2013 GEM4 BioNanotechnology Summer Institute

Cancer Nanotechnology and Cellular Mechanics

an NSF-IGERT and NIH-CNTC collaboration

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University of Illinois at Urbana-Champaign

MECHANOBIOLOGY 1 LAB MODULE (MechB1):
Scanning Epithelial Cells in Air
Location: L512 Digital Computing Laboratory (DCL)
Lead Instructor: Jenny Amos, Bioengineering
Lab Assistant: Brittany Weida, Bioengineering

Purpose and Expected Outcome:
Examine epithelial cells extracted from cheeks of participants. Students will measure and analyze mechanical properties of the surface of epithelial cells as well as observe optical and topographical appearance.

Overview of AFM:
The structure of eukaryotic cells is controlled by a dynamic balance of mechanical forces exerted by the cytoskeleton. Growth, cell cycle progression, gene expression, and other cell behaviors are sensitive to changes in the cellular mechanical force balance. Measurements of the spatial distribution and changes in viscoelastic properties of living cells will provide valuable insights into these processes. The atomic force microscope (AFM) can be used to image living cells under physiological conditions in a nondestructive manner [1]. The AFM can also be used to study material properties by collecting force curves on the surface of the cell. A force curve is a plot of the force applied to the AFM tip as the sample is approached and pushed against the tip. In principle, this plot gives the force required to achieve a certain depth of indentation (deformation) from which viscoelastic parameters can be determined. By performing multiple force curves along the surface of the cell, we can create high-resolution 2-D maps of mechanical properties such as stiffness and adhesion [2].

<ins>Head</ins>: this is what contains the optical detection system and controls the Z piezo actuation of the cantilever; it contains thumbwheel adjustments to position the laser and zero the deflection on the position sensitive diode/ detector (PSD); the top view optics position knobs and focus wheel to view the tip from above; has three independent legs that adjust height of head relative to sample. Use two hands to lift the head due to its weight.

<ins>Controller</ins>: The digital controller contains a DSP and FPGA, and software controlled analogue 'cross point switch' for rerouting internal and external signals for custom experiments. The front panel of the controller is where: the power switch is; the key to turn the 'laser' on/ off; the 'Hamster' wheel is located to fine tune imaging/ control parameters; BNCs for advanced input/output signal access. The controller communicates with the PC via USB interface.

Software

To use the Mode Master, just click on a function button- for example, say you want to imaging in Contact mode: Click 'Topography' button; this will bring up a panel with all the different topography acquisition modes.

Equipment, Materials, and Supplies:

Glass slide   Willing cheek cell donor   AFM Tips – AC mode, Force constant 5 N/m

Sample Prep:

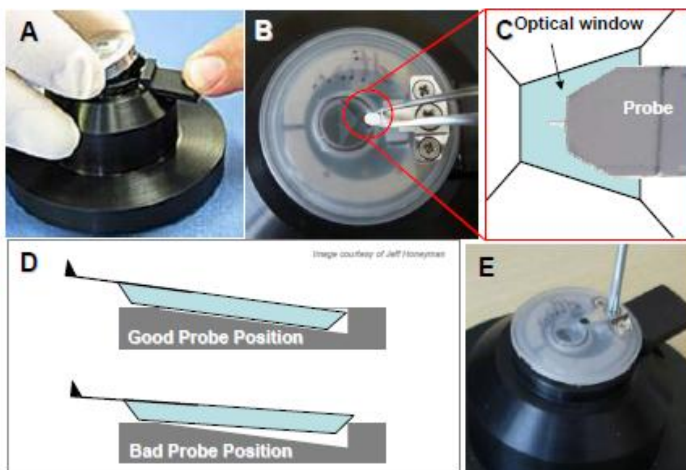
For the first experiment, we will simply image epithelial cells in air. The sample preparation is very simple and requires almost no facilities. Take a Q-tip and scrape the inside of your cheek (some physicists have been known to use their finger). Rub the saliva-soaked Q-tip on a glass slide. Use a marker to delimit the area of interest by drawing a circle on the other side of the glass. Let the sample dry for five minutes. If you inspect the slide under an optical microscope and a 20X objective, you should be able to see the cells.

After drying, you can position the cells under an AC mode cantilever and image them using the following standard protocol for air imaging.

Procedure:

Step 1: Loading the probe

The MFP-3D™ cantilever holder accepts most brands of commercially available probes. The quartz window is resilient from tweezer scratches. It can be cleaned by spraying with 70% Ethanol and spraying dry with canned air.

