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BMEN 5210 Final Lab Report

I. **Project title:** Cell Culture of Cardiac Fibroblasts Using Different Stiffness of Hydrogel

II. Methods:

A. Materials:

- Corning T75 culture flask
- Corning Matrigel (for the ECM coating)
- ATCC cardiac fibroblast
- Gibson DMEM
- Fetal Bovine Serum (FBS)
- 1ml pipette tips
- Aluminum foil
- 15ml Falcon centrifuge tube
- 4% paraformaldehyde solution in DPBS
- .5% TritonX-100
- 4% BSA in DPBS
- Mounting Solution
- F-actin staining kit
- Plastic film
- Polyacrylamide hydrogel with 2kPa, 8kPa, and 50kPa stiffnesses

*Equipment:

- Centrifuge
- Water bath
- Incubator
-Bright field light microscope
-Keyence fluorescent microscope with CCD
-Biohood
-Biosafety cabinet
-Freezer/ refrigerator
-Computer
-Coverslips
-Personal Protective Equipment (PPE)
-Sanitation spray bottle
-Forceps
-P-20 and P-200 pipettes

B. Experimental Procedure:

1. Cleaning: Before starting any of the experiments, it was required to leave any personal items outside the lab room and put on the proper PPE. It was then required to sterilize the workspace with the sanitation spray bottle that UNT provided following the COVID guidelines. The items sterilized includes the biosafety cabinet, biohood, equipment used for culturing the cells, and PPE latex gloves. Also, after every experiment, the stations were clean once again with the sanitation disinfectant spray bottle and all material put back to their prospective storage areas.

I. Culture cardiac fibroblast cells in culture flask

2. Prepare culture flask: The first protocol in this experiment was to culture cardiac fibroblast cells in a Corning T75 culture flask. To begin, a 10ml working Matrigel solution was created as an Extracellular Matrix (ECM) coat for the cells to attach to the hydrogel culture flask. To make this solution, 100ul of the Corning Matrigel working solution, on ice, was placed into a 15ml Falcon centrifuge tube. The working solution was then diluted with 10ml of DPBS and aspirated three times. The diluted solution was then pipetted into the T75 culture flask and placed in the incubator for 30 minutes.

3. Cell growth medium: While the coated culture flask was incubating, the cell growth medium was prepared in a 50ml Falcon tube by adding 45ml of DMEM, 5ml of FBS, and .5ml of 1% antibiotics. (Yang, BMEN 5210, instruction to seed cells on cell culture). The FBS solution was used to help promote cell growth and the antibiotics were used to minimize cell loss due to contamination. The DMEM solution was then place into a water bath set at 37 degrees Celsius for 20 minutes.

4. Prepare cells in culture flask: To prepare the frozen vial of cardiac fibroblasts for cell culture, the vial was placed in a 37-degree Celsius water bath outside the biohood to thaw. The vial was quickly stirred under the water until all the ice was gone, then dried with paper towel and sanitized. (Yang, BMEN 5210, instruction to seed cells on cell culture)

5. Culture medium: Back in the biohood, 9ml of the DMEM culture medium and the cells from the thawed vial were added in a 15ml centrifuge tube and aspirated three times with a P-20 pipette. The centrifuge tube was placed in the centrifuge along with along with another centrifuge tube filled with water about the same weight to keep the centrifuge at balance. Both vials were centrifuged for four minutes. The vials were taken back to the biohood and the supernatant was removed from the centrifuge tube with the DMEM medium and discarded in a 50ml tube labeled waste. Next, 15ml of the DMEM culture medium was added to the cell pellet and resuspended.

6. Trypan blue staining for cell imaging counting: The last step for cell culturing in the T75 flask, was to stain the cells with trypan blue dye and image cells under a bright light microscope. To staining the cells, 20ul of trypan blue dye was added to 20ul of the cell culture medium from part e and aspirated three times. Using a p-20 pipette, 10ul of the cell/dye mixture was placed onto a cell counting slide.

