

## **APPENDIX C**

# Calcium Analysis Protocol

## How to get into the analysis program:

1. On the computer desktop, double click on the **Image Master icon**.
2. From Image Master screen, choose **Analysis** button

## Analysis:

1. On the top of your screen now, chose the **Image pull down tab**, and choose **Load Image**

Choose your directory, depending on what folder you have created for these images.

C:\...\default\imagedata\ your folder

*\*\*All files created for 1 experiment have to be kept in 1 file folder*

2. From your folder, choose an image.  
If you double click with out hitting ok, a preview of your image comes up. If you click on ok, the image is chosen.  
If you accidentally choose an image, and you want a different one, go back to the Image pull down tab on the top of the page.
3. Once you have your image chosen, you need to run a movie of all the images to see if any of the cells sheared off during fluid flow.  
You do this because you will want to make sure that these cells aren't analyzed, as they won't be able to be looked at through out the entire process.

## To watch the movie:

1. Go to the **Macro pull down tab** on the top of your screen
2. Choose **Load Macro**
3. Choose **Ratmovie** in the list of options that comes up.

When you chose ratmovie, another screen will pop up. **You need to fill in:**

File path—find your folder where you images are

Read Root —Root name: Mena and Start number 001

Background images..1. mbkaa001

2. mbkaa002

Repeat Loop—1/2 of total images. If you have 276 images, then repeat loop is 138 ( $276/2=138$ )

4. Click **Run**

\*\*\*You can move the pop up screen out of the way so that you can see the entire screen of the movie. **Watch the movie for** cells that move off the picture due to the fluid flow, or cells that float through the picture, that detached somewhere else. You will want to note where these cells are and when they happened, so that you can avoid

them in the next step. (Exmp...if a cell detaches right after fluid flow is started; you will want to take a later image so that the cell isn't there.)

### **Create an ROI (Region Of Interest)**

1. You will have to go in and choose an image again after the movie.
  - \*\*If all the cells on the movie stayed attached, choose an earlier image.
  - \*\*You can choose which ever wave length is easier for you to see the cells in to start with
2. Go to the **Measure pull down tab** on the top of your page.
3. Choose **ROI**—a screen to the side of your image will pop up

#### **In this new screen,**

- a. In the **shape** pull down tab, choose **Free-hand**
- b. Then click on the **Add** button.

(If this is not the first image you are looking at for the day, an old ROI will automatically come up...simply click on the clear all button and then hit the Add button and continue with the rest of the procedure)
- c. Now go to your image and while holding the left mouse button down, draw a circle around one of the cells on the image. When you are done with the circle, let go of the mouse button. Your circles won't be perfect but do the best you can.
- d. You will need to right click to be able to get out of the image area and go and add more cells
  - \*\*Continue b, c, and d until all of the cells in your image are circled.
  - \*\*If you need to delete a circle or redraw it, select the number of the circle you wish to delete and hit the delete button on the computer screen
- e. Once you have circled all of your cells. Go to the **Save As** button.
- f. The Save screen will come up. You will want to save the file as the **Rootname.roi**
  - \*\**Make sure that you are saving the file into the same folder as the rest of your images are in and not the default ROI folder.*

#### **\*\*Note on choosing cells:**

- Look that the cells at both wave lengths to ensure that you are finding all of the cells, this way you can see some of the cells which might appear as only shadows in one wavelength.
- Some cells may appear as only shadows. You can check both frequencies to see if on one, the cell appears, but the cells might appear as shadows on both frequencies, you can still circle these cells.
- If cells are bunched up or on top of each other, you don't want to choose them because the computer won't be able to tell what is the response of 1 isolated cell.
- If the cells are really close together but you are able to circle just one, circle it.
- If there are a lot of cells...more than 50, you will need to pick and choose the cells so that you get an even coverage of the whole image. The ImageMaster

system will only allow you to add up to 50 cells. I had problems getting back into the images if I went to 50, so I usually stopped at 48.

If you wanted to look at any of the cells at the other wavelength image,

Choose the image from the **Image pull down tab**, then go to the **Measure pull down tab** and choose **ROI**. When the screen comes up, click on the **load button** and find your ROI that you have saved.