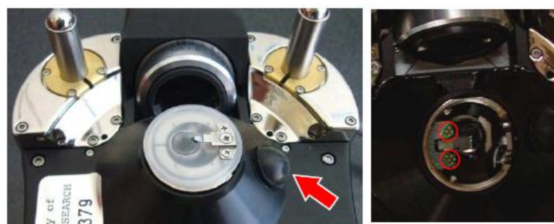


Proper probe loading:

A. Load the cantilever holder into the cantilever holder stand.
   1. Loosen tongue clamp screw with provide Phillips head screwdriver
B. Use tweezers to position cantilever in middle of polished quartz window.
C. Proper position of probe in quartz window.
D. Proper position of probe in pocket. For best results, DO NOT push probe chip substrate all the way back in the pocket: it can cause the probe chip to lift off the floor of pocket, compromising the deflection signal
E. Screw tongue clamp- ***no more than finger tight*** - with a Phillips (00x40) screwdriver.

Step 2: Install cantilever holder into MFP-3D head:

▪ Put cantilever holder into the MFP-3D™ head- it is easiest to put the ball bearing on release lever side (red arrow in figure) first, then ease the holder in from the back. Make sure the cantilever holder is parallel to the top of the head; otherwise it is not properly seated. The 'pogo' pins (red circles) used to get signals from the cantilever holder can be easily bent with excessive force.



Step 3: Instrument set-up and imaging

Keep in mind that these values are only guidelines, and need to be adjusted to each sample, each condition (air, buffers...) and each cantilever. Here are some general guidelines to follow:

Setting Up the AFM for AC Mode in Air

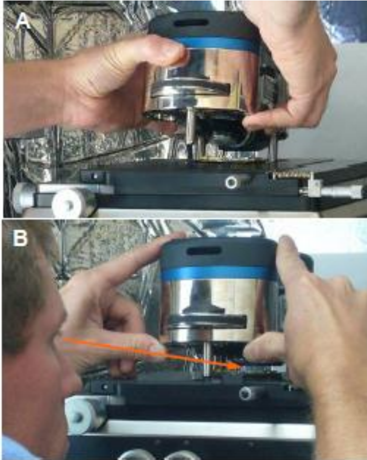
For AC imaging in air, we will use a silicon cantilever (AC150)

1. Center the cantilever in the holder as in "Loading the Probe".
2. Return the cantilever holder to the head.
3. Flip the head over and place head on scanner stage.

Placing Head on Scanner Stage:

Once a probe is properly installed in the cantilever holder, the superluminescent diode (SLD) can be aligned using the CCD camera.

1. Lift the head with two hands and place the back two legs in the kinematically machined divots on the MFP-3D baseplate.



2. Move hands so thumbs are under the front of head, and slowly lower head towards stage using back legs as pivot point; continually monitoring the tip – sample separation. If it looks as though the tip will crash, lift/ pivot head back up and adjust legs down to increase tip-sample separation, and repeat process.

Installing the head onto base plate over sample:

A) use two hands to lift head, set back two legs onto base plate first

B) gently lower front leg down, constantly monitoring tip sample distance to avoid tip crash. * Position the head above the sample, lowering the head until it is 1-2 mm above the sample surface. Make sure the head is approximately leveled.

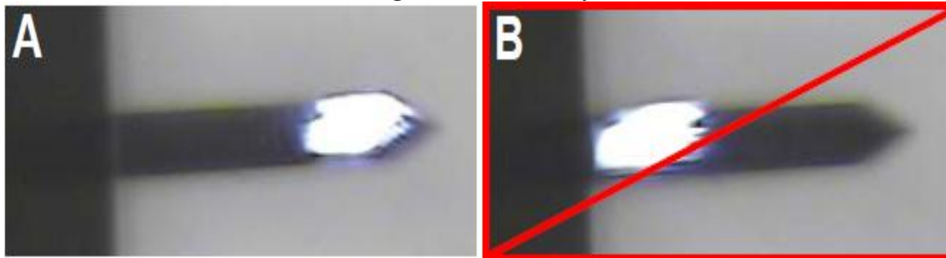
3. Position the spot on the end of the cantilever. We will use top view optical alignment.

4. Locate the SLD spot on the substrate

▪ Move LDX thumbwheel towards probe chip- the SLD spot is now reflecting off the cantilever; there is a great deal of refracted light, presumably due to the low fiber light illumination level. Slight adjustment in LDY may also be needed to maximize 'Sum' voltage in S&D meter.

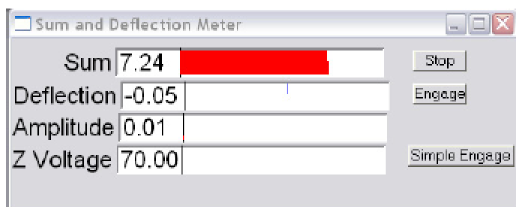
▪ When increasing the fiber light illumination, the spot is more apparent, and the amount of refracted light in the CCD image decreases.

