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Development of an In Vitro Platform for the Exploration of Skeletal Muscle Cell Mechanobiology

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1. Abstract

Muscles have the natural ability to regenerate when damaged. This works to keep the body functioning, unless this ability is overwhelmed. Volumetric muscle loss (VML) occurs when a substantial amount of a muscles mass is lost. This leads to the development of scar tissue, and often a loss of function. In order to find a solution for this condition, *in vitro* models are needed to explore the mechanobiology of skeletal muscle cells. Two platforms were developed in this study, to explore 3 factors: orientation, strain, and supporting structure. One platform used fibronectin stamped lanes on PDMS to encourage cell growth at specified orientations, which were then subjected to tension. There was increased myotube formation in samples where the stamped lanes aligned with the direction of strain. The strain resulted in an increased fusion index, though it was discovered that there was a limit to the strain that the cells could favorably function under. The second platform was used to determine if a supporting scaffold helped the regenerative process. Longitudinal sections of decellularized human skeletal muscle (hDSM) were taken on slides for porosity measurements, and on PDMS/coverslip constructs to test cell viability. An average porosity measurement was calculated from the slide samples, while the construct sample were cultured with cells and exposed to two different conditions. Both were cultured with C2C12 cells in growth media for three days, but while one set of samples was fixed at that point, the other set was cultured in differentiation media for an additional four days. There was successful cell proliferation on the samples, and an increase in growth could be seen in the 7 day samples. The future direction of this study is to combine these two platforms, and subject aligned hDSM samples to tension to better determine beneficial components that will lead to insight into skeletal muscle cell mechanobiology.

2. Introduction

The muscles in the body have a natural ability to regenerate when damaged. This regeneration is successful in building muscle after exercise, and repairing damage due to injury or strain. There is a limit to this regenerative ability however, in cases such as Volumetric Muscle Loss (VML) *(1)*. VML is characterized by the significant loss of a muscles mass, and is usually caused by trauma, surgery, or degeneration *(2)*. This causes the body's regenerative ability to be overwhelmed, and results in dense regions of scar tissue, and a loss of function *(3, 4)*. There is a solution needed for when VML occurs, and *in vitro* platforms are necessary to lay the groundwork for this research *(5)*. Most current research involving VML repair strategies are *in vivo*, and while these platforms lead to valuable insights, they also result in barriers in progression *(6-8)*. *In vivo* testing consists of uncontrollable variables that may affect experiment outcomes, while *in vitro* testing offers a more controlled environment. *In vitro* platforms are useful because they do not need animal models, the care and regulations that accompany them, and the experiments are more easily controlled and modified.

This research project worked to develop *in vitro* platforms to determine the relationship between the different variables that contribute to skeletal muscle regeneration. Orientation, strain, and supporting structure are just a few factors that can give further insight into the mechanobiology of skeletal muscle regeneration. One platform was developed to test the first two factors, and consisted of culturing C2C12 cells on lanes stamped with fibronectin on PDMS. This caused the cells to grow in aligned patterns, and after being placed on a stretcher, helped to determine the relationship between alignment and strain on fusion index. Since one platform was already developed to determine the contribution of orientation and strain, another was developed to understand the impact of a supporting structure *(9)*. To do this decellularized human skeletal

muscle (hDSM) was used. There are artificial scaffolds being developed to help assist in VML repair, but it was thought that it would be more favorable to use components already found in the body *(10)*. This way the scaffold would offer biocompatible assistance to the body's natural regenerative abilities. This hDSM was used to determine the validity of cell proliferation on the hDSM structure, as well as its continued proliferation on PDMS. These two platforms could be combined in the future to give a more complete insight into the mechanobiology of skeletal muscle, as well as potentially reveal more factors that could be tested for VML repair.

3. Materials and Methods

3.1 Chamber Assembly

Polydimethylsiloxane (PDMS) sheeting was cut into 7 cm X 8 cm rectangles, and silicone tubing was cut into 2 cm sections. The PDMS pieces were then laid onto petri dish lids or bottoms, carefully placed so that there were few bubbles, and smoothed to eliminate any bubbles or ridges that would affect chamber adhesion. Sylgard® 184 Silicone Elastomer base and curing agent were used in a 10:1 ratio to attach the silicone tubing to the PDMS pieces. For a normal trial this consisted of a mass of 2 to 4 g of base, and a mass of .2 to .4 g of curing agent. The mixture was mixed with a micropipette tip, and one end edge of the silicone tubing was coated in the mixture. This was done with the pipette tip, and just enough was used to cover the edge, but not enough to run down the side. Two of the silicone tubing pieces were glued to each PDMS piece by placing them equally spaced from the edges with about ¼ inch in between the silicone chambers, elastomer lined edge side down. They were placed so that the two chambers were beside each other in the 8 cm long direction of the PDMS (**Figure 1**). These were then checked for any spaces or unsealed holes, patched if needed with more elastomer mixture, and set in an 80 °C oven to cure for 24 hours. When removed from the oven, DI water was placed in

the chambers to check for leakage, dumped, and the membranes were ready to be attached to the brackets.

3.2 Bracket Assembly

To attach the membrane chambers to the brackets a piece of paper towel was laid in the bottom of the peg tray to prevent the PDMS from sticking to the tray. The bottom portion of one of the brackets were set on the pegs, and the 8 cm long edge of the PDMS square was laid flush with the grooves on top of the bracket bottom (**Figure 2**). The top was then placed onto the pegs, and tightened down with three screws. To attach the other bracket, the first assembled one was removed, and set up against the pegs with the attached edge downward, to prevent tension when attaching the second bracket (**Figure 3**). The PDMS edge was placed in the bracket the same way as the first one, and tightened down. Both brackets were set onto the pegs, and viewed from the side to verify that the chambers and PDMS did not sag. They should have been pulled tight enough to not bow or sag, but not tight enough to wrinkle the PDMS (**Figure 4**)**.** Once all four sets of chambers were assembled on the brackets they were ready to be prepped for stamping.

3.3 C2C12 Cell Culture

The cells used in this experiment were C2C12 cells. The growth media consisted of 500 mL of DMEM:F12 nutrient mixture, with 5 mL of Pen/Strep as well as 50 mL of Fetal Bovine Serum (FBS). The differentiation media was made with 500 mL of DMEM:F12 nutrient mixture with 5 mL of Pen/Strep and 5 mL of Horse Serum. The cells were started from a frozen aliquot, and cultured in a T175 flask using growth media. Once reaching adequate confluency to passage, the cells were trypsinized for 7 minutes, centrifuged at 125g for 7:30 minutes, the cell pellet was resuspended in 2 mL of media, and the cells were counted to determine seeding volumes. For the fibronectin stamped chambers a volume containing 200,000 cells was pipetted into each

chamber, and 2 mL of growth media was added (**Figure 5**). The samples were incubated with the chambers covered with mini petri dish tops and bottoms, and the media was exchanged every other day. A culture was also restarted in a flask, to continue the culture for further trials. For the hDSM trial a volume containing 300,000 cells was pipetted into each well onto the hDSM sample (**Figure 6**). These were then incubated for 30 minutes, before adding 2 mL of growth media into each well. The media was exchanged every other day, and one 6 well plate was fixed after 3 days. In the other 6 well plate, the media was replaced with C2C12 differentiation media, exchanged every other day, and was fixed after an additional 4 days.

3.4 Preparation and Sterilization

The bracketed chambers were placed in a beaker with 70% ethanol made by filling a 600 mL beaker with 280 mL of 190 proof ethanol, and 120 mL DI water. The silicone stamps were also placed in a 600 mL beaker with 70% ethanol with the lanes facing upward. A notch was taken out of the corner of the non-lane side of the stamps to more easily identify the orientation of the stamps. Both beakers were covered with plastic wrap, and sonicated for 30 minutes. The hDSM samples in the 6 well plates were sterilized using ETO.

