UCHC Guide to FACS DiVa

LSR II instruments

Start Up:

Turn on the instrument using the Green power button.

Fluidics pump power, LSR II-B & C

LSR II-B and LSRII-C-> Turn on the fluidics pump and ensure that it is <u>running</u>; listen for the motor.
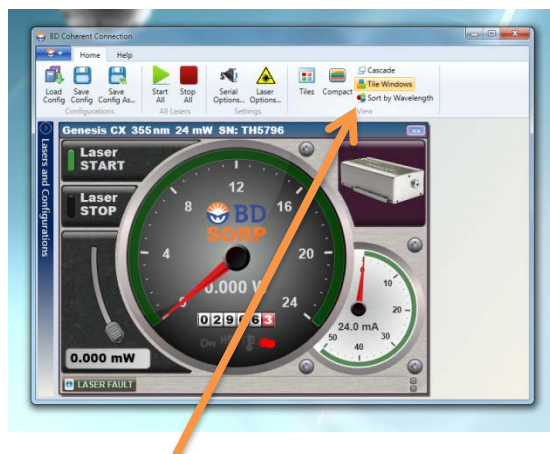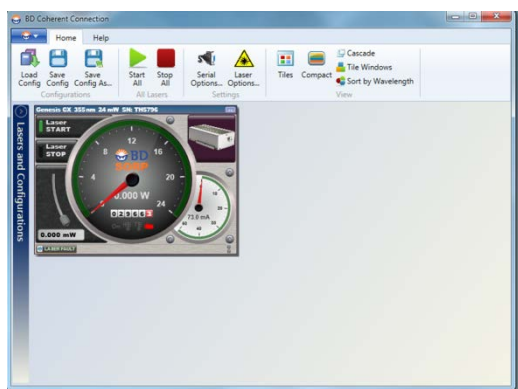
Turn on or log into the computer.

> User name=LSRII-A, LSRII-B, or LSRII-C

> Password =BDIS

Turn on (or confirm) Coherent Connection 2 laser control software.

> Coherent Connection 2 is set to startup automatically upon login.

> The first user of the day will presented with the following screen:
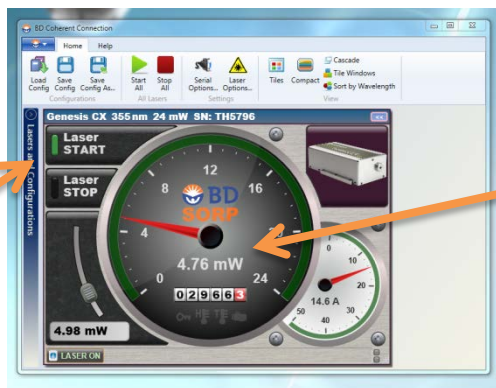

Resize the UV laser window by selecting "Tile windows"

Click on Laser STOP

Enter 5mW in the power window
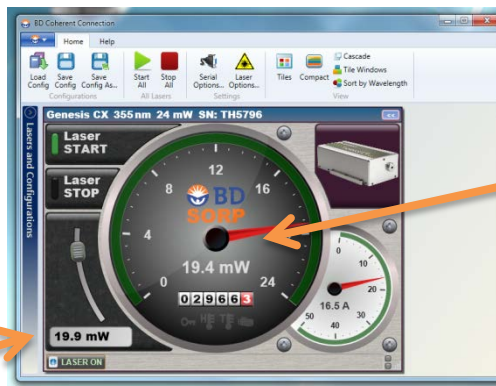

The "speedometer" should move to 5 mW ± 0.5

Click on Laser START

The "speedometer" should move to 20 mW ± 0.5

Enter 20mW in the power window

The UV laser is now on and at full power. You may now ignore the Coherent Connection 2 window.

PLEASE LEAVE THE WINDOW OPEN!

Check sheath fluid level. Make sure there is sheath pressure and remove bubbles from the bubble filter and intake line.

Bubble Filter Bleeder

Secondary Sheath Bleeder

Move sample fine adjust to center or 1X speed (5 rotations)

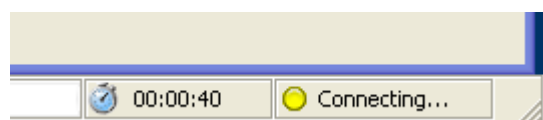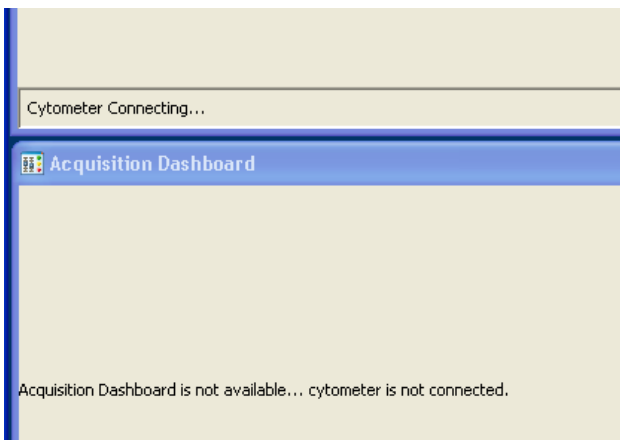Prime the instrument twice with no tube and arm to the side.
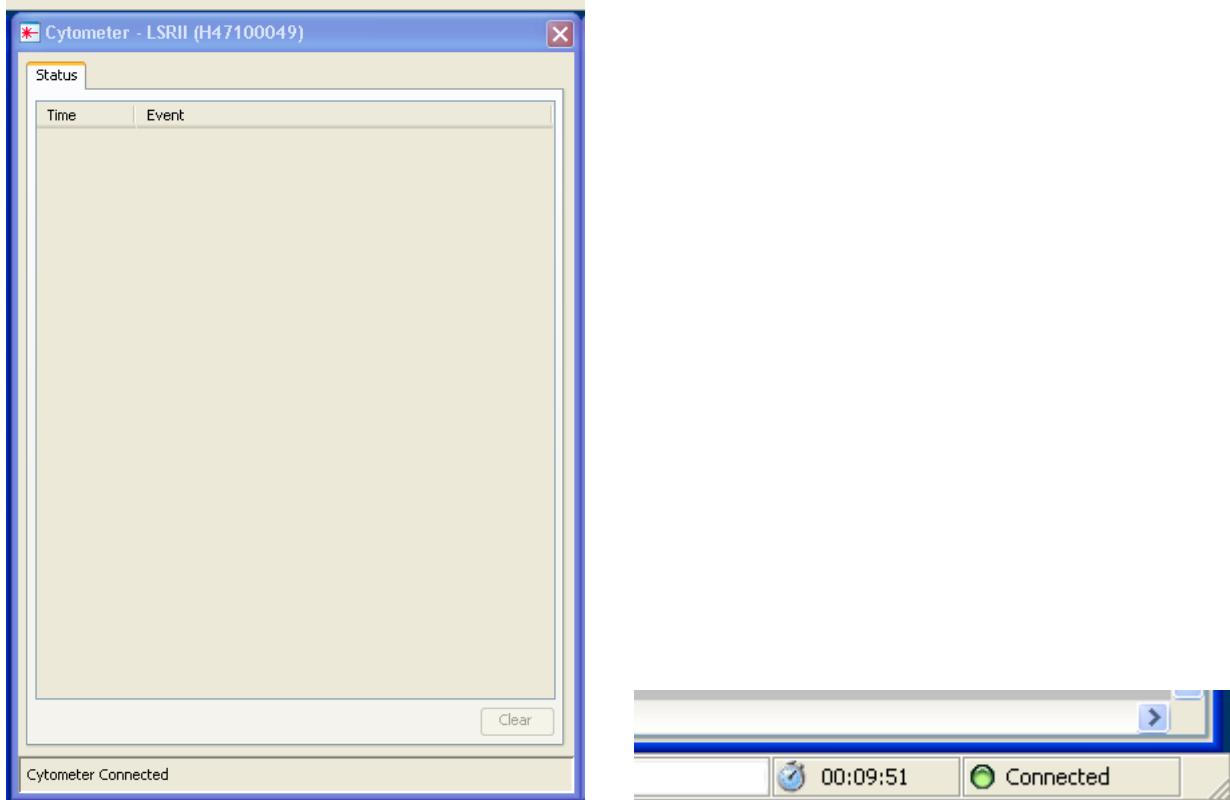