4. When your ROI is completed you will need to make a graph of the ratio of the two wavelengths against the baselines

### **To make a graph:**

1. Go to the **Macro pull down tab** (where the ratmovie was)
2. Choose **load macro**
3. One the screen that pops up choose LearnMacro

Another screen will pop up. In this screen you need to **fill in the following:**

File path

Read Root—Root name: menaa and Start number 001

ROI set up—choose the one you have saved

*\*\* (this is why everything has to be in the same file because if your ROI isn't in the same folder as your images and background images, your path name won't be correct and you won't be able to select it)*

- ✓ When you choose your ROI, another screen will pop up, make sure that Average and Time are chosen and click ok

Both of your background images 001 and 002

Repeat Loop

4. Click on Run. The graph will make itself.
5. Once the graph is made, pull down the **File tab**, on the Graph's window, and choose **Save Data File As: rootname.flx**  
*\*\*Hit cancel on the Summary Information window that pops up.*
6. Now you will need to go in to the Felix program (minimize the ImageMaster, and go back to the desk top)
  - Start→Programs→Felix Or use the icon on the desktop

### **In the Felix program:**

1. Open a file, and find the graph you just saved.
  - \*\*You might have to find your path name first because Felix won't come up in the folder you were just working in.  
image data→folder→file
2. Once you have the graph opened. Go to **File**, and **Save As** data as a text file: rootname.txt
  - \*\* (make sure you are saving all of this into the same folder)
3. Now you will need to open up Microsoft Excel.
  - Start→Programs→Excel

### **In Excel:**

*\*\*When you open Excel a Text Import Wizard pop up screen will come up, just keep hitting the next button until it is finished.*

1. Open the text file you just saved from Felix.

*\*\*You will need to find your way all the way in to the file*

- C→Imaging→Default→Imgdata→Folder→File
- Accept all default settings for file transfer. (Tab Delimited)

2. When the data comes up you will need to **highlight every X column** (they will all have the same numbers in them) **EXCEPT**, leave the first X column.

*\*\*Hold down the CTRL button to select multiple columns*

3. **Delete** all the other X columns

4. Go to **File: Save As** and save this document as an excel file: rootname.xls

*\*\*To do this, you will just need to change the file type to .xls and then it will change the file name)*

Now you are done with one experiment ☺

## CALCIUM BLOCKING (Thapsigargin-Tg)

Purpose: To rid the stimulation effect of cytosolic calcium oscillations by fluid flow through the blocking of intracellular calcium stores using the pharmacological agent Thapsigargin. Thapsigargin inhibits  $\text{Ca}^{2+}$  ATP pump in ER and directs  $\text{Ca}^{2+}$  release from  $\text{IP}_3$  receptor and GTP activated  $\text{IP}_3$  receptor ER stores effectively removing 85% of ER  $\text{Ca}^{2+}$  pools within the cell.

### Short Term Calcium Studies:

**Order of Operations** – for specifics on anything not pertaining to cell loading with Tg refer to the Short Term Calcium Studies Protocol.

- A. Add Thapsigargin 1mM stock Solution to Flow Media (98% DMEM w/o phenol red and 2% FBS) to obtain a final Tg concentration of 50 nM. 150 ml of flow media is usually needed to satisfy 1 day of calcium imaging studies. For this amount it would be necessary to add 7.5 ul of 1 mM stock Tg solution to give a 50 nM Tg concentration in the flow media. *Currently it is necessary to soak gasket in flow media to create a good seal between flow chamber parts. Thus, the Tg addition to the flow media and the filling of the flow system has to occur before loading of Fura 2AM to ensure a good gasket seal is obtained before cell modification occurs.*
- B. Wash slide with DMEM w/o phenol red x 2
- C. Load Meniscal Cells with Fura 2AM for 20 min incubating at 37 °C.
- D. Wash cells loaded with Fura 2AM with DMEM x 2, and on the second wash leave DMEM covering slide.
- E. Place slide containing cells on flow chamber, wash flow media past cells to incubate them with Tg and to remove air bubbles from system, and view cells to find a satisfactory area to image (make this brief).
- F. Incubate cells on stage in flow media containing Tg for 30 min before imaging. Be sure shutter for light entry is closed during this time.
- G. Image Cells

### Long Term Flow Studies:

**Order of Operations** – for specifics on anything not pertaining to long term flow refer to The Long Term Flow Protocol.

