically inherited muscular dystrophies, specifically Facioscapulohumeral muscular dystrophy (FSHD). This thesis describes the approach taken towards developing both 2D and 3D in vitro models. Here we investigate biomaterial surface modifications that facilitate the development of mature myoblasts for long-term 2D culture as well as the inhibition of fibrin hydrogel degradation for the development of 3D models.

Chapter 1: Introduction

Skeletal muscle is the most abundant tissue in the human body comprising approximately 40% of total body mass [1]. Skeletal muscle plays a role in a number of different functions including controlling voluntary movement, breathing, and maintaining posture [2]. Disorders such as muscular dystrophies are inherited diseases linked to abnormal genes and lead to progressive muscle weakness that can severely impact functional capacity [2]. One such dystrophy is Facioscapulohumeral Muscular Dystrophy (FSHD), which leads to progressive muscle weakness of the facial muscles, upper back, and dorsiflexors of the foot [3].As the disease advances individuals afflicted by FSHD may lose the ability to walk or stand unassisted. In the most extreme cases patients may become visually impaired and experience the inability to completely close the eyes during sleep [4]. FSHD has been linked to a mutation of the D4Z4 macrosatellite located in chromosome 4 that leads to the expression of the typically repressed DUX4 gene [5]. The D4Z4 region is normally hypermethylated and keeps DUX4 and DUX4-like genes inactive. However, in patients afflicted with FSHD chromosome 4 has a shortened number of D4Z4 repeats and leads to the expression of the DUX4 gene, resulting in the progressive muscle weakness associated with FSHD [5]. Currently, the initiation mechanisms of FSHD are unknown. In order to develop better treatments for FSHD further research is required.

Previous studies have used in vivo animal models to represent the disease at the physiological and functional levels [6]. An animal model for FSHD requires a stable and controllable expression of the DUX4 gene, however, this is only strongly conserved in primates [6].To introduce the human DUX4 gene into non-primates transgenic animals were created, but results indicated that these methods did not fully represent all aspects of FSHD [6]. In vitro models employing human pluripotent stem cells have been shown to have similar gene expression profiles and functional properties to biopsy-derived cell lines. However, the protocols involved to

achieve these models are labor intensive and offer limited scalability [7]. Utilizing immortalized cell lines that the Yokomori lab has developed provides a solution to these issues. An immortalized cell line will allow for a cost effective and simple to use method of generating a 2D skeletal muscle model that enables the study of FSHD disease progression [8]. Integrating these cell lines into an in vitro model would allow for the examination of functional and structural properties of skeletal muscle [8].

A 2D in vitro model of skeletal muscle allows for complete control of the cell's microenvironment as well as facilitates rapid drug screening studies [9]. To develop a model that mimics functional skeletal muscle, skeletal myoblasts are seeded onto PDMS substrates that are coated or micro-contact printed with an extracellular matrix. Previous studies have demonstrated the importance of spatial cues in creating aligned skeletal muscle for engineering mature myotubes in 2D [10]. However, the prolonged cell culturing of skeletal muscle has proven to be a challenge due to myotube delamination. Delamination prevents long- term study of disease development and provides limited data on chronic conditions. Surface functionalization techniques may offer a solution by providing improved conditions for cellular adhesion and proliferation[11].

Another approach to investigating FSHD initiation mechanisms is through the development of a 3D in vitro model. Methods have been developed to create a biomimetic microenvironment that allows for the creation of 3D cell cultures that promote rapid tissue generation and function [12]. Traditionally, these models have been generated by seeding myocytes at high density into 3D scaffolds [13]. Previous studies have utilized both natural and synthetic scaffolds such as collagen, fibrin, poly-L-lactic, and polyurethane [14]. Natural scaffolds offer the advantage of being inherently biocompatible but tend to degrade too quickly. The degradation rate of these scaffolds can be regulated with the use of aprotinin and tranexamic acid. In Maffioletti et al. an

adapted cardiac tissue engineering platform was used to create a human skeletal muscle model where a fibrin hydrogel was created between elastic polydimethylsiloxane (PDMS) posts and seeded with induced pluripotent stem cells from patients with muscular dystrophies [15]. To study the disease mechanisms and progression of FSHD, this method can be modified to make use of the immortalized cell lines provided by the Yokomori lab. This 3D muscle bundle could potentially be used to measure force for the evaluation of functional performance in response to therapeutic treatments.

This thesis will discuss the contributions made towards developing 2D and 3D in vitro skeletal muscle models. The following chapters will discuss the methods applied towards extending the culture time of 2D skeletal muscle constructs and growing mature myobundles for disease relevant timescales.

Chapter 2: 2D Model

Section I: 2D Model Methods

Coverslip Preparation:
Glass coverslips (Fisher Scientific Company) were sonicated in 95% ethanol for 30 minutes and placed in a 65°C oven. Polydimethylsiloxane (PDMS) was prepared in a 1:10 ratio of crosslinker to base and spin coated onto the glass coverslips. PDMS coated coverslips were cured overnight in a 65°C oven. Diamond scribes (VWR) were then used to cut coverslips into smaller squares.

Silicon Wafer Preparation:
Stamp designs were created using Adobe Illustrator and etched onto a 5 inch X 5 inch chrome with soda lime glass masks. Silicon wafers were created through SU-8 deposition in the BiON facility at UCI.

Stamp Creation:
PDMS was prepared in a 1:10 ratio of crosslinker to base. 65mg of prepared PDMS was then poured onto a silicon wafer in a petri dish. The petri dish was placed uncovered in a dessicator to remove air bubbles. After 30 minutes or until all air bubbles were removed the petri dish was left in a 65°C oven for 2 hours or overnight to allow PDMS to cure. Following cure time, the PDMS was removed from the wafer and cut into stamps.

Microcontact Printing:
PDMS stamps were sonicated in 95% ethanol for 30 minutes. Following sonication, stamps were dried using compressed nitrogen. 250 µL of Geltrex (ThermoFisher, A1413201) or Fibronectin solution was deposited onto the surface of the patterned side of the stamp.Geltrex was left to incubate overnight at 4°C. Fibronectin was left to incubate for 1 hour at room temperature. After incubation, excess solution was removed from the patterned side of the stamp and dried with compressed nitrogen. Stamps were then pressed onto coverslips. Next,

stamps were carefully removed from coverslips. Printed coverslips were placed in a 12 well plate and submerged in 2 mL of Phosphate Buffered Saline (PBS). 12-Well plate was wrapped in parafilm for storage. Fibronectin stamped coverslips were subsequently placed in a 4°C refrigerator. Geltrex stamped coverslips were incubated for 1 hour at 37°C prior to placing in a 4°C refrigerator.

PDMS Surface Modification with Genipin:
5mg of genipin were weighed and dissolved in 1mL of Millipore Milli Q water. Genipin solution was vortex mixed for 10 minutes to completely dissolve solution. Coverslips were ultraviolet-ozone treated for 8 minutes and transferred to the culture hood. For each coverslip, a 200 µL droplet of Genipin solution was pipetted onto a petri dish. Coverslips were placed PDMS-side face down onto the droplets and moved to a 37°C incubator for 4 hours. Following incubation time, coverslips were placed in a 12-well plate, submerged in 2mL of PBS, and moved to a 4°C refrigerator for storage.