▪ Regardless of how the SLD spot is aligned on the back of the cantilever, what is desired is to have the spot towards the end of the cantilever to maximize the optical lever sensitivity. The sum should be set to roughly 95% of the maximum value for the highest sensitivity.



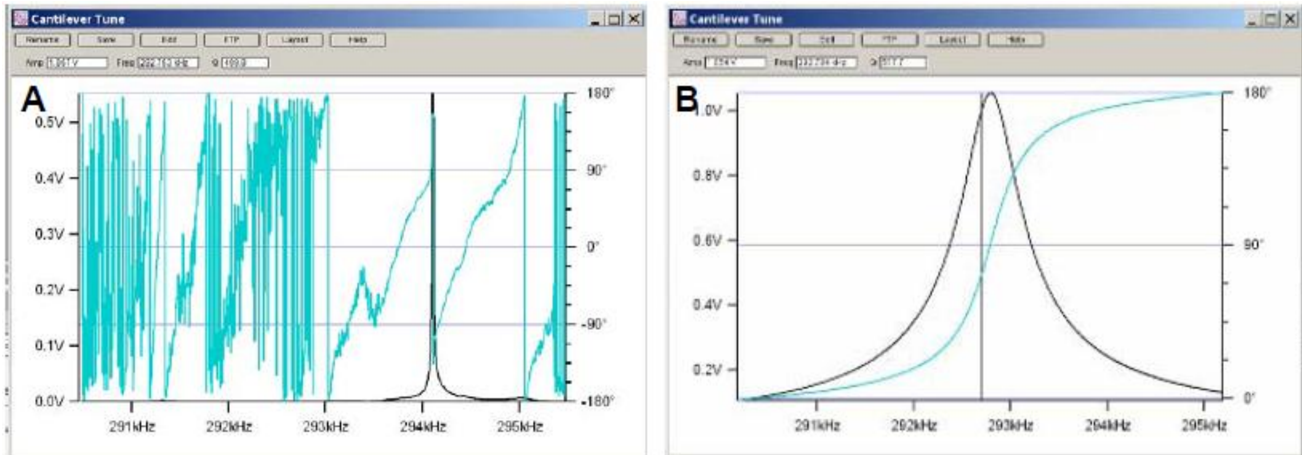
5. Zero the deflection signal using the Photo-Detector (PD) thumbwheel on the side of the head.

At this point, the sum and deflection meter should look something like the following.



6. The next step is to tune the cantilever. First, click on the “Tune” tab on the Master Panel. You should see the panel to the right appear:

For an AC150 lever, set the “Auto Tune Low” value to 250 kHz and the “Auto Tune High” value to 400 kHz. Enter “-5” for the target percent. Once those values have been entered, click on the “Auto Tune” button. After a few seconds, a graph similar to the one below should appear:
Note that the target amplitude can be chosen before tuning by entering a value in the “Tune” panel. *This value should be of about 1V in the case of biological samples.*



This is what a proper tune should look like. **A** shows how it will look during the tune, **B** shows the final product.

7. In the Main panel, select a setpoint of 0.8 Volts (if your target was 1V) and a feedback IGain of 10. Click the “Engage” button on the “Sum and Deflection Meter” panel to turn on the feedback loop. You should see the Zpiezo voltage extend towards the surface (red bar) as the feedback loop attempts to drive the amplitude towards the setpoint of 0.8 Volts
8. Using the large front thumbwheel, lower the head. When the amplitude reaches 0.8 Volts, the computer beeps. You should see the Z Voltage indicator on the “Sum and Deflection Meter” panel decreased from its maximum value of 150 volts. Lower this value until the red line is almost completely gone. This allows the greatest range of motion in the positive and negative directions.
At this point you are ready to start imaging. Click “Do Scan” on the “Main” panel and off you go!

Tuning image parameters:
Once the tip is engaged on the surface, imaging can commence.
Click the ‘**Do Scan’** button on the Main tab of the Master panel. This will move the tip to the corner of the scan area, and begin scanning.
Once scanning commences, the tip’s tracking of the surface tip is generally very poor. This can be seen in the individual Trace and Retrace fast scan lines below each of the image channels where one side of the feature has very poor tracking. Typically, three parameters should be adjusted first in this order

1. The **Set Point** voltage generally must be adjusted. Decreasing the Set Point voltage value increases the force applied to the sample. Higher Set Point voltages (lower force), will help preserve the tip apex, but may not allow proper tracking of the surface.

