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Designing 2D and 3D models to investigate function and structural properties of human skeletal muscle

THESIS

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by

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ABSTRACT OF THE THESIS

Designing 2D and 3D Models to investigate function
and structural properties of Human Skeletal Muscle

By

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Master of Science in Biomedical Engineering

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Professor Anna Grosberg, Chair

A consistently reproducible 2D and 3D model of human skeletal muscle would provide the necessary tools to investigate the relevant structural and functional properties observed in muscle tissue. For dystrophies, including Fascioscapulohumeral muscular dystrophy (FSHD), a model would act as a tool to investigate the symptoms of such an affliction. The importance arises in investigating the mechanisms involved with muscle degradation and reduction in motility. Using microcontact printing to pattern myocytes allows us to investigate the possibility of adapting a muscular thin film protocol to better understand the observable mechanisms in 2D. Alternatively, adapting a more biomimetic 3D model through a hydrogel-produced myobundle would allow for a comprehensive investigation of the structure at various layers, as well as contractility studies with stronger twitches than that of a 2D model. A platform to study human skeletal muscles *in vitro* has been optimized to improve reproducibility and consistency.

INTRODUCTION

Advancements in genetics and mechanobiology have allowed for various breakthroughs in understanding dystrophic muscular disorders. Dystrophies often lead to altered structures and mechanisms within the muscle; consequently, these alterations would cause hindered function and development. More particularly, Facioscapulohumeral dystrophy (FSHD) has led to symptoms, including muscle degradation and decreasing motility [1]. Recent research has discovered an association between FSHD and the DUX4 gene being actively transcribed in skeletal muscle [1]. DUX4 would normally remain repressed in the D4Z4 repeats; however, an overexpression has led to neighboring genetic regions to be more susceptible to gene expression [1]. With the pathophysiology of FSHD in mind, research has generally shifted towards developing experimental models to better explore genetics, as well as, drug delivery and therapies [1]. Current therapies have been unable to halt the degradation of muscles, and are only able to slow down an inevitable loss of functional motility among those who are afflicted [1,2,3]. A reproducible and closely biomimetic model is required to improve the understanding and opportunity of therapies for FSHD.

To pursue observing functional properties of muscle cells undergoing dystrophy, a 3D experimental protocol may be designed in conjunction with a 2D model for comparison. 2D and 3D cellular models *in vitro* have both been beneficial to muscle research, and taking such an approach with FSHD may lead to a consistent and readily reproducible model; however, there are challenges in creating a reproducible model.

Primary cells from patients often vary in quality and lose their proliferation and differentiation capabilities over time as they go into senescence. An effective model would need

to be capable of producing a timeline of the condition. An immortalized cell line may prove to alleviate this issue, as they are capable of longer lifetimes, and indefinite growth under the right circumstances [4]. The Yokomori lab has developed such cell lines along with a differentiation protocol, which will allow for consistent cells, preliminary expectations, and an investigation of the mechanisms involved with FSHD [5,6,7]. The cell line that is afflicted with FSHD would allow a comparable control between the cell line that is unafflicted. Creating a potential timeline throughout the emergence of FSHD, by following its development compared to a control, becomes more viable. The integration of these cell lines into a model would allow for a consistently reproducible test for new treatments, with capabilities of evaluating physical properties *in vitro*.

A foundation for our 2D model can be established through the "heart on a chip", which utilized microcontact printing in conjunction with muscular thin films to examine cellular structure, alignment, and functionality [8,9]. Microcontact printing would allow for patterning cells in a manner more representative of real biology when compared to unpatterned; however, a pattern would need to be adapted for skeletal muscle cells. Research from the Fienberg group have provided parameters for a compatible pattern, as well as guidelines for ECM that are beneficial to such skeletal muscle cells [10]. To expand the possibilities for a model, the adoption of a 3D model may prove to be well suited for observing FSHD. A myobundle model, as introduced by the Brusac group, may fulfill this direction by providing a framework for a 3D model design *in vitro*, capable of measuring contractile forces [11].

This thesis will share the progress made in adapting a pattern to better support skeletal muscle cells for the microcontact printing protocol, as well as progress made in optimizing a myobudle protocol for the cell line.

METHODS

Coverslip preparation[12]

Glass coverslips (Fisher Scientific Company) underwent sonication in a 95% ethanol bath for 30 minutes before being placed in a 65^{o}C oven to evaporate excess ethanol. They were then coated with Polydimethylsiloxane (PDMS (Ellsworth Adhesives)) that was prepared in a 10:1 ratio between the base and crosslinker. They were coated by spin coating under a designated program. With their coating applied, they were left to cure overnight in the 65^{o}C oven. Diamond scribes (VWR) were then used to cut the coverslips into smaller squares using a transparent grid to measure individual squares.

