Role of Vimentin in dynamic stretching of fibroblast cells

A Major Qualifying Project Report

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Degree of Bachelor of Science in Physics

by

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Abstract

In this project, an experiment was conducted in order to examine the effects of vimentin on cellular dynamics. The response and relaxation process of normal fibroblast cells and vimentin knockdown cells were tested and compared. The result shows that the vimentin-knockdown fibroblasts are much easier to change orientation than the normal fibroblasts. However, the contamination problem could affect the accuracy of the experiment, and it is advised for future researchers to refine the protocol in order to eliminate contamination which might lead to more accurate results.

1 Introduction

1.1 cellular response to external mechanical stretch

Human body is constantly subjected to external mechanical forces which could affect organ, tissues and cells at all levels. Muscle pulling tendons and bones is a classic example. Another example is the contraction of heart pumping blood throughout our body. The central player of this process is the mechano-sensitive cells inside our body, such as the fibroblast in ligaments and skins, chondrocytes in articular cartilage, and endothelium of blood vessel [1].

All of the mechanical forces inside human body can be roughly classified into three different categories: the tensile force, the compression force, and the shear force [1]. The tensile force refers to the force that is pulling outward from the surface perpendicularly. The compression force refers to the force that is pushing inward into the surface perpendicularly. And the shear force refers to the force that is pulling parallel to the surface [2]. Different types of cells are subjected to different mechanical forces, either it be in one form or as a combination of them. For example, the endothelia cells in blood vessel constantly reorient due to the shear stress caused by changes in blood pressure. It's also subjected to a combination of tensile forces as a result of vessel expansion and hydrostatic pressure [1]. Fibroblast cells, which are found in connective tissues, are typically aligned with its long axis parallel to the tendon or ligament. Therefore it is constantly under uniaxial (one direction) tensile force in vivo [1].

The purpose of this project is to investigate how vimentin could affect cellular response to external stretching by using fibroblast cells. By stretching PDMS substrate cyclically, it has been proved possible to mimic fibroblast in vitro condition.

1.2 Previous experiment in cell alignment under uniaxial stretching

The fibroblast response to external stretch is totally different depending on the dynamics of stretching. There are two types of fibroblast's uniaxial condition of stretching in vivo: the static stretch and the cyclic stretch. In static stretch, the cells tend to align parallel to the direction of stretching, whereas in cyclic stretch, the cells tend to align away from the direction of stretching. One interesting condition of cyclic stretch is the uniaxial stretch at high frequency (around 1Hz). In this case, the cells align perpendicularly to the direction of stretching [3]. Figure 1 shows a picture of such scenario from this project. This picture was taken immediately after the fibroblasts were stretched uniaxially for 4 hours. The direction of stretching was horizontal. It is clear from the picture that cells are typically aligned perpendicular to the direction of stretching. There are some variations, but it is clear that the fibroblasts' average positions are perpendicular to the direction of stretch.

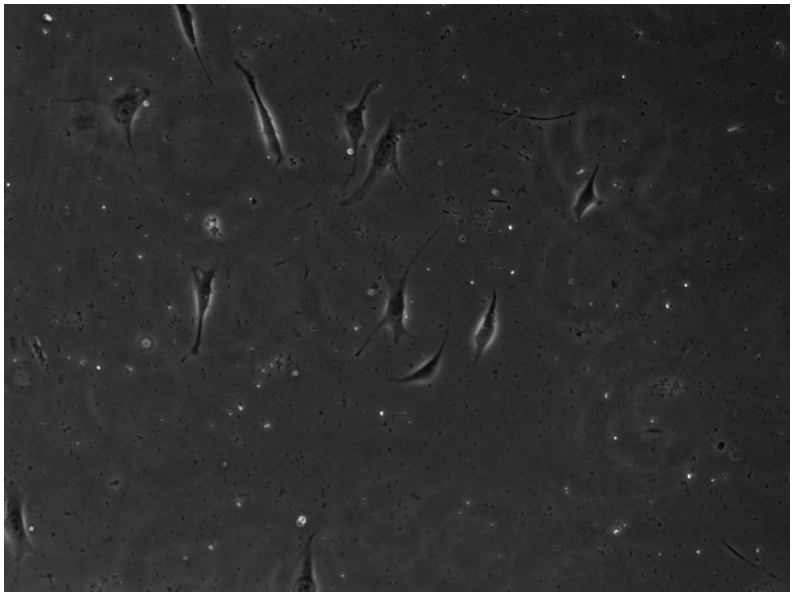


Figure 1 picture showing normal fibroblast cells after 4-hour stretching at 1 Hz

One very interesting analogy of such fibroblast response is electric dipole in an electric field. If an electric dipole is placed in a static electric field, it tends to be aligned parallel to the direction of the field as shown in figure 2 [8]. If an electric dipole is placed in a cyclically oscillating electric field, it tends to

oscillate accordingly. However, the average position of oscillation is perpendicular to the electric field. The fibroblast response to external mechanical stretch is exactly the same as the electric dipole model.

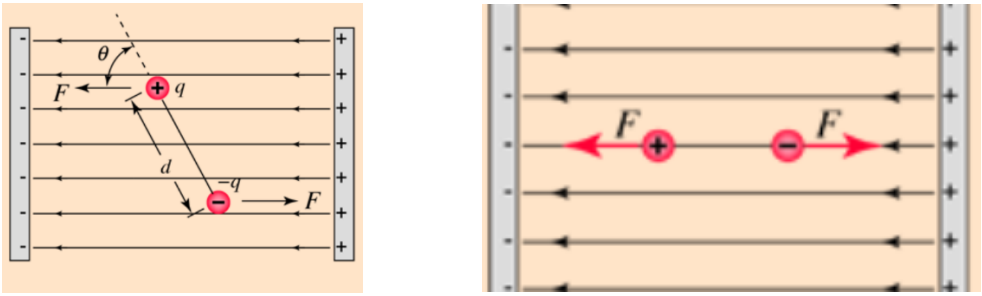


Figure 2 schematic of electric dipole in electric field [8]

In the case of static stretching, the fibroblast cells align parallel to the direction of stretching; and in case of cyclic stretch at frequency of around 1Hz, the fibroblast cells align perpendicular to the direction of stretching.

1.3 Role of cytoskeleton in cell response to stretching

According to De, Rumi, at low frequency (corresponding to the scenario of static stretch), cells have sufficient time to relax and therefore aligns parallel to the direction of stretching [3]. However, cells at high frequencies align perpendicularly in order to minimize the force exerted on them since the cells cannot adjust to the magnitude of applied force in a timely manner. This results from the interaction between cellular activity and stretching which does not occur under low frequency since the cells have time to adjust its focal adhesion and stress fibers. Cytoskeleton is the key structure that drives this cellular behavior [3]. Cytoskeleton is a complex cellular network extending throughout cytoplasm and can transmit force from cell membrane to nucleus (which prompts the cell to react to external forces). It plays a critical role in maintaining cell shape and provide mechanical resistance to cellular deformation [4]. Cytoskeleton mainly consists of actin filaments, microtubules, and intermediate filaments (IF). When cells are constantly under external cyclic stretch, the cytoskeleton cannot settle down to a fixed position.

As a result the cells tend to be fixed at an average position which is perpendicular to the direction of stretch.

Vimentin is a type III Intermediate filaments protein inside cytoskeleton. Vimentin is believed to be the key component that maintains cell integrity and provides cells with a resilience in the absence of actin filaments and microtubules. It has been found that cells with vimentin knocked down tends to become delicate when subjected to external forces [5].

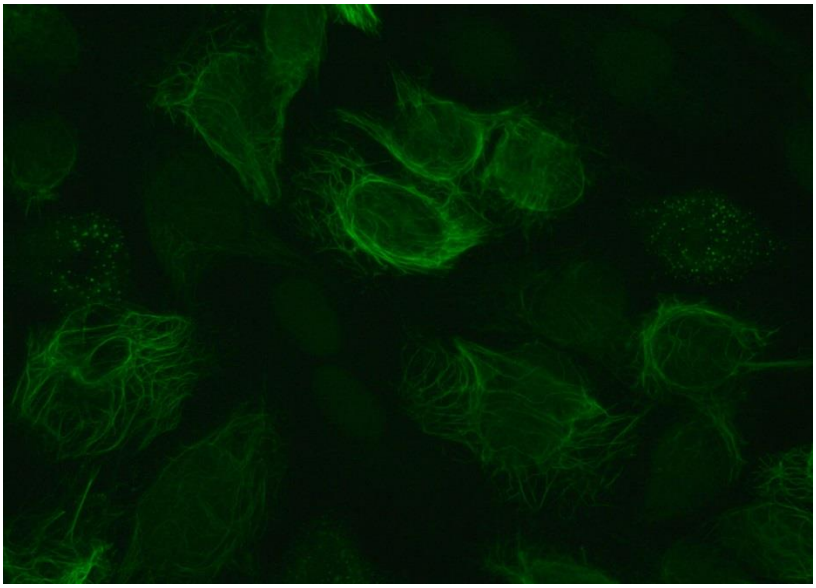


Figure 3 Immunofluorescence staining pattern of vimentin [7]

The primary task of this project is to investigate how the knockdown of vimentin could affect cellular dynamics to external stretch. The normal fibroblast tends to be aligned perpendicularly when it is subjected to cyclic stretch and remains roughly in the same alignment position after stretching. This project intends to address the question if the fibroblast would still exhibit the same dynamical behavior provided that the vimentin inside fibroblast is knocked down,

2 Experiment Procedure

2.1 General procedure

Figure 4 shows the general procedure of the experiment. The entire experiment is roughly divided into seven parts. First, a PDMS substrate is made with the mold provided by Professor Billiar from Department of Biomedical Engineering. Then alignment markers on the PDMS substrate is drawn in order to identify the area of uniform strain. This step is necessary since the PDMS substrate is not under uniform strain during stretching. It has been found that only the central part of the PDMS substrate, which has an area of 0.64 square centimeters and can be roughly identified as a square sided at 8 millimeters, is under uniform strain [2]. The third step is to put a layer of collagen on the PDMS substrate to assist cell attachment. Then cells are cultured on the PDMS substrate. The PDMS substrate is stretched and imaged subsequently. Finally the images are analyzed in ImageJ.

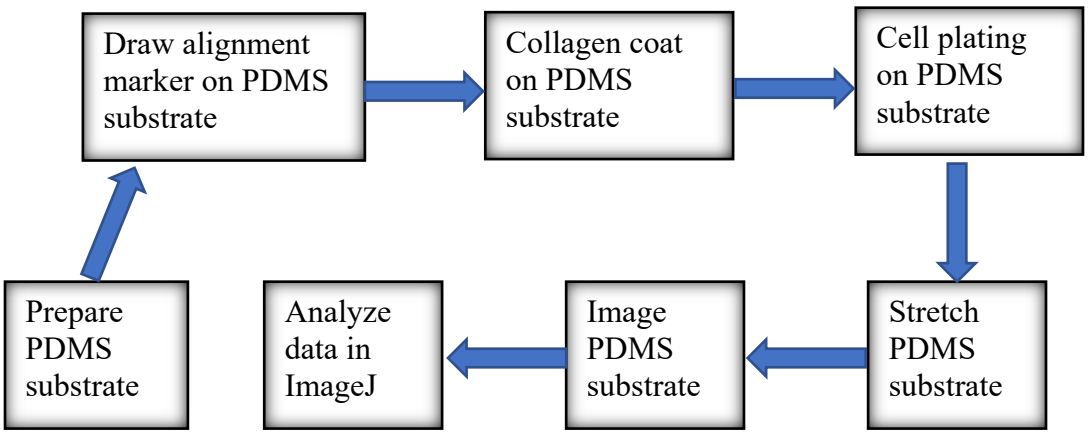


Figure 4 experiment procedure flow chart

2.2 Cell line

There are two cell lines used in this project. They are called 3t3 cell line and 117 cell line respectively. 3t3 is the standardized fibroblast cell line established by George Todaro and Howard Green at New York University [6]. 117 cell line is the vimentin knockdown version of 3t3 cell line. In order words, the only difference between 3t3 cell line and 117 cell line is whether vimentin had been knocked down. (3t3 is the

normal fibroblast and 117 cell line is the vimentin knockdown cells). Figure 5 shows the morphology of 3t3 cell line (left) and 117 cell line (right) respectively.