7. Cell counting: A bright field microscope was used to count the cells on the slide by taking the average number of cells from the 4-corner square graph and then multiplied by 20,000. Lastly, the culture flask was taken out of the incubator to seed the rest of the resuspended cells. The medium was replaced after 2 days of incubation.

II. Cell passage and seeding of cardiac fibroblasts on varied hydrogel stiffness

8. Prepare hydrogel for cell culture: The same steps were followed for preparing the Matrigel solution as explained in “cell culturing in a culture flask”. The Matrigel working solution was then added onto various hydrogel stiffness dishes (two at 2kPa, two at 8kPa, and two at 50kPa), instead of the culture flask, and then incubated for 30 minutes. During incubation, more DMEM culture medium was prepared, following step 4 in “cell culturing in a culture flask.”

9. Passaging the cells from the culture flask onto the varied stiffness hydrogels: The first step for passaging the cells was to remove all the culture medium from the culture flask. The unwanted liquid was put into a 50ml Falcon tube labeled waste. After all the culture medium was removed, 10ml of PBS was added to the flask to rinse off any of the residual medium and discarded in the 50ml Falcon waste tube. With a new pipette tip, the entire cultured surface was covered with 5ml of .25% of Trypsin enzyme and then incubate for six minutes and then aspirated 3 times with a new pipette tip.

10. Enzyme mixture: The cell/enzyme mixture was then put into a 15ml centrifuge tube, along with 5ml of the DMEM culture medium, and aspirated 3 times to neutralize the digestion enzyme. (Yang, BMEN 5210, instruction to How to passage cells and seeding on the hydrogel at varied stiffness) The centrifuge was set at 1200 revolutions per minute (rmp) for 4 minutes with another centrifuge tube filled with water to keep the equipment balanced. When finished, the supernatant was discarded and resuspended with 5ml of DMEM culture medium until the pellet is completely dissolved.

11. Cell counting: The same steps for "Trypan blue staining," were once again used for cell counting. However, 50ul of the cells and 50ul Trypan blue dye were added together instead, and then 50ul of that mixture was used for cell imaging.

12. Seeding: After imaging the cells, the Matrigel liquid was removed from each hydrogel dish and put in the 50ml Falcon tube labeled waste. Next, 500ul of the DMEM and cell/enzyme mixture were added onto each of the 6 hydrogel stiffnesses (two 2kPa, two 8kPa, and two 50kPa). All six seeded hydrogels were placed in the incubator, and after three days the medium was changed. (Yang, BMEN 5210, instruction to How to passage cells and seeding on the hydrogel at varied stiffness)

III. Cell fixation

13. Cell fixation: After the workstation had been sanitized, following step 1, the surface culture medium was removed from all six hydrogel dishes and discard in the 50ml Falcon tube labeled waste. Next, 1ml of DBPS was added to each dish, which acts as a buffer to maintain the cell medium.

14. 4% paraformaldehyde: The hydrogel dishes were then moved over to the "chemhood," where the 1ml of DBPS was discarded and 1ml of 4% paraformaldehyde was added onto each dish, which helps the cells forms crosslinks between each other. For 15 minutes, the hydrogels were incubated at room temperature. *As a note, the class was told that paraformaldehyde is toxic by skin contact and should be handled with caution.* When the timer was up, the 4% paraformaldehyde was discarded, and 1ml of DBPS was added onto each dish. The addition and removal of 4% paraformaldehyde was repeated three additional times.

15. Imaging: When this step was completed, one last 1ml of DPBS was added to each dish and then the cells were imaged under a bright field microscope. When the imaging was done, the dishes were put back in incubator until the next class period.