Silicon Wafer Preparation[12]

100x20 line designs for stamps were created on Adobe Illustrator (Adobe Systems Inc). The designs were etched into a mask by an outside business (FrontRange Photo Mask Company). With the glass masks, silicon wafers were made through SU-8 deposition at the Bio-Organic Nanofabrication Facility found at UCI. The silicon wafer was then salinized to prevent any PDMS adhesion. The silicon wafer was now used to create PDMS stamps by pouring over a particular quantity of PDMS over the wafer in a petri dish. Amount of PDMS depends on the desired thickness of the stamps. The petri dish was placed in a vacuum desiccator

to remove air bubbles from the PDMS and ensure impressions were full and complete. With no more air bubbles, the petri dish was placed in a 65°C oven overnight to cure the PDMS. The PDMS layer was carefully cut and removed from the wafer, and then cut into the individual stamps for the various patterns.

Cell Culture

Cells used for these experiments were procured and cultured by the Yokomori group [13]. The immortalized cell line was created through the use of hTERT with $p16^{INK4a}$-resistant R24C mutant CDK4 and cyclin D1 on human primary myoblasts that were cultured as an unafflicted control. Cells were sorted utilizing magnetic-activated cell sorting (MACS) by targeting CD56-positive cells with anti-CD56 antibody (Miltenyi Biotec). After screening 70 single cell-clones, 30 had expressed the typical doubling time expected from primary cells - around 28 hours.They were also confirmed to have an efficient differentiation into myotubes, with 2 clones going as far as showing spontaneous twitching after 7-14 days.

KYD0-11T cells underwent differentiation as described in a previous protocol [5]. The cells were seeded onto coverslips that were either patterned or unpatterned at a seeding density of around around ~250,000 cells/ml, with 2ml of growth media (Dulbecco's Modified Eagle Media (DMEM (Gibco)) with 20% Fetal bovine Serum supplement (FBS (Omega, Scientific, Inc.)), 1% Pen-Strep (Gibco) and 2% Ultrasor G (Crescent Chemical Co.)) going into each well in a 12-well dish. After ~16 hours, differentiation was then induced through the use of DMEM media with 2% FBS and Insulin-Transferrin-Selenium (ITS - insulin 0.1%, 0.000067% sodium selenite, 0.55% transferrin, Invitrogen).

2D Model Methods

Microcontact Printing

Geltrex (ThermoFisher) was placed in a beaker of ice for at least one hour. PDMS stamps, described previously in our methods, were sonicated in 95% ethanol for at least 30 minutes. Geltrex was then mixed with DMEM to a selected ratio by gently pipetting the geltrex into a DMEM aliquot, the mixture was left into an ice bath until use. PDMS stamps that have been sonicated were then dried by using compressed nitrogen. 250ul of the Geltrex mixture was pipetted onto the patterned surface of the PDMS stamps. When unpatterned coverslips were desired, ultraviolet-ozone (UVO) treated PDMS coated coverslips were placed over 250ul droplets of geltrex mixture, so their PDMS surface was making contact. Either the stamps or coverslips in contact with geltrex were then wrapped in a petri dish with parafilm to be transported to a 4ºC fridge to incubate. The coverslips were incubated for 30 minutes, while the stamps were incubated for 90 minutes. After incubation, PDMS stamps were transported back to be placed over new UVO treated PDMS coated coverslips. Geltrex mixture was removed from the stamp surface and briefly blasted by compressed nitrogen to remove excessive geltrex mixture, and was then placed patterned-side down onto the PDMS surface of the coverslip. The stamps were carefully and delicately removed to not hinder the pattern left on the coverslips. Unpatterned and patterned coverslips share the next steps of the procedure, where both coverslips were then placed into various wells in a well-plate, treated side up, so that they can be washed by 1% pluronic solution (5g Pluronic F-127 (Sigma Aldrich Inc.) dissolved in 500mL sterile water for 5 minutes). This blocked regions on the coverslip that were untreated, including the well itself from allowing cells to adhere to those regions. After 5 minutes, the coverslips then

went through three washes of Phosphate Buffered Saline (PBS (ThermoFisher)) to remove excess pluronic solution as it would degrade the geltrex layer. After the third wash, coverslips were left submerged in PBS and incubated for 30 minutes at 37ºC to solidify Geltrex pattern. With incubation completed, the coverslips could immediately be seeded with cells or stored in a 4ºC refrigerator for up to 2 days.

Fixing and Immunostaining

Seeded coverslips that have been cultured for a desired amount of days have their media aspirated from the wells, and were then washed three times with warm PBS. A solution of 4% paraformaldehyde ((PFA)VWR) was mixed with 0.0005% Triton X-100 (Sigma-Aldrich) and left to warm until the washes were completed. The coverslips were then submerged in the warmed fixing solution and allowed to incubate for 10 minutes at room temperature. The coverslips will now have the permeability to undergo immunostaining. 4,6-Diamindino-2-Phenylindole Dihydrochloride (DAPI (ThermoFisher)), AlexaFluor 488 Phalloidin (ThermoFisher), and Mouse monoclonal anti-alpha-actinin (Sigma Aldrich Inc.) were used as primary antibodies for nuclei, actin, and sarcomeric alpha-actinin, respectively. Coverslips were placed over droplets of the primary antibody solutions, mixed with PBS, in contact with the fixed cell surface, and left to incubate for 1 hour at room temperature. The coverslips were then washed by PBS three times to reduce any background staining before being stained with secondary antibodies. Goat anti-mouse IgG secondary antibodies (Alexa Fluor 633 (ThermoFisher)) were used as the secondary for the alpha-actinin primary antibody. The coverslips were placed over droplets of the secondary antibody solution, mixed with PBS, in