3.5 Fibronectin Stamping

After the sonication of the chambers and stamps, the two full beakers, tweezers, four petri dishes, sterile DI water, and two 100 μL aliquots of fibronectin were taken to a hood with a nitrogen nozzle. The nitrogen was used to dry the membranes, and after drying they were placed on petri dish lids so that there were little to no bubbles in the chambers bottoms. This was achieved by laying the membranes down gradually, and using a small amount of the 70% ethanol on a fingertip to smooth from the inside of the chamber. The petri dish bottoms were then placed over the top of the chambers. Each stock fibronectin was diluted with 900 μL of DI water to

bring the total volumes up to 1000 μL of solution. This resulted in a concentration of 50 mg/mL, but some trials were done using double that concentration. A micropipette was used to place 200 μL of the fibronectin solution in drops onto the lane side of each stamp. Once all of the stamps were covered in the designated volume, the pipette tip was used to smooth the fibronectin so that it covered the whole stamp, while making sure that the pipette tip didn't touch the lanes. The covered stamps were set into a large petri dish and left in the hood until the chambers were ready. The chambers were taken to UV ozone for 1 hour, and the bottoms of the petri dishes were removed and placed under the lids so that the chambers were open to the treatment.

Once the chambers were finished with treatment, the bottoms were placed over the chambers again, and taken back to the hood. The fibronectin was shaken off of one stamp at a time, and the nitrogen nozzle was used to dry the stamp just until it was no longer wet, making sure not to over-dry it. The tweezers were used to place the stamp lane side down into one of the chambers (**Figure 7**). This was done at specified orientations of control, 0, 45, and 90 degrees. The stamp was set in the correct orientation, and the tail end of the tweezers was used to tap the stamp down. This was done by letting the weight of the tweezers drop from around ¼ inch above the stamp in an X pattern, then going around the edges of the stamp. Any areas that were not covered by this pattern were tapped, and the rest of the wells were done in a similar fashion. One orientation was chosen per set of wells. Once the last well was stamped, the first stamp was peeled off by gripping it with the tweezers, and lifting from one side in a continuous motion. The stamps were stored lane side up in a large petri dish for future use, and the bottom of the petri dishes were replaced over the chambers. To treat the lanes before seeding, a volume of 1 mL of 1% pluronics was added to each well, and let sit for 20 minutes. This was aspirated off, and the chambers were ready to be seeded with cells and cultured.

3.6 PDMS/coverslip Construct Assembly

PDMS sheeting was cut into 22mm X 22mm squares. The PDMS squares and the coverslips were placed into beakers with 70% ethanol, covered with plastic wrap, and sonicated for 30 minutes. The beakers were then taken to a hood, and the constructs were assembled by placing one PDMS square onto one coverslip. These were stored in a petri dish, coverslip side down, on kimwipes to prevent sticking together. At least 12 constructs were assembled for a full trial.

3.7 Decellularization of Human Skeletal Muscle

Human tibialis anterior muscle was placed into a container with 1% SDS fluid for decellularization. It was placed on a rocker plate, and the fluid was exchanged every day for 2 weeks. The SDS fluid was then replaced with DI water, and placed on the rocker plate for 30 minutes. This was repeated 3 times, and then stored in a 4 degree refrigerator for 3 days to remove excess SDS. To remove the excess water from the hDSM, the muscle pieces were placed into a scintillation vial with OCT for 2 days (**Figure 8**), and placed in a cassette and frozen in the cryostat to prepare for cryosectioning (**Figure 9**).

3.8 Cryosectioning

A cryostat was used to take 10 μm longitudinal sections of the hDSM. Some sections were collected on slides, while others were collected on the PDMS side of the PDMS/coverslip constructs (**Figure 10**). Twelve samples were taken for the latter, and the constructs were placed into two six well plates.

3.9 Staining

The fibronectin stamped lanes with cells, as well as the hDSM with cells, were both stained with DAPI and Phalloidin. This was done by aspirating off the media once it was decided

that the samples were to be fixed, which was either a timeframe or a decision based on predicted success of the trial. The samples were fixed by adding 5 mL of formalin to each chamber or well for at least 30 minutes. The samples were then rinsed with 5 mL of PBS 3 times, at 5 minute intervals on a rocker plate. After aspirating off the last PBS the samples were stained with DAPI by placing drops of the stain on the sample, covering the sample and letting it sit for 7 minutes, then recollecting the DAPI stain. The samples were rinsed with PBS 3 times, at 5 minute intervals on a rocker plate. Next, the samples were stained with Phalloidin by pipetting drops onto the samples, leaving covered for 7 minutes, and recollecting the stain. The samples were then rinsed with PBS 3 times at 5 minute intervals on the rocker plate, and the PBS was aspirated off. The samples were stored covered to reduce light exposure to maintain fluorescence, until they were imaged using a Nikon Eclipse C*i* microscope at 100X. The hDSM samples that were taken on slides were stained using Hematoxylin and Eosin (H&E). This was done on non-seeded samples to be imaged to determine structure and porosity.

4. Results and Discussion

Results from both platforms proved that they were adequate for the exploration of skeletal muscle cell mechanobiology. The fibronectin stamped lane study proved that mechanical stimulations along the direction of alignment were beneficial in myotube formation (**Figure 11**). The proliferation of the aligned samples (0 degrees) was increased as compared to the unaligned samples (45 degrees and 90 degrees). It was also determined that fusion was strain dependent, though there was a limit to the benefits. The 10% strain was most favorable, though both strain samples yielded more progressed myotube formation as compared to the static samples. The 20% strain reached detrimental levels, causing decreased development as compared to the 10% strain.

These observations were quantitatively characterized using the fusion index, which is the percent of cells fused together to form myotubes.

The second *in vitro* platform that was tested was to determine the impact of using hDSM as a supporting structure for increased regeneration. A measure of porosity was done on the hDSM slide samples, using ImageJ. The H&E stained samples that were taken on slides were imaged, and an area fraction was calculated after thresholding. This resulted in an average porosity of 43.776%, and a standard deviation of 4.751% (**Figure 12**). With more trials, a correlation could potentially be found between porosity and proliferation or fusion index. The samples that were taken on the PDMS/coverslip constructs were used to test the viability of cell growth on hDSM. There were two 6 well plates used for a single trial, which resulted in 12 total wells. Each of the 6 well plates was treated with 3 days of growth media, while one plate was treated an additional 4 days with differentiation media. This resulted in 3 and 7 day samples, of which 11 of the 12 wells successfully maintained proliferation. The degrees of proliferation varied, though the most consistent throughout were the 3 day samples. The 7 day samples had one well which didn't result in cells, though 2 of the wells showed greatly increased proliferation as compared to the 3 day samples (**Figure 13**).

5. Discussion of Future Directions

These two *in vitro* platforms were used to determine different components of cell mechanobiology. The first platform was used to understand the effect of directionality on the level of regeneration, and to determine if strain made a significant impact in myotube formation. The second platform was used to determine if having a supporting structure helped cell proliferation, using hDSM. These two platforms covered three components of regeneration to

determine muscle cell mechanobiology, but can be combined to give further insight into the impact of these components. The next platform to be developed in this *in vitro* study will be to seed cells onto hDSM in the PDMS bottomed silicone chambers, and subject it to stretching on a bioreactor (**Figure 14**). This would lead to insight into structure under tension, and the validity of decellularized skeletal muscle as a scaffold for regeneration. This could help to pave the way for using scaffolds for VML, and determining optimal strain and orientation for improved regenerative ability. This platform could give the information needed to introduce promising techniques in animal models, and help in the progression of VML repair.

6. Conclusion

The goal of this research was to develop *in vitro* platforms that led to insight into skeletal muscle cell mechanobiology. The specific factors that were tested were orientation, strain, and a supporting structure. One platform determined that using fibronectin stamped lanes at different orientations and strains yielded successful results. Alignment played a part in increased fusion index, and while strain was beneficial in this aligned state, it was discovered that there was a limit to the degree of strain in which the cells could favorably proliferate and fuse. Another platform was used to determine the validity of using hDSM as a supporting structure for increasing cell growth. The C2C12 cells proliferated on the hDSM samples, and showed indication toward fusion in multiple samples. Both of these *in vitro* studies were beneficial in determining factors of cell mechanobiology, and by using them to develop future platforms, could give a more comprehensive view of the part that each factor plays in the overall regenerative process.