2. The **Drive Amplitude** can also be adjusted to increase the amount of Drive Amplitude applied to the shake piezo (and hence, cantilever); advantages of increasing this can be maintaining the tip during the scan (especially when imaging sticky samples).

The trade off by increasing the Drive Amplitude is beating the tip apex harder against the surface causing oscillations in the image (especially at feature edges); The tradeoff of lowering the Set Point voltage is applying more force to the sample.

3. The **Integral Gain** should be adjusted such that the surface is tracking well; things to avoid are decreasing it to a value that doesn't allow the feedback to track the surface well, OR too high that oscillations are apparent in the image and trace/retrace scan lines below the images. One of the best approaches to adjusting the Integral gain is to increase it until there is a 'ringing' seen in the (re)Traces lines below the height image. Then decrease it until this ringing goes away.

Monitor how the tip's tracking improves by looking at how well the trace and retrace line scans compare to each other. Note that they do not have to overlap exactly (because they are slightly offset in the software display), but they should have similar shapes/slopes per given surface feature.

What were your final settings for
Setpoint:______________________

Drive Amplitude:_______________

Integral Gain:__________________

Other observations:____

____

____

►Perform a 50 µm scan and a 10 µm scan within that scan area.

►Make a 3D plot from your height data by clicking "3D" on the image menu bar once scan has completed.

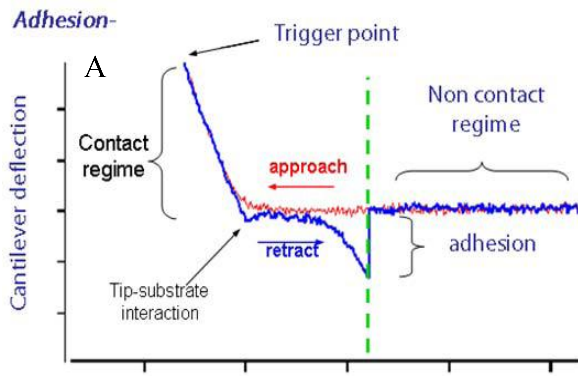
Igor Layout
We will create reports using the Igor layout feature of the software. All windows have a send to layout option either through "export to layout" buttons or "Ly" on any scanned image.

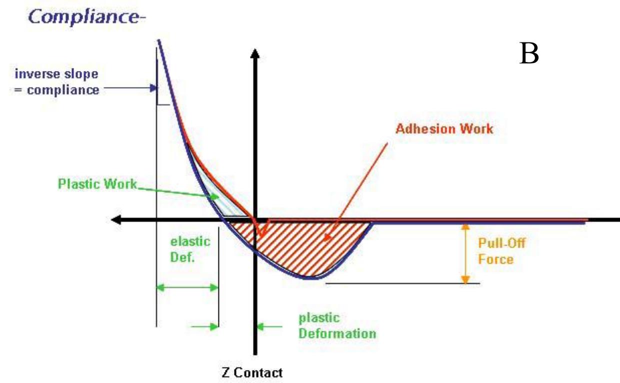
Save Graphics:
▪ If you happen to just a want a screen shot of one of the windows, you can make it the forward most window, and goto File **Save Graphics**. You can alter the size and resolution in the panel.

►Include images of your height trace, and 3D plot in your Igor Layout

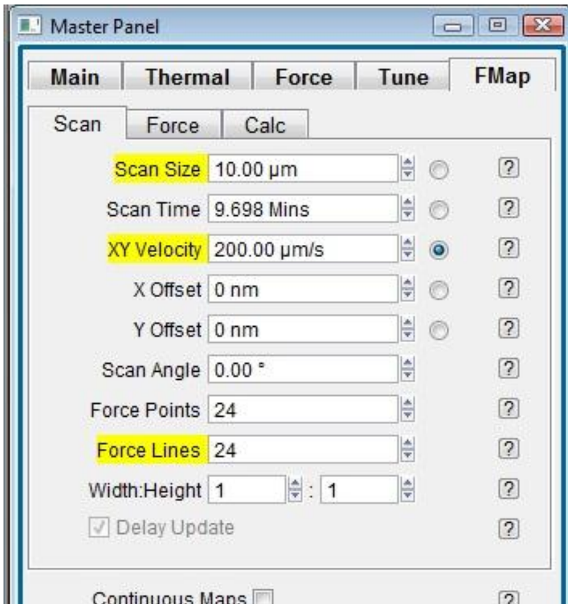
Step 4: Force Mapping
There are two major classifications that most force spectroscopy experiments measure:

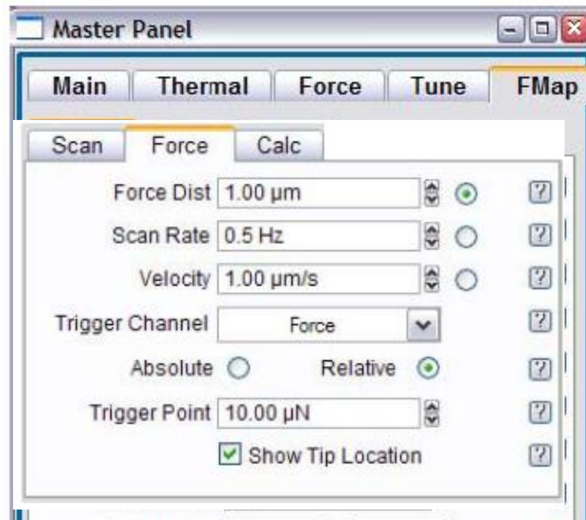
1. A pulling event in which the tip interacts with the surface, and some adhesion dissociation event between the tip and surface is measured on the retract cycle (A).
2. The tip pushes into the (material on the) surface to measure compliance (B).

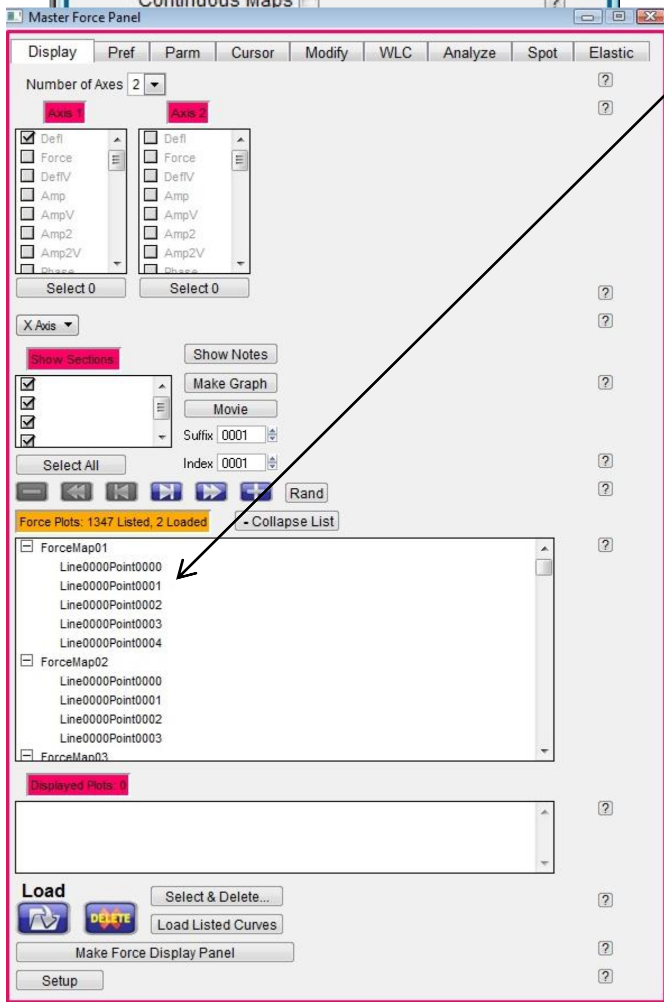




Using the Mater Panel, go to FMap. Select a scan size of 10 µm. Add a name to the file such as "GroupName_FMap". Adjust the settings on the Force subtab to look like the following:







* If you are having trouble collecting force data, you should try to increase the force distance so that you achieve a full withdrawal between each data point. This happens with sticky or tall samples (like cells).

Calculate the stiffness in the form of Young's Modulus for each data point.

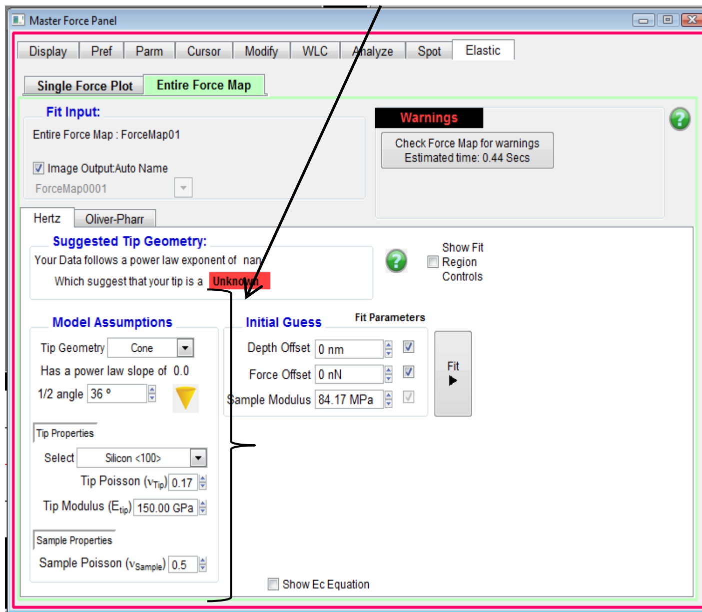
1. Go to the Master Force Panel. In the "Display" tab, select your data by browsing the directory until you find the name of your forcemap.