PDMS Surface Functionalization using APTES and Genipin:
A 1:2 solution of APTES:Ethanol was prepared. For 6 coverslips, 4.5mL of APTES and 9mL of ethanol were used. 2mL of Ammonia solution per coverslip was placed into a 15mL conical. Coverslips were ultraviolet-ozone (UVO) treated for 8 minutes and placed into a 12 well plate. Coverslips were submerged in 2mL of APTES:Ethanol solution for 7 minutes. After 7 minutes the solution was aspirated and replaced with 2mL of Ammonia solution for 3 minutes. Ammonia solution was then aspirated and the well plate was transferred to a dessicator and dried in vacuo for 1-2 minutes. Coverslips were then removed from the well plate and placed on a petri dish to dry overnight at room temperature.

Cell Culture:
Skeletal myocytes

Healthy human primary myoblasts were immortalized by transduction of hTERT onto p16 resistant R24C mutant CDK4 and cyclin D1 as described previously [17]. Myoblasts were proliferated in growth media containing Dulbecco's Modified Eagle Media (DMEM) with 20% Fetal bovine serum (FBS), 1% Pen-Strep, and 2% Ultroser G. Differentiation was induced using media that contained high glucose DMEM supplemented with 2% FBS and Insulin Transferrin Selenium (ITS).

Cell Fixing:
Media was aspirated from wells and washed with warm PBS 3 times. A solution of warmed 4% paraformaldehyde and 0.005% Triton X-100 was added to each well and incubated for 10 minutes at room temperature. Following incubation, the PFA:Triton X-100 solution was aspirated and the wells were washed with PBS 3 times with a 5 minute wait between washes.

Immunostaining:
4,6-Diamidino-2-Phenylindole Dihydrochloride (DAPI (ThermoFisher)), AlexaFluor 488 Phalloidin (ThermoFisher) and mouse monoclonal anti-alpha-actinin were used as primary antibodies for nuclei, actin, and alpha-actinin, respectively. Coverslips were placed cell side down onto droplets of primary antibody solution and incubated for 1 hour at room temperature. Following incubation the stamps were washed with PBS 3 times before staining with goat anti-mouse IgG (Alexa Fluor 633 (ThermoFisher) secondary antibody solution for 1 hour at room temperature. Coverslips were washed with PBS 3 times prior to mounting.

Mounting:
A drop of Prolong Gold Antifade Mountant was placed onto a glass slide. Next the samples were placed cell side down onto the drop of prolong gold. A coating of nail polish was set around the edges of the coverslip and left overnight to dry. The microscope slides were then stored in a -20° C freezer.

Imaging:
Cell culture samples were imaged using the IX-83 inverted microscope.

Section II: 2D Results

A 2D in vitro model can provide valuable insight into the structure and function of skeletal muscle [8]. However, attempts at culturing and differentiating myocytes have resulted in cell delamination issues that prevent mature tissue from forming. During culture, cells will begin to delaminate from the substrate as early as 1 day after seeding with a significant reduction in adhered cells by day 5 (Figure 1A). To overcome this limitation a crosslinking protein, such as genipin, can be used to act as intermediary between the substrate and the ECM by providing cells a hydrophilic surface to adhere to.

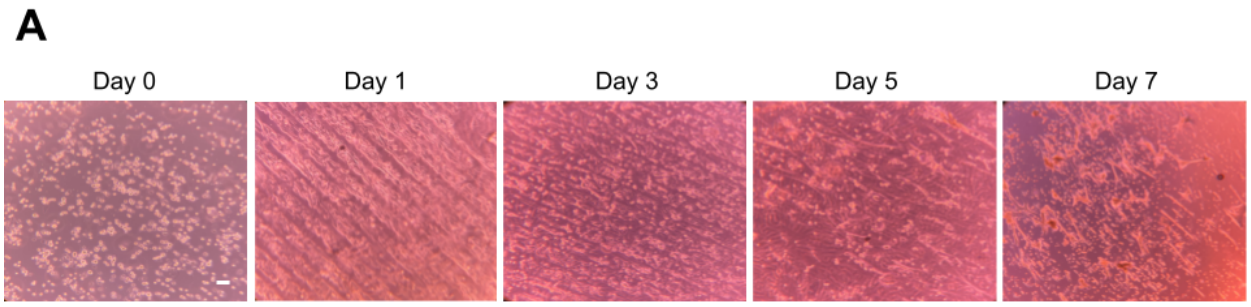

Figure 1: **Cell Delamination (A)** Skeletal muscle myocytes delaminating as culture time increases

PDMS coated coverslips were treated with varying concentrations of genipin solution followed by a microcontact printed layer of fibronectin (Figure 2B). Myocytes were seeded on these genipin functionalized coverslips and underwent differentiation. It was observed that concentrations of genipin of 3 mg/mL, 5 mg/mL, and 7 mg/mL had facilitated the formation of myotubes early on in the differentiation protocol but continued to display significant levels of cell delamination by day 7. Furthermore, it is noted that cells cultured on coverslips treated with a 5mg/mL solution of genipin displayed signs of myotube formation approximately 2 days earlier

than the 0mg/mL, 3mg/mL, and 7 mg/mL samples. Thus, 5mg/mL was determined to be the optimal genipin concentration for generating mature myotubes.

In order to prolong culture time beyond 5 days, several modifications were made to coverslips in addition to treating with genipin. Coverslips prepared with a double coating of genipin and fibronectin were found to have no beneficial effect in the development of mature myotubes or in extending culture time. As shown in Figure 3A, cells seeded on double coated coverslips resulted in a reduction in the number of myotubes formed during differentiation when compared to the standard single coat (Figure 2B(i)).