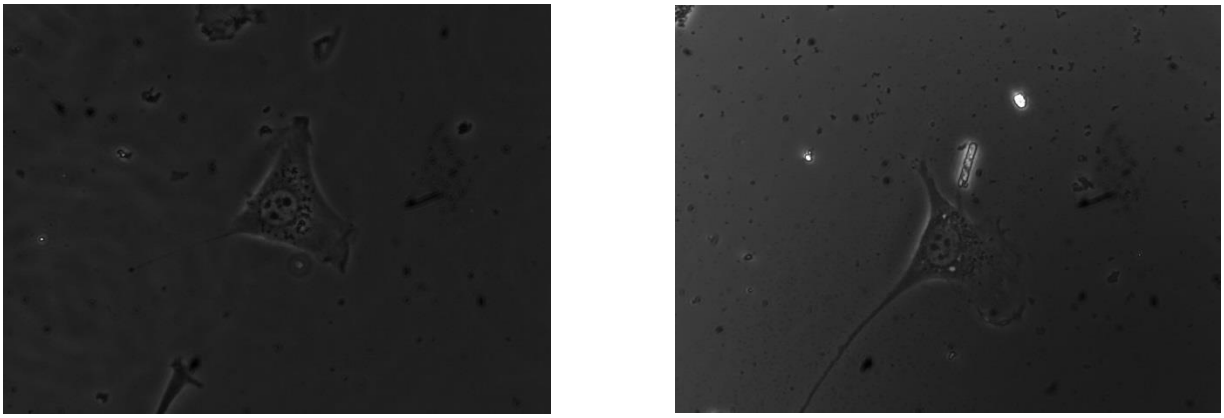


Figure 5 images of 3t3 normal fibroblast cell (left) and 117 vimentin-kockdown fibroblast cells3.3 Prepare PDMS substrate

2.3 PDMS Substrate

2.3.1 Material and Equipment

a) PDMS mold

b) Silicone Elastomer Base and Silicone Elastomer Curing Agents

c) Screw driver, fliers, chemical spoon and scissor

d) Vacuum pump and 70 Celsius oven

2.3.2 PDMS mold

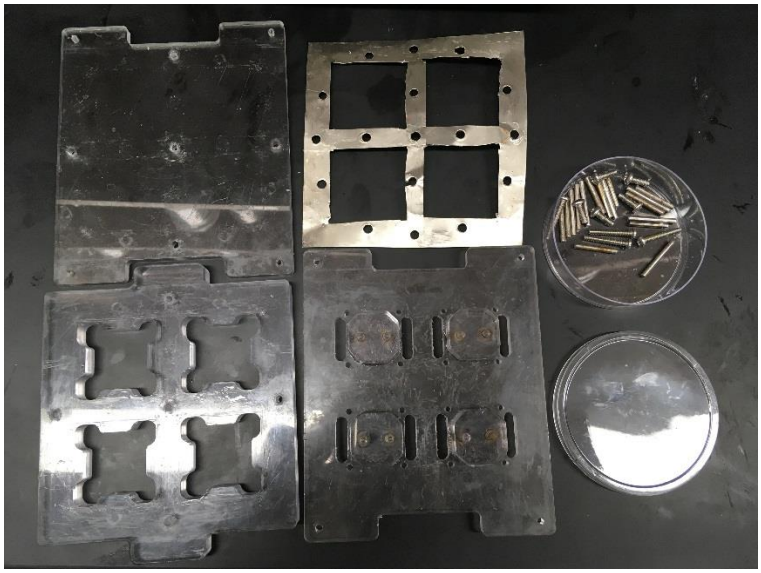


Figure 6 Complete set of PDMS mold

Figure 6 shows the complete set of PDMS molds. It mainly consists of five parts (clockwise starting from the top-left component): the base, the iron sheet, screws, well, and cap. There are three types of screws as shown in figure 6: five short screws, four long screws, and sixteen pins. Each type of screws has one specific usage. Figure 6 also shows an assembled PDMS molds without cap.

Figure 7 three types of screws (left) and PDMS mold (right)

2.3.2 PDMS solution

The PDMS solution is a mixture of two different types of solutions. The first solution is called “Silicone Elastomer Base”, or simply referred to as the “base solution”. The second solution is called “Silicone Elastomer Curing Agents”, or simply referred to as the “curing agents”. None of the two solutions are detrimental to human health. However, it is still advised to operate with gloves on since they are highly viscous (especially for the base solution). The ratio between the base solutions to curing agents is 10:1. In this project, 30 grams of base solution and 3 grams of curing agents are used to make 4 PDMS substrates.

2.3.4 Protocol

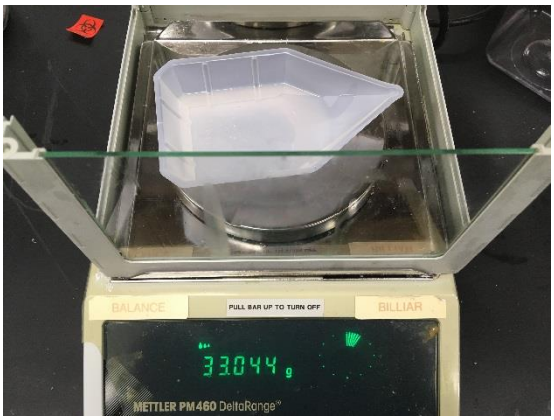


Figure 8 Cup with PDMS solution on a scale

1) Prepare base solution and curing agent solution in a plastic cup. Put the cup on the scale before putting solution in it. Zero the scale.

Figure 9 PDMS solution before mixture (left) and PDMS solution being mixed with a chemical spoon (right)

2) Pour 30 grams of base solution inside the cup, then pour 3 grams of curing agent solution inside the cup. Stir the container with a chemical spoon in order to completely mix the two types of solution.

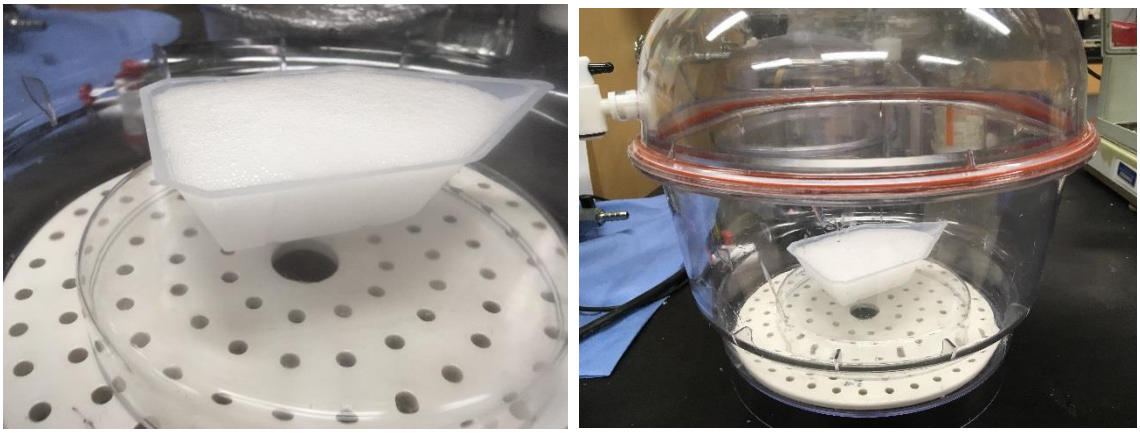


Figure 10 PDMS solution in vacuum chamber. A culture dish cap is placed beneath the cup to keep the cup leveled

3) Put the cup with PDMS solution inside a vacuum container. Turn on the vacuum valve. Once the bubble nearly gets spilled outside the cup, turn off the vacuum vale and pump air back into the container. This process needs to be repeated for a number of times in order to completely remove the air inside the solution. In order to keep the cup leveled, it is suggested to put the cup on top of a culture dish cap as shown in figure 10.

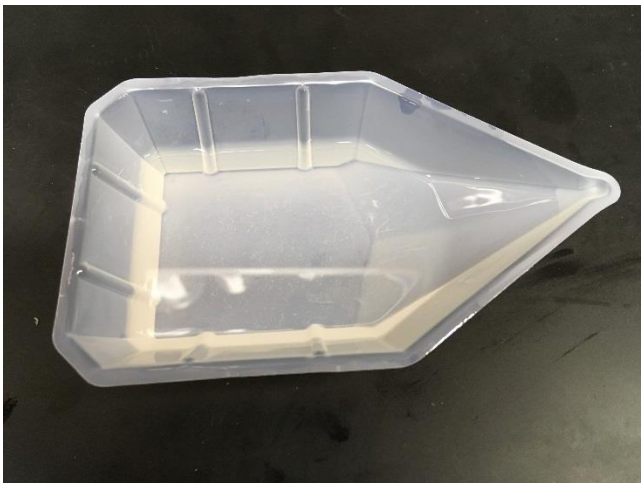


Figure 11 PDMS solution with air removed

4) Once all bubbles have disappeared, put the cup on the stage. A solution fully devoid of air is shown in figure 11.

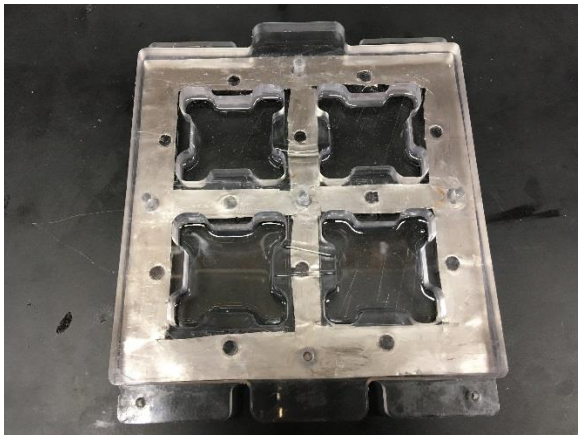
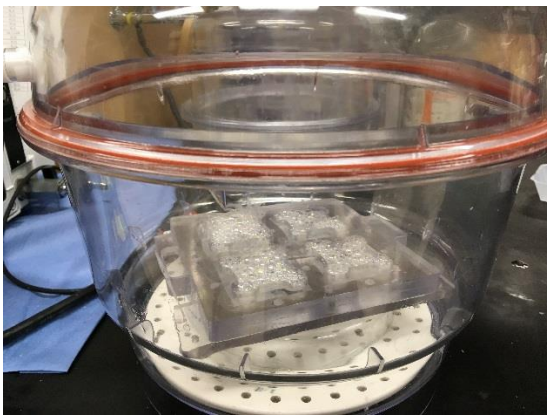


Figure 12 Assembled PDMS mold without cap

5) Assemble the PDMS mold.[1] Pour the PDMS solution into the four wells located in the mold. Since we still do not have a dependable method to measure the amount of PDMS solution poured into each space, please operate carefully.