contact with the primary stained surface and left to incubate for 1 hour at room temperature. The coverslips were washed by PBS three times, once again to reduce background staining. Coverslips were mounted onto glass microscope slides by placing the coverslips over ProLong Gold Antifade Mountant (ThermoFisher) in contact with the stained surface, and clear nail polish was used to seal the coverslip by going along the edges with a brush. They were left overnight before being stored in a freezer to ensure the nail polish solidifies.

Stamp Pattern Validation

In order to validate our new patterns, we seeded cardiomyocytes, harvested from Neonatal Sprague-Dawley rats (Charles River Laboratories), onto stamped coverslips. Stamps varied from the previously described protocol in our methods, as they were coated in fibronectin (Fisher Scientific), instead of geltrex, so that they would be immunostained for fibronectin. Fixing and immunostaining was carried out as previously described in our methods. However, the primary antibody polyclonal rabbit anti-human fibronectin (Sigma-Aldrich) was used to stain fibronectin. Goat anti-rabbit IgG secondary antibodies (Alexa Fluor 750 (Thermofisher)) were used for the secondary stain as well.

3D Model Methods

Frame and Channel Assembly

Molds were provided by a previous graduate student, Kirby Fibben. Channel molds were designed on software, and 3D printed out of plusCLEAR resin by a Asiga pico plus27 3D printer

(Asiga). Molds were then washed and sonicated for 30 minutes in 95% ethanol to eliminate any remaining resin. Following a 45 minute post cure under UVO, the molds were salinized to not allow any PDMS adhering to the mold.

PDMS in a 10:1 ratio with base:crosslinker can now be prepared and degassed before being added to the molds, where they were then placed in a vacuum desiccator to degas further until air bubbles were not visible. They were then placed in a 65^{o}C oven for at least 24 hours before getting pried out of their frames. The frames were made by pouring PDMS at the same 10:1 ratio into a large petri dish, where they were then also degassed in a vacuum desiccator. They were left in a 65^{o}C oven to cure overnight. They can then be removed from the petri dish so that frames can be punched out with a large circle punch, for the outer frame, and a smaller 11m x 11m square punch, for the inner frame. At this point, what type of velcro or adhering material may be varied for the inner frame. When using a more traditional velcro, it was fixed into place with a 2:1 ratio PDMS mixture. When using a fastener with various protruding stands, the stands were shaved off to give space to the channel to be placed later. The shaved regions were then painted over with a 10:1 ratio PDMS mixture to become one complete unit with the frame.

Hydrogel creation and implementation

Preliminary steps to create the hydrogel required a 1mL DMEM (Corning) and 20ug fibrinogen mixture to be made the day before, so that it was stored in a 4^{o}C refrigerator overnight. As well as allowing matrigel and thrombin aliquots to defrost overnight in the same refrigerator. PDMS frames and channels were sonicated in 95% ethanol for 30 minutes to ensure their sterility as well as removal of any debris that cells may adhere to. The channels were placed

in pluronics for 45 minutes to prevent any adhesion, especially from the hydrogel later in the experiment, and then washed in PBS 3 times. The frame and channels were then assembled together. Cells were counted and concentrated by centrifuging the mixture to a pellet, for a calculated media to be added, which obtained the desired quantity of $1.33x10^6$ myoblast for every 34ul of growth media. A master mixture was then prepared by combining per bundle: 20ul Matrigel, 20 ul Fibrinogen mixture, 22ul Growth media, 34 ul cell mixture. This was done with cold pipette tips that were left in a fridge before use. The next step required cold pipette tips that were kept cold by leaving them in a box surrounded by ice, but not in close contact to the tips to maintain sterility. 96ul of a master mixture was mixed with 4ul thrombin one-by-one for each bundle. This was done quickly and carefully as to avoid gelling before seeding into the channel. Once transferred into the channels, the hydrogel was left to incubate at 37^{o}C for 45 minutes to allow gelling to complete before submerging in warmed media. After 4 days of culture, the channels were then removed and the media exchanged to differentiation media. The bundles were left for at least 14 days with daily media changes.