Start BD FACSDiva software

Log in using your Lab's credentials

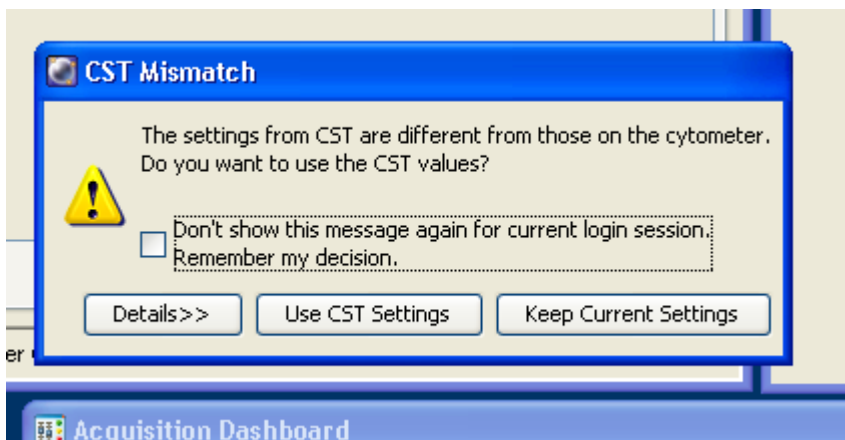Allow the Cytometer to connect to the computer
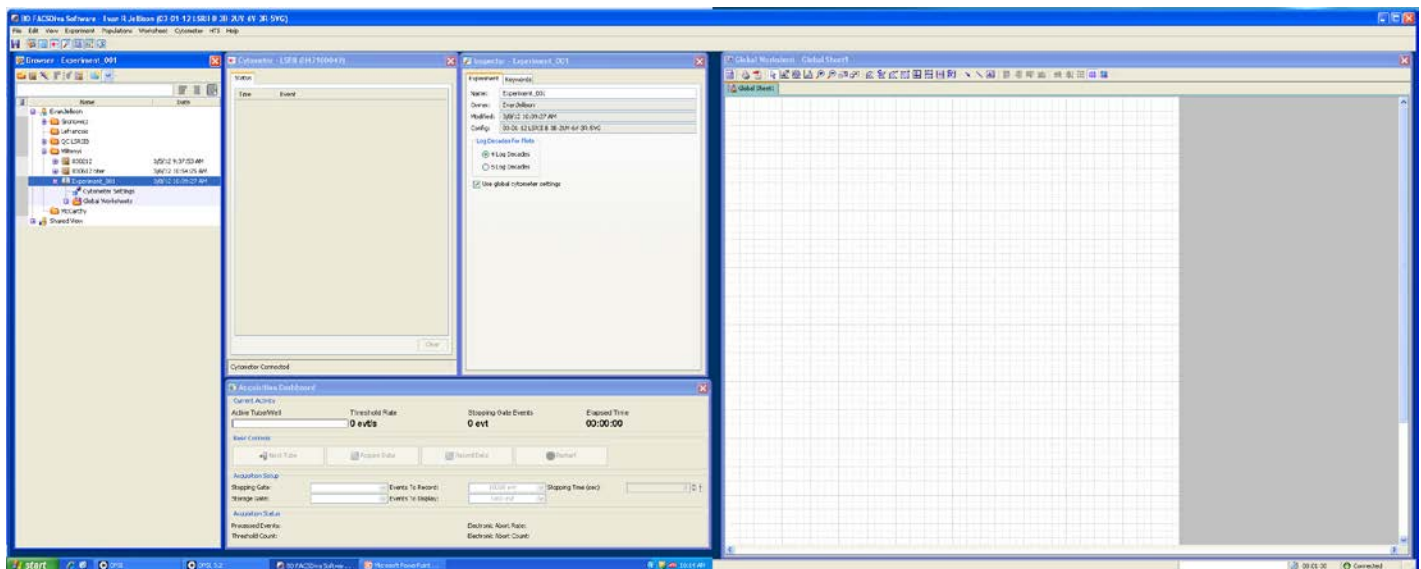
Once connected you should see:

If the cytometer does not connect, restart both the cytometer and computer.

If prompted, choose Use CST Settings.

Welcome to DiVa!

The above windows should all appear. They are the **Browser, Cytometer, Inspector, Acquisition Dashboard, and Worksheet.** If one is missing, navigate to view and select the missing window(s) or use the shorcut buttons on the menu bar.
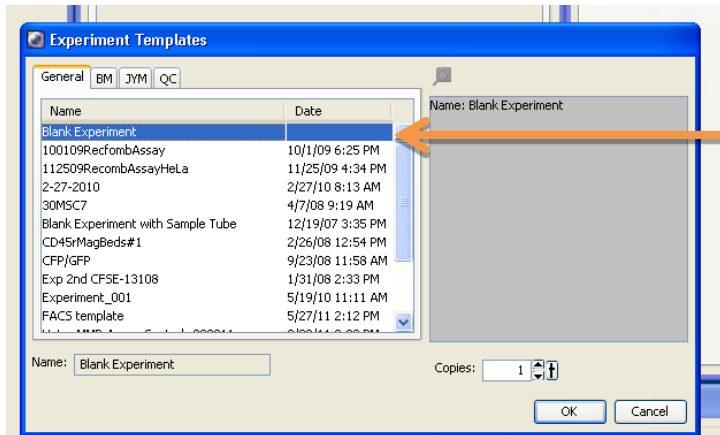