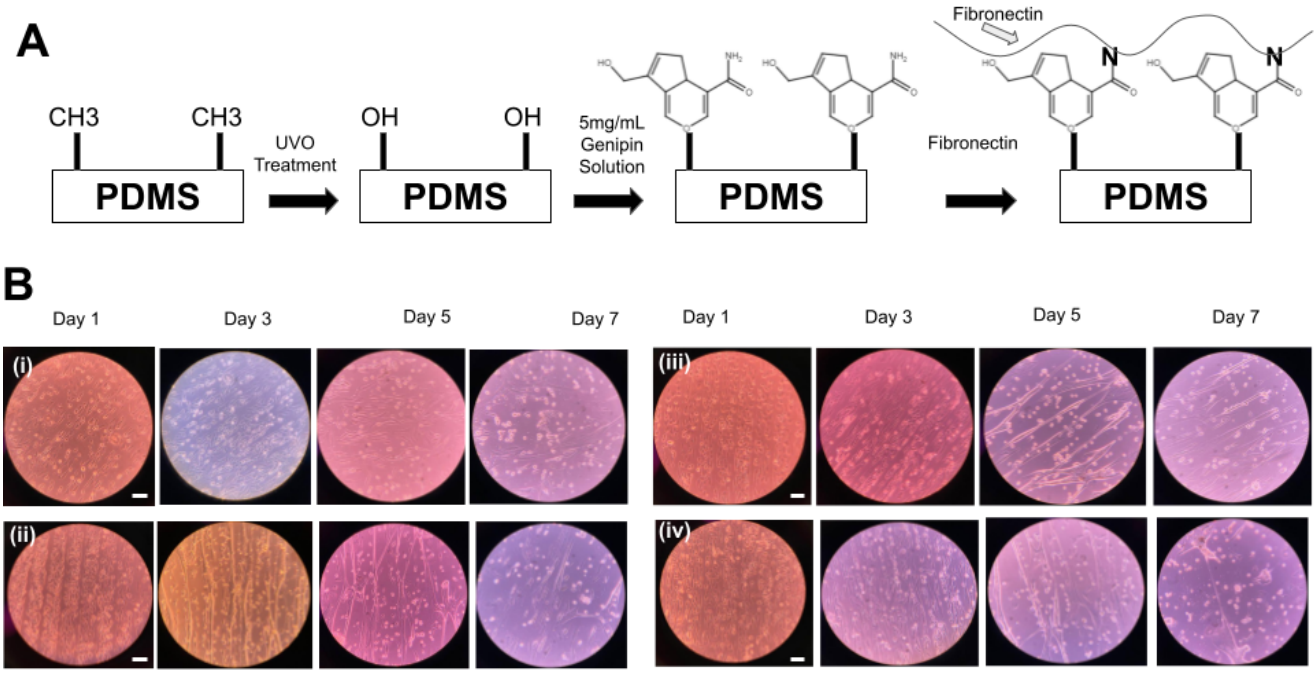

Figure 2: **PDMS surface modification with genipin (A)** Illustration of procedures performed for PDMS surface functionalization **(B)** Cells cultured on functionalized PDMS coated coverslip with varying concentrations of genipin; (i)7mg, (ii)5mg, (iii)3mg, (iv)0mg. Scale bar = 100 µm

It has previously been reported that skeletal muscle will develop approximately 20% longer myotubes when seeded on stiff substrates. [17]. Therefore, coverslips were prepared using a 5:1 PDMS ratio in order to provide a stiffer substrate in an attempt to improve differentiation

(Figure 3A). However, results depicted that there is no improvement in myotube formation or reduction in cell delamination. Similar to previous trials, significant cell delamination occurred at day 5 and worsened as time progressed.

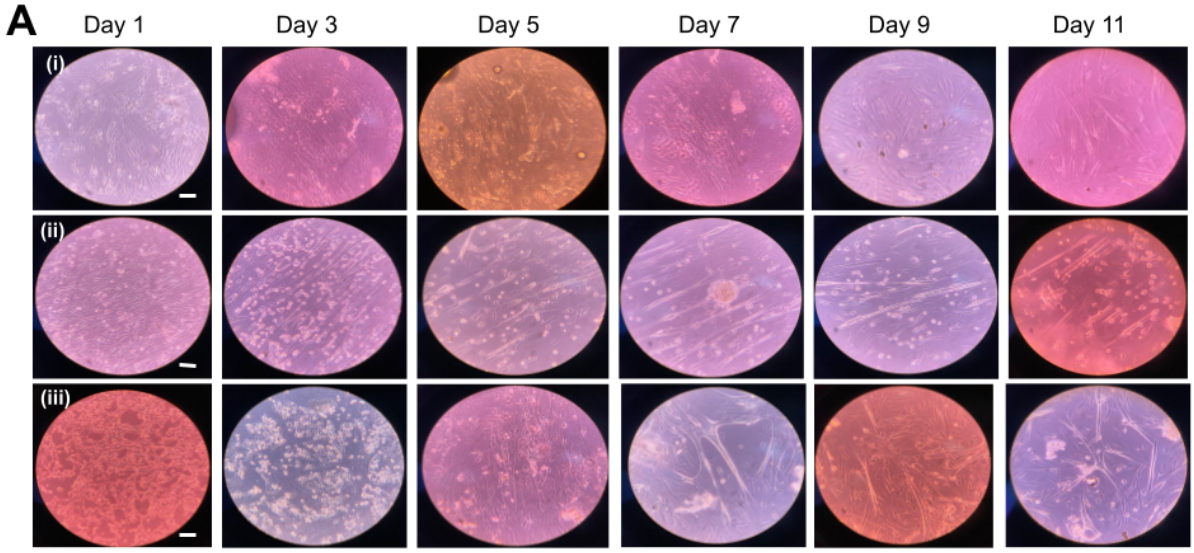

Figure 3: **Different conditions to prolong culture time of skeletal myocytes (A)** Coverslip modifications include: (i) double coating of genipin and fibronectin, (ii) 5:1 PDMS ratio, (iii) use of geltrex as ECM. Scale bar = 100 μm

Previous work has revealed that differentiating myocytes prior to seeding on coverslips results in a higher number of observed myotubes [18]. In an attempt to prevent cell delamination and prolong culture time, myocytes underwent differentiation prior to seeding on genipin-treated coverslips. Under these conditions, myotubes were successfully maintained for 7 days. Results showed a significant increase in attached myotubes after a week in culture, however no twitching was observed. After 7 days in culture samples were stained for alpha-actinin (red), actin (green), and the nuclei (blue) (Figure 4B).