Fixing and Immunostaining

To view preliminary findings, the bundles, while still being adhered to the fasteners, were submerged into a fresh PBS wash 3 times, before being submerged into a 4% PFA and 0.0005% Triton-X100 solution and left overnight in a 4^{o}C refrigerator. The bundles were then placed over droplet solutions of the previously mentioned DAPI, Alexa Fluor 488 Phalloidin, and mouse monoclonal anti-alpha-actinin primary antibodies. They were incubated for 4 hours at room temperature, followed by a PBS wash three times to prevent any background staining. The

bundles were then placed over the previously mentioned Alexa Fluor 633 secondary antibody for 4 hours at room temperature. Followed by another 3 washes in PBS, they were placed over a droplet of prolong gold in an imaging well-plate, and stored in a -20°C freezer before imaging under a confocal microscope.

RESULTS

2D Model Results

Our stamp patterns were validated and compared by stamping three different patterns with fibronectin as the ECM, so they could be immunostained. Images showed expected width increase between line and gap regions, when comparing the 25x15µm, 50x20µm, and 100x20µm lines (Fig 1). Fibronectin lines were also intact throughout the coverslip, validating the integrity of the silicon wafer and the impressions left on the PDMS to create a stamp (Fig 1). The

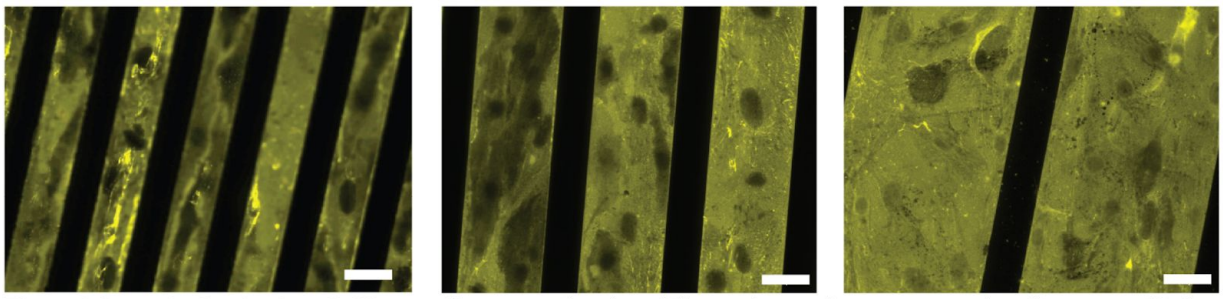

Figure 1. Stamp Authentication. Cy7 Immunofluorescence imaging of fibronectin lines from PDMS stamps. Stamped coverslips were seeded with cardiomyocytes (Scale bar 25µm). From left to right: 25x15 µm, 50x20 um, 100x20 µm lines

microcontact printing protocol was carried out under the previously described protocol to create both patterned and unpatterned coverslips that would be observed under two differing conditions (Fig 2A). First, myocytes were seeded onto both patterned and unpatterned coverslips and underwent differentiation protocol. The second condition was to seed myotubes that were on day

7 of their differentiation protocol, from a 2D culture, onto both types of coverslips. Both conditions were observed for up to 14 days. For the first condition, a fairly confluent culture was