Go to the Elastic Tab and click on Force Map.

Make sure that "Hertz Model" is selected.

Enter your tip geometry, material, and enter a guess for the Poisson ratio (0.5 is a good starting point).

2. Click "Estimate" and check for any warnings and then "Fit".



- The computer will calculate the elastic modulus for each data point – this may take a minute, a plot will be added to your forcemap data when it is complete.

3. Check that data points were all calculated – any that were not will appear maroon on the greyscale plot.

- If there were many data points not fitted, you should scroll through the fit for the data points and perhaps lower the max force fit through the Hertz model.

4. Repeat until you achieve desired fit.

Create a 3D image of your cell with the force data overlayed.

1. Click 3D on the menu bar.
2. Use the top left drop down box to select your cell image and select the "height" in the right hand top drop down menu.
3. Click the second row image for another layer
4. Select the forcemap in the left have drop down menu and then the Young Modulus for the data. Click "do it".

Related References:

1. Schaus and Henderson, 1997 S.S. Schaus and E.R. Henderson, Cell viability and probe-cell membrane interactions of XR1 glial cells imaged by atomic force microscopy, *Biophys. J.* **73** (1997), pp. 1205–1214.
2. Emad A-Hassan, William F. Heinz, Matthew D. Antonik, Neill P. D'Costa, Soni Nageswaran, Cora-Ann Schoenenberger, Jan H. Hoh, Relative Microelastic Mapping of Living Cells by Atomic Force Microscopy, Biophysical Journal, Volume 74, Issue 3, March 1998, Pages 1564-1578.
3. Fuierer, Ryan, Procedural Operation 'Manualette', Asylum Research, Santa Barbara, CA, 2008.

MECHANOBIOLOGY 2 LAB MODULE (MechB2):
Response of Cardiac Cell Beating to Substrate Stiffness
Location: 3116 Digital Computing Laboratory (DCL)
Lead Instructor: Andrew Smith, Bioengineering
Lab Assistant: Haden Duke, Mechanical Science and Engineering

Purpose and Expected Outcome:
The purpose of this module is to provide participants with hands-on experience in the synthesis of hydrogels and understanding of how the hydrogel stiffness properties correlate to the beating of cardiomyocytes. Participants will be able to handle hydrogels of varying stiffness, learning to differentiate between gel stiffness and hypothesize on cell response to extracellular surroundings.

Additionally, participants will explore cardiomyocyte response to commercial reagents used in current medicine practices in relation to cardiovascular disease.

Background

Substrate Types:

It is desired that the substrates be chosen so as to match the cardiomyocyte's natural environment (match stiffness/elastic properties). However, due to time constraints, we must choose a gel/hydrogel which will provide representative measures of stiffness – i.e. they can be handled by the participants. In this module, participants will make 6 hydrogels of stiffness varying from 1 kPa to 40 kPa.

The prepared hydrogel serves the purpose of mimicking the extracellular matrix, which is the cell's natural environment. It has been shown at the cellular level that changes in substrate stiffness (elasticity) influence cell behaviors, including locomotion, adhesion, spreading, etc (Tse & Engler, 2010). In this module, participants will fabricate matrix substrates of varying stiffness from polyacrylamide (PA) – while hydrogels can be made from many different polymer types (such as Agarose, PMMA, etc.), PA serves well to demonstrate property changes correlating to mixing stepwise amounts of acrylamide (%) and bis-acrylamide (%).

Some things to note about PA hydrogels [13]:

1. The modulus of elasticity of the substrate can be varied by changing relative concentrations of acrylamide and bis-acrylamide.
2. The surface chemistry of PA can be kept constant while changing its mechanical properties.

3. PA is generally non-fouling – only adhesive molecules chosen to be covalently attached to the surface of the gel can serve as ligands for cell attachment.
4. Immunofluorescence is made possible at high magnifications because of the thin, translucent quality of PA gels.