- A. Add Thapsigargin 1mM stock Solution to Flow Media (48.5% DMEM/F12 w/ phenol red 2% FBS, and 1% P/S) to obtain a final Tg concentration of 50 nM. 250 ml of flow media is usually needed to satisfy 1 day long term flow studies. For this amount it would be necessary to add 13 ul of 1 mM stock Tg solution to give a 50 nM Tg concentration in the flow media.

- B.** Fill flow system with media containing Tg w/o the flow chamber attached.
- C.** Attach bottom of flow chamber to top, and fill with media containing Tg; check for leaks and then place the slides within the chamber.
- D.** Put flow chamber containing slides in flow system and work bubbles out of system by rotating chamber about central axis fairly rapidly.
- E.** Incubate cells in media containing Tg for 30 min before introducing fluid flow.
- F.** Run flow test for desired time; may have additional air bubbles present once flow is introduced. Be sure to remove the air bubbles before initiation of timed flow period.

### **Preparing Stock Solution Thapsigargin**

1. Tg crystals came in 0.5 g quantity. Added 769 ul of DMSO to 0.5 g Tg to give 1 mM Tg in DMSO.
2. Aliquoted Tg solution into 25 ml volumes contained in microcentrifuge tubes.
3. Aliquots and stock solution stored in  $-20^{\circ}\text{C}$  freezer.

## CALCIUM IMAGING STUDIES PROTOCOL

- Look at slides under 100X. Need to start with 80% confluence of cells on quartz microscope slide.
- Place all necessary stock solutions and existing flow media in water bath and warm contents to 37° C. Takes approximately 10 minutes.

### Concocting Flow Media (2% FBS)

1. For 100 ml solutions obtain 98 ml of DMEM (MEM for MC3T3 cells) w/o phenol red from refrigerator. Will use 49% DMEM/F12 once Ham's F12 becomes available without phenol red. Currently, there is a 1:1 mixture of F12 to DMEM offered by Cellgro that does not contain phenol red. This should be used in future studies.
2. Add 2 ml of Fetal Bovine Serum (FBS). **Must be done in hood to remain sterile!**

### Preparing Fluorescent Dye (This should be done in the fumehood)

1. Obtain 50 µl Dimethyl Sulfoxide (DMSO) with a 50 µl syringe. **(Wear Gloves!)**
2. Remove vial of Fura-2AM (molecular probes for intracellular measure) from the dry chemicals container in freezer.
3. Once thawed, add 50 µl DMSO to the Fura-2AM vial and tap vial until mixture turns yellow. Vortex and tap vial a few times to ensure thorough mixing.
4. Leave mixture in vial wrapped in aluminum foil on table or bench if using for multiple trials. Be sure to label aluminum foil.

**Note: Dye is light sensitive, protect at all times by wrapping in aluminum foil. 1 vial prepares ~ 6 slides.**

### Loading Cells with Fluorescent Dye

1. Place 700 µl DMEM w/o phenol red in 2ml micro centrifuge tube using 100-1000 µl pipette. Unsterile pipette tip traditionally used.
2. While in the hood with blower and light on; remove media from cell dish with vacuum aspirator. Unsterile pipette tip may be used in vacuum tubing.