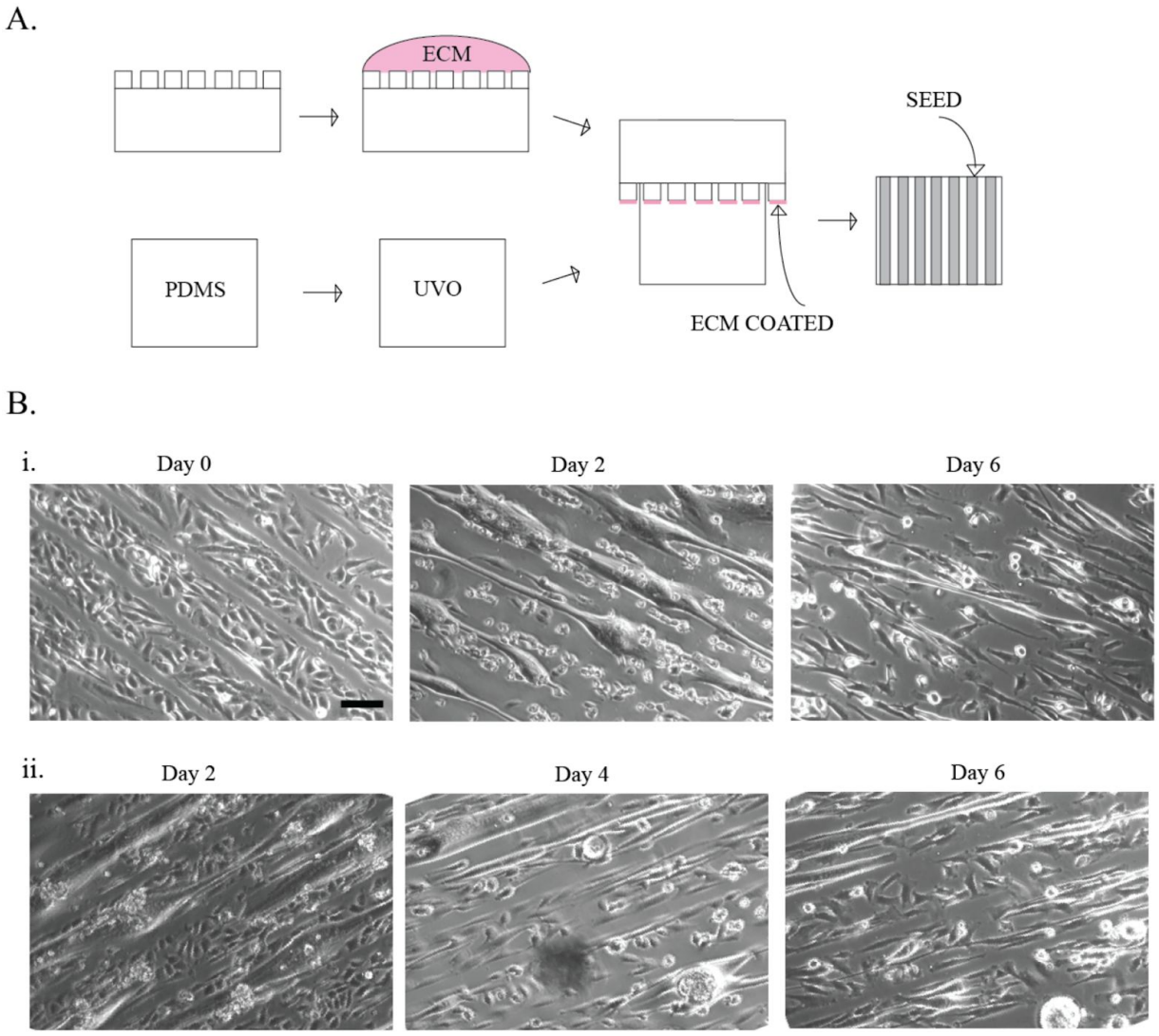

Figure 2. Stamping Design (A) Schematic of microcontact printing protocol, where PDMS coated coverslips are imprinted on by a geltrex coated PDMS stamp, before seeded with cells. (B) Myocytes seeded on Patterned 100x20µm lines (Scale Bar 100µm). (i) Myocytes seeded day 0 that then go through differentiation protocol (ii) Myocytes that have undergone 7 days of differentiation before being seeded.

observed on day 0, followed by a decrease in myocyte population with potential myotube formation on day 2 (Fig.2B i). Looking further at day 6, we find there to be great cell detachment

with a reduced population of potential myotubes, and potentially unhealthy myocytes (Fig.2B i). In the case of the second condition, we observe myotubes starting to detach by day 2, with potential myocyte proliferating without differentiating, dominating the space (Fig.2B ii). By day 4, the myotube population has greatly diminished with some myocytes still potentially differentiating; however, by day 6, we see the continuous detachment of any potential new myotubes (Fig.2B ii.) Beyond day 6 both conditions showed no improvement and would only continue with detachment, some samples were taken from day 8 to be immunostained.