IV. Cell staining with F-actin on varied hydrogel stiffness

16. Prepare F-actin stock solution: When it was time to stain the cells for fluorescence imaging, the dishes were taken to the "chemhood" and prepared using the F-actin staining kit with GFP conjunction phalloidins. These phalloidins help produce a low fluorescence background by binding the to the F-actin molecules ("Acti-stain 488). *Also noted that phalloidin is toxic and should be handled with caution.* To begin, the F-actin stock solution was centrifuged for four minutes, along with another centrifuge tube filled with water about the same weight. A 14uM solution was then created by taking the F-actin product at the bottom of the centrifuge tube and adding it to 50ul of 100% DPBS. The solution was then aspirated 3 times and divided into 12 centrifuge tubes with 10ul of the solution in each tube, then put in the refrigerator for storage.

17. Preparing cell culture for staining: For each hydrogel dish, remove the medium from the cultured dishes and discard solution in a 50ul Falcon tube labeled waste. When this was complete, 10ml of a solution called TrixonX-100 was added onto the cultured dishes to permeabilize the cells for staining and let sit for ten minutes in the chemhood. When the ten minutes was over, the cultured cells were rinsed with 1ml of DBPS and then discarded into the waste tube. Next, 1ml of 4% BSA in DPBS was added to each dish and incubated at room temperature for 20 minutes (Yang, BMEN 5210, instructions how to cell staining). The BSA was then removed and rinsed again with DPBS. The cultured dishes were then wrapped in aluminum foil and put in the refrigerator until the staining working solution was prepared.

18. Staining: To prepare the working solution, 3.5ul of F-actin stock solution from earlier was diluted with 500ul of PBS and aspirated three times. The cells were taken out of the refrigerator and three drops of the staining solution was added to each dish and incubated at room

temperature for 30 minutes. The dye was then removed, and the culture was rinsed with DPBS three times for two-minute intervals. A plastic film was added on top of the culture and sealed with the stock solution (DAPI). The dishes were once again wrapped in aluminum foil and placed in refrigerator until it was time to image the cells with the fluorescent microscope.

V. Imaging cardiac fibroblasts with fluorescent microscope on varied hydrogel stiffness

19. Imaging with fluorescence microscope: To image the cells on each stiffness, the Keyence fluorescence microscope, was set at 10x magnification and switching the GFP filter to the green fluorescence first. The images were taken on the computer and saved in a class file. The DAPI filter was then used to image the cells with the blue fluorescence. The images were once again taken on the computer and saved to a class file which were uploaded on Canvas to use later for data analysis.

III. Data analysis:

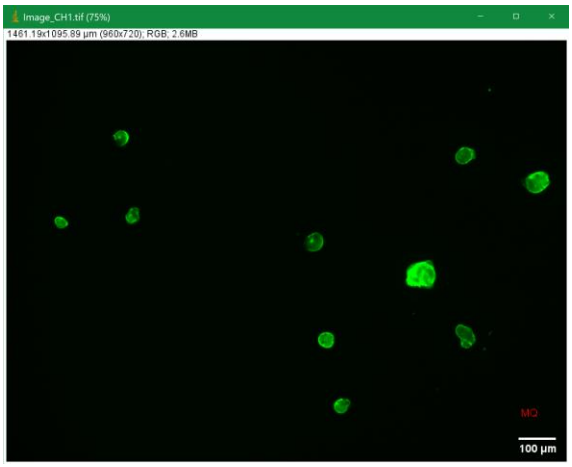
The data analysis for this experiment was conducted using the Keyence fluorescent microscope with CCD to take multiple images of the cells on each hydrogel stiffness. The magnification of 10x and 40x was used to capture the cells on a desktop computer for later data analysis. The green fluorescence was used to capture the cell sizes and the blue fluorescence was used to capture the cells nuclei. A general image without fluorescence was also taken for observation. After the cell images were distributed to the class, the software Image J allowed for further data analysis of the cell size, cell orientation, and nuclei size of the cells on each hydrogel stiffness.