Create a folder with your name by using the create folder icon or by using the experiment drop down menu. Folders help keep labs with multiple users organized.
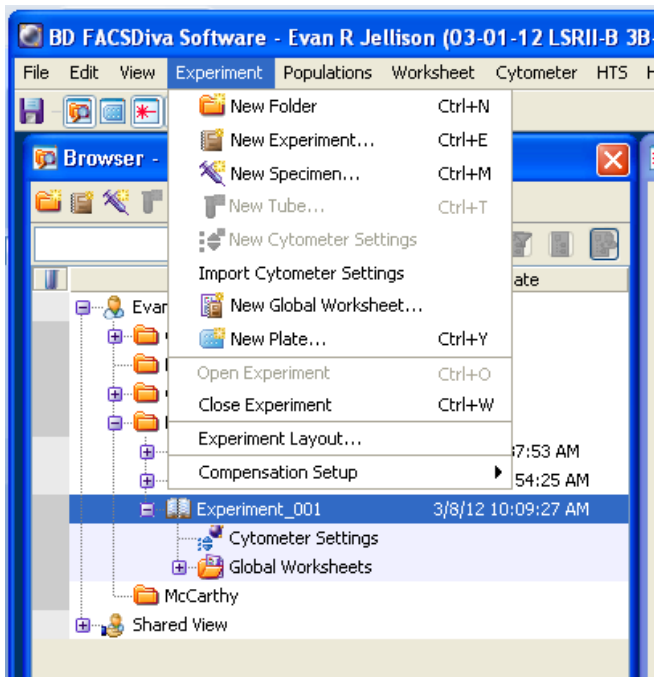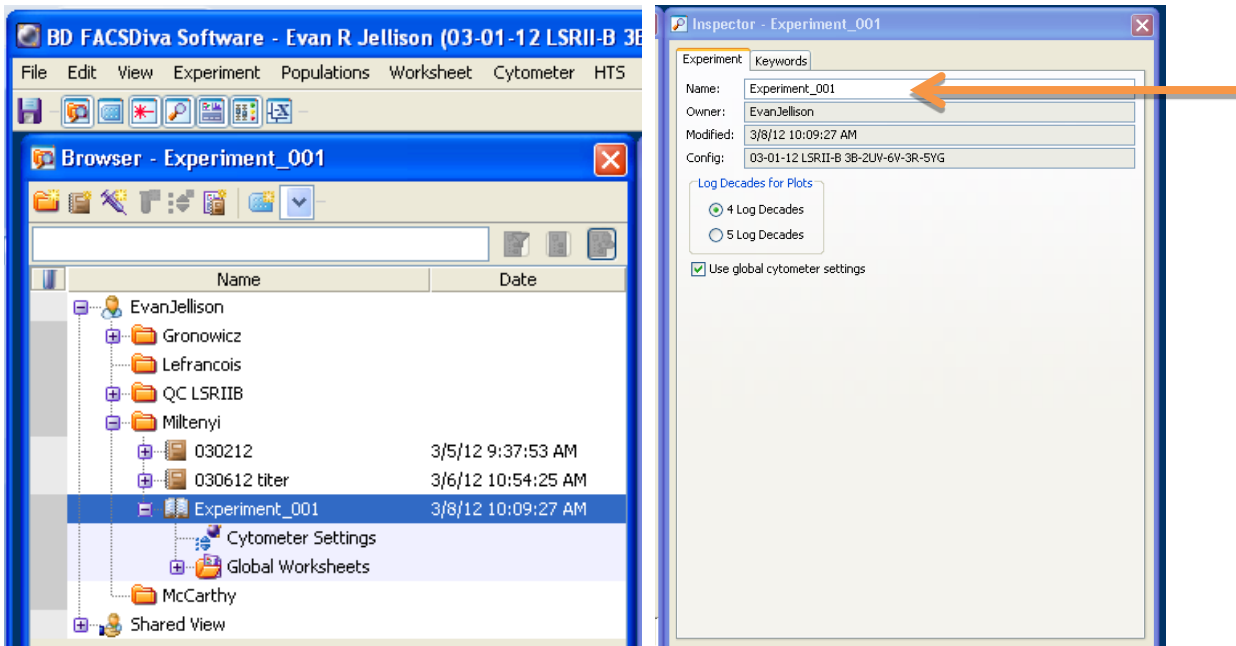
Make an experiment by selecting the new experiment icon or by using the drop down (as above) and selecting blank experiment.

Blank Experiment

A new experiment will appear in the Browser window. Note that the icon is a notebook. Active or in-use experiments are open notebooks, inactive or not-used experiments are closed notebooks

You may rename your experiment by right clicking the experiment and selecting rename, or by selecting the experiment and renaming in the inspector window. This step is highly recommended for organization purposes.

Cytometer Settings:

Select cytometer settings under your experiment name. In the Inspector window, change the names of parameters as they relate to your experiment by clicking on the name and selecting the appropriate label from the drop down menu. If your fluorochrome is not listed, choose a fluorochrome with similar excitation/emission properties; fluorochrome names are only for our benefit, not the instrument's
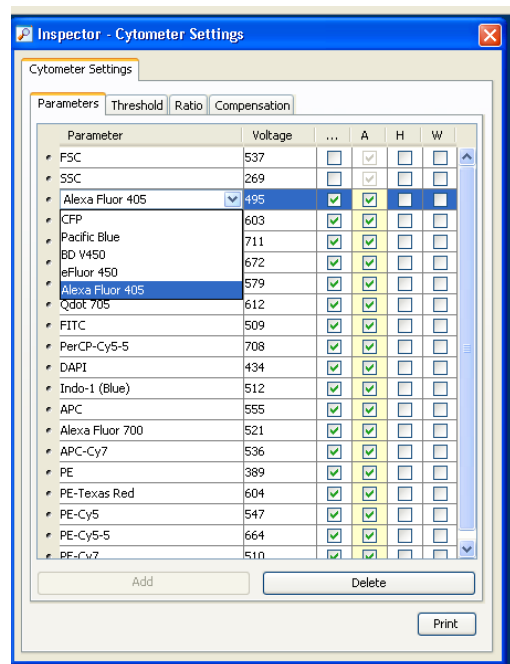

Delete parameters that you do not have by highlighting them and selecting delete. Multiple parameters can be selected by holding down the Ctrl key.
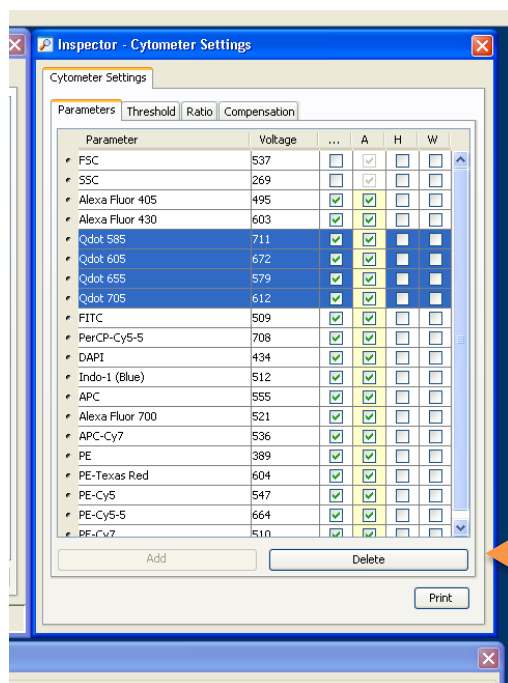