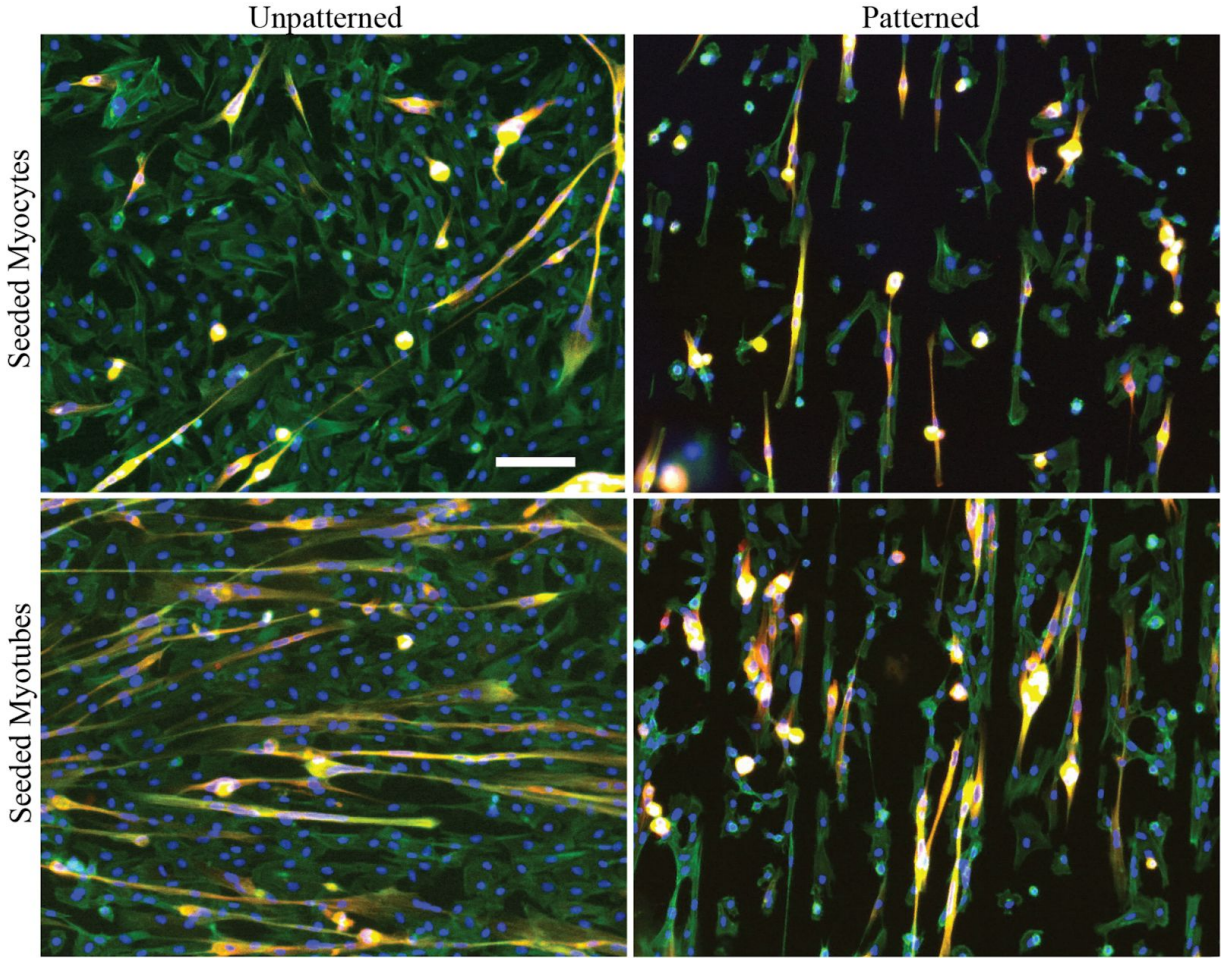

Figure 3. Immunostained myocyte/myotube seeded coverslips (Scale Bar 100μm). Stained for alph-actinin (red), actin (green), nuclei (blue). Top row: Day 8 of myocytes undergone differentiation protocol. Bottom row: Day 8 of myotubes that were seeded after 7 days of differentiation, continued protocol.

Coverslips that were patterned and unpatterned, from both conditions previously described, were fixed and immunostained by day 8 for alpha-actinin (red), actin (green), and nuclei (blue). Those that were first seeded as myocytes showed few potential myotubes in their population on both patterned and unpatterned cover slips; however, the patterned coverslips showed excessive overall detachment (Fig 3). The second condition, where myotubes were seeded first, was found to display more potential myotubes when observed on both patterned and unpatterned coverslips, although there is still a great amount of detachment observed on the patterned coverslip (Fig 3). Myotubes that were seeded onto the unpatterned coverslips also showed some alignment, where potential myotubes were parallel to one another. This alignment adds to the identification of elongated cells being myotubes [14].

3D model results

Myobundle experiments were carried out with various materials sampled to find what would be easily fitted to our frames, as well as what would best allow myobundles to adhere to. A velcro-like fastener was settled on, where the stand-offs were shaved off to allow the channel to fit into the frame (Fig 3A). Hydrogel was seeded into the channel and by day 4 the channel was removed, leaving the myobundle adhered to both sides of the frame (Fig 3A ii). Experiments were carried out with both a regular velcro and the described fastener, both proving to successfully hold the myobundle from both ends (Fig 3A iii). Upon closer investigation under a microscope, a differing behavior in binding to the materials may be observed, where we see finer individual connections with the individual hooks found on the typical velcro, and more concentrated individual connections with the stand-offs on the fasteners. Observing these

myotubes under a differentiation protocol for as long as 14 days had never shown any observable spontaneous contractions; however, they did remain adhered to frames.