To analyze the area of the smaller cells and nuclei, the 10x magnified images were converted to um. The threshold of the images was changed to a black and white scale to make a clearer image of the cell. The images were then converted to mask, the holes were filled and watershed. Analyze Particles was then used to obtain the results for each cell area and major and minor axes. This was done for the cells and nuclei images on 2kPa hydrogel stiffness and 8kPa hydrogel stiffness. To analyze the cell area of the larger cells on the 50kPa, the cells in the green fluorescence image were measured using the fit ellipse tool on the Image J software. The data collected from Image J was then put in an Excel spreadsheet to create bar graphs that would allow for further analysis for comparing the areas of the cell.

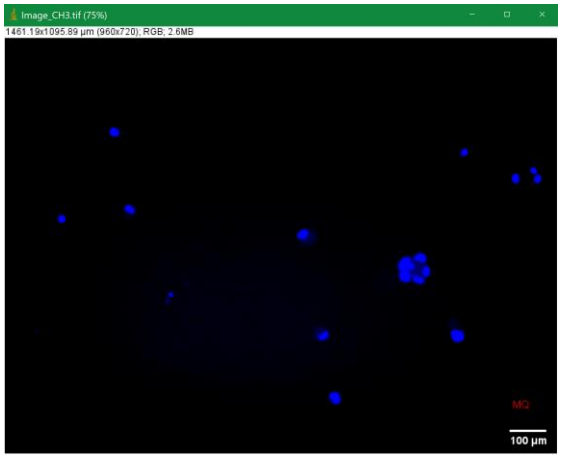
III. **Results**:

Figure 1: Each stiffness has one green, one blue, one merged of green and blue, and one phase-contrast (the grey image) image.