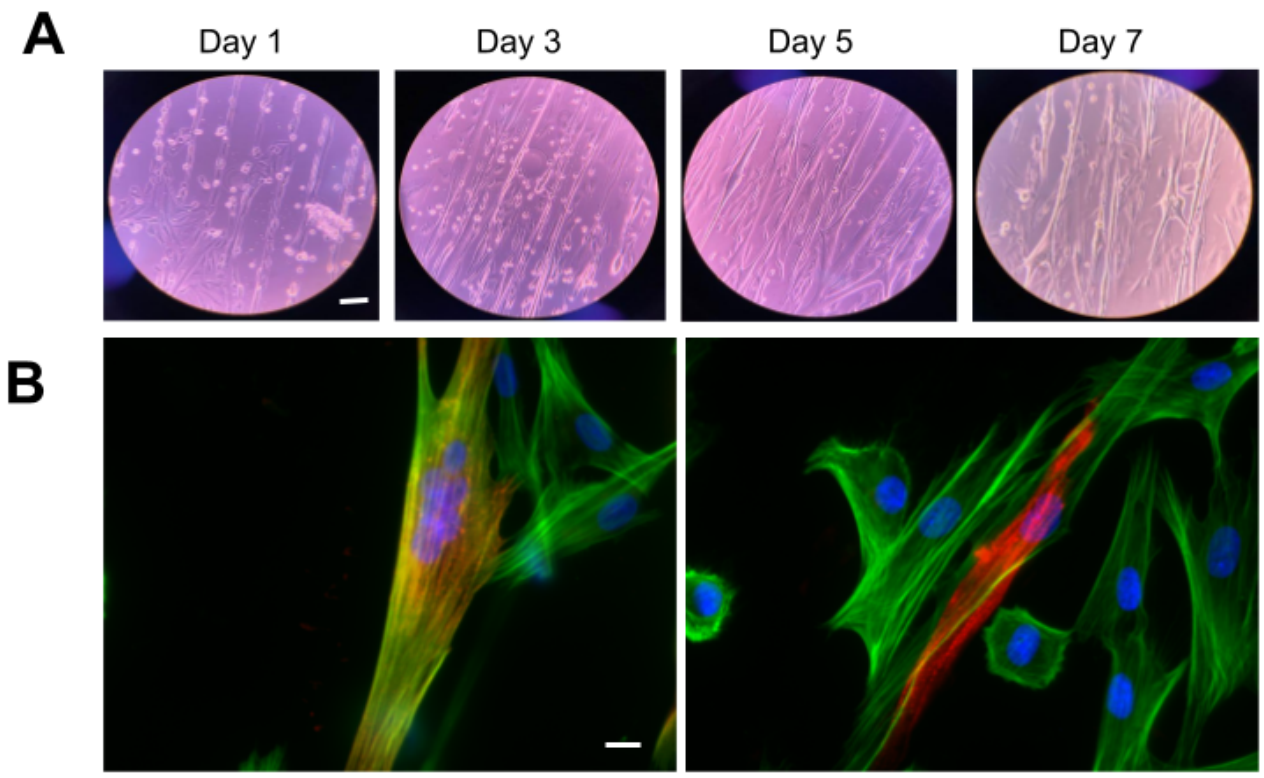

Figure 4: **Differentiated skeletal myotubes seeded on coverslips (A)** Myotubes seeded on genipin coated coverslips for 7 days. Scale Bar = 100μm **(B)** Immunostained myotubes seeded on genipin coated coverslips. Stained for alpha-actinin (red), actin (green), and nuclei (blue). Scale Bar = 10 μm

Modifying PDMS substrates with genipin alone proved to have a limited effect on extending culture time of human skeletal muscle. Thus, an additional silanization step was performed to keep cells adhered to coverslips (Figure 5A). Coverslips stamped with both geltrex and fibronectin were successfully kept in culture for 7 days without experiencing significant levels of cell detachment (Figure 5). However, no twitching was observed.

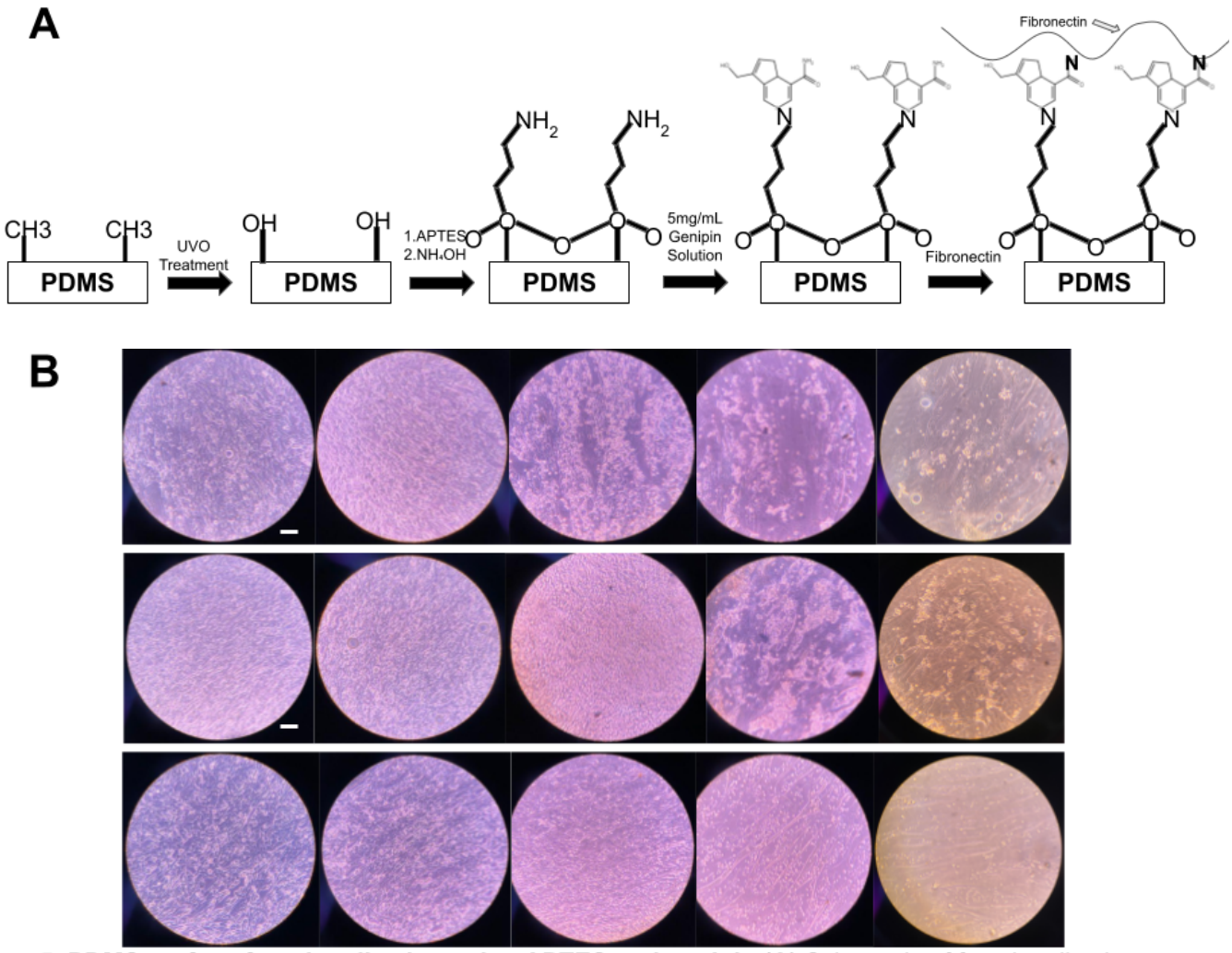

Figure 5: **PDMS surface functionalization using APTES and genipin (A)** Schematic of functionalization procedure **(B)** Cells seeded on functionalized coverslips and Control [No functionalization]: (i) APTES functionalized coverslips with stamped Fibronectin, (ii) APTES functionalized coverslips with stamped Geltrex, (iii) Stamped geltrex [No functionalization]

Section III: 2D Discussion And Future Work

2D models of human skeletal muscle have the potential to serve as physiological relevant and cost-effective alternatives to animal models and human trials. However, cell delamination has proven to be a major obstacle in developing mature in vitro muscle tissue. In this work we observed significant improvements in early myotube formation when coverslips were treated with a 5mg/mL solution of genipin, although cell delamination was still observed at 5-6 days

after seeding. Genipin is meant to serve as an intermediary between the hydrophobic surface of PDMS and the hydrophilic surface of the cells, therefore preventing cell detachment. However, cell delamination after initial myotube formation may indicate that micro contractions occurring in developed tissue leads to detachment. Thus, the PDMS surface functionalization protocol was modified to include a silanization step that would immobilize the substrate to prevent cell delamination. It was observed that silinating the coverslip surface did in fact prolong cell culture time when stamping with either geltrex or fibronectin. However, no spontaneous contractions were observed in either case.