3. Wash cells with 5 ml DMEM w/o phenol red, and remove. (x 2) It will be helpful if as much liquid as possible is removed from the dish. This will help keep the 700 ul of media on in step #6. It is ok to be vigorous with the slide to remove any liquid that is present, even lifting of the slide to remove media from underneath.
4. Add 3.5  $\mu$ l Fluorescent Dye solution, with 1-10  $\mu$ l pipette with unsterile tip, to 700  $\mu$ l DMEM w/o phenol red with the tip of the pipette submerged in solution.  
**Make sure to push all dye out of pipette!**
5. Mix solution with pipette.
6. Drip mixture onto slide being sure that all fluid remains on slide only! **Make sure there are no bubbles in the pipette when doing so.** *If the fluid spills off the slide at anytime before the 10 minute incubation period the fluid must be sucked up, making sure to leave no fluid behind, and placed back on the slide.*
7. Place dish, containing slide, into the incubator for 15-30 minutes depending on confluency for meniscal cells. Normally 20 minutes is sufficient. The more confluent the longer the incubation time. (45 min for MC3T3 cells). Check dish at 15 min to be sure that cells are not balling up signifying cell death (at this point we believe the DMSO is toxic to the cells).
8. After incubation with Fura, wash extra Fura from dish with DMEM w/o phenol red. (X2) Keep slide submerged in DMEM after second wash. Allowing the slides to dry out will make cleaning them difficult.

**Note: View cells to be sure they are healthy and have the right confluency.**

### **Preparing Imaging System**

1. Make sure all of the computers are turned off as well as their monitors. (Igniting the arc lamp can destroy the computers if they are on.)
2. With dial on right of faceplate (current dial) in vertical position, turn on power button on arc lamp (box under desk). Digital display should read 55 (between 54-56 is satisfactory). Wait 15 seconds (arc lamp will automatically ignite).
3. Turn on power to monochromater(box next to computer (picture)).
4. Turn on computer.
5. Open Imagemaster program icon.

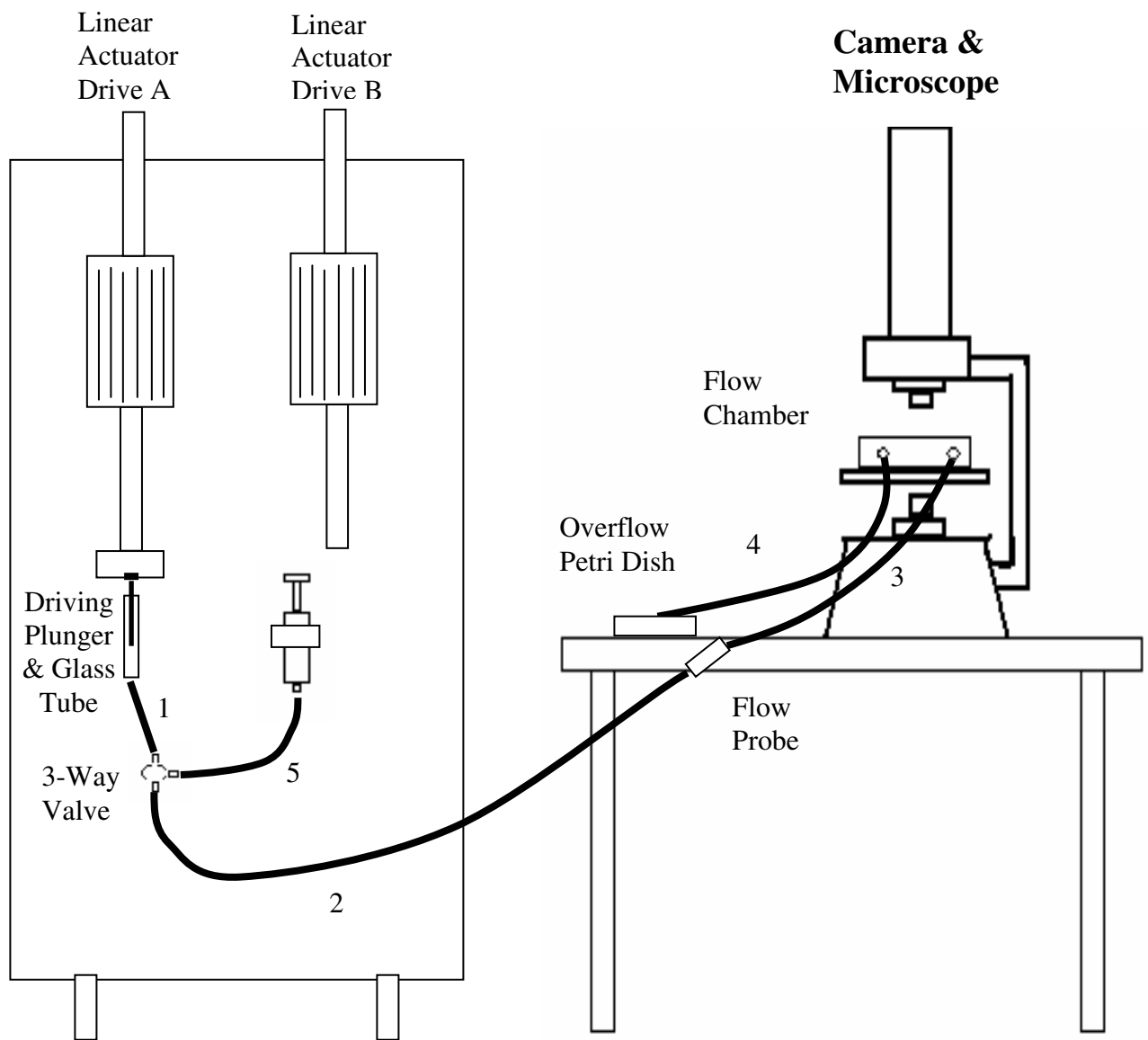
6. Click on Acquisition, with furatio.acq selected. Dial readout on monochrometer should be same as that displayed on computer screen at the start-up of Imagemaster. If it is not, change the display value and click OK.
7. Darken room (fix curtain, turn lamp on low, turn off overhead lights, have flashlight handy).