7. Acknowledgements

I would like to thank Dr. Jeffrey Wolchok for being my thesis advisor for this project, and guiding me throughout my research. I'd also like to thank PhD student John Kim, who acted as a mentor, and offered invaluable advice and direction throughout the time that I conducted research. A thanks to the Biomedical Engineering Department, Engineering Research Center (ENRC), and Honors College, whom all provided facilities and resources to make this possible. This included an Honors College research grant for the Spring and Fall 2017 semesters. Thank you to all who assisted me in the completion of this endeavor.

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Appendix

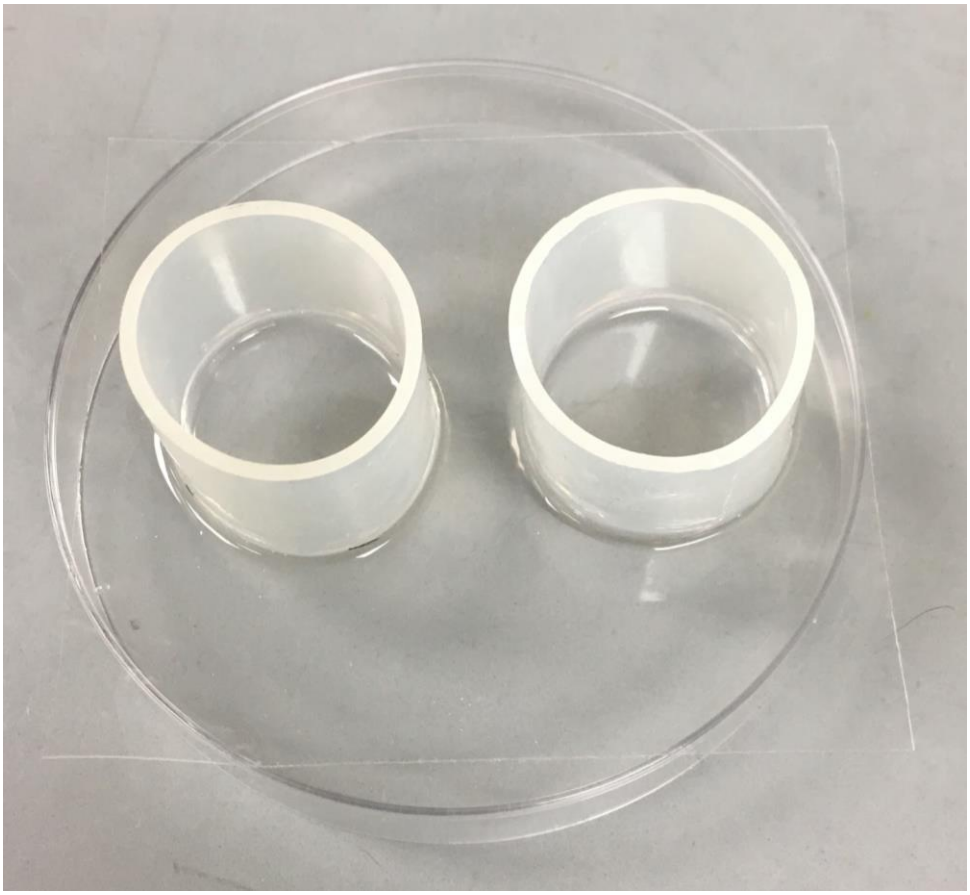

Figure 1: PDMS membrane on the bottom of a petri dish with two silicone chambers affixed with elastomer. PDMS measurements measure 7 cm vertically, and 8 cm horizontally.

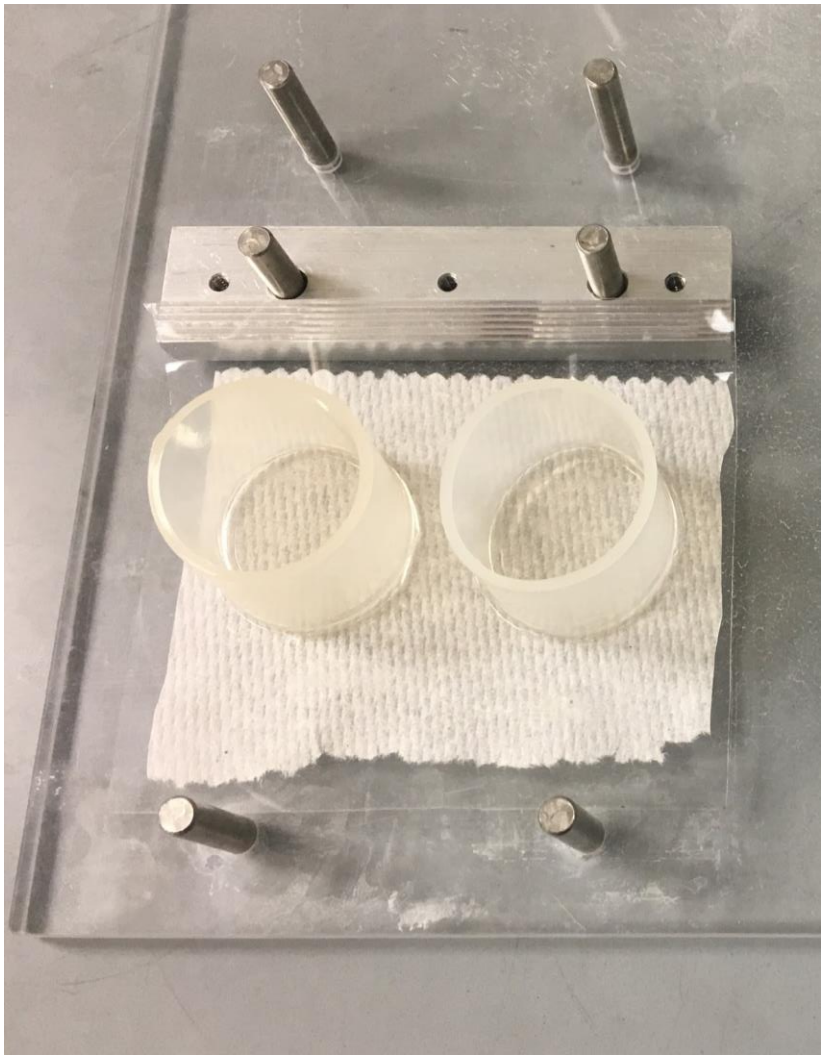

Figure 2: Paper towel underneath PDMS membrane with chambers to prevent sticking. PDMS membrane is placed with the 8 cm long edge meeting the back ridges of the bottom bracket piece. The top bracket piece would next be added, and tightened down with screws.