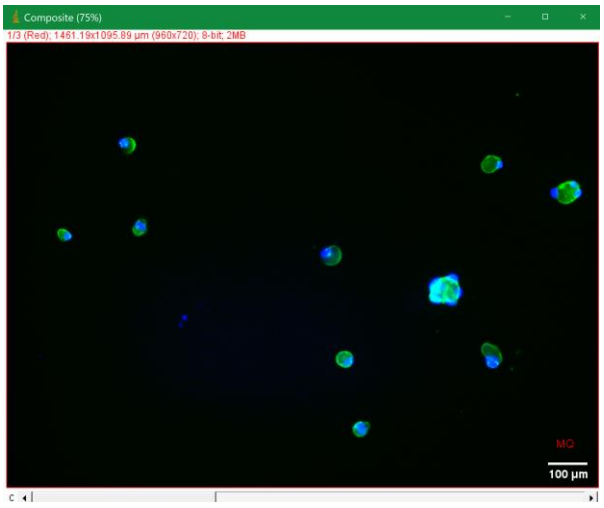
2kPa



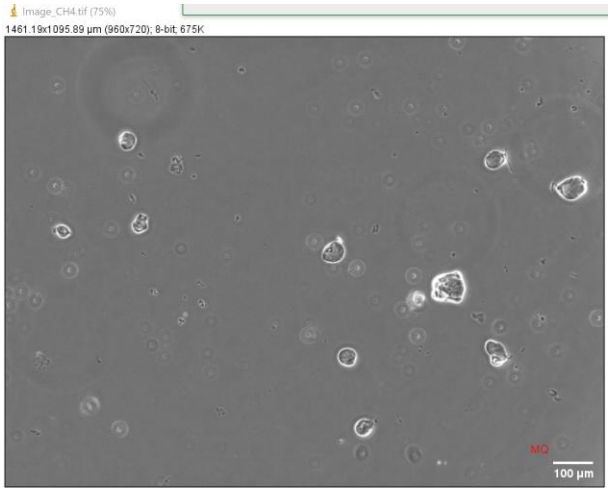
Green



Blue

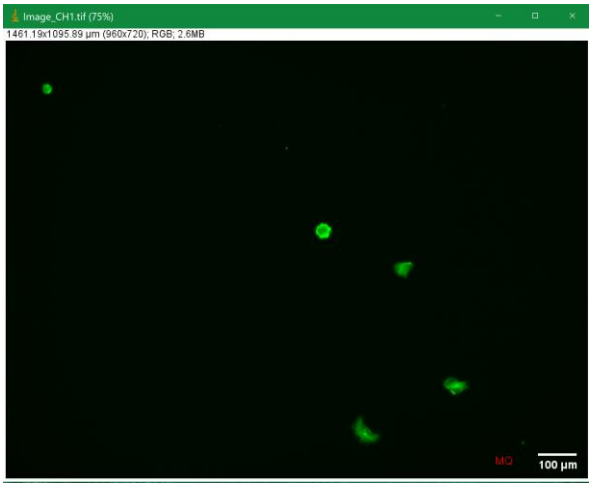


Merged

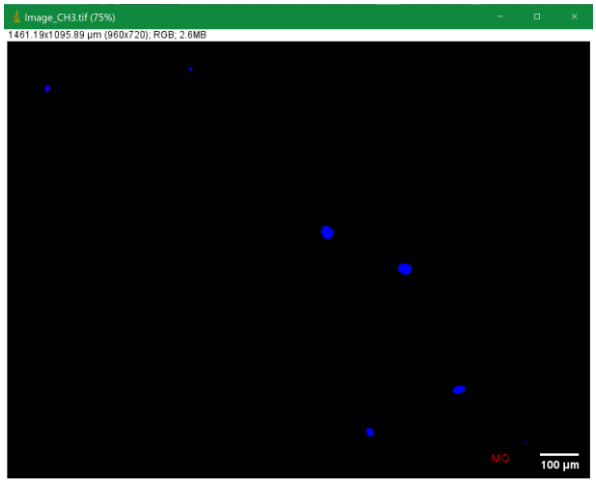


Phase-Contrast

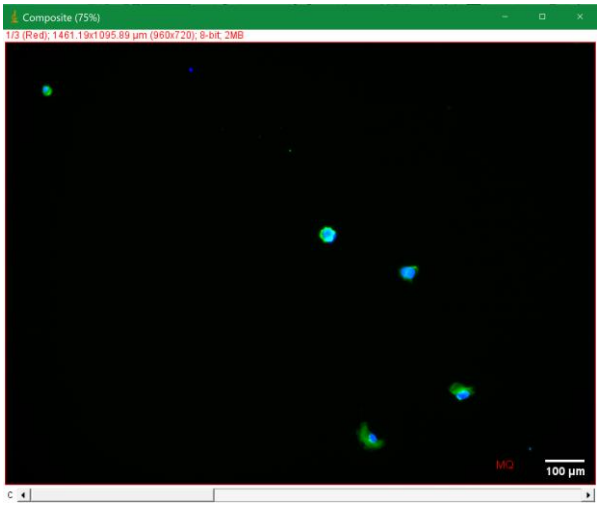
8kPa



Green



Blue



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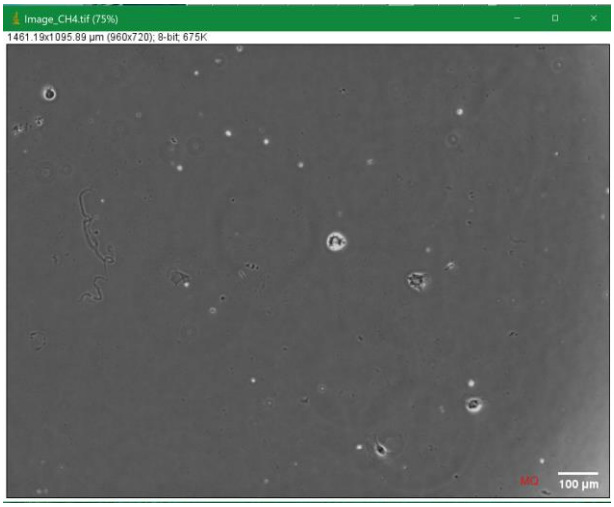
