A. Sign in to the log book and turn on the following numbered switches in this order:

1. Turn on switches ① thru ④ on the boxes underneath the air table.

2. Turn on the stage controller box ⑤, switch is on the backside of the box.

3. Turn on the computer ⑥.

If you are going to use any of the electrophysiology components of this system, such as the Sutter manipulators, please see the separate appendix for these components.

B. When the pGina login window appears on screen, wait until it reads “connected” on the line above the user name box, then login using your VUnet ID and password.

C. Start the FV software ⑦.

D. From the drop-down list, choose “guest” and no password, OR choose your saved setup file (ask CISR staff about how to set one up).

E. After the software loads, close any windows you will not use, such as the “Stimulus Settings” window.
II. SETTING UP THE MICROSCOPE FOR YOUR SAMPLE

1. If you are going to use the superfusion system and the heated slice chamber, set this up first. Details of using the heater controller, superfusion pump, vacuum collection and CO2 tank are covered elsewhere.

2. The DeepSee laser requires a warmup of about 5 minutes so turn it on early in the set up procedure. Click the laser box in the “Acquisition Setting” window and in the new window click “On”. 

3. Raise the objective with the remote focus controller before changing the specimen holder in order to prevent damage to the objective. The buttons on the right side of the controller either quickly raise or lower the lens.

4. Choose a specimen holder appropriate for your sample and secure it in the stage.
5. There are 4 objectives available. The 25X/1.05 NA objective is mounted on its own fixed position carrier while the 10X, 40X and 60X objectives mount 2 at a time on a flip carrier. All objectives are water immersion lenses with long enough working distances to permit electrodes between the lens and specimen.

6. To change carriers, loosen the set screw on the side of the carrier with the allen wrench stored in the scope stand.

7. The top image on the right shows the 25X lens in place and the second image shows the flip carrier with the 10X and 40X lenses in place. Both carriers have a slider that puts a DIC polarizing filter in the light path when pushed in. This should only be used in ocular mode or when using the IR camera for patching cells. It must be pulled out all the way when scanning with the 2-photon laser. After changing the lens, re-tighten the set screw and return the allen wrench to its slot.

The objectives not in use are stored in the red tool cabinet in the anteroom.

8. The 25X lens is equipped with a collar to adjust for refractive index differences between samples. For example, a coverslipped slide will need water as immersion fluid and the collar set to 1 over 0.17 as shown. However, for live superfusion imaging with no coverslip, the collar should be set at 0 over 0.17. These are both nominal settings and should be further refined by gradually turning the collar during the image optimization process.

NOTE: the 25X lens is a high performance lens that must be handled with special care. Any damage to this lens will be charged to the user/PI ($23,000 replacement cost).

9. If you change objectives, you must inform the software of the change by choosing the correct lens from the drop-down list in the “Acquisitions Settings” window.
9. Two reflector cubes are available for doing 2 color imaging with the 2-photon laser; blue & yellow or red & green. These cubes are separate from the single color cubes used in the ocular mode. The appropriate 2 color cube for your application must be manually inserted into the light path thru a slot on the left side of the scope head.

10. To find out which cube is currently in the scope, look in the storage box marked “REFLECTOR CUBES” to see which one is not in the box.

11. To change cubes, open the slot cover (2 finger screws) and loosen the set screw in the bottom of the slot with the same Allen wrench used for the lens carrier. Reach into the slot and grab the small handle on the side of the cube and slide the cube out. Do not touch any of the glass surfaces of the cubes. Fingerprints or dust can ruin them. If you see anything on the cubes, please contact CISR staff BEFORE passing any laser light thru the cubes.

12. Each cube is labeled on the side. MRG/RW=red/green and MRC/YW=blue/yellow. Slide the cube you need into the slot as far as it will go, retighten the set screw and replace the cover.

13. If you have changed the cube, you will also need to inform the software of this change. For instructions, see next section of this quickguide.

14. Return the cube not being used to the “REFLECTOR CUBE” box.
III. ADJUSTING THE LIGHT PATH & VIEWING THE SPECIMEN

1. There are several sliders that direct light to either the eyepieces (ocular mode), the camera used for patching cells or the detectors for imaging mode.

2. The slider on the right side of the scope directs light to either the eye pieces (pushed in 1) or to the camera (pulled out). On the left side, the sliders work together to direct light to the eye pieces (both pulled out 2), to the regular RXD scan detectors (both pushed in 3) or to the high sensitivity GaAsp detectors (top in, bottom out 4).

3. Mount your specimen on the stage, set the sliders for ocular mode, lower the lens to within about 1 mm of your specimen, add immersion water if it is a dry sample and turn on either the white 5 or the fluorescent light source 6 using the buttons in the “Image Acquisition Control” window in the software.

4. The DIC and single color reflector cubes are switched in the “Microscope Controller” menu. If this menu did not load on startup, click the “Device” dropdown 7, then click “Microscope Controller” 8.