[1] Order of each component from bottom to top: base, iron sheet, and well. Use only the five short screws for now.



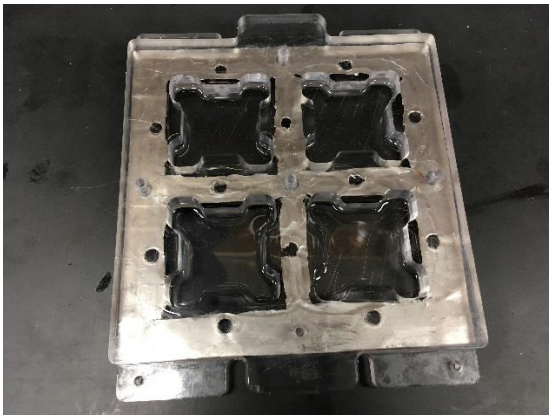


Figure 13 PDMS solution in vacuum chamber (left) and PDMS solution devoid of air inside the mold (right)

6) Put the mold with the PDMS solution inside the vacuum container. Turn on the vacuum valve. It is okay if the PDMS solution bubbles get spilled outside the spaces. My experience has shown that it is impossible to completely remove air from the PDMS solution once it is poured into the mold. But ten minutes inside the vacuum container is enough to reduce air concentration in the PDMS solution to a minimal level. In order to keep the mold leveled, it is suggested to put the mold on top of a culture dish cap as shown in figure 13.

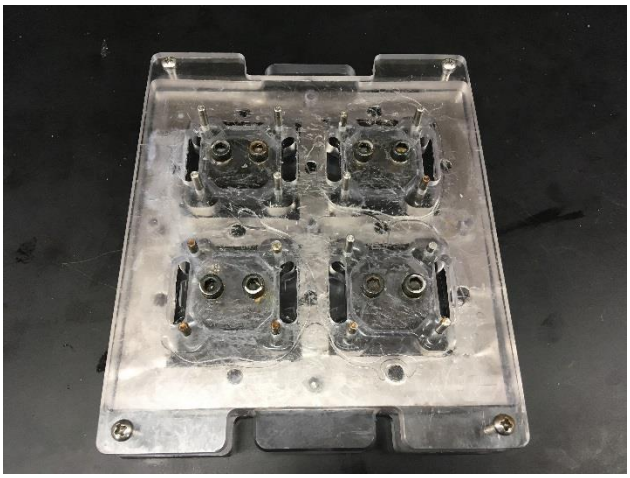


Figure 14 assembled PDMS mold with cap

7) Put on the cap. Use screwdriver to tighten the four long screw at the four corners of the mold. Insert the pins into the corresponding 16 slots on the mold respectively. Press the pins to make sure they are fixed in position.

Figure 15 PDMS mold inside 70 Celsius oven. A cell culture dish is put beneath the mold to keep it leveled

8) Put the assembled mold inside 70 Celsius oven for two hours. It is suggested to put the mold on top of a culture dish cap in order to keep the mold leveled.

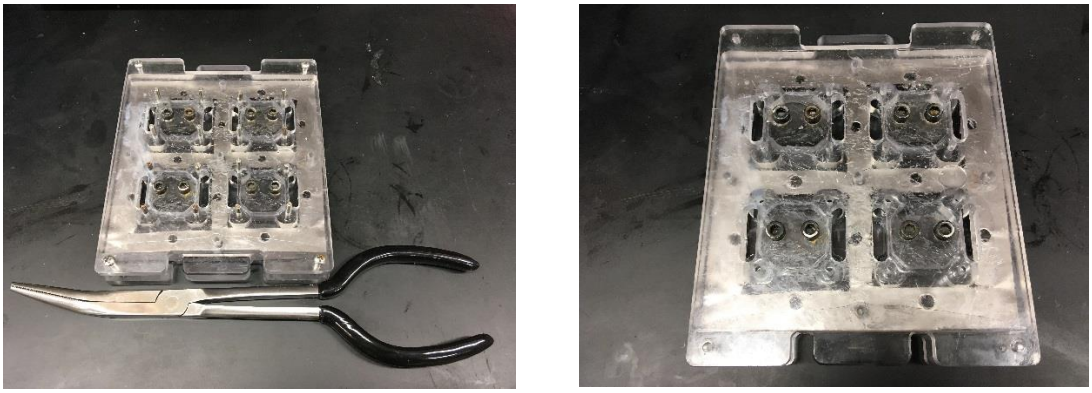


Figure 16 PDMS mold immediately after heating (left) and PDMS mold with screws removed (right)

9) After two hours inside the 70 Celsius oven, take the mold outside. The PDMS solution should have already gelled up. First use pliers to remove the 16 pins from the mold. Then use the screw driver to remove the four long screws from the four corners of the mold.

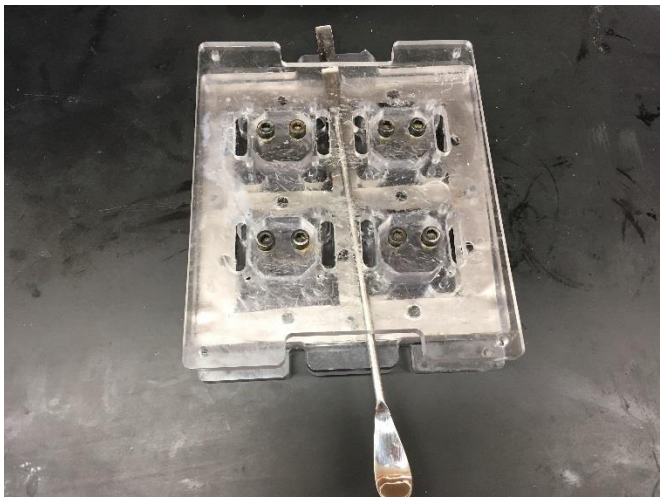


Figure 17 Use chemical spoon to prize up the PDMS mold cap

10) Remove the cap. Since it might be glued tightly to the mold, it is suggested to insert chemical spoon between the well and the cap.

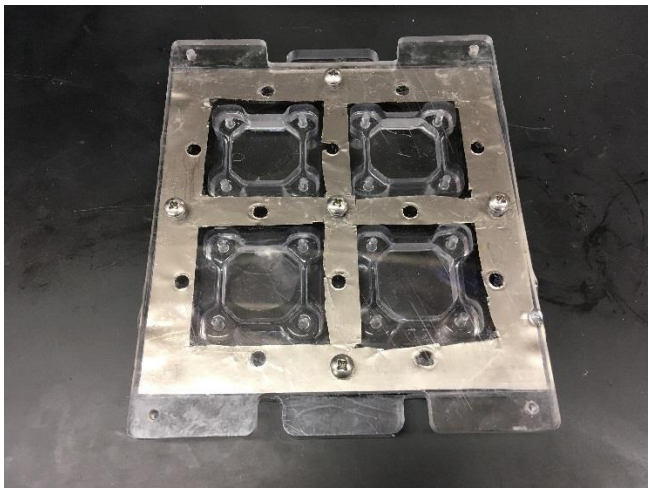


Figure 18 flip PDMS mold to remove the five short screws

11) After mold cap is removed, flip the mold. Use screw driver to remove the five short screws.

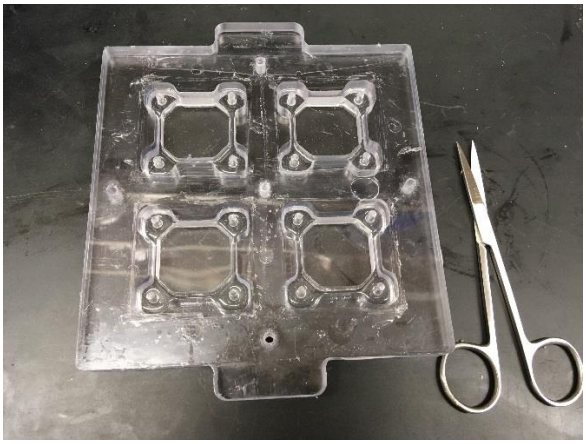


Figure 19 PDMS well with PDMS substrate

12) Remove the base and the iron sheet. It is suggested to use a scissor to assist this procedure.

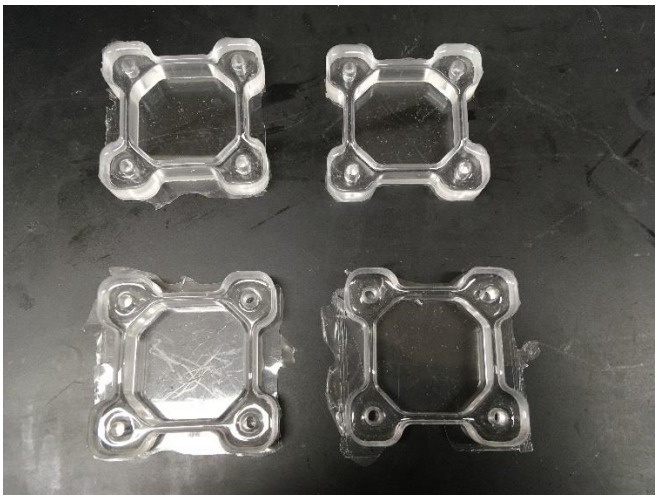


Figure 20 Completed PDMS substrate. The top- right has been trimmed by scissor. The other three substrates are yet to be trimmed.

13) Carefully remove the four PDMS substrates from the well. Please be careful as the PDMS substrates are very susceptible to breaking. After the PDMS substrates are successfully removed from the well, it is suggested to use scissor to cut any unnecessary parts. The top right PDMS substrate in Figure 19 has been fully trimmed. The other three PDMS substrates are yet to be trimmed.

2.4 Draw alignment markers on PDMS substrate

2.4.1 Material and equipment

a) One PDMS substrate

b) One 60mm culture dish cap and one 100mm culture dish

c) 70% ethanol

d) Ruler and Pen

e) Hood

2.4.2 Protocol

1) Completely submerge the PDMS substrate and culture dish cap inside 70% ethanol for at least one hour.

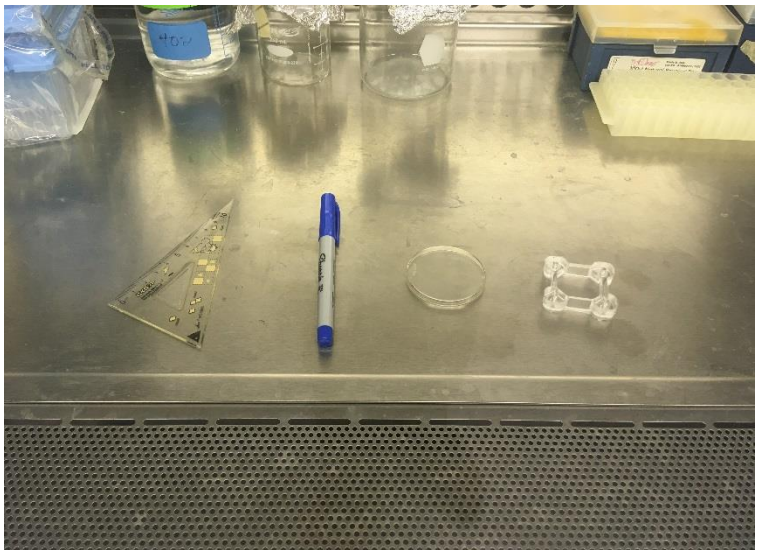


Figure 21 put the ruler, pen, culture dish cap and PDMS substrate inside the hood under UV light for sterilization. Please note this picture does not show UV light as it is harmful to human eyes.