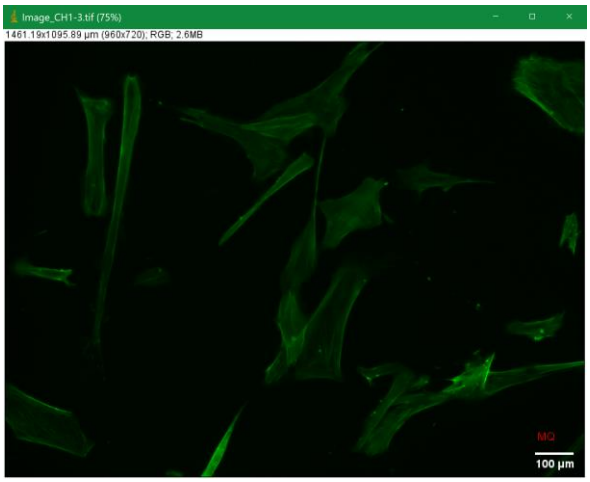
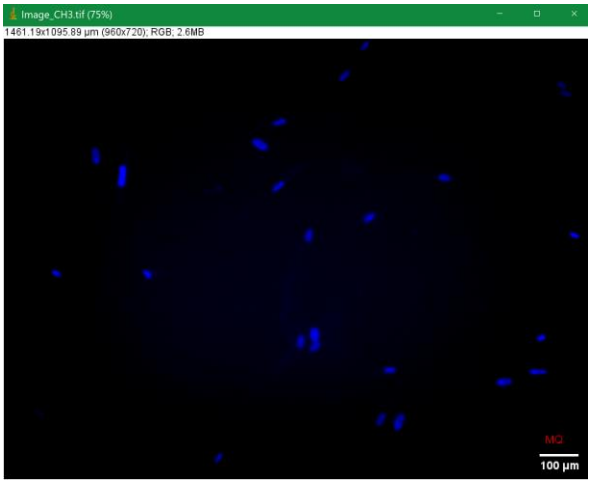


Phase - Contrast

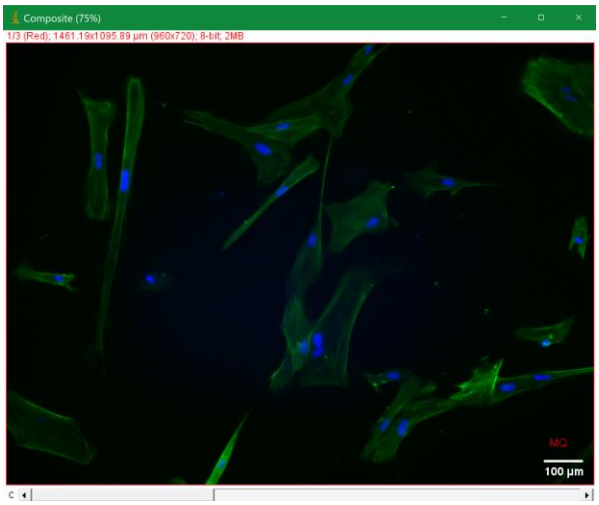
50kPa



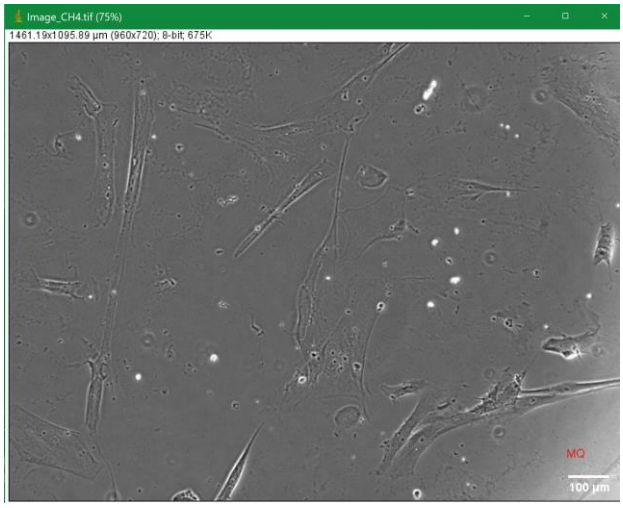
Green



Blue



Merged



Phase - Contrast

Figure 2: Average cell size (area) distributions of 2, 8, and 50 kPa compared in one bar graph with standard deviation.