Now that cell delamination no longer impedes tissue formation, it is necessary to gain insight into the level of maturation of the tissue formed. This can be done by characterizing the cell layer via immunocytochemistry to reveal if well defined sarcomeric development is present, as indicated by striations in the tissue. Furthermore, preliminary stimulation studies may reveal the level of functional maturation present in the developed tissue by determining if cells contract synchronously with stimulation pulses or not. Tissue that is capable of contracting in response to stimulation is evidence of a high level of maturation and functional capacity [22].

Chapter 3: 3D Model

Section I: 3D Model Methods

Cell Culture:
Skeletal myocytes

Healthy human primary myoblasts were immortalized by transduction of hTERT onto p16 resistant R24C mutant CDK4 and cyclin D1 as described previously [17]. Myoblasts were proliferated in growth media containing Dulbecco's Modified Eagle Media (DMEM) with 20% Fetal bovine serum (FBS), 1% Pen-Strep, and 2% Ultroser G. Differentiation was induced using media that contained high glucose DMEM supplemented with 2% FBS and Insulin Transferrin Selenium (ITS).

Hydrogel Generation:
Myoblasts are counted and adjusted to reach cell density of 1.1 million cells/72.8 µL of media for each bundle. A 50mg/mL aprotinin solution is prepared by dissolving 2mg of aprotinin in 32µL of water. Fibrinogen (Sigma Aldrich) is removed from the -20°C freezer and brought to the culture hood to thaw. For a set of 4 bundles, 291.2 µL of cell solution and 104 µL of Fibrinogen are added into an Eppendorf tube. Ice is added to Ziplock bags to create "ice packs" and placed into an empty beaker. Matrigel is removed from the -20°C freezer and moved into the culture hood. Fibrinogen and cell media are pipetted into the matrigel eppendorf tube and kept on ice. 8µL of aprotinin solution is added. 126.8µL of hydrogel solution is aliquoted into eppendorf tubes for each bundle.

Molds were prepared by dissolving agarose in PBS and microwaving for 45-60s. 1.6mL of the warmed agarose was placed into a sterile 24 well plate followed by adding the teflon spacer. Well-plate with spacer was left alone for 10 minutes to allow agarose to set. The spacer was then carefully removed. 5.2 µL of thrombin was added to each eppendorf tube and briefly mixed. Then 130 µL of the hydrogel mixture was pipetted into the molds for each bundle. Posts are

carefully inserted into the molds and placed in a 37°C incubator. After 2 hours, 300 µL of growth media was added to each well before being placed back for another 2 hours. After incubation, posts were carefully removed and placed in a new 24 well plate with 2mL of growth and 2µL of aprotinin solution for each bundle.

Myoblast embedded hydrogels were cultured in growth media for 3 days.On day 4 media was switched to differentiation media until day 14 with media changes every other day. 20µL of 1% ITS supplement and 50 ug/mL solution of Aprotinin was added to differentiation media at each media change.

Electrically Stimulating Hydrogel Bundles:

Following 2 weeks of culture, bundles are moved to an empty 12-well plate. 2mL of tyrode solution is then added to each well. The well plate is then transferred to the stereoscope for recording. Myopacer (IonOptix) leads are connected to an electrode and carefully held in the tyrode solution without disturbing the hydrogel bundles. Settings are adjusted to deliver 10-40v at a frequency of 1hz.

Cross-Sectioning

Bundles were transferred to cryostat in a styrofoam container filled with ice. Cryostat was set up with the following settings: Temperature: -18 to -22°C, Large Thickness: 20µm, Fine Thickness 7µm. A layer of OCT was placed on a chuck and sample was set on top. The chuck and sample were left in the cryostat to freeze. A blade was then inserted into the blade mount. Once frozen, the chuck with the sample was placed on the chuck clamp. The chuck angle was adjusted and secured tight. The stage distance was adjusted to get it close to the sample. Using the large thickness setting, the wheel was turned until the sample was reached. Once the sample is visible in cut sections, the thickness setting was changed to fine (7µm). The wheel was rotated

and the sample was slid towards the stage with a bush as it was sliced. A microscope slide was dropped on a sectioned sample to mount.

Fixing and Immunostaining:
Bundles were fixed using either methanol or 4% PFA fixation with 0.005% Triton-X100. In either case, microscope slides were washed in PBS to remove OCT and then dried. Using a hydrophobic pen, a circular border was made around each sample. Next, a 200 µL drop of fixation solution was placed on each sample for 10 minutes. Microscope slides were then washed by submerging in PBS for 5 minutes.

Samples were stained with and without the use of a blocking agent (BSA). Samples that used BSA had a 200 µL drop deposited on top and left to incubate for 30 minutes. This was then followed by submerging in PBS for 5 minutes. Samples that did not use BSA omitted the BSA deposition and incubation step.

Next, both BSA and non-BSA samples were dried with a kim wipe. Primary antibody solution was prepared (DAPI, Phalloidin, Mouse monoclonal anti-alpha actinin). 200 µL of primary antibody solution was placed on samples and incubated for 1 hour. Samples were then submerged in PBS for 5 min and dried with a kim wipe. Secondary antibody solution was prepared (Goat anti-mouse igG). 200 µL of secondary antibody solution was placed on samples and incubated for 1 hour. Samples were then submerged in PBS for 5 min and dried with a kim wipe.

Section II: 3D Results

Bundles created using previous methods [19] have resulted in rapid hydrogel degradation that prevented culture past 1 week. To overcome this limitation, a 50 mg/mL solution of aprotinin was added to both the hydrogel solution and the media to facilitate prolonged maintenance of myocyte embedded hydrogels. Initially, bundles were seeded with a density of 800,000 cells and underwent a 2 week differentiation protocol (Figure 5A). Beginning on day 10, localized

spontaneous contractions were observed, however these contractions were not enough to create deflections in the posts. This may have been due to an insufficient number of cells present throughout the hydrogel.