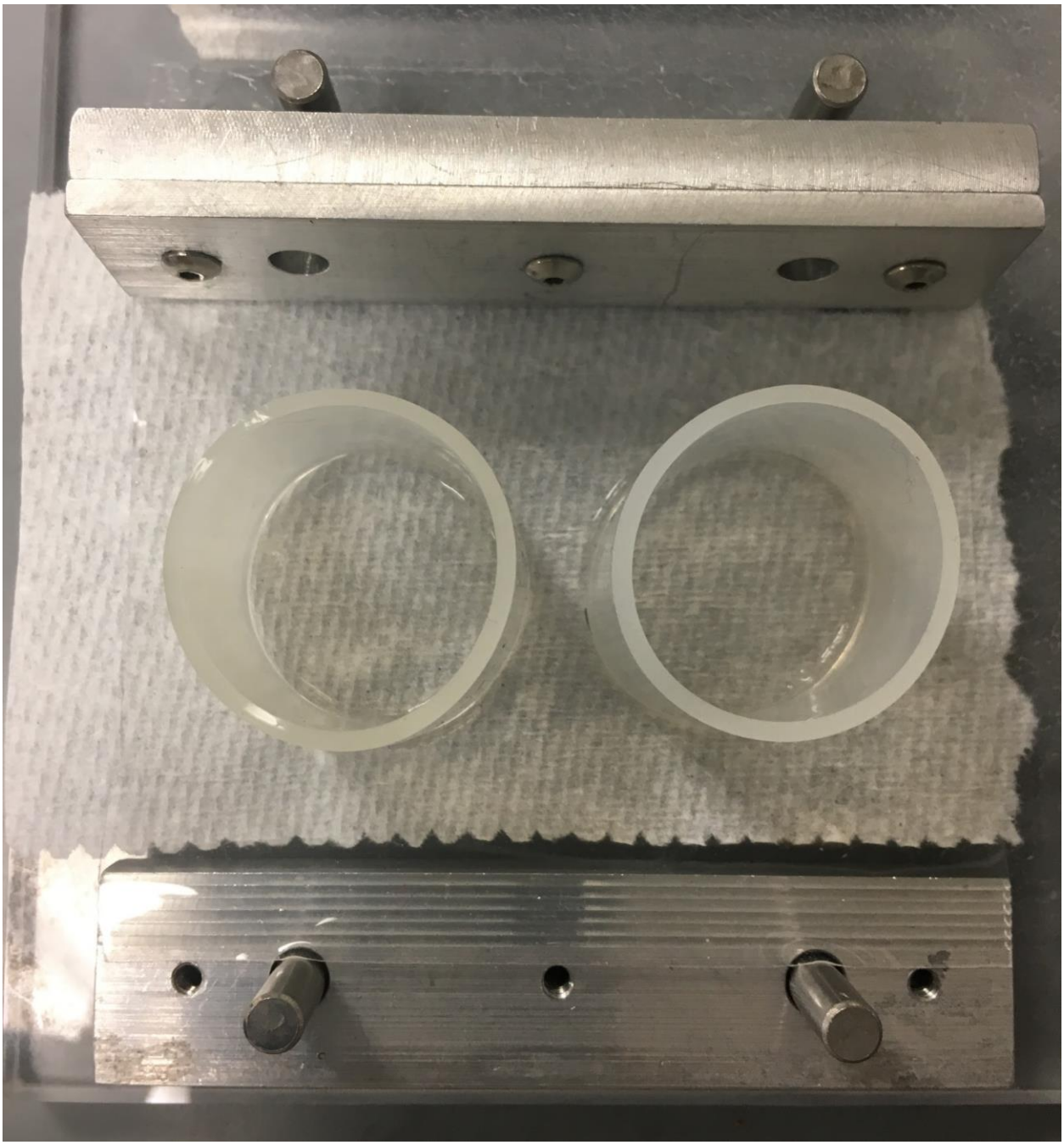

Figure 3: Attachment of second bracket, with first bracket removed from pegs and resting in downward position to reduce tension so that the second bracket can be attached securely.

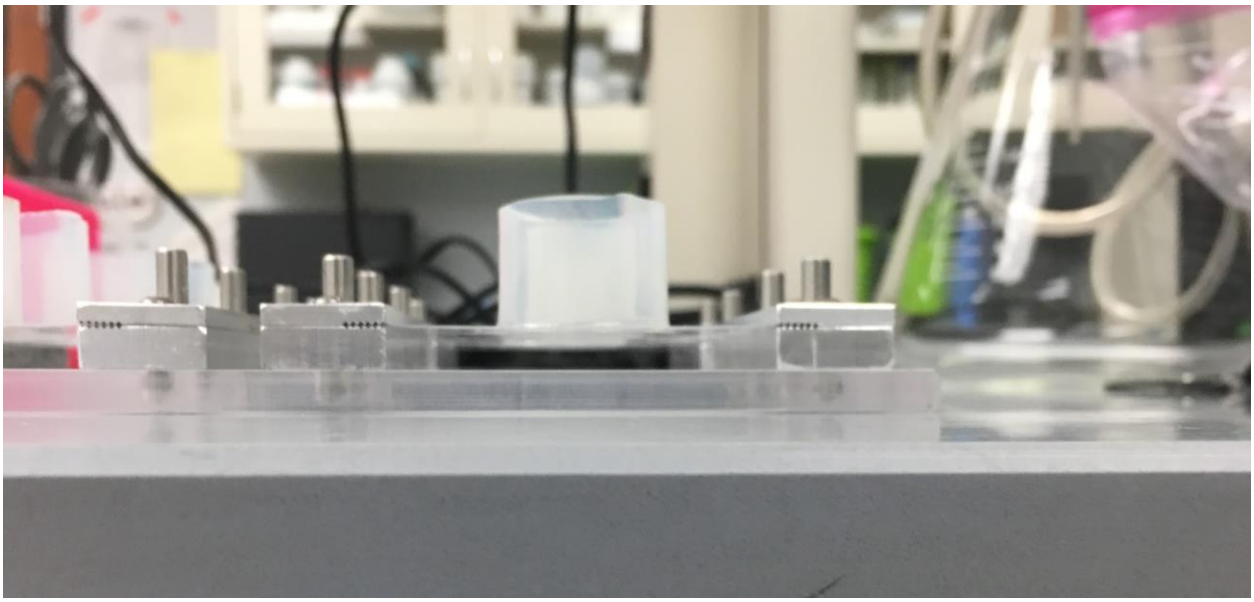

Figure 4: Side view of PDMS with chambers with correct tension. Membrane was not sagging, nor pulled taut enough to cause wrinkles in the membrane.

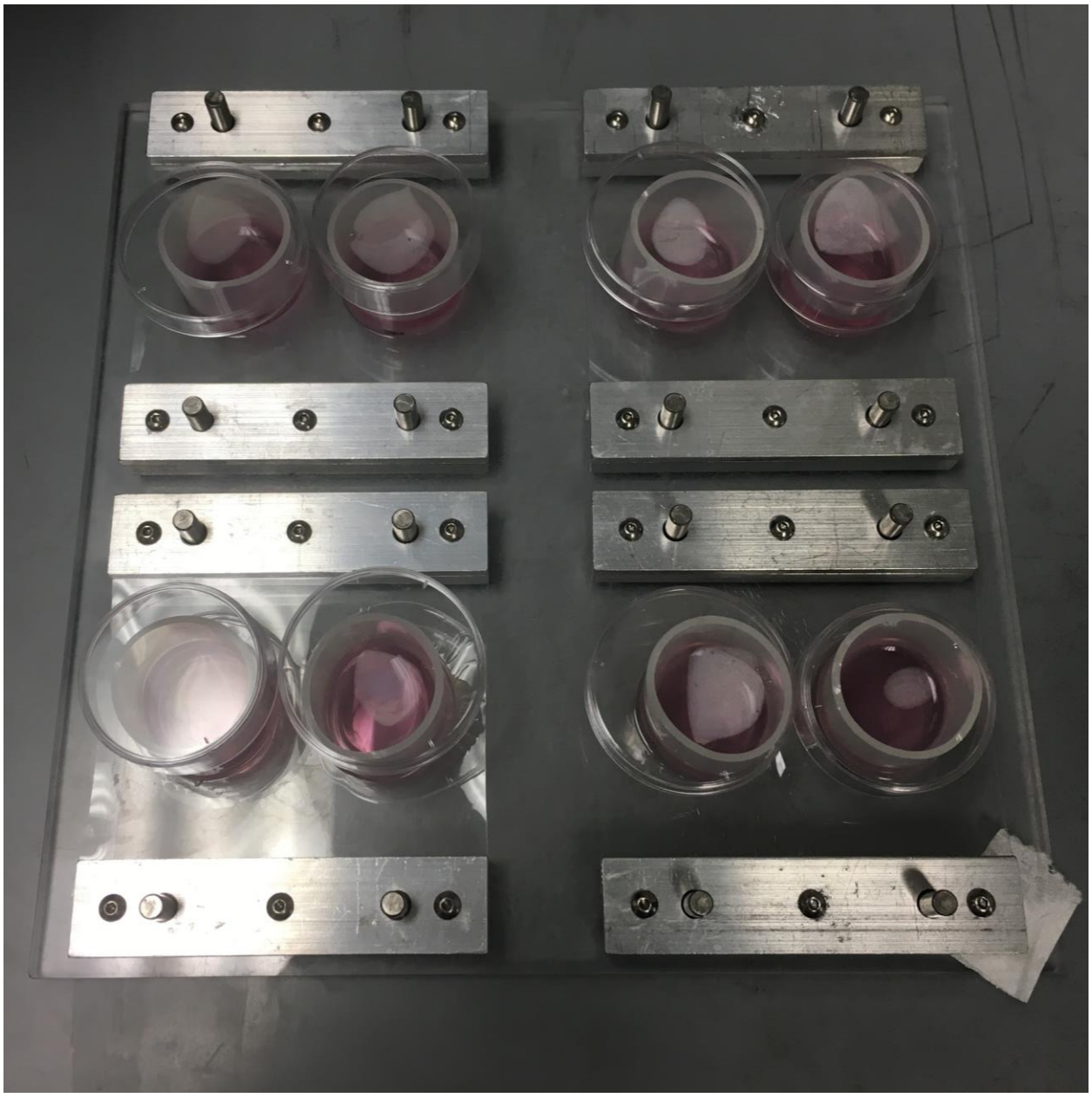

Figure 5: Fibronectin stamped chambers after cell seeding. Chambers have 2 mL of growth media, and are covered with mini petri dish tops and bottoms to control cross contamination between samples.