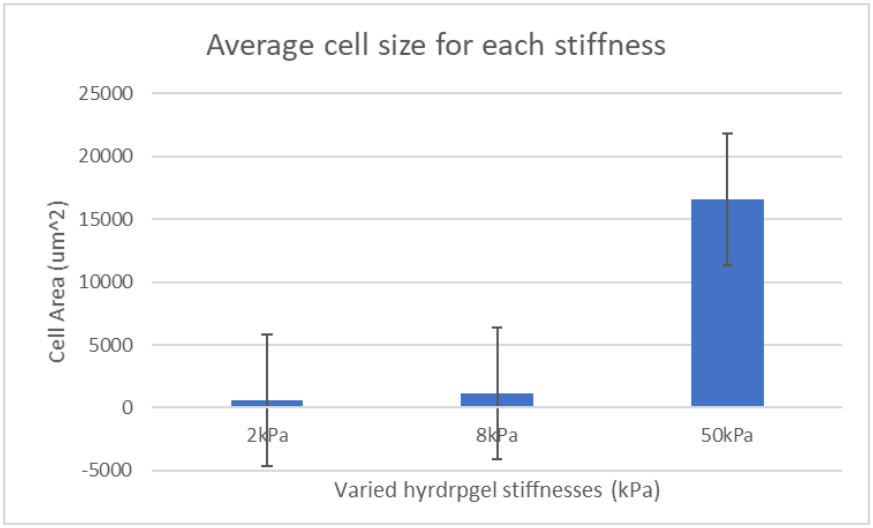


Figure 3: Average cell orientation in the ratio of major to the minor axis of 2, 8, and 50 kPa compared in one bar graph with standard deviation.

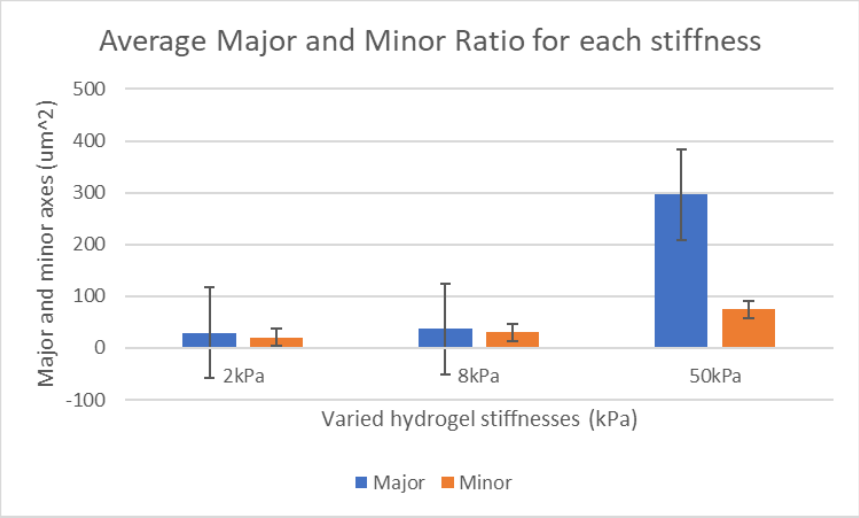
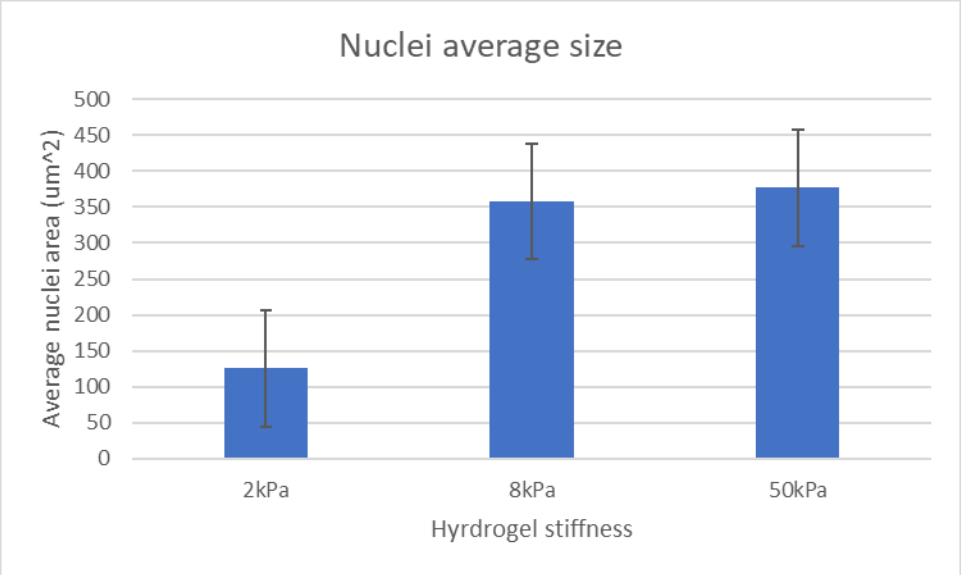