### **Preparing Flow System**

1. Tubing System starting with driving pathway:
  - a. Plunger is secured in circular platens attached to linear actuator rod.
  - b. Glass tube slid around plunger and locked in vertical position in green clamping arms of fixture.
  - c. Short piece of tubing (#1) connected to glass tube and to three way valve.
  - d. Long tube (#2) attached along linear path to three way valve and the other end to small flow probe.
  - e. Long Tube (#3) attached to flow probe and to inlet of flow chamber (when chamber face up, right most nozzle is inlet port).
  - f. Long Tube (#4) connected to outlet port (left most nozzle).
  - g. A petri dish is used to collect media issuing from the outlet tube.
  - h. Purging syringe, small mouth, attached to flow cart by Velcro at same level as plunger.
  - i. Small piece of tubing (#5) connected to syringe and three way valve perpendicular to other tubes.
2. Flow Chamber Assembly:
  - a. Put gasket on plastic flow chamber, make sure there are no air bubbles in gasket, especially between the inner flow chamber and the vacuum canal. Check gasket for tears, and be sure that there is an extra gasket available.
  - b. Put on metal piece with 4 Allen screws until they are just snug, do not want to have gasket position compromised by having the screws too tight.
  - c. Fill Petri dish  $\frac{1}{4}$  full with distilled water to house blank slide, and to keep it moist between trials.
3. Fill System with Media:

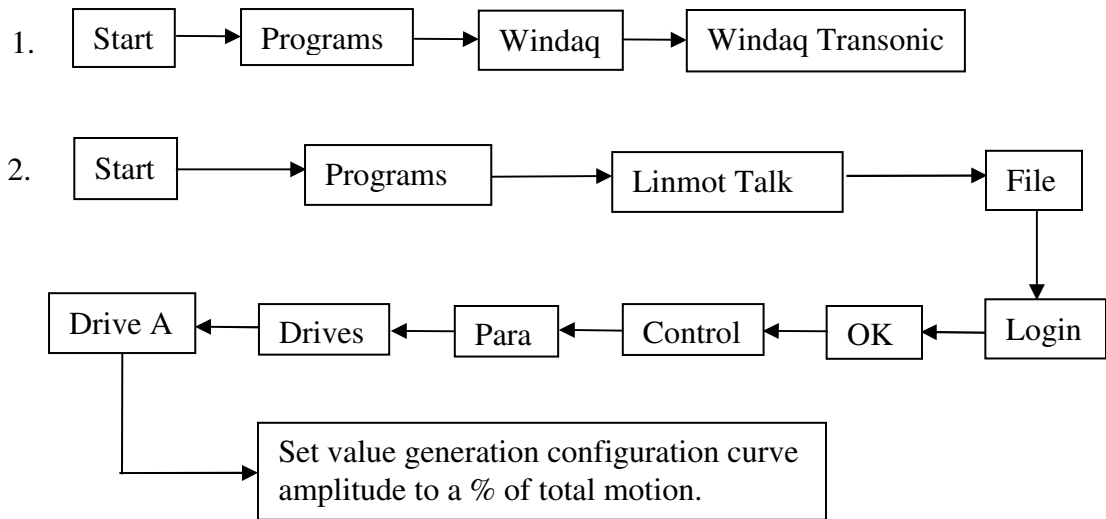
- a. Fill purging syringe with media by removing plunger. Use purging syringe plunger to force media through tubing connected to glass tube. Remove air bubbles from glass tube and slide over driving plunger and lock in place.
- b. Close valve to glass tube and force fluid through tubing connected to flow chamber with purging syringe.

**Figure 1. Flow System Diagram with Flow Cart and Microscope Set-Up**  
**Flow Cart**



- c. Turn on vacuum and place blank slide in chamber. Use fluid from purging syringe to rid system of air bubbles. If ER and a number appears on the Windaq Transonic flow meter, there are most likely bubbles present in the probe. Tap the probe to remove the bubbles.
4. Turn Linmot cutoff switch on before turning on the master power switch. Allows system to recognize actuator.
5. Flow ComputerSet-Up: Turn on master power strip for cart and turn on computer.

**Fig. 2. Flow Chart for accessing actuator control.**



By following these steps the LinmotTalk window will display the sinusoidal flow rate given by the actuator detected by the flow probe. The flow rate can then be used to determine the shear stress seen by the cells using the following equation based on parallel plate fluid flow.

Reference: Saunders MM, You J, Trosko JE, Yamasaki H, Li Z, Donahue HJ, Jacobs CR. *Gap junctions and fluid flow response in MC3T3-E1 cells*. Am J Physiol Cell Physiol. 2001 Dec;281(6):C1917-25., and is widely accepted by the scientific community.

**Shear Stress Equation:**

$$\tau = \frac{6Q\mu}{bh^2} = 1.579 \cdot Q$$

$\tau$  = shear stress (dynes/cm<sup>2</sup>) 10 dynes/cm<sup>2</sup> = 1 Pa  
 $\mu$  = viscosity of fluid = 0.921 E<sup>-3</sup> kg/ m·sec (water)  
 Q = flow rate (ml/min)

b = width of flow area = 8.0 mm

h = height of flow area = height of gasket = 0.27 mm

**Note: The viscosity is subject to change based on room and fluid temperature, which can significantly alter the above shear stress equation. Room Temperature should be monitored for each experiment, and the viscosity and above shear stress equations modified accordingly.**

6. Fill system, using syringe, with flow media.
7. Open valve to glass plunger tube from the syringe and push media out of glass tube to remove bubbles from the system.
8. Turn on vacuum.
9. Place blank slide on the flow chamber and clear system of air bubbles. **Make sure the slide is sealed tightly by vacuum! If flow travels back to the flow chamber from the output tubing there is a leak in the system.**
10. Windat Transonic
  - a. Follow the instruction in the flow system manual to calibrate the Windaq Transonic system and probe.
  - b. To find Peak to Peak amplitude of wave corresponding to Peak flow rate, go to Set Limits, and change values to those desired for chosen shear stress level.
11. Hit start on computer, and adjust amplitude % of plunger stroke to achieve desired flow rate/shear stress. Confirm water movement in output tube. *If the actuator does not move, turn off and then on master power switch so that system recognizes actuator.*