5. The microscope controller has a default cube setting of “TXRed” so after turning on the light, click the appropriate cube button, for example “DICT” 9 for white light, CFP for blue dyes, GFP for green and yellow dyes, TXRed for red dyes.
III. ADJUSTING THE LIGHT PATH & VIEWING THE SPECIMEN

6. Focus on your sample by moving the lens up from your initial position. All of the lenses have a focal point above the approximate 1 mm recommended starting point. This way of focusing helps to prevent crashing the lens into the sample during initial focusing, a big risk on this scope.

7. Focus is done very fast with the up ① and down ② buttons on the remote focus module. Fast (course) and slow (fine) focus speeds are done with the knob on the focus module ③. To switch between fine and course, click the dropdown button on the “Microscope Controller” menu ④.

8. In ocular mode, the fluorescent light source is the mercury lamp located on the rear right corner of the air table ⑤. This lamp has a manual shutter ⑥ that can be used to reduce light intensity for samples that may bleach easily. The shutter should be closed at the end of the imaging session and therefore needs to be opened at the beginning of your session.

9. Bring your sample into focus, use the joystick stage controller to move around. Turn out the light when ready to scan.

10. Change the light path sliders to send the light to either the RXD or GaAsp detectors and flip the plastic sheet down over the front of the scope/faraday cage ⑦. Turn off room lights before doing any scanning, especially important when using the GaAsp detectors because they can be damaged by too much stray light.
1. If not done already, turn on the laser as described on page 2.

2. Change the laser excitation wavelength by clicking the laser box and then using the slider in the Laser Emission menu or the drop-down list of preset wavelengths.

3. The laser wavelength will be used in all detector channels by default. For two color imaging in which different wavelengths are needed for each fluorophore, click the sequential box and then set the wavelengths in each channel separately.

4. Click the “Sequential” box, turn on both channels and put one channel in “Group 1” and the other channel in “Group 2”. If both channels are initially in Group 1, left click and drag channel 2 into Group 2.

5. Click the drop down arrow of the laser in the channel you want to change and then click the MP line. A new menu will appear that can be used to change the laser wavelength by moving the arrow along the bar. Click the close button. The laser wavelength should now be changed in only that channel. Note that while scanning a two color image using two different laser wavelengths, there will be a delay of up to several seconds between scanning each channel while the laser re-adjusts the excitation wavelength.
4. If you changed the reflector cube, click on the Light Path window icon ①.

5. The window shown to the right is set for the green/red cube (FV10 MRG/RW). To switch to the correct setup for the blue/yellow cube, click on any of the boxes along the light path ② that opens the window shown below ③. From the "Cube Set" drop-down list ④, find the correct cube (in this case FV10-MRC/YW ⑤), highlight it and click “OK” ⑥. This is enough to change all filter settings for all detectors.
IV. IMAGE OPTIMIZATION

6. With your sample in focus and the correct light path sliders in place, use the “Image Acquisition Control” menu for optimizing each color. If unsure which pair of detectors to use, try the RXD detectors first (both sliders in).

7. Set the initial laser power to < 5%. Optimize one color at a time by clicking “on” the first or second detector of the pair you want to use, where detector “1” will image the shorter wavelength (either blue or green depending on the chosen reflector cube) and detector “2” will image the longer wavelength.

8. Start scanning using either the “XY Repeat” button or the “Focus x2” (twice as fast but 1/2 the resolution) or “Focus x4” (four times as fast but 1/4 the resolution). Refocus your sample.

9. Adjust the HV and laser power to achieve a bright image that is not over-saturating more than a few pixels. The HV has an approximate limit of 700, above which it will add noise to your image. Laser power of 5% or more will burn up a coverslipped slide mounted sample. Higher laser power can be used to image samples immersed in a fluid that can dissipate heat but samples may bleach. To check for too much saturation, click the LUT icon in the image window. Click the “Hi-Lo” button. Or use the keyboard shortcut “ctrl/h”.

10. The example image below is in Hi-Lo mode where red pixels are saturated and blue pixels are no signal. Adjust HV and laser power to get a few red pixels. Use “Offset” to get a few blue pixels in sample areas where there should be no signal.
IV. IMAGE OPTIMIZATION

11. To turn off Hi-Lo, hit ctrl/h again or go back to the LUT window and click on the preferred color. If you are satisfied with the resulting image, unclick the detector you have optimized, click on the other detector and repeat the optimizing process.

12. There are two additional adjustments that may increase brightness and resolution of your sample. Testing these parameters need only be done in one channel. First, if using the 25X lens, change the correction collar as discussed previously (page 3). If done carefully, you can turn the collar while imaging to see if the image improves, keeping in mind that you will also have to refocus when you turn the collar.

13. The second adjustment is to the dispersion compensation. This is a laser setting that is adjusted for the refractive index (RI) of the sample. The default is for water. If your sample RI is different than water, change the dispersion compensation using the handheld controller that is attached to the pump laser unit in the far right corner of the room.