Figure 4 Average nuclei size (area) for 2kPa, 8kPa, and 50 kPa compared in one bar graph with standard error (SE). The Student's t-test was also used to compare the stiffnesses from each other.



T-test:	2 and 8	2 and 50	8 and 50
	0.021097	0.000607	0.877507

IV. Discussion:

This experiment was conducted to understand how controlling hydrogel stiffness is important for cell culture, because different tissues and cells have unique reactions under various hydrogel stiffness. Depending on the goals of the project, controlling hydrogel stiffness for cell culture could influence cell differentiation, cell-to-substrate interaction, fibril alignment, tissue regeneration, cell migration, cell spreading, and other cellular behavioral factors (Caliari, 2016). According to the results from this experiment, it can be concluded that each stiffness had a different cellular response in the context of fibrosis. This can physically be observed with the fluorescent microscope images in Figure 1. Both the 2kPa and 8kPa hydrogel stiffnesses are around the same size and do not have the long strains of fibroblasts as seen in the 50kPa. The differences of cellular response can also be observed graphically, with 50kPa having the largest cell area and major and minor

orientation compared to the 2kPa and 8kPa. Interestingly, the nuclei for both the 8kPa and 50 kPa were on average almost the same size with similar standard deviation errors. Also having a similar in vivo environment using the Extracellular Matrix coating on the cultured flask, may have also aided in cell growth, cell distribution, and migration.

This experiment was also helpful in understanding the relationship between collagen stiffness and other cardiovascular pathophysiological conditions, such as cardio fibrosis. From further research, cardiac fibrosis is known to be an abnormal thickening of the heart valves due to the activation of cardiac fibroblasts, which in turn produces an irregular buildup of the cardiac Extracellular Matrix (Herum, 2017). With this condition, the activation is caused by the increased stiffness which may stretch the cells similar to those seen in Figure 1 for the 50kPa hydrogel stiffness.

V. Conclusion:

In conclusion, this experiment helped to demonstrate how hydrogel stiffness affects the behavior of cardiac fibroblasts. By seeding and passaging the cardiac fibroblasts cells onto different hydrogel stiffnesses, we were able to stain the cells and image them under a fluorescent microscope and analyze the structures of the cultured cells. The analysis showed that an increase in hydrogel stiffness, lead to an increase in the major axes of the cells. Learning how to conduct similar experiments, this could eventually help to understand how different cardiovascular pathophysiological conditions may be treated in the future. Overall, this was a very intellectually stimulating experiment where I have not only learned how to experimentally conduct human cell culture, but I also learned new techniques to analyze the collected data.

VI. References.

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