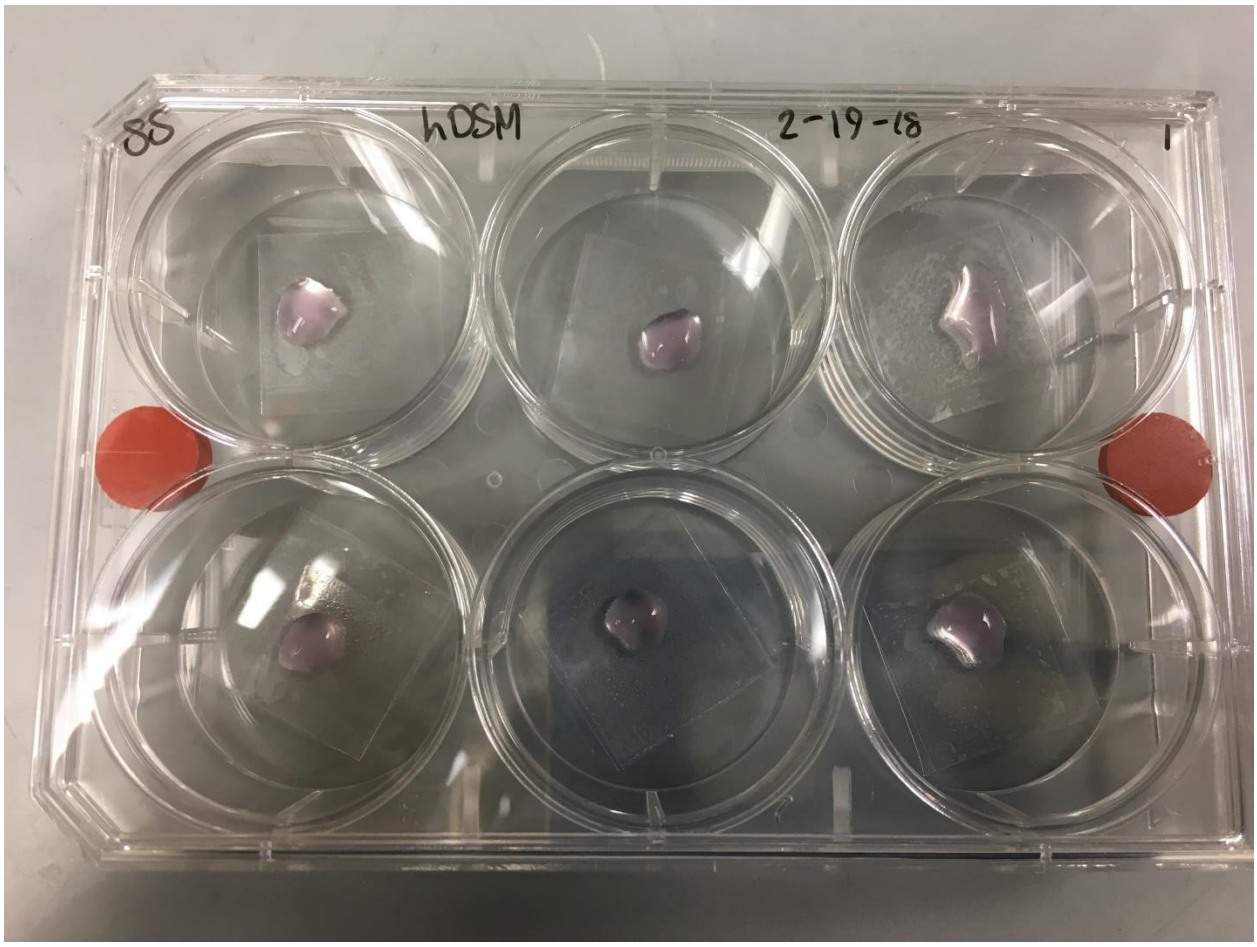

Figure 6: Seeding volume of 300,000 cells on hDSM samples on PDMS/coverslip constructs. This trial resulted in a seeding volume of 37 µL of cell solution places directly on sample, this was then incubated for 30 minutes before adding media to culture.

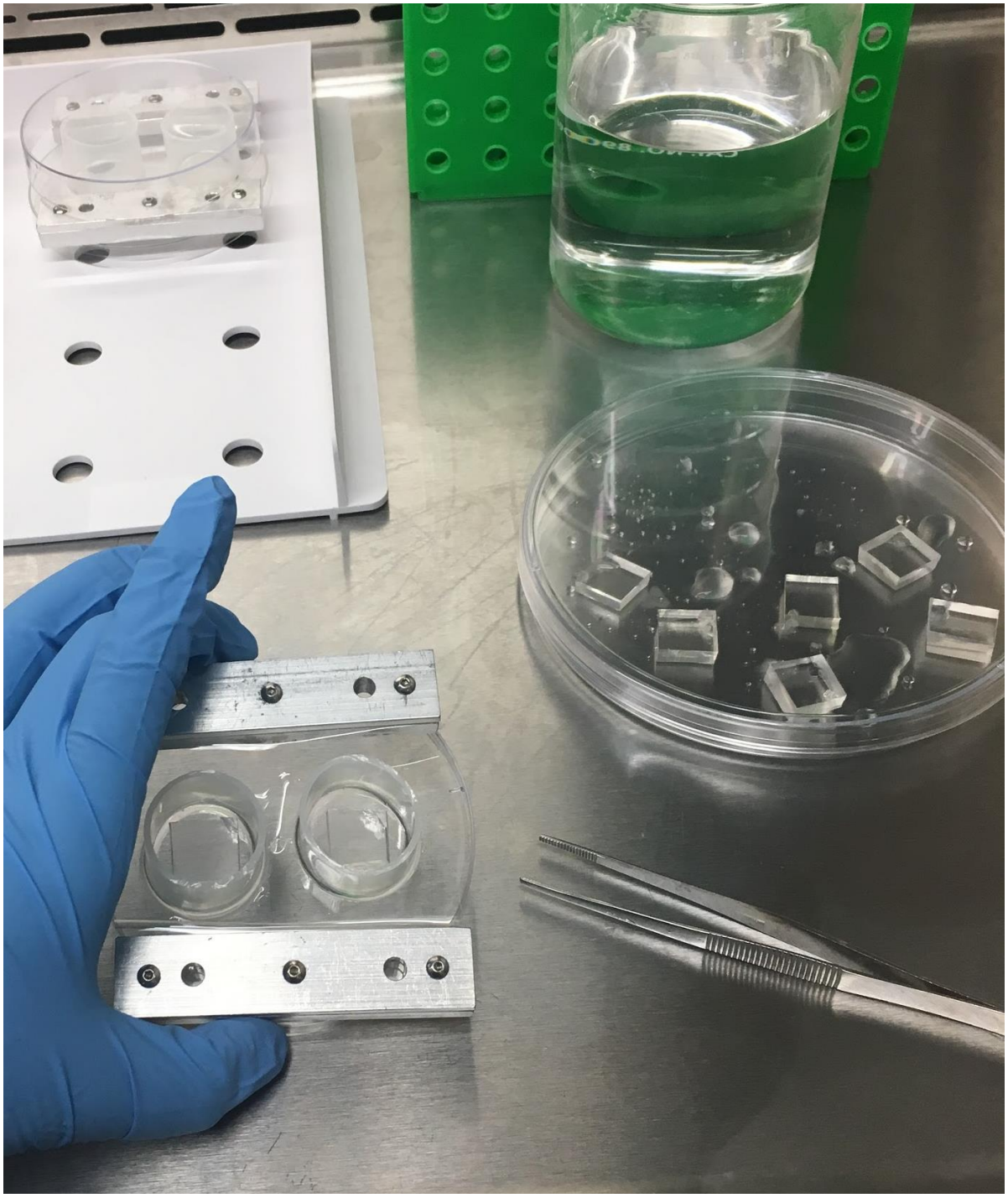

Figure 7: Stamps placed in chambers to achieve fibronectin stamping. Stamps are placed at varying orientations, with these being at the 0 degree orientation, which would be running vertical in this image. The 0 degree orientation aligns with the direction of strain.