2) Put the ruler, PDMS substrate, culture dish cap and pen inside hood. Turn on UV light[2] for at least 30 minutes to completely sterilize the aforementioned items.

[2] Try to avoid staring straight into the UV light as it is harmful to human eyes

Note: Step 3 to step 7 must be operated inside hood. In the figures the items were placed outside hood. This is only for illustration purpose and should not be regarded as norm.

Figure 22 picture showing step 3

3) Flip the PDMS substrate so that the bottom faces up. Keep the ruler aligned with one pair of two inner sides of the PDMS substrate. Draw one alignment marker at 10 millimeter from one side, and another alignment marker 8 millimeter from the first marker.

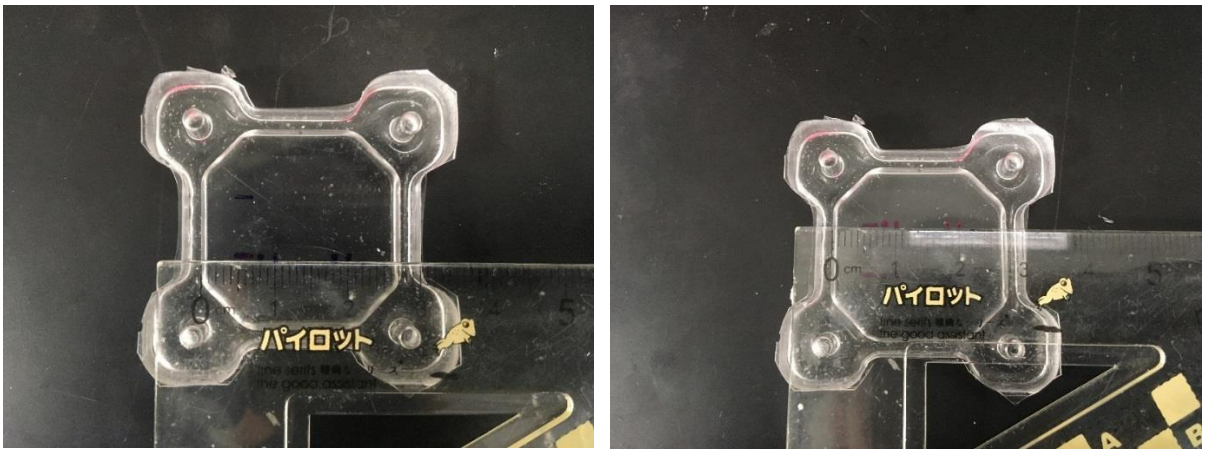


Figure 23 picture showing step 4

4) Rotate the PDMS substrate counterclockwise for 90 degrees. Keep the ruler aligned with one pair of two inner sides of the PDMS substrate. Keep the ruler also aligned with one of the alignment

marker drawn in step 1. First draw one dot located 10 millimeter from one side, then draw the second dot located 8 millimeter from the first dot. Draw the third dot located 2 millimeter on the left side of the first dot, and draw the fourth dot located 2 millimeter on the right side of the second dot. Repeat the above steps at the second alignment marker to draw another four pairs of dots.

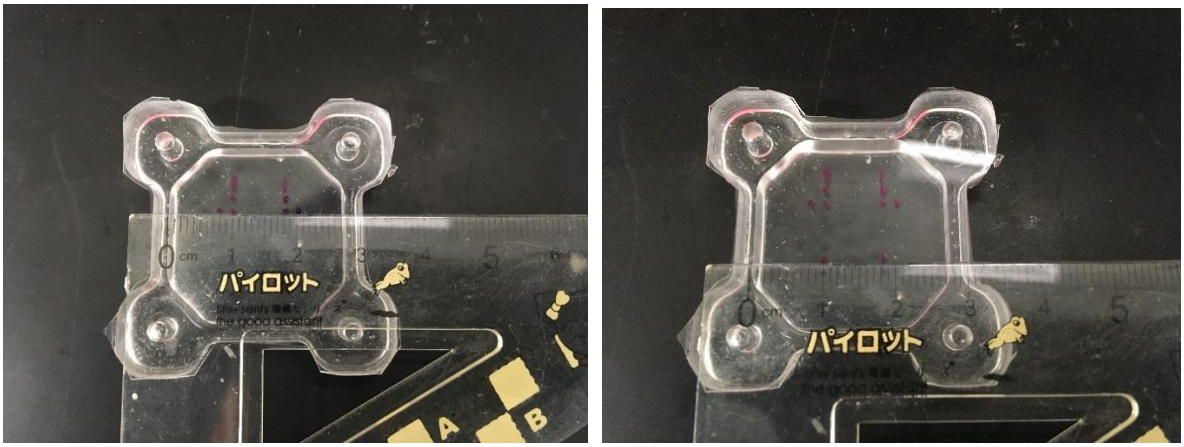


Figure 24 picture showing step 5

5) Again rotate the PDMS substrate clockwise for 90 degrees. Keep the ruler aligned with one pair of two dots drawn in step 2. Draw one dot located 2 millimeter on the left side of the left dot, and draw another dot located 2 millimeter on the right side of the right dot. Repeat the above steps for the second pair of two dots drawn in step 2.

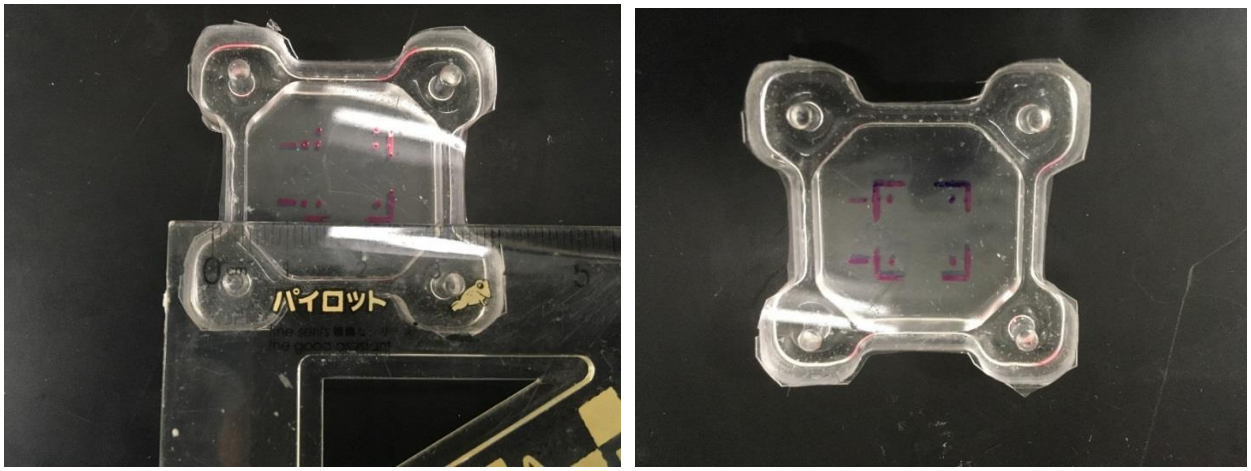


Figure 25 Picture showing step 6 and completed pen alignment markers on a PDMS substrate

6) Connect the eight dots located close to edge as shown in figure 24. The inner four individual dots represents the area of uniform strain which is also the area for imaging.
7) Put the PDMS substrate with alignment markers on top of the 60mm culture dish cap. Then put the entire setup inside a 100mm culture dish. The 100mm culture dish is large enough to contain the PMDS substrate in order to insulate it from outside contamination. The 60mm culture dish cap is necessary. [3]

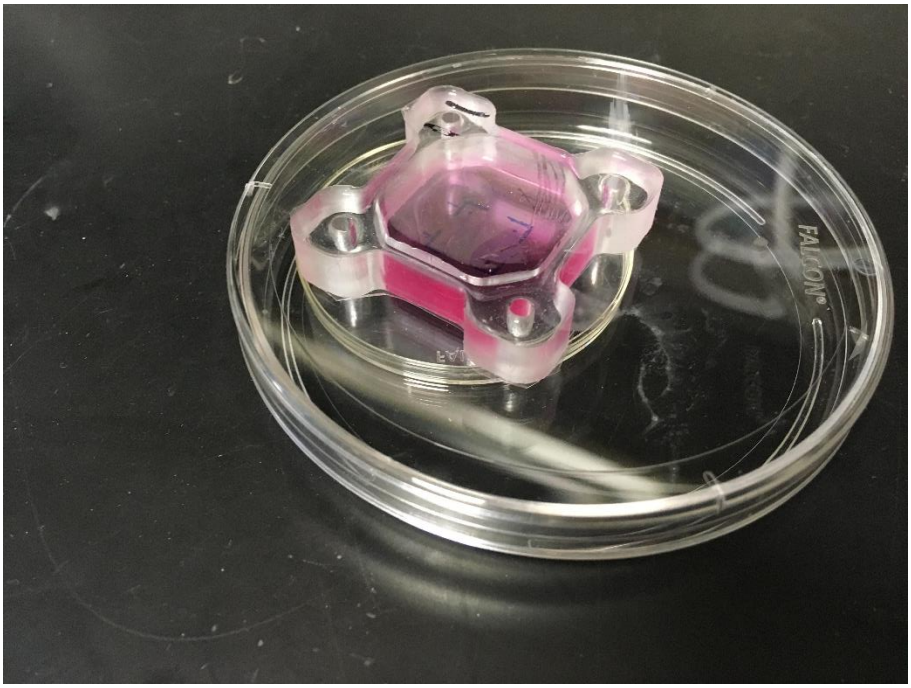


Figure 26 PDMS substrate inside the 100mm culture dish. It is placed on top of a 60mm culture dish cap in order to preserve the alignment markers.

2.5 Collagen Coat on PDMS substrate protocol

1. Submerge the culture dish cap and PDMS substrate completely inside ethanol for 1 hour. This procedure is to kill all existing germs attached on the PDMS substrate surface and cap.
2. Bring DPBS to the Hood

[3] This is because if the PDMS substrate is placed directly on top of the 100mm culture dish, the alignment markers could be detached from the substrate and attached to the culture dish instead.

3. Prepare 0.1 mg/ml Collagen-PBS solution.

    i) Take out 3 mg/ml Collagen bottle from fridge and put it inside Hood.

    ii) Dilute it 30 times with DPBS.[4] Usually prepare 1240 µl of Collagen-PBS solution. Put 1200 µl DPBS in a 1.5 ml Eppendorf and add 40 µl collage solution into the Eppendorf. Then vortex the Eppendorf for 10 seconds.