Delete button

At a minimum, select the Area **(A)**, Height **(H)** and Width **(W)** parameters for **FSC** in order to eliminate doublets from your analysis. FSC sould be collected using linear scale. SSC-area (A) is all that is necessary on the LSRII, however SSC-H and SSC-W may also be used for additional doublet discrimination. SSC can be collected in either linear or log scale, depending on the needs of the experiment or preference of the lab. All fluorescence parameters should be collected in log scale unless DNA cell cycle analysis is to be performed. Area should be selected for fluorescence parameters on LSR II instruments.
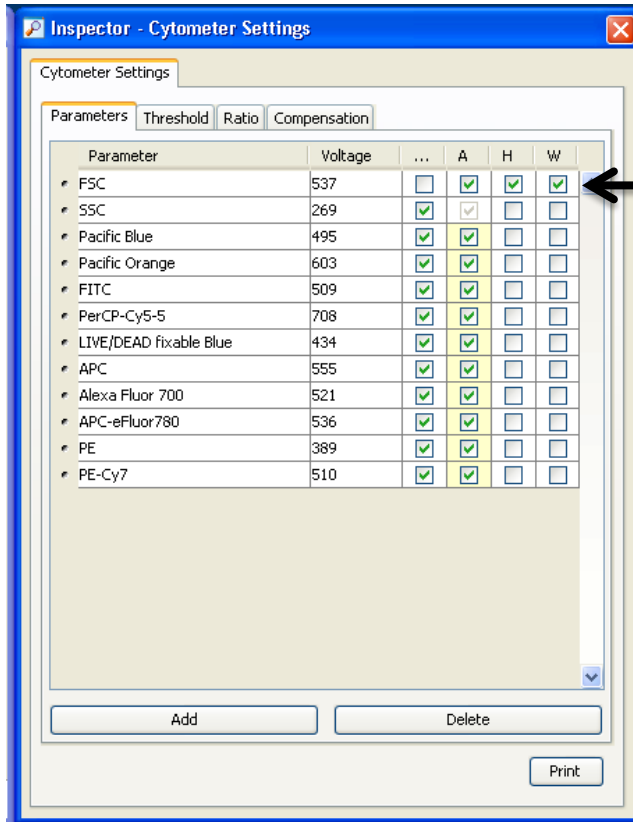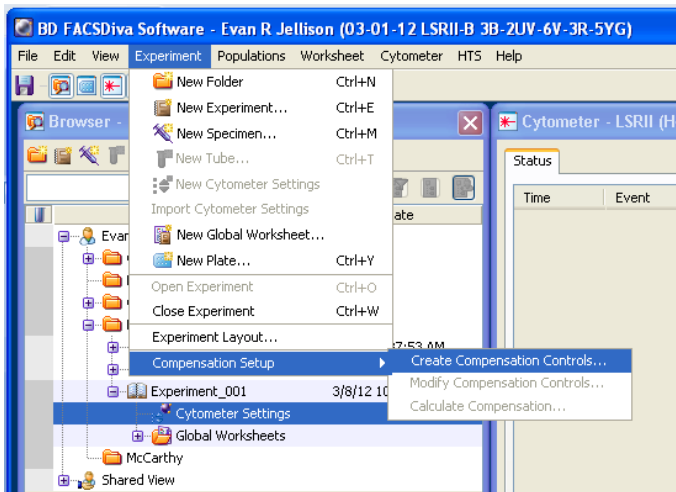

Select A, H, & W for FSC

Example of the same data collected in SSC-Linear and SSC-Log:
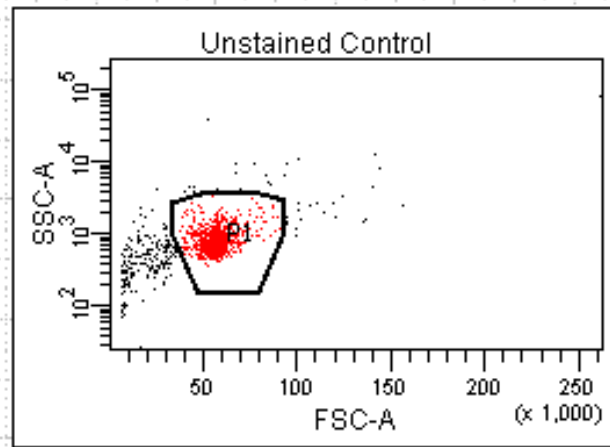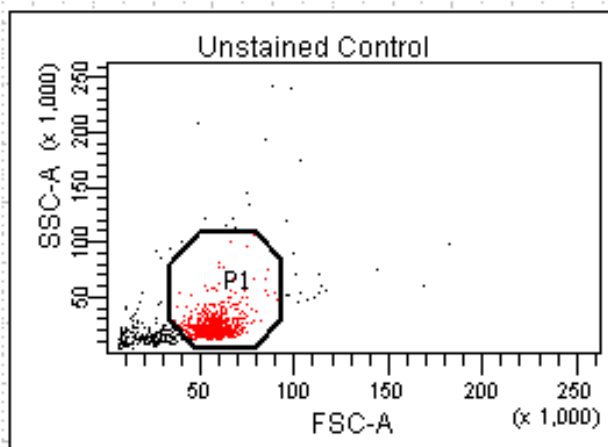
Automatic Compensation:

Create compensation controls by selecting the Experiment dropdown> Compensation Setup> Create Compensaton Controls