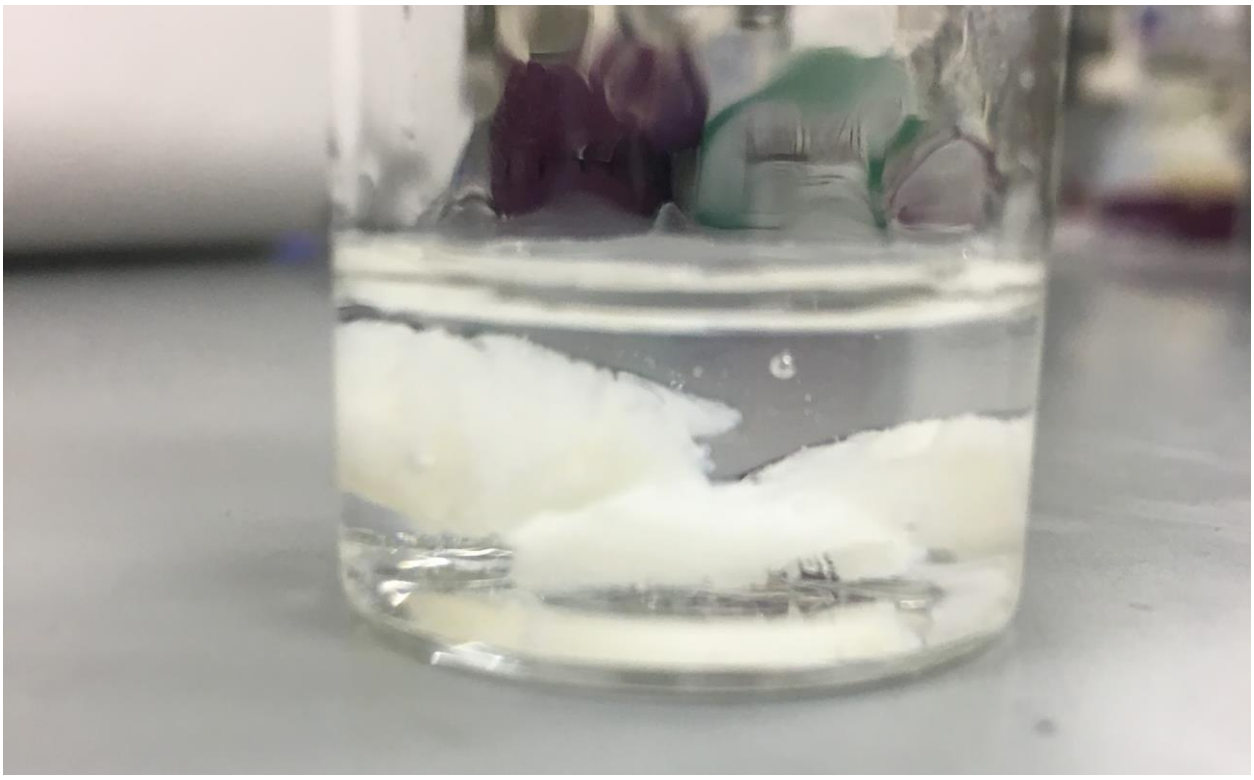

Figure 8: After the skeletal muscle was placed into 1% SDS fluid for 2 weeks, it was transferred to DI water, then to a scintillation vial with OCT as pictured above. The samples were stored like this for 2 days to infiltrate the tissue and remove excess water.

Figure 9: A decellularized piece of TA was placed into a cassette with OCT to be frozen for cryosectioning. The samples were laid as aligned as possible, so that large longitudinal sections could be taken.

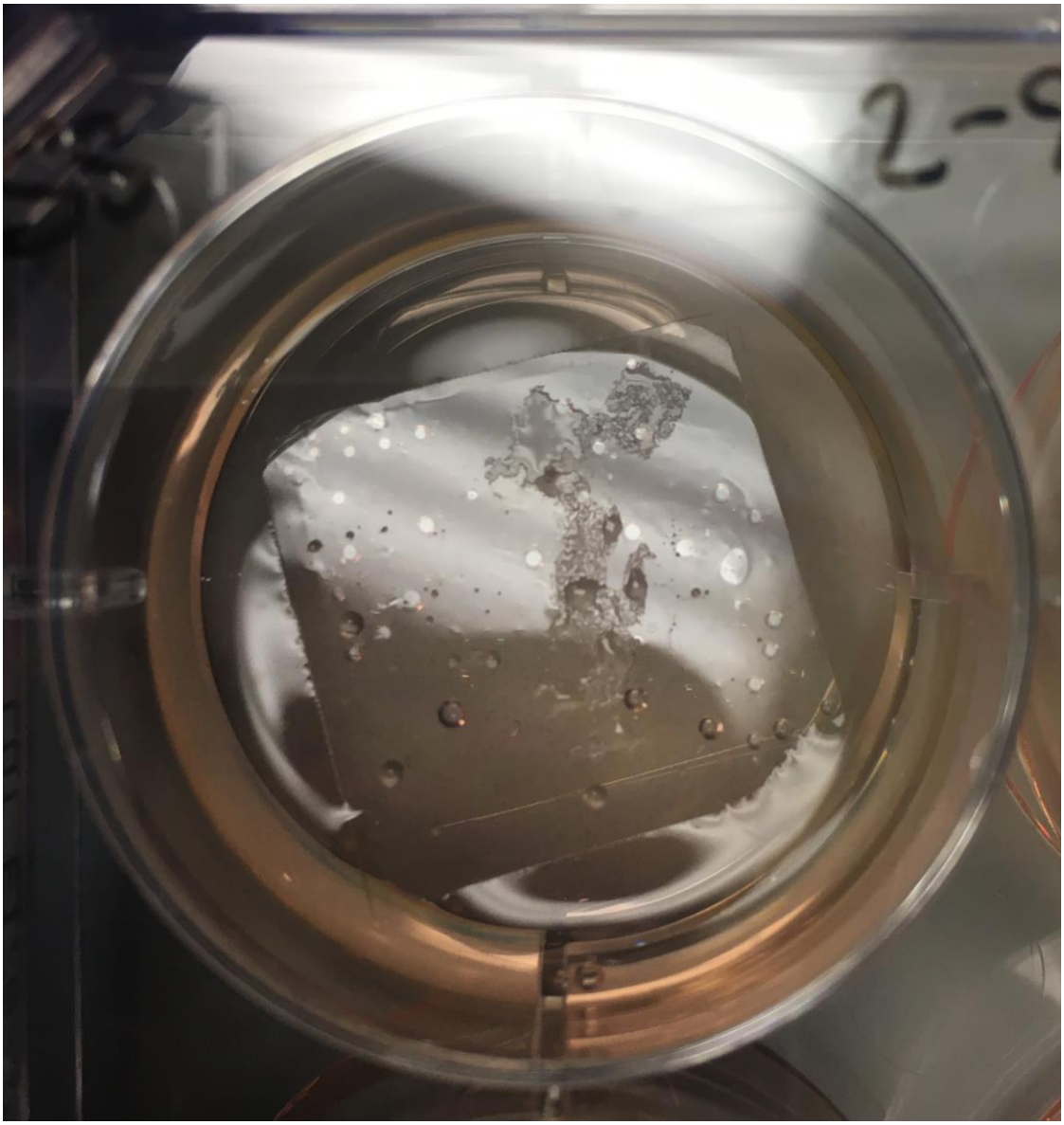

Figure 10: hDSM pieces were cryosectioned and collected on PDMS/coverslip constructs. A piece of hDSM can be seen, sitting in differentiation fluid.