### **Prepare Cells for Viewing**

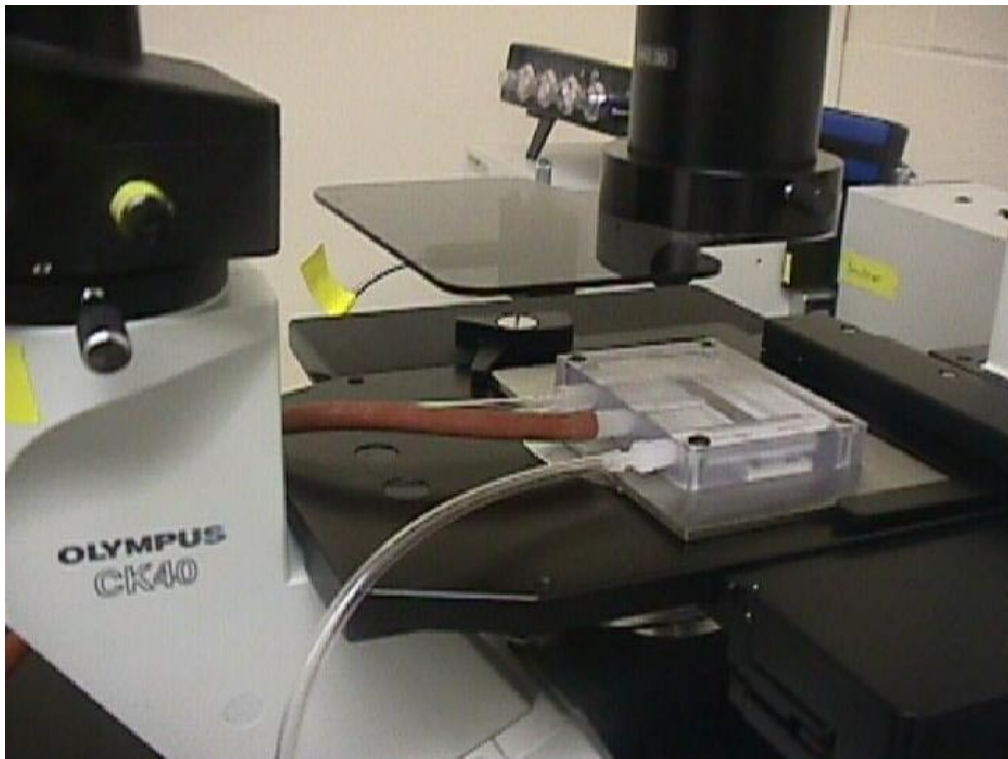
1. Flush cell plated slide with flow media. Check cells under microscope for any change in confluence or shape. Dead cells appear balled up.

### **Mount Slide in Flow Chamber**

1. Make sure vacuum is on. (toggle switch at base of flow cart).

2. Make sure shutter of microscope is closed (you only want to open it when you need to view or image cells, otherwise keep it closed to avoid photobleaching the dye).
3. Remove blank slide from flow chamber.
4. Push some flow media into chamber.
5. Place loaded slide onto chamber (cell side down), and dab the edges of the slide against the gasket to insure a good seal.
6. Push a small amount of flow media through the tubing again to make sure that there are no bubbles present in the chamber. Flash light onto the underside of the chamber to search for bubbles in the system.
7. Turn chamber upside-down so that the cells are facing down, and be sure that the microscope objective is all the way down to make sure that the chamber does not come in contact with it (Figure 3).

**Figure 3. Flow chamber placement on stage with flow and vacuum tubing.**



8. Place flow chamber on microscope stage.

### View Cells with Fluorescent Microscope

1. Turn up power on arc lamp to 65 Watts. (Under Desk)
2. Set monochromater to 380nm (currently this is done using the camera fast focus or blacklevel setting).
3. Set light path on microscope to 100% eyepieces (slider in).
4. Open shutter.
5. Focus on a nicely fluorescent group of at least 20 cells that are spread out and adherent to the slide (not balled up). Use stage adjustment knobs or hands to adjust slide/chamber location on microscope stage. Make sure that flow chamber is tight against stage.
6. Close shutter immediately after viewing to avoid photobleaching the dye.