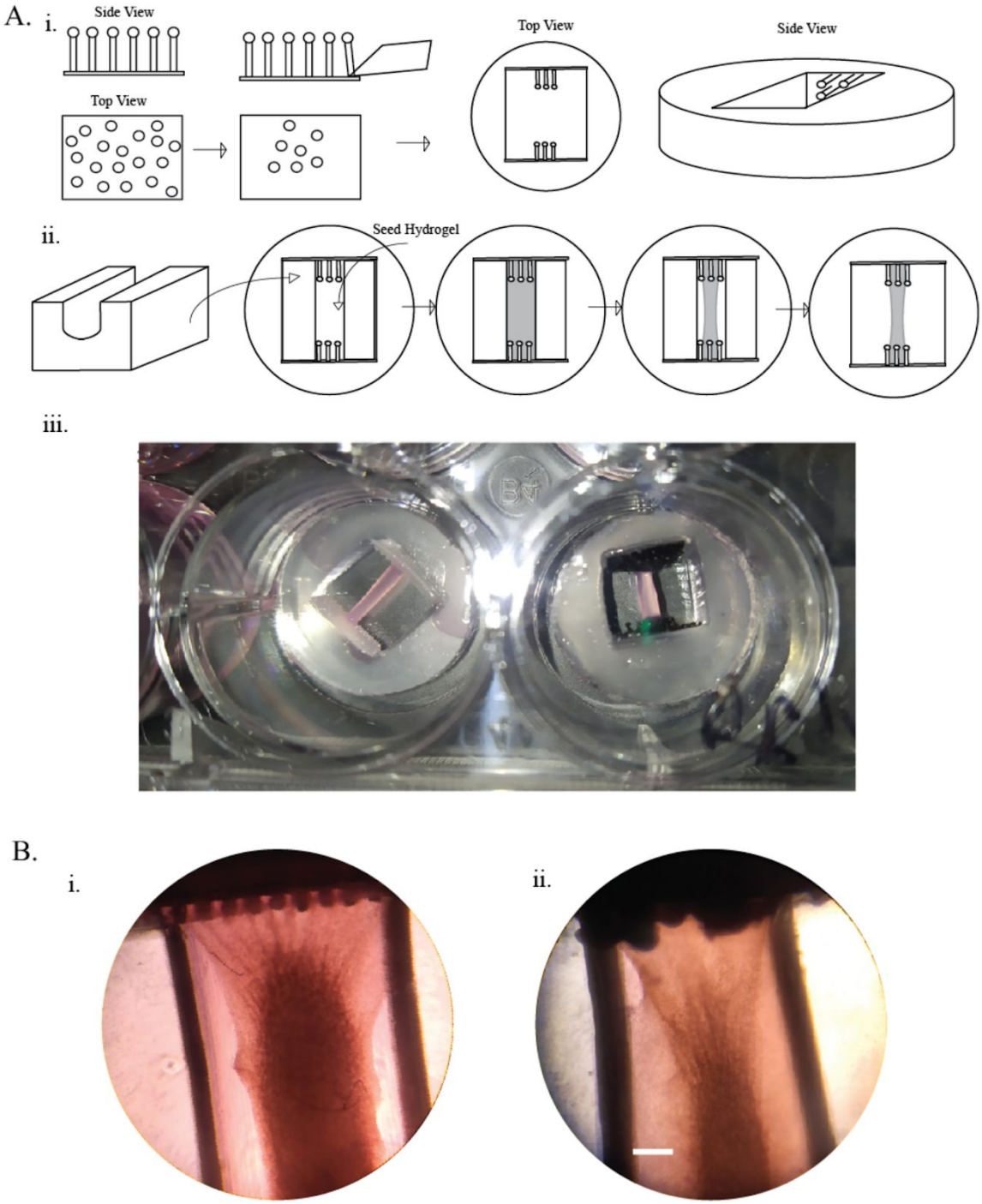

Figure 4. Myobundle Design. (A) Schematic of frame and channel creation. (i) Velcro-like fasteners are shaved down to fit channels and adhered to frame. (ii) Channel is place into frame, where hydrogel can be seeded. Channel is removed before starting differentiation. (iii) View of bundles using different binding material, velcro (left) and fastener (right). (B) Images comparing the frame binding sites, velcro (left) and fastener (right) (Scale Bar 1mm).

DISCUSSION AND FUTURE WORKS

Previous attempts at 2D modeling were conducted with 25x15μm patterned lines, where detachment was still heavily experienced. This was potentially believed to be the problem of narrow lines, not allowing the culture to proliferate and differentiate healthily. An increase to 100x20μm lines would greatly increase that space (Fig 1). Although some improvement was observed, detachment was simply delayed. As this issue is not observed in other literature, a hypothesis may be made that the geltrex density, used for microcontact printing in our protocol, is more compatible with myocytes, rather than myotubes [10,15]. It would be beneficial to run a geltrex density trial and confirm whether detachment may be possibly reduced by these means. A trial with simply undifferentiated myocytes may also conclude any discrepancy on whether myotube differentiation causes the detachment.

The potential of exploring other extracellular matrices in combination with other methods, including genipin to improve cross-linking may prove to work as an alternative, if the geltrex protocol can not be resolved. Beyond issues with detachment, various literatures observe no spontaneous contractions on patterned myotube cultures [10,15]. After resolving detachment issues, and observing healthy sarcomere development and maturation through immunofluorescence, it will be valuable to observe if any electrical stimulation may stimulate contraction. By using the "heart on a chip" devices, contractile forces for the myotubes may be studied. If they contract from electrical stimulation, applied by field electrodes and a pulse generator, the curvature of a bendable muscular thin film layer can be observed and measured for contractility data. Contractility studies, which may be compared to our 3D model, as well as FSHD afflicted cells, could be possible.

Previous attempts at 3D model design have had issues with the gel and myobundles adhering to the channel walls. Revising individual steps in our protocol seemed to alleviate the issue. Reducing the time channels were exposed to open air, may have reduced any potential debris or dust from providing binding regions in the channels that pluronics fail to block. With that no longer being as prevalent of an issue, the next step was to reduce the frequency of tearing or detachment in our various experiment runs (Fig 5). Velcro originally showed promise, but perhaps individual hooks did not provide sufficient contact with the myobundle to be supported.