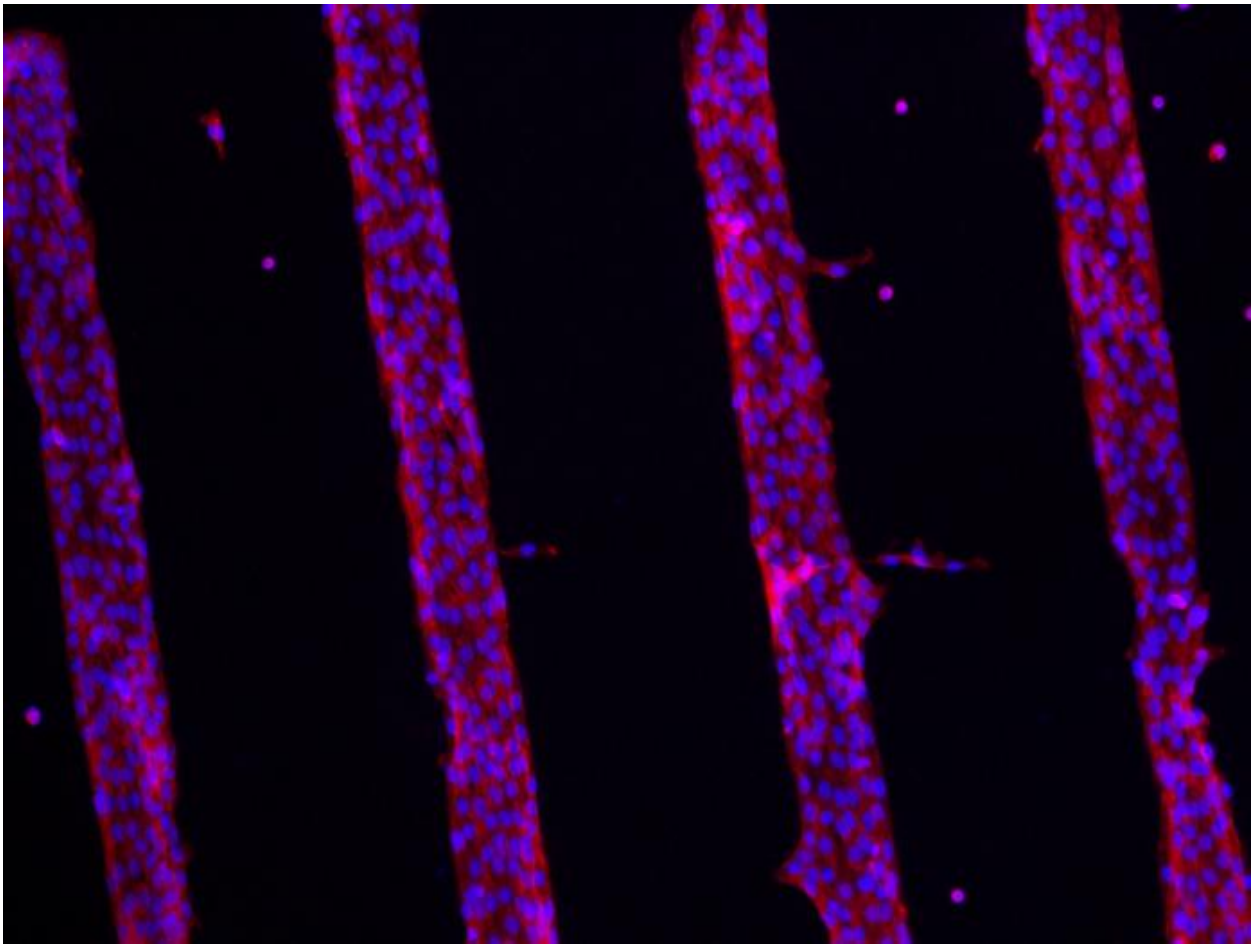

Figure 11: Fibronectin stamped lanes encouraged cell growth in specific orientations. After stamping the lanes were treated with pluronics to restrict the growth to the lanes, and cells were allowed to proliferate with the intention of forming myotubes.

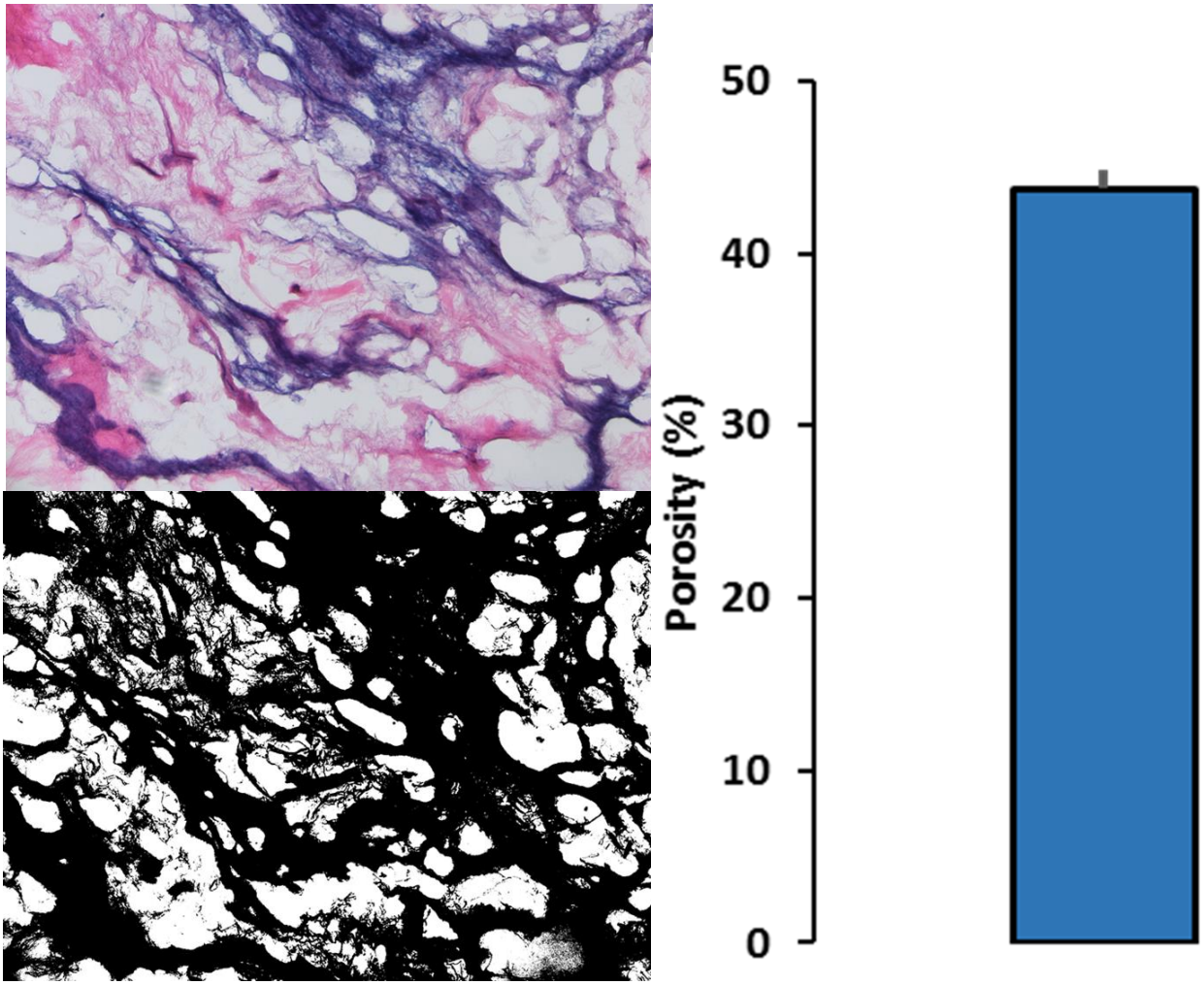

Figure 12: (A): H&E stained hDSM section collected on slide. (B): Thresholded image of (A) using ImageJ. (C): Average porosity measurement, with error bar, of samples acquired using area fraction restricted to threshold in ImageJ.