### View Cells with Camera

#### Adjust camera black level

1. Turn voltage on camera amplifier to 0 (all the way counterclockwise). Far left knob on blue box. *If the knob does not catch when turning to the left, 20 revolutions should be sufficient. Always try to avoid shining light directly on Monochrometer.*
2. Turn on amplifier (switch is on the back of the box).
3. Set light path on microscope to 20% eyepieces 80% camera (slider out).
4. Make sure that shutter is closed.
5. Click on Black Level.
6. Adjust Black dial (2<sup>nd</sup> knob from the left on the blue box) on camera amplifier till histogram is centered on zero.

#### Focus camera

1. Set Gain (Gain 2 knob on the far right of blue box) on camera amplifier to 10 (all the way clockwise).
2. Open shutter.
3. Click on fast focus (on shortcut toolbar).
4. Click on 380nm.

5. Turn voltage up on camera amplifier until center of the flattened out cells resemble a white cloud (about 7.5V), have to go past the point where the screen becomes black. Make sure you can tell the difference between the flattened cells and those that are balled up (and will be ignored).
6. Record the gain that is being used for every experiment.
7. To obtain a clearer image, adjust focus on cells with microscope adjustment.

Do not disturb chamber for 30 minutes. This allows the cells to recover from stimuli associated with the mounting and focusing procedure, and allows time for complete de-esterification of intracellular AM stores to the cell impermeant/ $\text{Ca}^{++}$  sensitive form.

**When not using camera, always be sure the the shutter is closed, and that the eyepiece control is at 100% eyes.**

#### Prepare to take pictures

1. Make sure that transonic system shows 0ml/min flow rate.
2. Set LinMot program to appropriate plunger stroke amplitude.
3. Check valves on flow system for proper flow set-up.
4. Be sure that there aren't any bubbles in the 250ul syringe body.
5. Check that monochromator is set to 380nm. If it is something close, but not quite (384, 375 etc.) close and reopen the Imagemaster software. When the box appears displaying the monochromator setting, change it to match the actual monochromator reading (see analog display on arc lamp housing).
6. Set the light path to 20% eyepieces 80% camera.
7. Open shutter.
8. Set root name of 5 letters, index starting with 001. Currently r#\_##. The first number is the rabbit specimen number, and the second set of numbers is the experiment number for the specific rabbit specimen.
9. Click on Free Run, and verify the image is acceptable.
10. Zero the Imagemaster timer.

#### Run flow experiment

##### **Meniscal Cells**

1. Click on Save Run, the timer will automatically start.
2. At 1 minute start flow.

3. Take 10 seconds of data with Transonic (the flow probe software). *Go to File pulldown menu and select record. Filename should be consistent with experiment name, accept default values from popup window. Record one screen set, and then go to File and choose Stop.*
4. At 4 minutes stop flow (This give 3 minutes of flow data in addition to one minute of baseline data).

### **MC3T3 Cells**

1. Same
2. Same
3. Same
4. at 3 min stop flow.
5. at 8 min stop taking data.

### **After Running Your Experiment**

#### **Take camera background snapshots**

1. Keep all camera amplifier settings, microscope focus (and everything else) the same as in your experiments.
2. Set flow chamber (filled with flow media with blank quartz slide in place) on stage.
3. Open shutter.
4. Select microscope light path to be 20% eyepieces, 80% camera.
5. Enter root file name. **Change name to something different from saved data!**
6. Click on Free Run to view image.
7. Click on Snapshot to take pictures at 340 and 380.

### **Clean Up & Shut Down of System**

1. Disconnect hoses, flush with water, then alcohol, then water, and blow air through them. Hang all tubing on rack to dry.
2. Turn off all equipment. **Shut off arc lamp and monochrometer before turning on any light source.**
3. To clean slides, rinse with distilled water, scrub with Sparkleen powder soap using fingers, and then rinse with distilled water and alcohol and place in Calcium

Slides container filled with ethanol. This will remove any cellular debris that could cause problems during next trial.

**\*\*\*Be very careful with the camera. It can NEVER be exposed to ambient light. Keep eyepiece shutter always open to eyes, not to camera at all times unless room is darkened.**