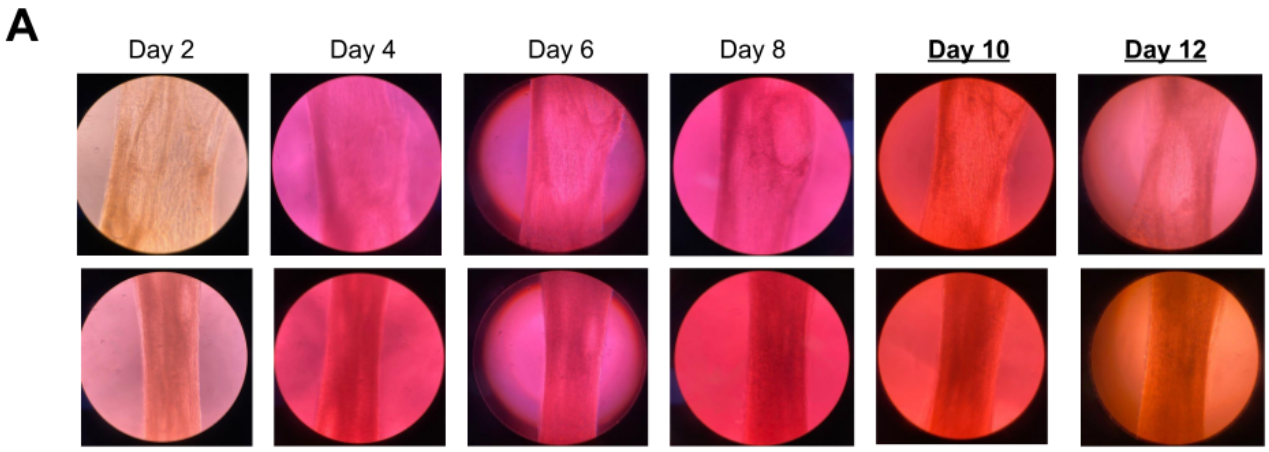

Figure 5: **Hydrogel Bundles seeded with 800k cells (A)** Bundles displayed spontaneous contractions starting on day 10. Hydrogel degradation was inhibited with the addition of 2uL of aprotinin solution to cell media

To determine optimal seeding density, hydrogel bundles were formed using 1.1 million and 1.6 million cells. On days 8-10, bundles seeded with 1.1 million cells showed strong spontaneous contractions that caused deflections in the posts. On days 10-14 only localized contractions could be observed. Bundles with an initial seeding density of 1.6 million cells did not display contractions strong enough to move the posts. On days 8-14 only localized contractions were observed.

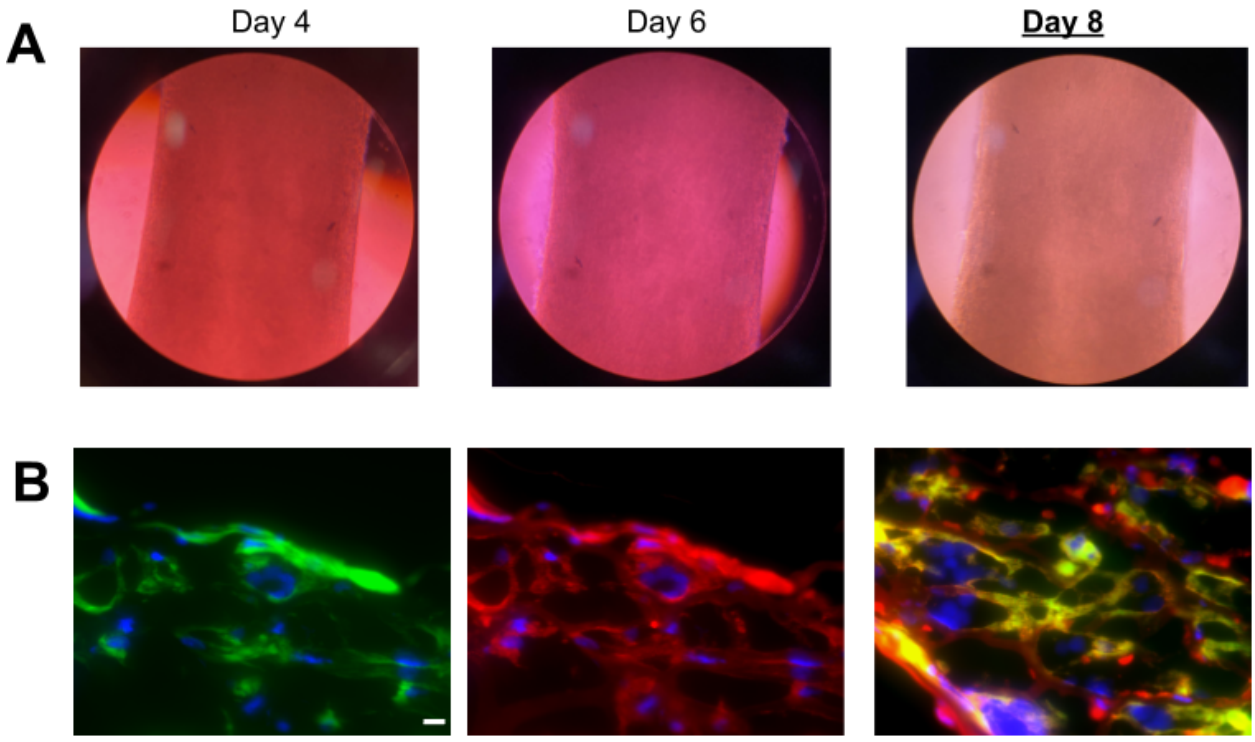

Figure 6: **Hydrogel Bundles seeded with 1.1 x 10^6 cells (A)** Bundles displayed spontaneous contractions starting on day 8. Post deformations were observed from day 8 to day 10. **(B)** Cross-sectioned hydrogel bundles. Stained for alpha-actinin (red), actin (green), and nuclei (blue). Scale Bar: 10μm

Following 14 days of culture the bundles were electrically stimulated with 15V at a frequency of 1hz. Movies of electrically stimulated bundles were analyzed to measure the distance that the posts moved during contraction. Forces generated by the myobundles were calculated using the equation $F = 3\pi E a^4 \delta / 4L^3$ [20], where the elastic modulus, length, area and deflection of the posts are denoted by E, L, a, and δ, respectively. Results indicated that bundles seeded with 1.1 million cells produced maximum contractile forces ranging from 65μN to 85μN (Figure 7C).