On the next screen, you can highlight and delete any parameters that you do not wish to compensate (because the positives will be excluded from your future analysis). Examples include autofluorescence parameters, Live Dead discriminators, or lineage marker dump populations. Selecting "include separate unstained control tube/well" will include a universal negative unstained control in your compensation controls. **DO NOT LABEL YOUR ANTIGENS ON THIS SCREEN.** Leave the labels as generic.
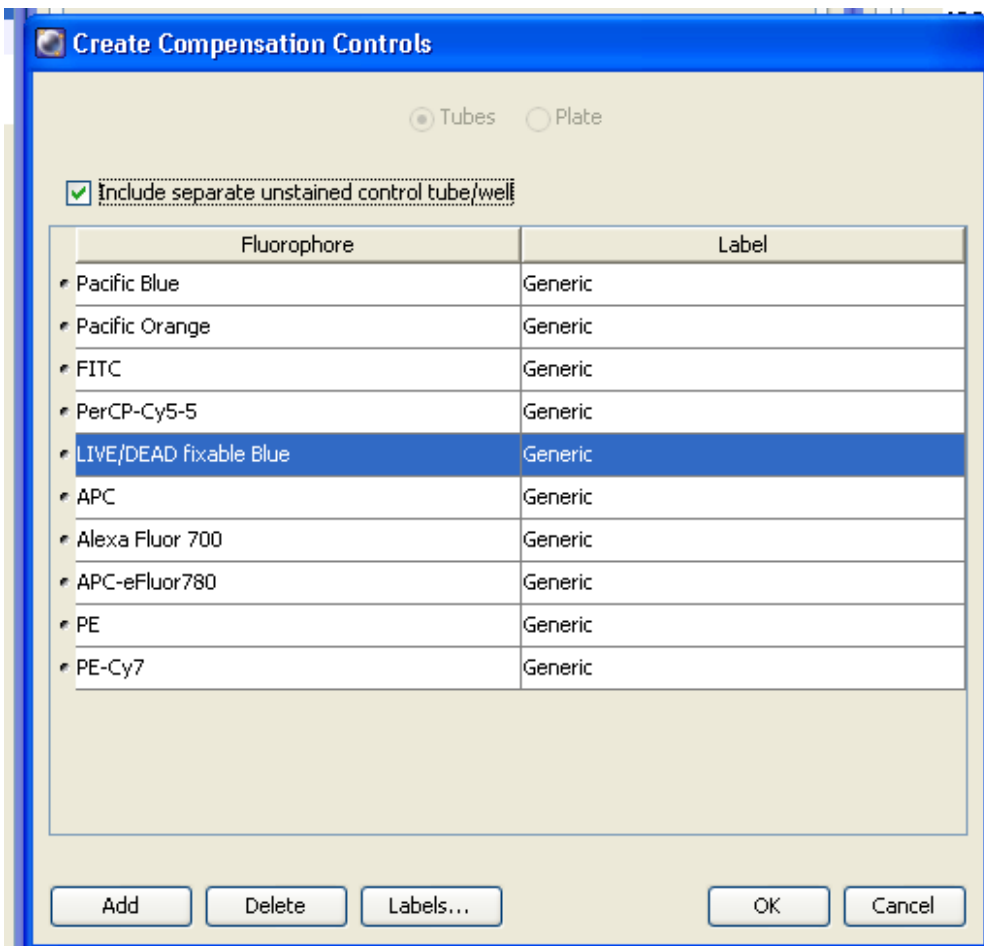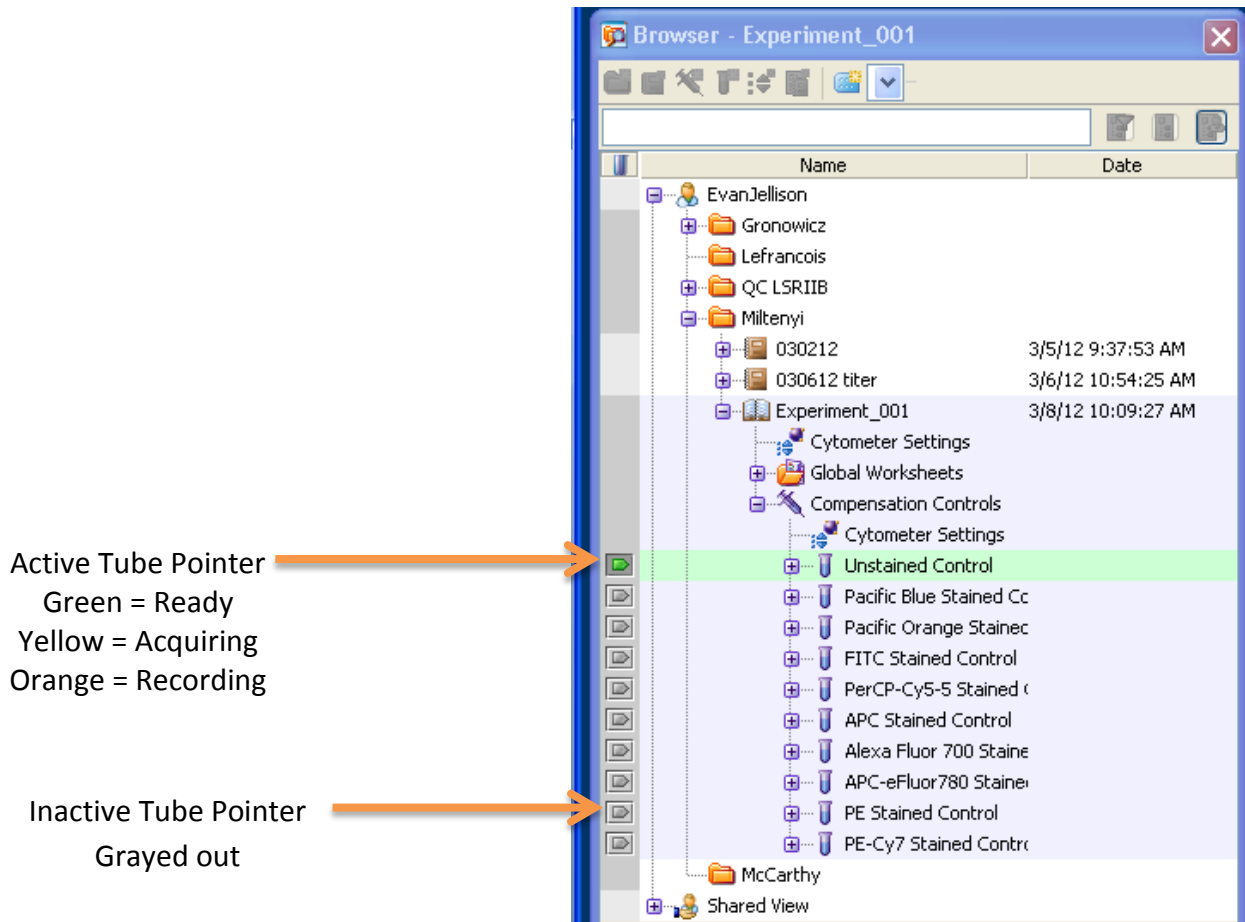
Select the OK button. You will see a specimen appear in the Browser window labeled Compensation Controls.
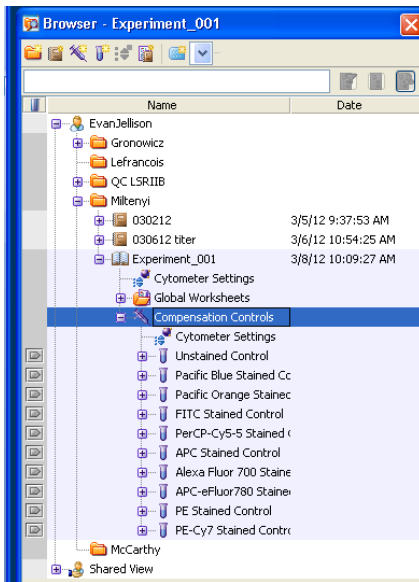
Expand the compensation contol specimen by clicking the **+** symbol.

Click on the Tube pointer next to the unstained control, it should turn turn green.

Active Tube Pointer
Green = Ready
Yellow = Acquiring
Orange = Recording

Inactive Tube Pointer
Grayed out

The Tube Pointer is the "eye of the LSR II". The Tube pointer tells you what the instrument is "looking at". As its name implies, it can only look at Tubes. Selecting (highlighting) elements in the browser does not tell the LSRII what tube to "look at". The LSR II can be looking at one tube even if you have selected or highlighted a different tube. The Tube Pointer should point at the the tube you want to Aquire/Record.

Note the Worksheet window now has plots that correspond to your fluorescent parameters. Highlight and move (click & drag) any plots that appear off screen into view. You will want to see all of the plots.
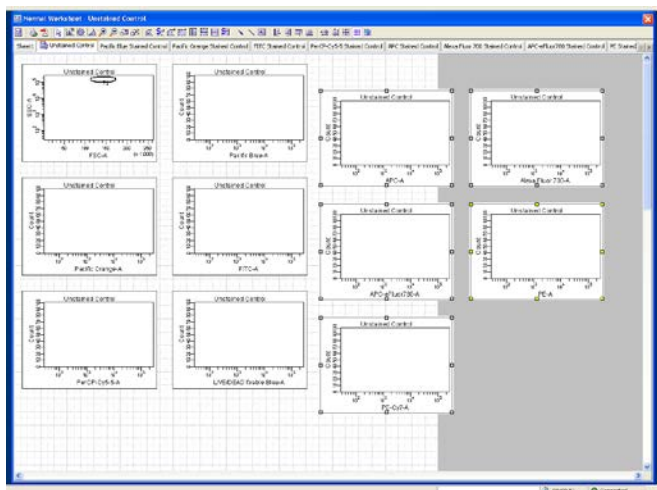
On the FSC x SSC dot plot, move the P1 gate into view. Adjust the FSC voltage to ~350±100. In the cytometer window, click on the number and either adjust the slider, click on the up /down arrows, or type in 350. Most cells @ UCHC should be on scale with this voltage. Larger cells will need a lower voltage and smaller cells will need a higher voltage.
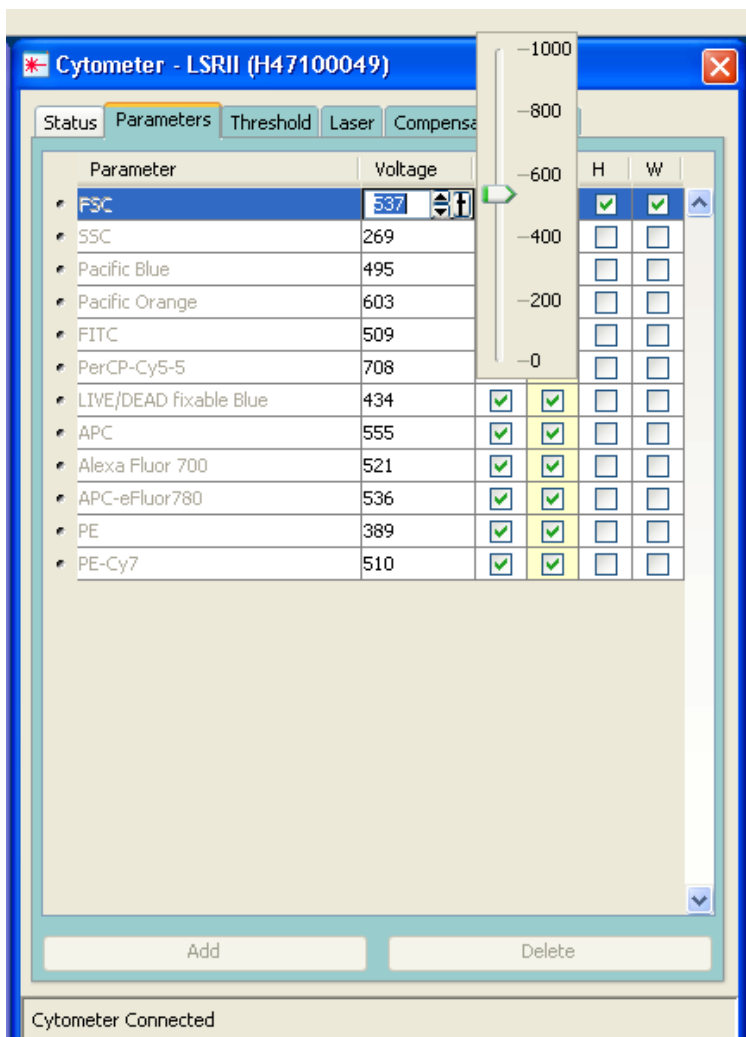
Put the instument in Run mode at low speed.