4. Lay the 1240 µl Collagen-DPBS solution in the PDMS substrate. Put the PDMS substrate inside a 100mm culture dish and parafilm the dish to insulate the PDMS substrate from outside contamination. Put the dish in the fridge and wait for 4-6 hours before plating cell.[5]

2.6 Cell Plating on PDMS substrate protocol

1. Prepare DMEM (culture solution), DPBS in 37°C water bath for 20mins. Put Trypsin in room temperature for 20mins.
2. While waiting for step 1, bring the collagen coated PDMS substrate from fridge and bring it inside Hood. Suck out the collagen.
3. Wash the Collagen PDMS substrate 3 times with DPBS. Wait for 10 minutes during the first wash. Wait for 5 minutes during the rest of two washes.
4. Lay 2000 µl DMEM on the PDMS substrate and put the dish in incubator for around 30mins.
5. After waiting for 20mins in step 4, start doing cell culture. Do everything in Normal Cell Culture[6] UNTIL finishing the step 7 in Normal Cell Culture.
6. Put 1000 µl cell liquid from the cell dish to a new 1.5mL Eppendorf. Centrifuge the Eppendorf for 5 minutes at speed of 1200 rev/min.
7. Take out the PDMS substrate from the incubator and suck out the DMEM in the PDMS substrate. Then add 1 ml DMEM into the PDMS substrate.[7]

[4] Pipetting the Collagen solution should be done very slowly as Collagen solution is very viscous.
[5] An alternative is to put the dish in room temperature and wait for only 1 hour.
[6] Please see Appendix for detailed steps of normal cell culture.
[7] Eventually the PDMS substrate should contain 2 ml cell culture solution in total.

8. Continue to do step 10 and step 11 of Normal Cell Culture. Transfer the 50 µl cell liquid from the Eppendorf to the PDMS substrate.
9. Check the PDMS substrate under 10X microscope. If the cell density is high, add 1 ml DMEM directly on top of the gel so that some of the cells would be washed away from the central part of the PDMS substrate. If the cell density is low, add 1 ml DMEM around the PDMS substrate so that the cells are kept above the central part of the PDMS substrate.
10. For vimentin-knockdown cells, add 0.4µl 1mg/ml Puromycin into the PDMS substrate. For further explanation please see step 13 of normal cell culture in Appendix.
11. Put the PDMS substrate with cells inside incubator and wait for 12 hours to do experiment.

2.7 Stretch

2.7.1 Materials and Equipment

a) Stretch device

b) Cell plated PDMS substrate

The stretch device is provided by Professor Kristen Billiar. It is the result of an MQP project finished in 2013 [2].

2.7.2 Protocol

As shown in figure 27, the entire cell stretch system consists of two major parts: the stretch device and the computer. And the computer includes the control box that transfers commands into the stretch device.

Figure 27 Left picture shows the entire stretching system. the right picture shows the computer and control box

1) Take the two foams and spray 70% ethanol on all sides. Dry the two foams by blowing. Then put them in 70 Celsius oven for 15 minutes to completely evaporate any ethanol left inside the foams.
2) Make sure the control box is turned off. Turn on the computer.

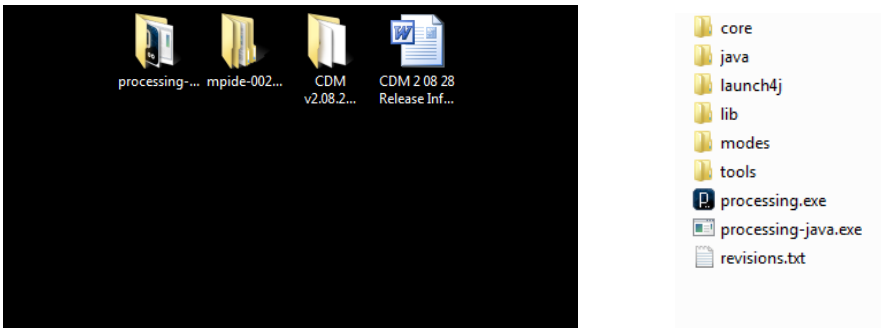


Figure 28 screenshot showing the location and content of the folder "processing-2.0b8"

3) On the desktop click on the folder "processing-2.0b8". Then click on "processing.exe".

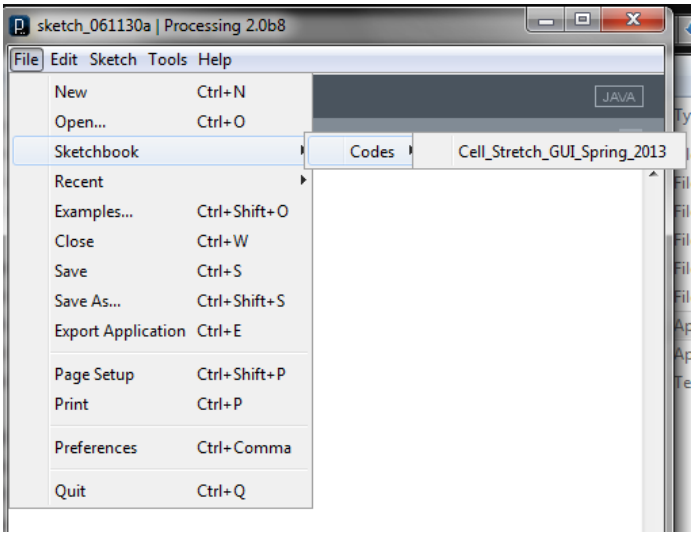


Figure 29 screenshot showing the position of the file "Stretch_GUI_Spring_2013"

4) After the program is turned on, click "File" →Sketchbook→codes→Cell→ Stretch_GUI_Spring_2013. Then click on "run" button. Another interface would appear as shown in figure 30.

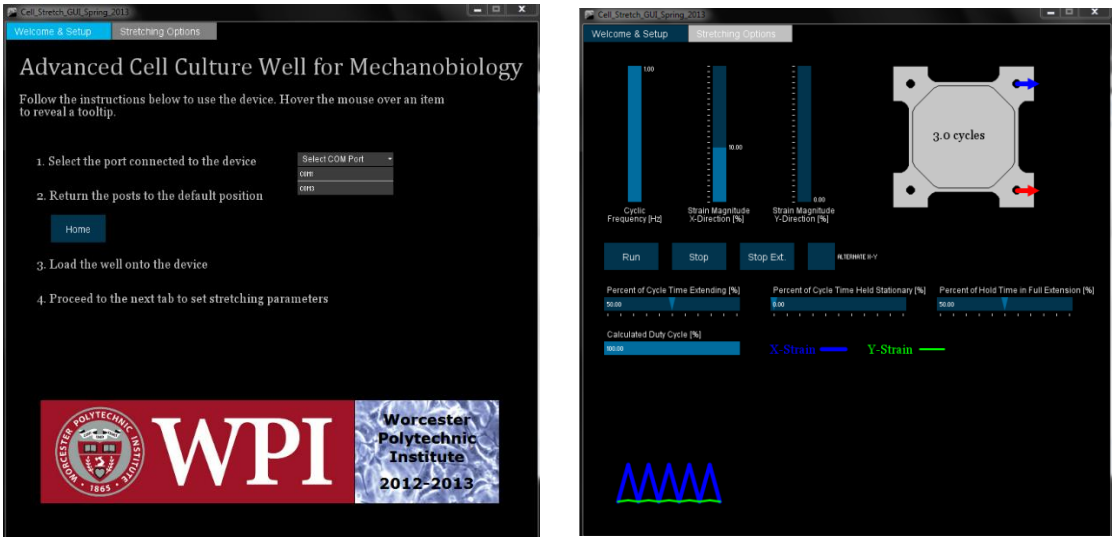


Figure 30 screenshot showing the operating page of the program

5) Connect the two portals with the stretch device as shown in figure 31. Turn on the control box.[8] Select "COM3" in the "Welcome & Setup" page. Then go to "Stretching options", select the desired stretch options. In this project the selected condition is "1 Hz, 10% strain in X-direction, 0% strain in Y-direction" as shown in figure 28. Click "Run" to check the stretch device is running correctly.

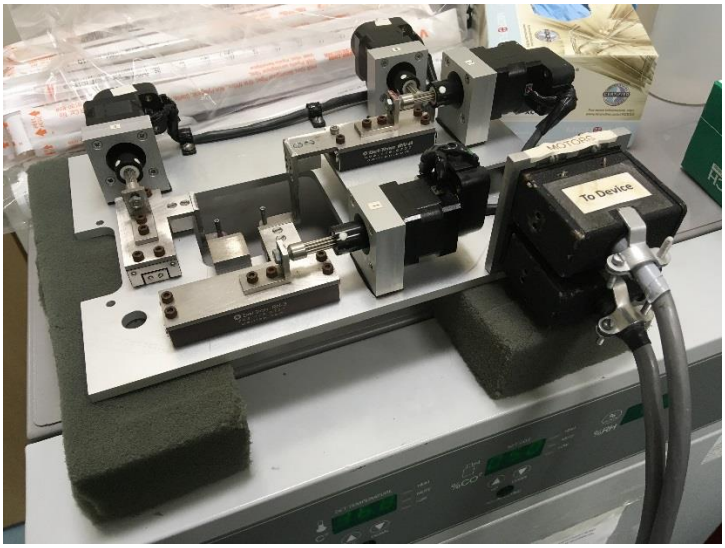


Figure 31 Stretch device with two portals connected.

6) Stop the stretch device. Put on the PDMS substrate with cells. Turn off the control box and disconnect the portals. Take out the foams from the 70 Celsius oven[9] and put in the incubator. Put the entire stretch device (with PDMS substrate) on top of the foams.

[8]Always connect the portals before turning on the control box. Otherwise the stretch device could be damaged.
[9] After around 10 minutes inside 70 Celsius oven, all ethanol left inside the foams should be evaporated already. This is necessary since ethanol inside incubator is harmful to the cells.

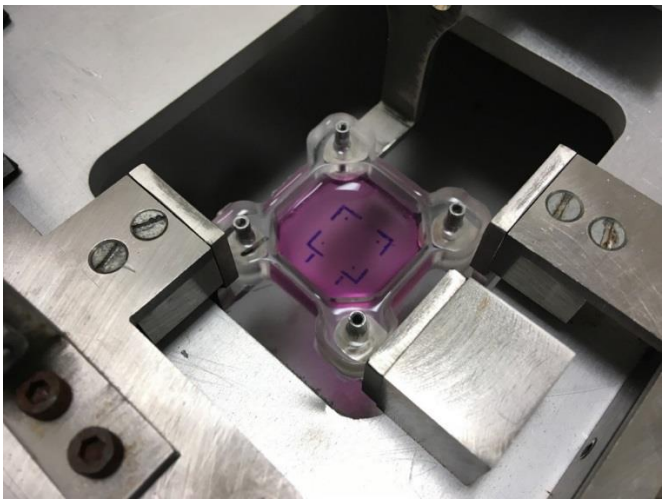


Figure 32 cell plated PDMS substrate mounted on the stretch device before stretching

7) Connect the portals and turn the control box back on. Click “Run” to start the process of stretching PDMS substrate
8) After 4 hours, click “stop” to stop the PDMS substrate stretching.
9) Immediately after stretch is stopped, start the procedure of microscope imaging.

2.8 Imaging on microscope

3.8.1 Materials and Equipment

a) Stretched and cell-plated PDMS substrate

b) Round coverslip with alignment markers and tape

c) Olympus IX-83 Microscope

d) Environmental chamber with heating and carbon dioxide.

e) 60mm culture dish cap

2.8.2 Protocol

1) It is recommended to turn on the microscope two hours before imaging. This is because it usually takes around two hours for the microscope Z-stage (the focus) to completely settle down.

Otherwise the drift in Z-stage could lead to blurry images. It's also recommended to turn on the carbon dioxide heating and environmental chamber heating two hours before imaging.

2) Use tape to fix the alignment coverslip on the microscope stage. It is recommended to do this step immediately after carbon dioxide heating and environmental chamber heating are turned on. Immediately after stretching, remove the PDMS substrate from the stretch device and put it on top of the stage. Make sure the alignment markers on the PDMS substrate bottom is aligned well with the alignment markers on the coverslip. For detailed explanation of the alignment coverslip please see Appendix. The alignment coverslip was prepared at the beginning of the project and had been used throughout the entire project with no changes.