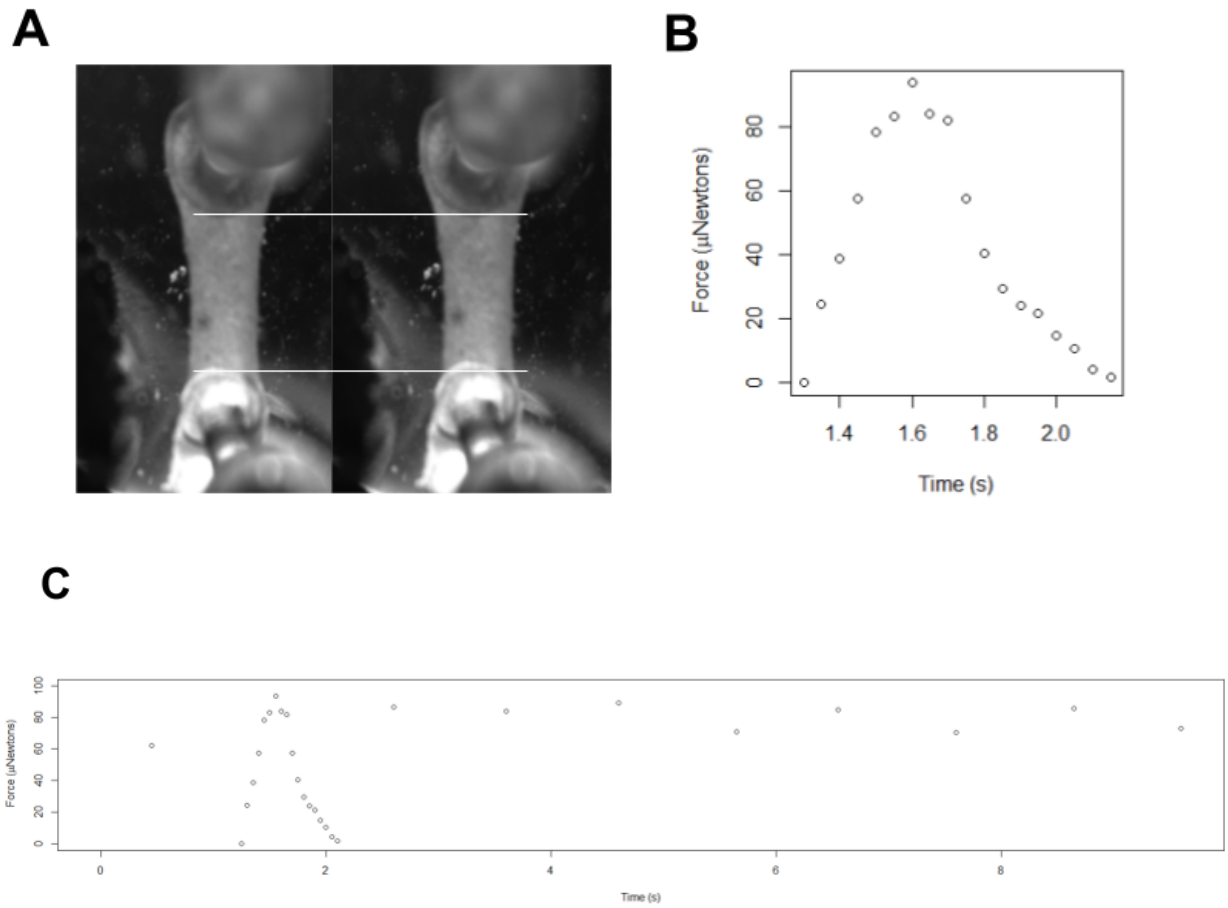

Figure 7: **Electrically Stimulated Hydrogel Bundles (A)** Bundles were stimulated with 15V at a frequency of 1Hz. **(B)** Force profile of contraction during electrical stimulation. **(C)** Peak forces plotted for 10 seconds of stimulation

Section III: 3D DISCUSSION AND FUTURE WORK

The development of a 3D model of skeletal muscle would allow for the creation of a microenvironment that mimics the conditions of native skeletal muscle tissue and facilitates the study of cellular architecture and function [12]. In this work we have successfully produced myocyte embedded hydrogel bundles that provide the ability to quantify force production of generated tissue. Using this 3D model, force measurements of both spontaneous and electrically stimulated contractions can be recorded.

Currently, hydrogel bundles are electrically stimulated following differentiation and subsequently fixed and stained for analysis. However, previous studies have demonstrated that intermittent stimulation during differentiation promotes myogenic activity, specifically through increased expression of MyoD and MyoG [21]. Furthermore, electrical stimulation of in vitro skeletal muscle has been found to promote cellular organization and growth Thus, it may be beneficial to incorporate some form of stimulation during culture to produce tissue that more closely resembles those found in vivo.

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Appendix I: 2D Protocol

Stamp Preparation

1. Pour 65g of Sylgard 184 elastomer base and 6.5 grams curing agent into cup
2. Mix and degas PDMS in Thinky Mixer
3. Pour PDMS on silicon wafer with 100x50 pattern
4. Place in vacuum desiccator until air bubbles are gone
5. Bake in oven for at least 2 hours
6. Cut out of petri dish, cut out each stamps

Coverslip PDMS functionalization

Day 1

1. Prepare a 1:2 ratio of APTES:Ethanol solution (2mL of solution per coverslip)
2. For each coverslip,place 2mL of ammonia solution in a 50mL conical
3. UVO treat coverslips for 8 minutes and transfer to hood
3. Place coverslips in a 12 well plate and submerge in 2mL of APTES:Ethanol solution for 7 minutes
4. Aspirate APTES:Ethanol solution and replace with 2mL of ammonia solution for 3 minutes
5. Aspirate Ammonia solution
6. Place an ethanol wipe at the base of a petri dish and transfer coverslips to petri dish (Coverslips will permanently adhere to the plastic well plate if left overnight and will be unable to be removed. The ethanol wipe provides a surface that guarantees the coverslips can be removed without difficulty)
7. Leave coverslips at ambient temperature overnight

Day 2

1. Weigh out 5mg of genipin in an eppendorf tube and dissolve in 1mL of sterile water
2. Place 200 µL of genipin solution on petri dish and place coverslips PDMS side down onto droplets
3. Incubate overnight at 37°C (Genipin has been characterized to readily bind with amine groups producing a dark blue pigment. Following incubation, genipin solution should look a dark blue color. If not, insure that previous steps were done correctly)

Stamping with Geltrex

1. Remove Geltrex (1:40 ratio, Geltrex:DMEM) aliquots from -20C and transfer to hood
2. Sonicate the stamps for 30 minutes
3. Move stamps to hood and place on petri dish patterned side up
4. Place 250 µL of Geltrex mixture on top of stamp and evenly distribute with pipette tip
5. Incubate stamps at 4°C overnight
6. After incubation remove excess genipin solution from coverslips with nitrogen gun
7. Remove excess Geltrex from stamps and dry with nitrogen gun
8. Place stamps, feature side down, on coverslips
9. Remove stamps and transfer coverslips to a 12 well plate

10. Submerge coverslips in 2mL of PBS
11. Wrap well plate in parafilm and store at 4°C

Stamping with Fibronectin

1. Remove fibronectin (1:20 ratio, Fibronectin:Water) from 4°C and transfer to hood
2. Sonicate the stamps for 30 minutes
3. Move stamps to hood and place on petri dish patterned side up
4. Place 250 µL of Fibronectin mixture on top of stamp and evenly distribute with pipette tip
5. Incubate stamps at room temperature for 1 hour
6. After incubation remove excess genipin solution from coverslips with nitrogen gun
7. Remove excess Fibronectin from stamps and dry with nitrogen gun
8. Place stamps, feature side down, on coverslips
9. Remove stamps and transfer coverslips to a 12 well plate
10. Submerge coverslips in 2mL of PBS
11. Wrap well plate in parafilm and store at 4°C

Seeding Coverslips

1. Rinse wells (with no coverslips) with 2 mL of pluronics
2. Let pluronics sit in well for 5 min
3. Aspirate and wash with PBS 3 times
4. Transfer coverslips to washed wells
5. Count cells and seed each coverslip with ~60k myocytes