14. Click the lowest button on the left side of the controller to move the arrow on the LED to the third line and note the number on the right end of this line. In this case, “45.67” is the default for RI of water, the 25X lens and the chosen excitation wavelength. While scanning, use the up/down arrow buttons on the right side of the controller to increase or decrease this number, and stop when your image is at its brightest. Turning on the Hi-Lo mode helps. No refocusing is needed.

V. SAVING YOUR IMAGE

1. After acquiring an image to be saved, click on “file/save as” and look for a server folder beginning with your Vunet ID followed by “cisrstore”, the name of the server. If you choose instead to save your data to the computer hard drive attached to the scope, please make a folder with your name on the data drive (D:) under “users”. Data saved on the scope computer will be deleted after one month.

2. The file format options are .oib and .oif. We recommend using the default format which can later be converted to other formats such as .tiff by ImageJ or a free version of the Olympus software. For a link to the free software, visit our website, click on FAQ and look for “How do I get relevant software?”
VI: Z-sectioning

1. In the **Image Acquisition** window, click the **depth** button ①. Note that now the XY button ② used to start the imaging is 3D (XYZ) instead of 2D.

2. Turn on the detector for the channel that is most appropriate for determining the z dimension size you want and turn the other off.

3. Start **focus x4** and, by focusing toward the wall (away from yourself), raise the objective turret and find the beginning of your z stack. Hit the **start set** button ③ in the “Microscope” section at the bottom of the **Acquisition Settings** window.

4. Now focus toward yourself to lower the objective and find the last optical section to be included. **BE CAREFULL NOT TO CRASH THE OBJECTIVE INTO THE SAMPLE.** The 25X lens can be safely lowered as much as 1.5 mm. Track how far down the lens has moved by watching the change in Z position shown in **RED** ④. Click the **end set** button ⑤. Click the “Step Size” box ⑥ and choose a step size of 0.5 to 1 um ⑦. The number of optical slices is now shown ⑧.

5. Turn on all channels to be used and start imaging by clicking the XYZ button ②.

6. When all images are complete, the “**Append Next/series done**” button ⑨ appears on top of the stop button in the **Image Acquisition** window. If satisfied with the series as captured, press the **series done** button to end acquisition. Or append the number of optical slices ⑩ and continue capturing more images.

7. Save the images as you would a single picture.
VII. IMAGE MANIPULATION

1. Use the icons/buttons along the top and left side of the image to change what you see on the screen without changing the underlying data file. Many of the icons are self-explanatory; just place the cursor over the button and wait for an explanatory note to appear.

2. Some buttons open menus or boxes. Commonly used buttons include:

   A. The LUT menu opens controls for changing contrast, intensity and gamma for each channel. Choose the channel of interest in the drop-down list. Change the color of a channel by clicking a colored box, or choose one of the special palettes, such as the Hi-Lo for saturation intensity. Note that the Intensity slider affects only one RGB color at a time: use the drop-down list to switch colors. Use the LUT histogram to gain an appreciation for how each slider changes the image. Remember that any changes that you want to keep must be saved as a separate file.

   B. The Overlay/split button used to either overlay all channels or split them into separate images.

   C. The Z slider that lets you scroll thru the images in a Z-stack.
D. The **pencil icon** opens options to draw regions on the image for cropping and adding overlays such as arrows or a scale bar. Click the scale bar button and then draw a line on your image.

E. The **2-D control panel** can be used to view specialty applications such as tiling, and manual settings of rows and columns for displaying z-stack images. This menu also has a very useful button for re-loading all of the optimized settings used in a previously saved image.

Acknowledgment:

Our funding depends upon your citing the **Cell Imaging Shared Resource** when you publish data obtained with equipment or services from CISR. This would include images from the microscopes, training or the use of data analysis software. The minimum acceptable acknowledgment should read:

“Experiments/Data analysis/presentation [include what you use]” were performed in part through the use of the VUMC Cell Imaging Shared Resource, (supported by NIH grants CA68485, DK20593, DK58404, HD15052, and Dk59637).
VIII. FV-1200 SHUT DOWN PROCEDURE

1. Check the calendar on the CISR website to see whether you are the last user of the day. If you are using the scope during regular work week hours (until 6 PM) and there is another user scheduled before 6:30, please leave all of the equipment turned on.

2. Open the laser control menu and click the “Off” button. Save your images and close the FV-10 software. When the warning prompt says that light will be emitted from the objective, close the manual shutter on the mercury lamp. Click “OK” and log off. Fill in the log sheet.

3. If you are using the scope in the evening or weekend and someone is scheduled to come within 15 minutes, proceed as described above.

4. Raise the objective as high as it will go, remove your sample and clean the lens with lens paper and distilled water.

5. If you are the last user of the day or it is an evening or weekend where the next user is not due to arrive for at least 15 minutes, proceed to shut the system down entirely. After logging out, turn off the computer followed by switches thru .

6. Clean up around the scope and computer bench so that the room is cleaner and more tidy than when you arrived. Thanks.