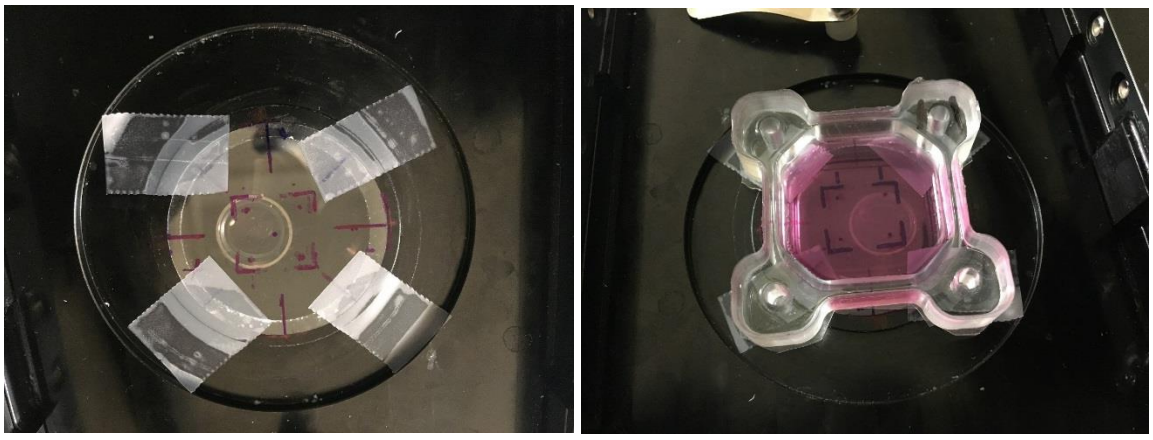


Figure 33 Left: Coverslip with alignment marker on top of the environmental chamber. Right: PDMS substrate mounted on top of the coverslip with markers aligned with each other

3) Open Slidebook. In the focus control dialogue, click on "XY". Then click on "Load" and select file "Yu 10% MQP 5".[10] Then 120 preset points with specific XYZ coordinates would be shown. Adjust Z coordinate for each point. After the Z coordinate of all 120 points have been adjusted[11], click on "Image Capture" .

[10] Please see Appendix for explanation of the file
[11] After the Z-plane of all 120 points have been adjusted, it is recommended to go over this process again as the Z-plan might still drift.

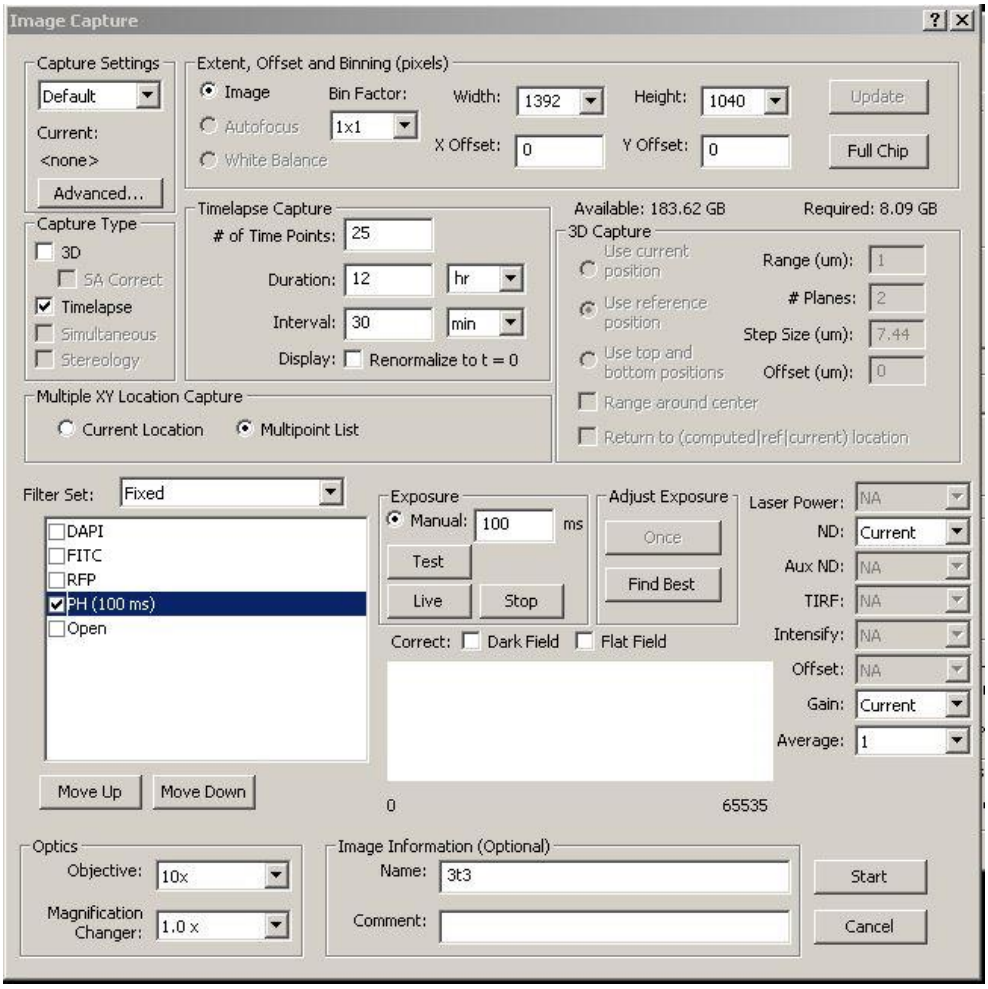


Figure 34 Screenshot showing the location of the file

4) In the Image Capture dialogue, select “PH” (phase contrast), “Multipoint List”, and “Timelapse”. Select “30mins Interval” for 25 time points. In this way, total imaging duration would be 12 hours.

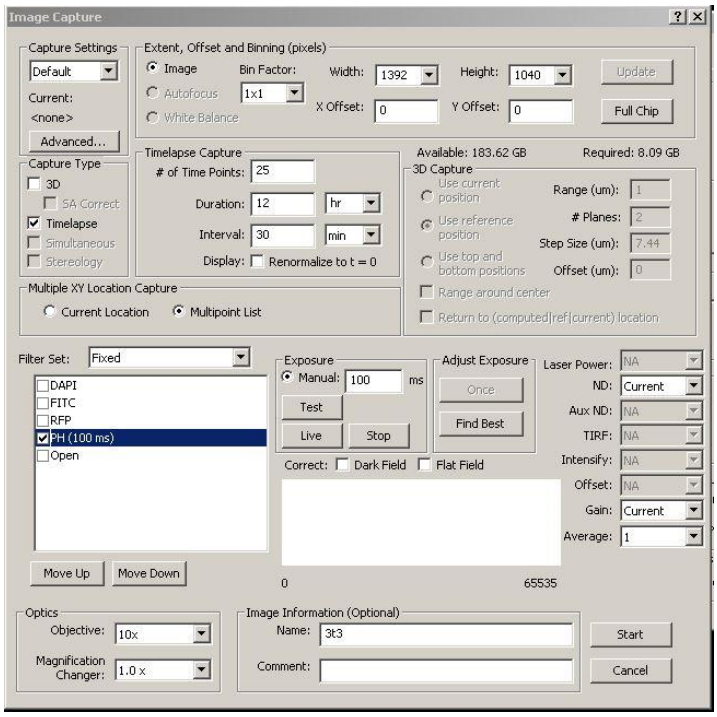


Figure 35 screenshot showing the location of the image capture dialog box

5) After the imaging process has completed, add bleach into PDMS substrate to kill the cells. Then Dump the PDMS substrate. Export the images, and images are good for analysis in ImageJ.

3 Results

3.1 Normal fibroblast cell response to cyclic stretch

The first step is to verify the normal fibroblast cellular response to cyclic stretch. Figure 36 shows the results of three sets of experiments. The condition of stretching was 0.65 Hz, 10% strain for 4 hours. In all three figures, X-axis represents the angle between the fibroblast polarization and the direction of stretch; and Y-axis represents the number of cells that fall into the corresponding angle range. In all three sets of experiments, it is clear that the fibroblast cells were aligning roughly perpendicularly to the direction of stretch. The average angles being 90.1 degrees, 88.7 degrees and 84.8 degrees respectively. These angles are roughly perpendicular to the direction of stretch, which confirms the conclusion of previous experiments as mentioned in section 1.2.

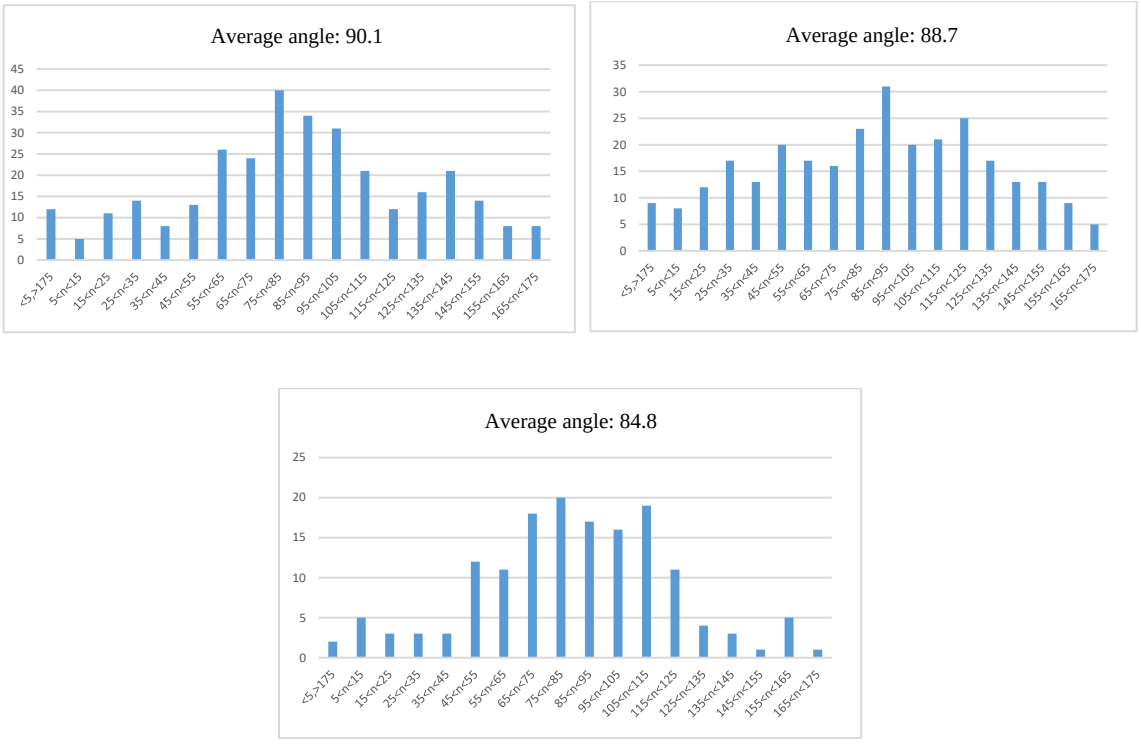


Figure 36 Figures showing results of three sets of fibroblast response to cyclic stretch at frequency of 0.65Hz and strain of 10% for 4 hours.