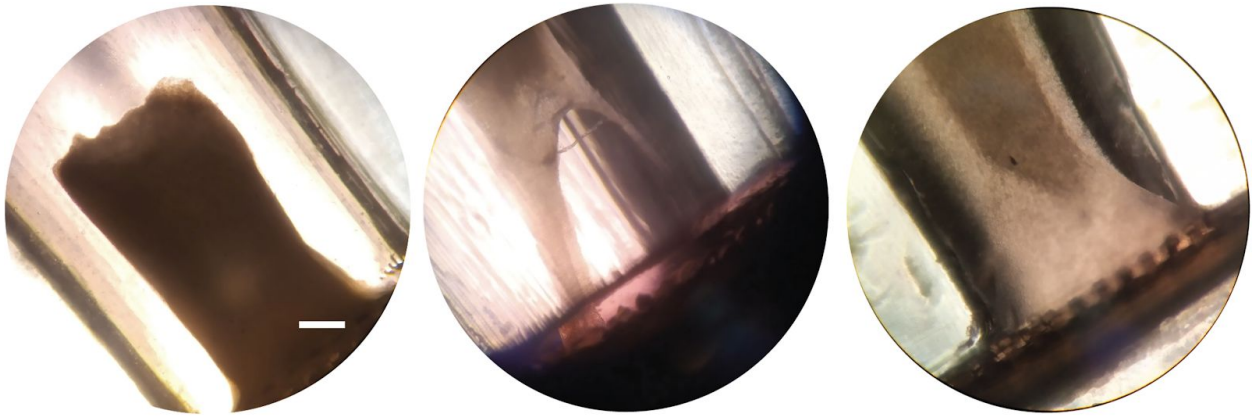

Figure 5. Myobundle comparisons. Images from left to right: Complete detachment from one end, partial tearing at one end, succesful concave shape at binding point. (Scale Bar 1mm).

Various materials were trialed, and previous literature had observed that the material may not only be related to supporting the tensile force of the bundle, but how well the bundle would form into a concave shape [15]. It was important to provide a material that would not interfere with the fit of the channel into the frame, and to ensure the frame is as flush as possible with the channel, so the concave myobundle structure can form [16]. The problem with velcro was that its thickness,provided by velcro hooks, had space where the channel walls would come into contact with the frame as opposed to the empty channel space that was meant to be covered. Introducing a fastener with a stand-off design, allowed for shaving off the unnecessary stand-offs to make a

more flush surface (Fig 4A i). Experiments with this setup had proved to provide more frequent concave myobundles as well as less detachment among the various samples.

The next issue to be explored was the lack of spontaneous contraction that other literature would typically expect to see around day 10 [15]. A sample was stained and fixed at day 10 to observe under a confocal microscope (Fig 6). Cell vitality could be confirmed by observable nuclei. It was expected that actin and sarcomere would be prevalent, as other literature had observed myotubes more localized near the surface [15]. However, these structures were not prevalently observed in the myobundle, indicating issues with stain permeation and/or the development and maturation of myotubes. In the future, myobundles could be imaged following better established 3D protocols, involving paraffin embedding and slicing [11]. It would also be worth investigating alterations in the current culturing protocol, as some literature have used some mix of growth media and differentiation media throughout the entirety of the culture time, as opposed to only differentiation media beyond some point [15].

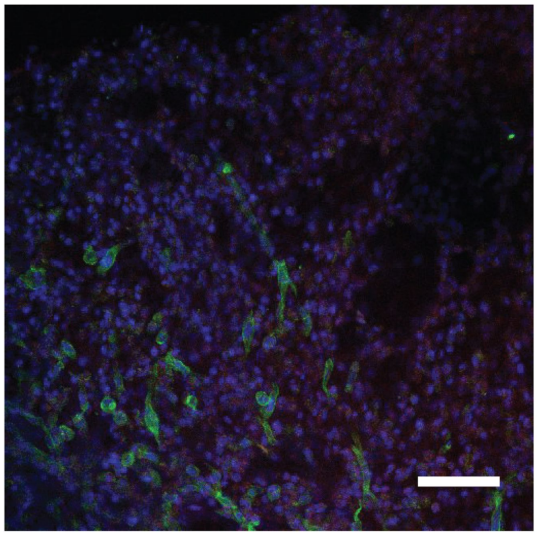

Figure 6. Confocal Image of Myobundle stained for alpha-actinin (red), actin (green), and nuclei (blue). Image is taken at the surface (No z-stack compiled). Scale bar 100μm.

If alterations do not produce spontaneous contraction, it will be important to confirm sarcomere development and maturation with immunofluorescent imaging, so that the vitality of the cells may be validated. Any confirmation of such sarcomere development would open the door for any potential contractility studies through the use of electrodes and a pulse generator,

similar to the previously described plans for the 2D model. A combination of our future 2D and 3D model data would provide a comprehensive understanding of how structure and function are affected by FSHD to start investigating both mechanisms and potential therapies for the affliction.

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