Module Workflow

Participants will learn about the beating of cardiomyocytes and how this is affected by hydrogel stiffness. At the conclusion of this session, each participant (or participant group) will be able to make and differentiate between hydrogels of different stiffness. Participants will be split into 6 teams of two individuals.

Participants will be actively interacting with each other and the instructor(s) by hypothesizing the result of modulating hydrogel stiffness. This session will conclude by showing videos which demonstrate stiffness-dependent beating of cardiomyocytes.

0-30 minutes – Briefing and introduction to section
30-90 minutes – Participants will synthesize gels
90-120 minutes – Video demo session and final remarks

Protocol

Section 01 - Hydrogel Synthesis

1. Mix acrylamide and bis-acrylamide to their desired concentrations in either distilled H2O or PBS. *The elastic moduli will be slightly lower if the solutions are made in water, due to gel swelling when placed in cell culture media*.
2. Degas the mixture under strong vacuum for 15 min to exhaust the solution of dissolved oxygen. *Dissolved oxygen in the solution will act as a sink for the subsequent free radical polymerization. Degassing the solution not only speeds up polymerization but ensures more uniform polymerization as well*.
3. Add 1/100 total volume of APS and 1/1000 total volume of TEMED to gel solutions.
4. Vortex the polymerizing solution.
5. Quickly pipet 25 µl of the gel solution (see Figure 1.4-1B) onto the treated side of the chloro-silanated glass slide(s) (see Figure 1.4-1C) and add the amino-silanated coverslip(s) with the treated side down (see Figure 1.4-1A). See Figure 1.4-1D for how the completed glass-gel-coverslip composite should appear. *Essentially, the setup resembles a "sandwich" in which the polymerizing solution sits in between the chloro-silanated glass slide and the amino-silanated coverslip.*

6. Allow the gel to polymerize for 5 to 30 min. Monitor the unused solution to determine when the solution is fully polymerized. *Shorter polymerization times may result in insufficient polymerization of all available monomers and may cause the mechanical properties of the hydrogels to vary from the values noted here.*
7. Remove the bottom glass slide and discard. Place the top coverslip-gel composite in a 35-mm petri dish or 6-well plate in PBS or dH2O depending on what was used to dilute the acrylamide. Make sure that the gel-coated side faces up.
8. To remove un-polymerized acrylamide rinse twice, each time for 5 min in PBS or distilled H2O depending on what was used to dilute the acrylamide. *These hydrogels can be stored for long periods of time without losing any of their mechanical properties. To store them, immerse the hydrogels in water or PBS to keep them hydrated and store them at 4◦C.*

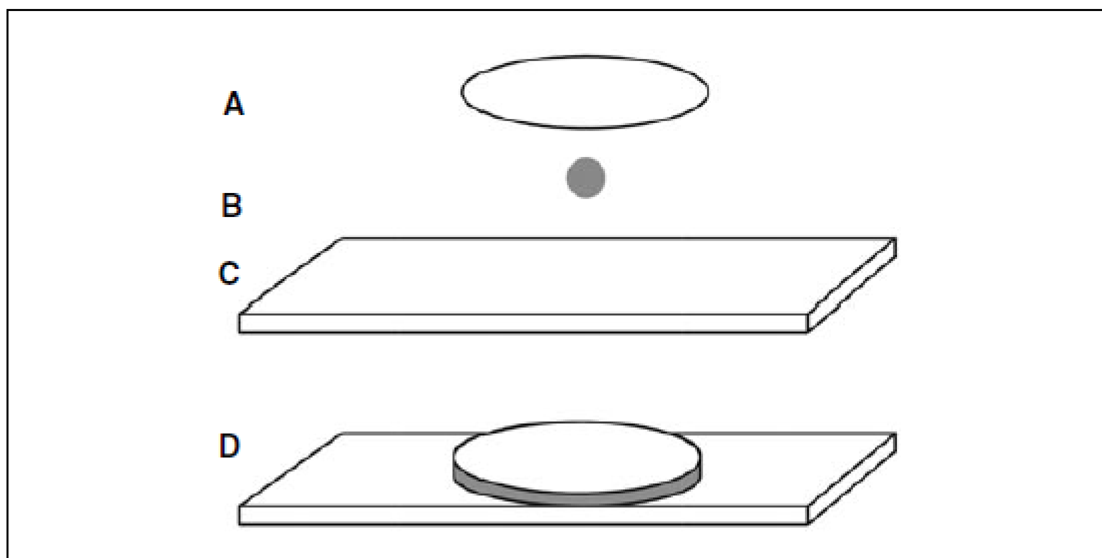


Figure 1: Exploded schematic of the setup for statically compliant PA hydrogels. The gel-glass composite includes (A) the amino-silanated coverslip, (B) polymerizing solution, and (C) chloro-silanated glass slide. (D) The completed setup is shown.