3.2 Fibroblast relaxation

After the fibroblast cells were stretched, the PDMS substrate was imaged every 30 minutes for up to 12 hours to document the relaxation process. Since the vimentin inside 117 cells have been knocked down, it is expected that the vimentin-knockdown fibroblast would show different dynamics than the normal fibroblast cells. The behaviors of normal fibroblast cells and vimentin-knockdown fibroblast cells have been shown in figure 38 and figure 39 respectively. In both figures, the histogram on the left side shows the cell orientation 30 minutes[12] after stretching, and the histogram on the right side shows the cell orientation 8 hours after stretching. In both figures, a quadratic fit was performed to measure the width of distribution. The quadratic equation fitting each histogram is also displayed. The standard quadratic equation function is $y = ax^2 + bx + c$. a is the quadratic term that determines the width of the function. The larger the absolute value of a, the narrower the function. The smaller the absolute value of a, the wider the function [9].

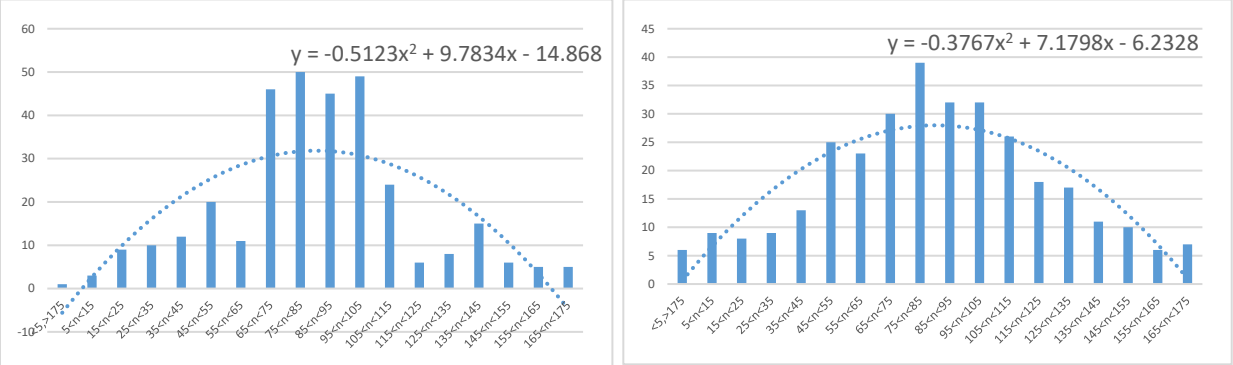


Figure 37 Histogram of normal fibroblast cells. The left histogram is the cell orientation 30 minutes after stretching, and the histogram on the right side is the cell orientation 8 hours after stretching

[12] Ideally it should be immediately after stretching. But adjusting the Z-plane of each points (mentioned in section 2.8) takes around 30 minutes.

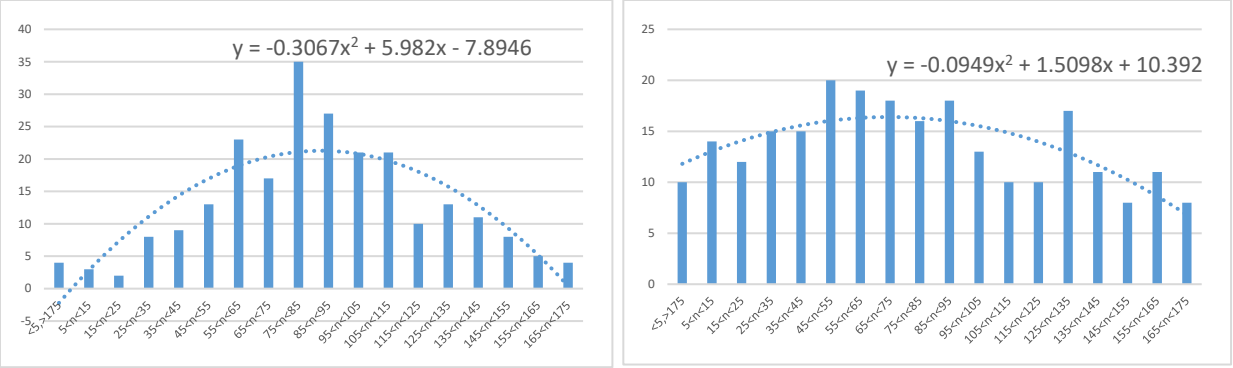


Figure 38 Histogram of vimentin-knockdown fibroblast cells. The left histogram is the cell orientation 30 minutes after stretching, and the histogram on the right side is the cell orientation 8 hours after stretching

According to figure 37, the quadratic term of the normal fibroblast orientation 30 minutes after stretching is -0.5123, whereas the quadratic term of the normal fibroblast orientation 8 hours after stretching is -0.3767. Eight hours after stretching, the quadratic term's absolute value decreased by 26.5%. According to figure 36, the quadratic term of the vimentin-knockdown fibroblast orientation 30 minutes after stretching is -0.3067, whereas the quadratic term of the normal fibroblast orientation 8 hours after stretching is -0.0949. Eight hours after stretching, the quadratic term's absolute value decreased by 69.05%, which is nearly three times than that of the normal fibroblast. Therefore it is concluded that vimentin-knockdown fibroblast is much easier to reorient than normal fibroblast. This aligns well with the theoretical explanation in Section 1.3. It has been found vimentin plays a major role in maintaining cell shape and integrity, and the experiment result from this project shows that knockdown of vimentin makes 117 cells much easier to reorient and lose its original polarization than normal fibroblast cells.

3.3 Contamination

However, the results in Section 3.1 may not be accurate and requires further testing due to contamination problem. The PDMS substrate was constantly contaminated by external bacteria. Figure38 is one typical picture of such scenario. In figure 38, each tiny dot represents a bacterium that could potentially affect cellular responses and thus disrupting experimental results. Therefore it is necessary for future researchers

to conduct more refined experiments comparing the cellular dynamics of normal fibroblast and vimentin-knockdown fibroblast.

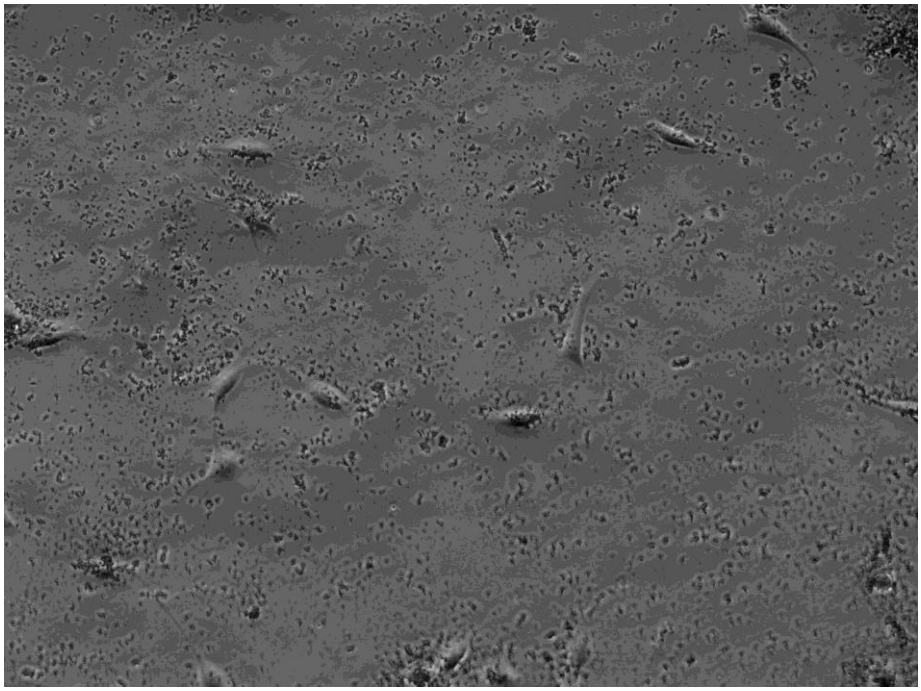


Figure 39 image of contaminated PDMS substrate

In this project, a number of tests have been conducted to eliminate the contamination. But so far there has been no major success. Instead of simply soaking PDMS substrate and culture dish cap inside ethanol, a 30-minute sonication was applied. Before drawing the alignment markers on the PDMS substrate, the PDMS substrate and culture dish cap were sonicated for 30 minutes inside ethanol. The sonication is expected to remove any dirt and biological residues attached on the substrate surface. Figure 40 shows the surface of a PDMS substrate immediately after sonication. It is clear from figure 40 that the PDMS substrate surface was free of bacteria. Then the PDMS substrate was drawn alignment markers. Afterwards the PDMS substrate was put under UV light for 1 hour in order to kill any bacteria still left on the PDMS substrate surface. After this step the PDMS substrate was collagen coated and seeded cells. However, after the cells have attached to the PDMS substrate, the microscope images show there is still contamination as shown in figure 41. The contamination could be from various sources. The process of collagen coat and cell plating are the most likely sources of contamination as these are the only two

procedures implemented between figure 41 and figure 42. Replacing the corresponding chemical solutions in these procedures could decrease contamination. Other methods of decreasing contamination include prolonged sonication inside ethanol and prolonged exposure to UV light.

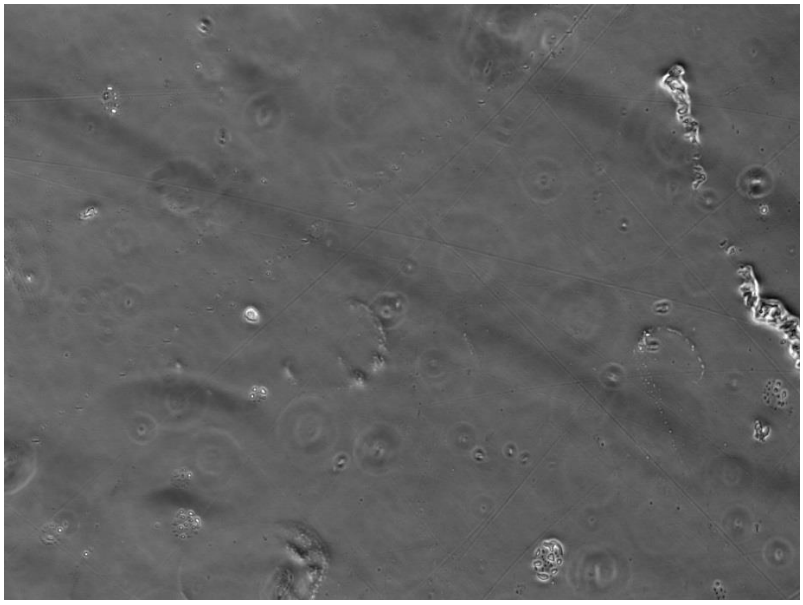


Figure 40 images of PDMS substrate immediately after being sonicated inside ethanol. It is clear that no bacteria exist on the surface.