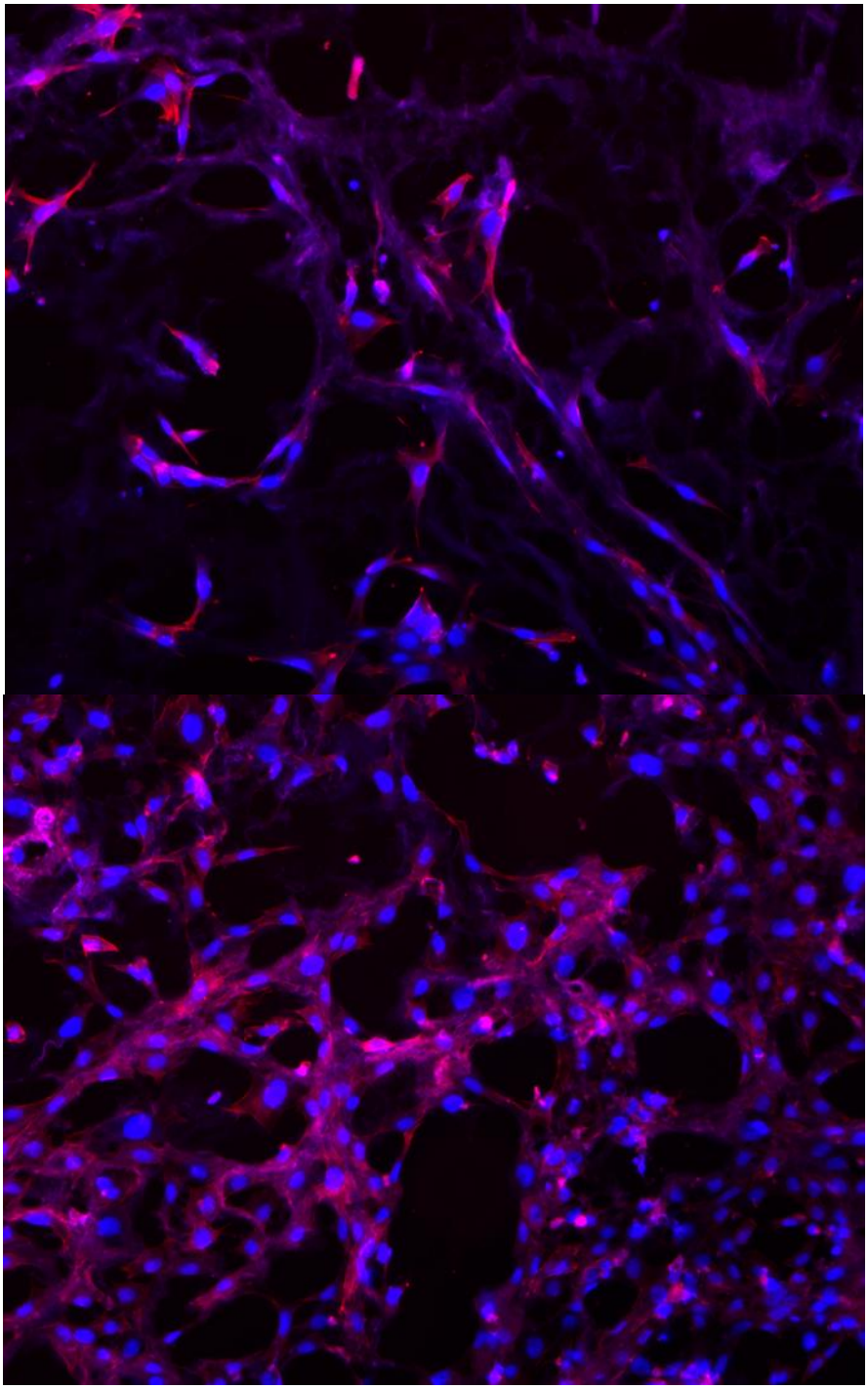

Figure 13: (A): DAPI and Phalloidin stained fluorescence image of 3 day growth of C2C12 cells on hDSM. (B): DAPI and Phalloidin stained fluorescence image of 7 day sample of C2C12 cells on hDSM.

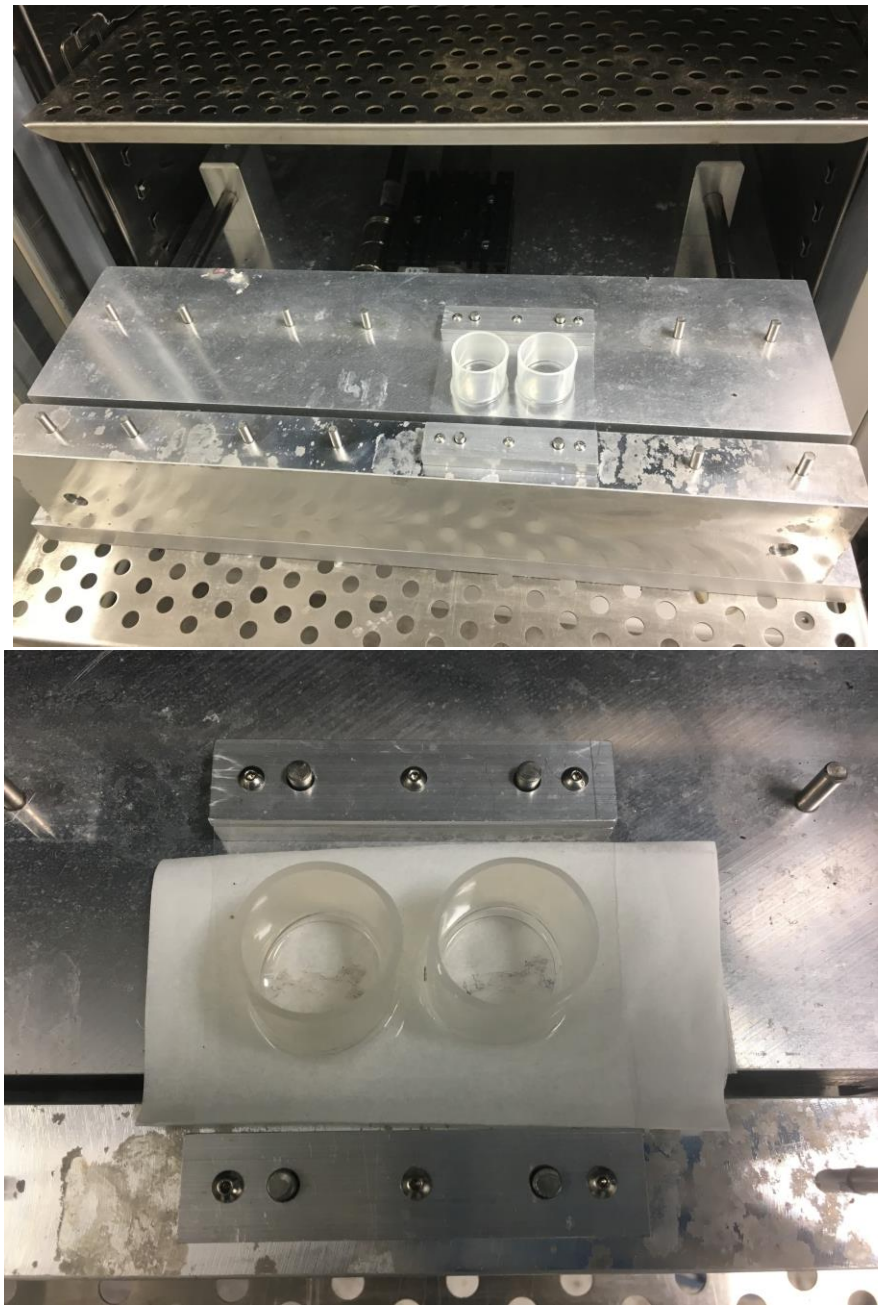

Figure 14: (A): Bioreactor inside incubator with bracketed chamber attached. (B): Close up image showing H&E stained samples of hDSM to demonstrate future direction of study.