Appendix II: 3D Protocol

Generating Hydrogel Bundles

Before Experiment

1. Wash and autoclave Racks and Spacers

Day of experiment

Set up and hydrogel mixture preparation

1. Set up hood and bring materials (e.g. eppendorf tubes, racks and spacers, conicals, 24 well plate) and non gelling reagents (e.g. fibrinogen, growth media, cells)
2. Count cells and prepare cell solution with $1.1x10^6$ cells/72.8µL for each bundle (One set of 4 bundles should have a total of $4.4x10^6$ cells)
3. Weigh out 2mg of aprotinin and dissolve in 32µL of sterile water
3.Grab Ziplock bags and fill with ice. Place in 150mL beaker
4. Transfer 104 µL aliquots of matrigel from -20°C freezer to hood and place on ice
5. Pipette 104µL of fibrinogen into matrigel tubes
6. Transfer 52µL of matrigel:fibrinogen solution into eppendorf tubes
7. Add 72.8µL of cell solution to eppendorf tubes
8. Add 2µL of aprotinin solution to eppendorf tubes

Agarose mold casting

1. Weigh out 400 mg of agarose in a small beaker and add 20 mL PBS. Microwave until bubbling. Stop every 10 seconds to swirl.
2. Move to hood and place 2mL in each well
3. Place spacer into agarose and allow it to gel for 10 minutes.
4. Remove spacers.

Hydrogel generation

1. Transfer thrombin from -20°C freezer to hood and place on ice
2. Add thrombin to one eppendorf tube containing hydrogel mixture and carefully pipette into agarose mold. **Avoid air bubbles**. (Tip: Setting the pipette to a larger volume than the hydrogel mixture and stopping short of any air bubbles will make the process easier.)
3. Repeat step 2 for each bundle
4. Once a row of hydrogel solution has been added to molds, carefully place the posts into the slot.
5. Place 24 well in incubator for 2 hours
6. Remove bundles after 2 hour incubation and add 300 uL of growth media to each well
7. Place back into incubator for 2 hours
8. After incubation add 2mL of growth media and 2µL of aprotinin solution to new 24 well plate
9. Remove bundles from molds and place in new 24 well plate
10. Move well plate with bundles to incubator

11. Feed with media every other day. First 3 days use growth media. On day 4 media is switched to differentiation media and supplemented with 20µL of ITS until end of experiment.

Bundles Reagent Volumes (per bundle):

Fibrinogen	26 µl
Matrigel	26 µl
Cells + Growth Media	72.8 µl
Thrombin	5.2 µl
Total	130 µl

Electrically Stimulating Bundles

1. After 14 days in culture,aspirate media and move bundles to a new well plate with 2mL of Tyrode solution
2. Move well plate with bundles to stereoscope for viewing
3. Connect electrode to myopacer
4. Carefully place electrode in Tyrode solution with bundles
5. Set myopacer to 15-40V at a frequency of 1 Hz.
6. Set up recording software and run myopacer for 10 seconds

Freezing and Sectioning

Freezing

1. Cut flaps on two sides of freezing molds for easier handling
2. Place OCT on freezing mold; use enough to cover the base
3. Remove bundles from rack and place on the OCT covered mold
4. Add enough OCT to cover bundle and fill the mold
5. Fill a dewar with liquid nitrogen
6. Chill 2-methylbutane by pouring into a metal container and dropping into liquid nitrogen filled dewar. This will take 3-5 minutes
7. Remove metal container from dewar.
8. Dip molds in 2-methylbutane until frozen. Handle using tweezers. OCT will turn white when frozen
9. Wrap molds in aluminum foil and store at -80°C until ready to section

Sectioning

1. For transfer, fill a styrofoam container with ice and place bundles inside
2. Set up cryostat with following settings:
    A. Temp: -18 to -22 °C (Adjust during sectioning to maintain optimal temperature)
    B. Large Thickness: 20µm
    C. Fine Thickness: 7µm
3. Place a layer of OCT on chuck and place sample on top. Wait for OCT to freeze
4. Insert blade into blade mount
5. Place the chuck with sample on the chuck clamp. Adjust chuck angle and secure tight
6. Adjust stage distance so that it gets as close as possible to the sample
7. Using the large thickness setting, turn the wheel until sample is reached.
8. Switch to fine thickness
9. Rotate wheel and slide sample towards the stage with a brush as it is cut.
10. Drop a microscope slide on sample to mount
11. Repeat steps 9 and 10 until enough sections have been collected

Fixing and Immunostaining

Methanol Fixation

1. Wash microscope slides containing mounted samples with PBS for 5 min to remove OCT
2. Dry slides with kim wipes
3. Using a hydrophobic pen, make a circular border around sample
4. For each sample prepare 200 µL of methanol with 1 µL of Triton-X100
5. Place a 200 µL drop of methanol solution on each sample for 10 minutes
6. Wash microscope slides by submerging in PBS for 5 min
7. Dry slides with kim wipes

4% PFA Fixation

1. Wash microscope slides containing mounted samples with PBS for 5 min to remove OCT
2. Dry slides with kim wipes
3. Using a hydrophobic pen, make a circular border around sample
4. For each sample prepare 200 µL of 4% PFA with 1 µL of Triton-X100
5. Place a 200 µL drop of PFA solution on each sample for 10 minutes
6. Wash microscope slides by submerging in PBS for 5 min
7. Dry slides with kim wipes

Immunostaining with Blocking agent

1. Place 200 µL of BSA for each sample in a conical
2. Pipette 200 µL drops of BSA onto samples
3. Incubate for 30 minutes
4. Wash microscope slides by submerging in PBS for 5 min
5. Dry slides with kim wipes
5. Prepare primary antibody solution (DAPI, Phalloidin, Mouse monoclonal anti-alpha actinin)

6. Pipette 200 µL drops of primary antibody solution onto samples
7. Incubate for 1 hour
8. Wash microscope slides by submerging in PBS for 5 min
9. Dry slides with kim wipes
10. Prepare secondary antibody solution (Goat anti-mouse igG)
11. Pipette 200 µL drops of secondary antibody solution onto samples
12. Incubate for 1 hour
13. Wash microscope slides by submerging in PBS for 5 min
14. Dry slides with kim wipes

Immunostaining without Blocking agent

1. Prepare primary antibody solution (DAPI, Phalloidin, Mouse monoclonal anti-alpha actinin)
2. Pipette 200 µL drops of primary antibody solution onto samples
3. Incubate for 1 hour
4. Wash microscope slides by submerging in PBS for 5 min
5. Dry slides with kim wipes
6. Prepare secondary antibody solution (Goat anti-mouse igG)
7. Pipette 200 µL drops of secondary antibody solution onto samples
8. Incubate for 1 hour
9. Wash microscope slides by submerging in PBS for 5 min
10. Dry slides with kim wipes

Mounting

1. Cut large glass coverslips to cover samples
2. Place a drop of Fluoromount G or Prolong Gold on sample
3. Place glass coverslip on mounting media
4. Incubate overnight
5. Seal with nail polish
6. Store in -20°C freezer