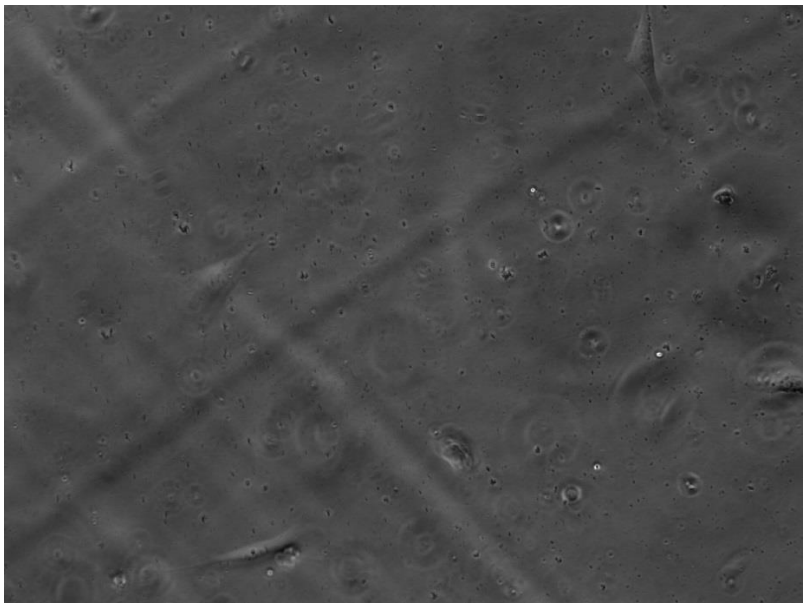


Figure 41 Images of the same ethanol-sonicated PDMS substrate after cells were cultured. Even before stretching there were lots of bacteria inside the PDMS substrate

4 Conclusion

In this project, an experiment was conducted in order to examine the effects of vimentin on cellular dynamics. The cellular response and relaxation process of normal fibroblast cells and vimentin knockdown cells were tested and compared. The cells were first cultured on a PDMS substrate, and then stretched at high frequency in order to keep the cells aligned in on direction. The PDMS substrate was subsequently imaged to document the relaxation process of the cells, and the images of normal fibroblast cells and vimentin-knockdown were analyzed and compared. The result shows that the vimentin-knockdown fibroblasts are much easier to change orientation than the normal fibroblast cells, therefore the project confirms the vimentin's role in maintaining cell shape and integrity. However, the contamination problem could have affected the accuracy of the experiment, and it is advised for future researchers to refine the protocol in order to get more accurate results.

5 Acknowledgements

I'd like to thank my advisor Professor Qi Wen who instructed me on this project, Professor Kristen Billiar who provided me with the stretch device and the PDMS mold, Zach who taught me making PDMS substrate, and Ho Thanh, Minh-Tri who helped me with cell culture solution and microscope imaging.

6 Appendix

6.1 Normal Cell Culture

1. Put DEMEM (the culture solution), DPBS inside 37°C water bath for 20mins. Put the Trypsin in room temperature for 20mins
2. Take out the cell dish and observe it under 10X microscope to check if there are cells attached to the dish's bottom. If there are cells attaching to the dish' bottom, then it's good.
3. Put the cell dish, DEMEM, DPBS and Trypsin in the Hood.
4. Suck out all of the liquid in the dish.

5. Lay 3 ml DPBS in the dish and wait for around 1 min. [13]
6. Suck out all of the DPBS from the dish. Lay 200 µl Trypsin in the dish. Tilt the dish to make the Trypsin spread evenly. Put the dish in the incubator for around 5 minutes.
7. Take the dish from incubator, check the dish under 10X microscope again to make sure the cells have already detached and floating. Lay 3 ml DEMEM in the dish and pipette the solution up and down for around 20 times to make the cells spread evenly. [14]
8. Transfer 1 ml cell liquid from the cell dish into a new Eppendorf. Put the Eppendorf in the centrifuge machine. Centrifuge it for 5mins at speed of 1200 rev/min.
9. While waiting for 8, take a new dish and label it. The P value should be added 1.[15] Write the P value, date, name and cell name on the cap. Add 2 ml DEMEM in the new dish.
10. Take the Eppendorf back into the Hood. The cells are now concentrated at the bottom of the Eppendorf because of centrifuge. Suck all of the liquid in the Eppendorf UNTIL a small amount is left at the bottom.
11. Put 1 ml DEMEM in the Eppendorf. Pipette up and down around 10 times in order to make the cells spread evenly inside the Eppendorf. Then transfer all liquid from the Eppendorf to the new dish (So the new dish would have 3 ml liquid in total).
12. Check the new dish under 10X microscope if the cells are floating. If there are cells floating, then it's good. Put the new dish in the incubator.
13. This step is only necessary for vimentin-knockdown cells. After it is confirmed that cells are floating, add 1mg/ml Puromycin[16] into the cell dish. For every 1000 µl cell culture solution, add 2

[13] This step is to wash the cells.
[14] Try to pipette slowly and avoid creating bubbles. Otherwise some cells could die.
[15] For example, if the old dish is P14, then the new dish should be labeled P15. The P value is the number of times that the cells have been split. If the P value is P14, then the cells have been cultured 14 times (namely split 14 times) already.
[16] The function of Puromycin is to kill normal fibroblast cells (3t3). This is because some of the vimentin-knockdown cells would turn into normal fibroblast cells after splitting. Adding Puromycin ensures no normal fibroblast cells would survive.

µl Puromycin. Typically a 60mm culture dish hold 3000 µl cell culture solution, so it would be 6 µl Puromycin in total.

14. Clean up. Suck all liquid left to the waste bottle.

6.2 Determine the area of uniform strain

The coverslip alignment marker is instrumental in keeping the PDMS substrate located at the same location during different experiments. Because only the specified 0.64 cm^2 square at the central part of PDMS substrate is under uniform strain when it is stretched, it is necessary to keep the PDMS substrate aligned accurately on the microscope. Minor changes in the PDMS substrate's location during each experiment could result in imaging an area that deviates from the square at the center which is not under uniform strain. Figure 42 shows the actual setup on the microscope stage. An exactly the same copy of alignment marker on PDMS substrate (shown in Section 2.4) is drawn on a 35 millimeter round coverslip. Schematics of the alignment markers are shown in figure 43. The alignment markers are drawn around the center of the coverslip. The area inside the four dots represents the area of uniform strain, and the four markers outside are used to align with the same markers on the PDMS substrate. The four dots form an 8 millimeters time 8 millimeters square, which is aligned with the area of uniform strain on the PDMS substrate.

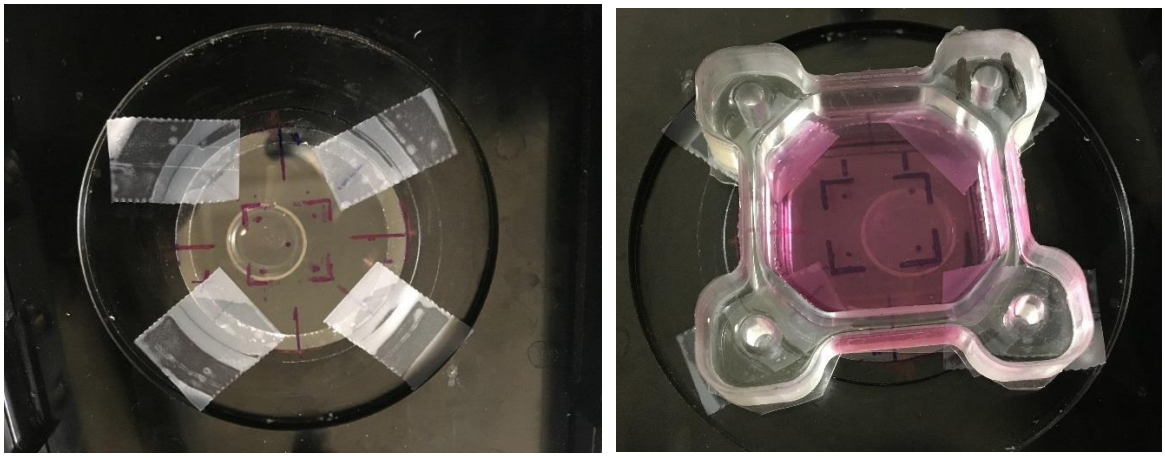


Figure 42 Alignment coverslip and PDMS substrate on the microscope stage

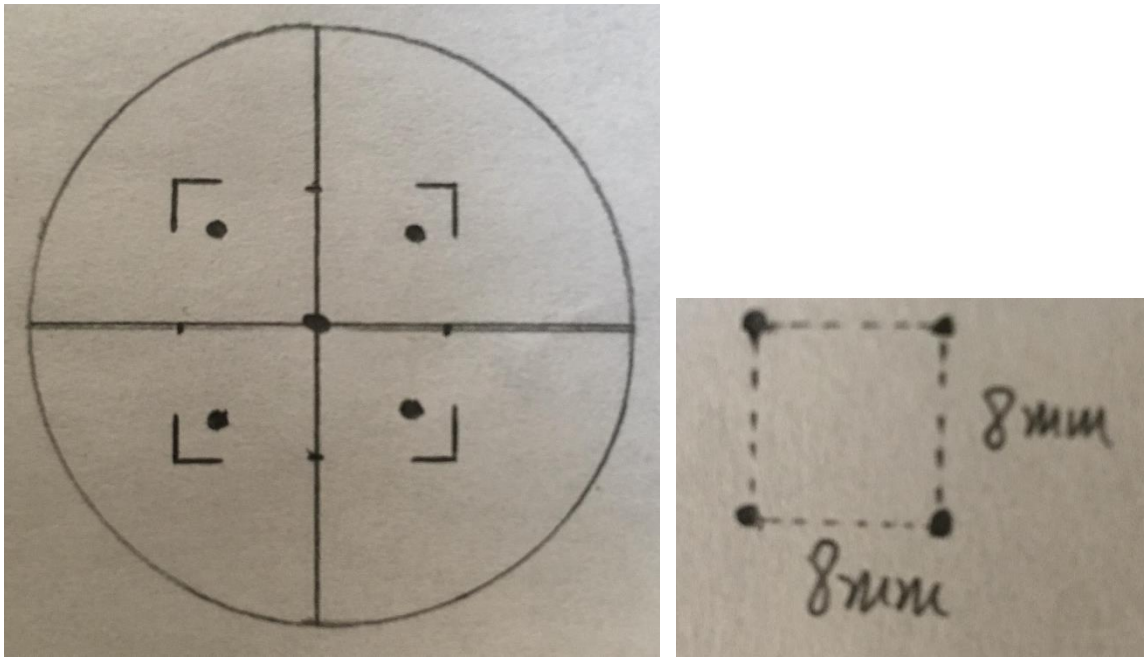


Figure 43 Schematics of the alignment markers on the 35mm round coverslip

Simply fixing the PDMS substrate in place is still not enough to make sure the microscope is imaging the same location during each imaging process. Another necessary step is to set up the coordinate of the microscope stage. The microscope must scan precisely over the area of uniform strain (which is the 8 millimeters time 8 millimeters square at the center of the PDMS substrate). Different microscope has different camera field. For the Olympus IX-83 Microscope with 10x objective used in this project, the horizontal length of each image is 898 μm, and the vertical length of each image is 671 μm. Therefore each image scans over an area of 898 μm × 671 μm. In this project, a 10% overlapping was kept in order to minimize the number of cells lost or split at the edge of each image. A 10% overlapping means each image scans over an area of 808 μm × 604 μm. In order to cover a square of 8 mm × 8 mm, it is clear that 10 points in horizontal direction (X coordinate) and 13 points in vertical direction (Y coordinate) are needed (130 location points in total).[17] Then find the alignment

[17] In the actual experiment only 12 points were taken in the vertical direction. This is because 120 points already provide around 200 cells which is enough for statistics. In addition, the more points taken for images, the longer it takes to complete the imaging. 120 points cost 8 minutes to complete the imaging process, which is already a quarter of the 30 minute interval between each set of images.

dot on the PDMS substrate and set up the X coordinate and Y coordinate of the 130 points to cover the entire area of uniform strain on the PDMS substrate. The 130 points' XY coordinates can be saved as a file (such as the "Yu 10% MQP 5" in section 2.8) so that it is ready to be loaded for the rest sets of experiments.

7 References

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