Place your unstained cells on the SIP tube and select Acquire in the Acquisition Dashboard window.

Note the number that appears under Threshold Rate. This should be maintained below 10,000 events per second to prevent clogs and reduce electronic abort events. If necessary, dilute your cells with saline. If there are too few events, you may elect to increase the flow rate using Medium, High, or the Sample Fine adjust. Remember that these adjustments reduce sample data resolution.

Examine the plots in the Worksheet window and adjust the FSC and SSC voltages to put your population of interest on screen. I prefer to have this population in the lower left quarter of the dot plot but you may want to put this population in the center of the dot plot.
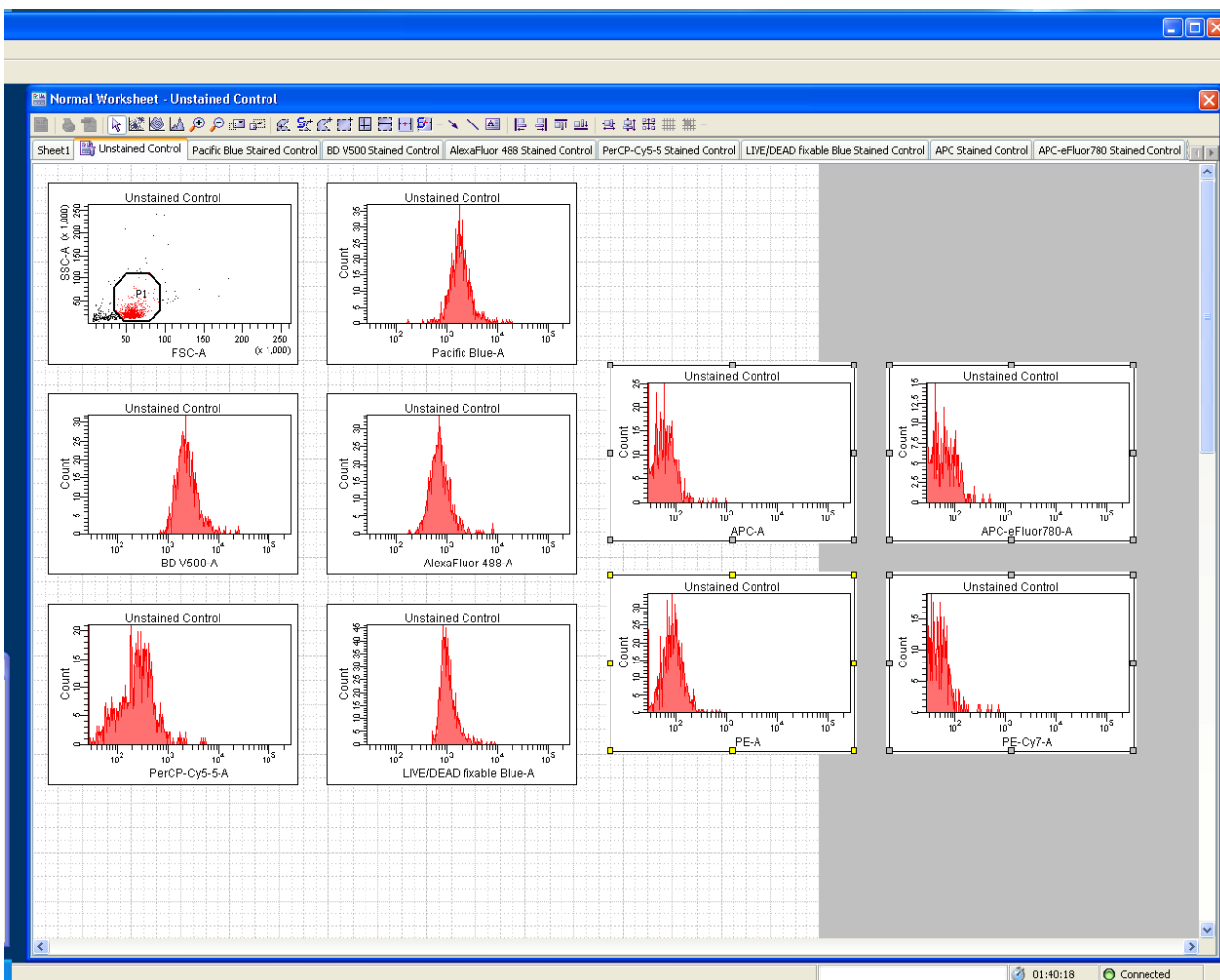
Adjust the P1 gate so that it surrounds your population of interest. Note where your cells are falling on the fluorescence histograms. **For some parameters, your population may not be all the way to the left of the histogram and that is OK (eg: Pacific Blue-above). For some parameters, your population may be somewhat hidden on the left edge of the histogram, this too is OK (eg: APC-eFluor780 and PE-Cy7-above).**

Without clicking Aquire, remove the unstained contol tube from the SIP and put on your first single color control. Look at the fluorescence parameter and examine where the positive cells fall on the histogram. **You should <u>only</u> adjust the voltage of your fluorescent parameters if the positive is off scale.**

Example **Figures A, B, & C below**:

Figure A, AlexaFluor488 <u>positive</u> cells are "on scale" of the histogram. Note that the negatives **are not** all the way to the left of the histogram.
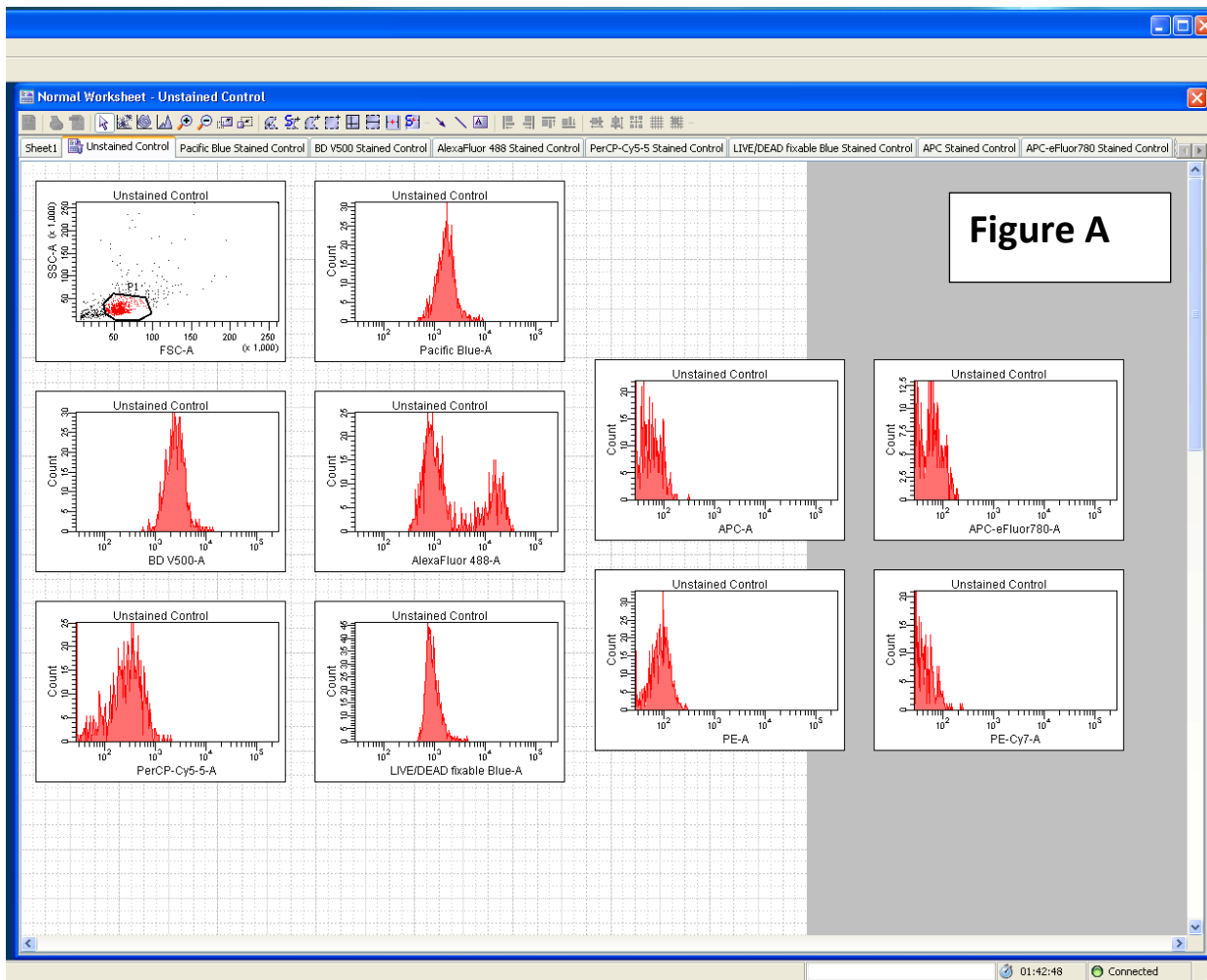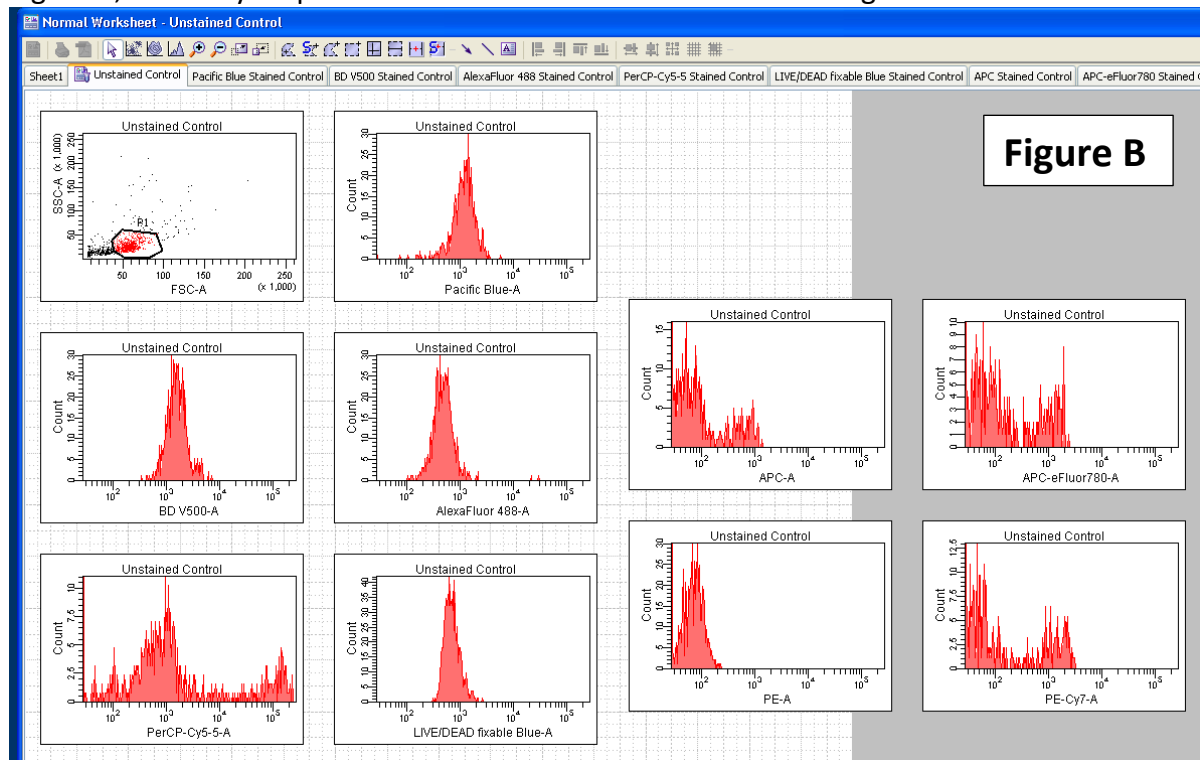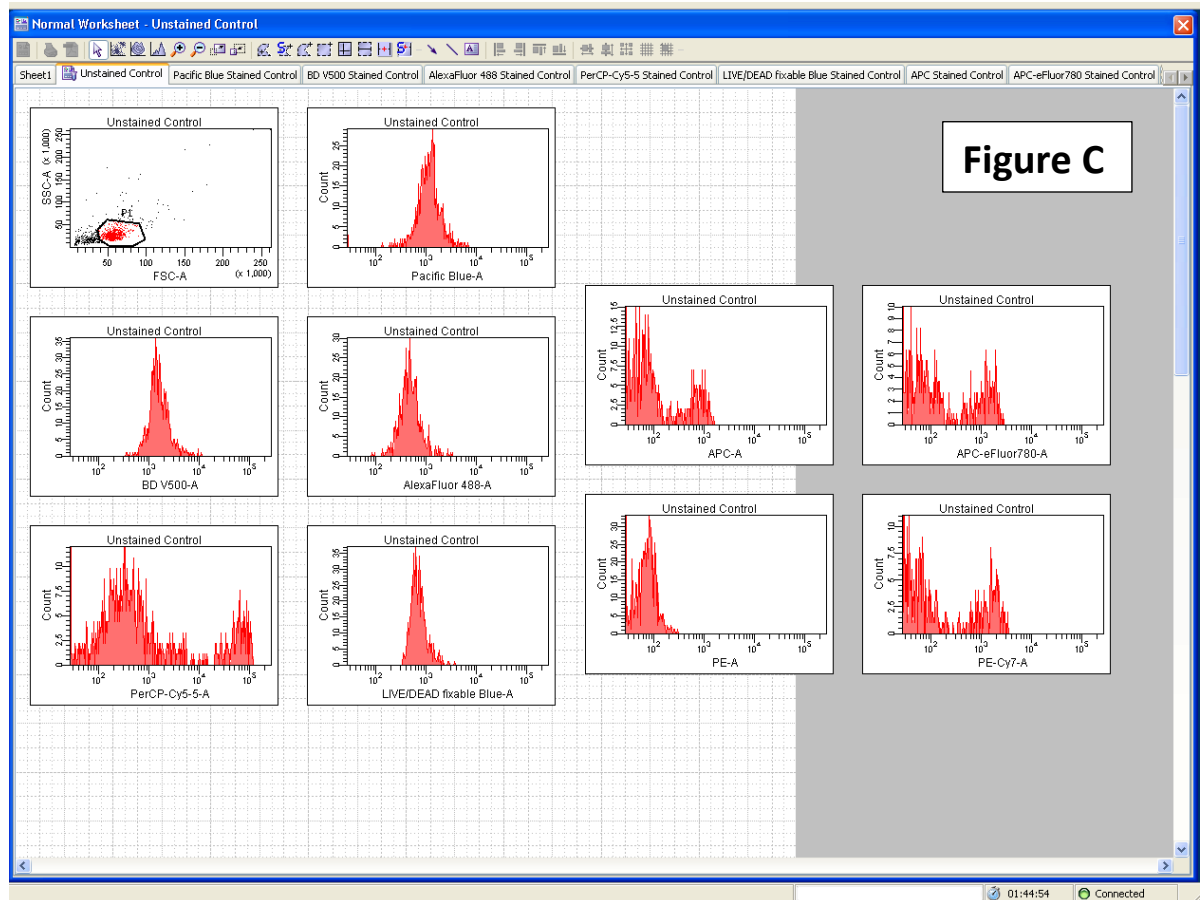