Section 02 - Imaging

Live imaging will either be conducted by lab assistants with participants observing, or will be shown as videos in the *Briefing and introduction to section* and/or *Video demo session and final remarks* portion of the module.

Suggested References

[1] Bajaj, P., Tang, X., Saif, T. a, & Bashir, R. (2010). Stiffness of the substrate influences the phenotype of embryonic chicken cardiac myocytes. *Journal of biomedical materials research. Part A*, *95*(4), 1261–9. doi:10.1002/jbm.a.32951

[2] Chung, C., Anderson, E., & Pera, R. (2012). Hydrogel crosslinking density regulates temporal contractility of human embryonic stem cell-derived cardiomyocytes in 3D cultures. *Soft matter*, *8*(39), 10141–10148. doi:10.1039/c2sm26082d

[3] Engler, A. J., Carag-Krieger, C., Johnson, C. P., Raab, M., Tang, H.-Y., Speicher, D. W., Sanger, J. W., et al. (2008). Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. *Journal of cell science*, *121*(Pt 22), 3794–802. doi:10.1242/jcs.029678

[4] Gonnerman, E. A., Kelkhoff, D. O., McGregor, L. M., & Harley, B. A. C. (2012). The promotion of HL-1 cardiomyocyte beating using anisotropic collagen-GAG scaffolds. *Biomaterials*, *33*(34), 8812–21. Retrieved from http://dx.doi.org/10.1016/j.biomaterials.2012.08.051

[5] Hazeltine, L. B., Simmons, C. S., Salick, M. R., Lian, X., Badur, M. G., Han, W., Delgado, S. M., et al. (2012). Effects of substrate mechanics on contractility of cardiomyocytes generated from human pluripotent stem cells. *International journal of cell biology*, *2012*, 508294. doi:10.1155/2012/508294

[6] Hersch, N., Wolters, B., Dreissen, G., Springer, R., Kirchgeßner, N., Merkel, R., & Hoffmann, B. (2013). The constant beat: cardiomyocytes adapt their forces by equal contraction upon environmental stiffening. *Biology open*, *2*(3), 351–61. doi:10.1242/bio.20133830

[7] Rask, F., Mihic, A., Reis, L., Dallabrida, S. M., Ismail, N. S., Sider, K., Simmons, C. A., et al. (2010). Hydrogels modified with QHREDGS peptide support cardiomyocyte survival in vitro and after sub-cutaneous implantation. *Soft Matter*, *6*(20), 5089. Retrieved from http://pubs.rsc.org/en/content/articlehtml/2010/sm/c0sm00362j

[8] Shapira-Schweitzer, K., & Seliktar, D. (2007). Matrix stiffness affects spontaneous contraction of cardiomyocytes cultured within a PEGylated fibrinogen biomaterial. *Acta biomaterialia*, *3*(1), 33–41. doi:10.1016/j.actbio.2006.09.003

[9] Smith, A. W., Segar, C. E., Nguyen, P. K., MacEwan, M. R., Efimov, I. R., & Elbert, D. L. (2012). Long-term culture of HL-1 cardiomyocytes in modular poly(ethylene glycol) microsphere-based scaffolds crosslinked in the phase-separated state. *Acta biomaterialia*, *8*(1), 31–40. doi:10.1016/j.actbio.2011.08.021

[10] Tang, X., Bajaj, P., Bashir, R., & Saif, T. a. (2011). How far cardiac cells can see each other mechanically. *Soft Matter*, *7*(13), 6151. doi:10.1039/c0sm01453b

[11] Young, J. L., & Engler, A. J. (2011). Hydrogels with time-dependent material properties enhance cardiomyocyte differentiation in vitro. *Biomaterials*, *32*(4), 1002–9. doi:10.1016/j.biomaterials.2010.10.020

[12] Yuan Ye, K., Sullivan, K. E., & Black, L. D. (2011). Encapsulation of cardiomyocytes in a fibrin hydrogel for cardiac tissue engineering. *Journal of visualized experiments : JoVE*, (55), e3251. Retrieved from http://www.jove.com/video/3251/encapsulation-cardiomyocytes-fibrin-hydrogel-for-cardiac-tissue

[13] Tse, J. R., & Engler, A. J. (2010). Preparation of hydrogel substrates with tunable mechanical properties. *Current protocols in cell biology / editorial board, Juan S. Bonifacino ... [et al.]*, *Chapter 10*(June), Unit 10.16. doi:10.1002/0471143030.cb1016s47