Figure A

Figure B, PerCP-Cy5.5 positive cells are "off the scale" of the histogram.


Figure B

Adjust the PerCP-Cy5.5 voltage down to get the PerCP-Cy5.5 positive cells "on scale" (Figure C).

Figure C

Repeat this process with all of your single color control samples.

Whenever possible, adjust antibody titrations or the concentration of fluorescent dyes in order to avoid voltage adjustments. Unfortunately, fluorescent constructs like GFP and mCherry will always need to be adjusted if the positive cells are off scale.

DiVa advanced: If you plan to repeat this experiment, this is the point at which you export Application Settings

Record your unstained contol tube.
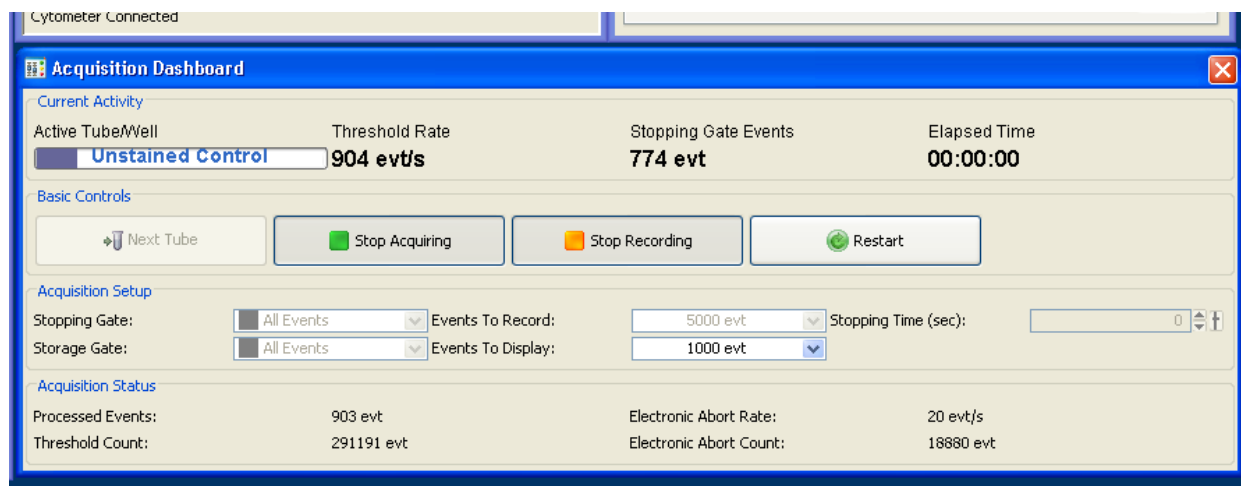
DiVa sets the events to record at 5000. You may elect to increase this number by selecting the dropdown or typing the number of events you want to record in the box. Note the progress bar in the Acquisition Dashboard and Stopping Gate Events as they relate to recording your sample.
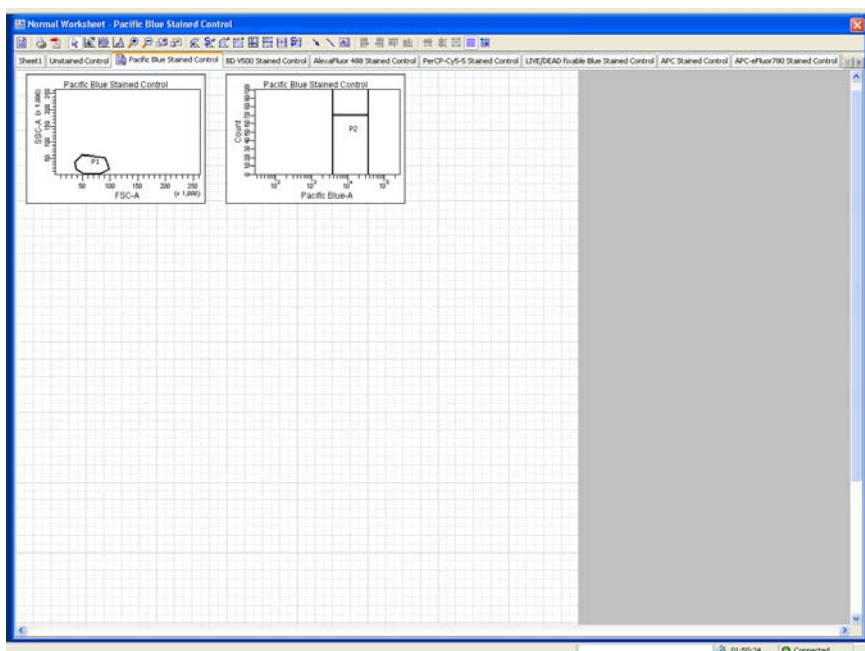
Remove your sample from the SIP.
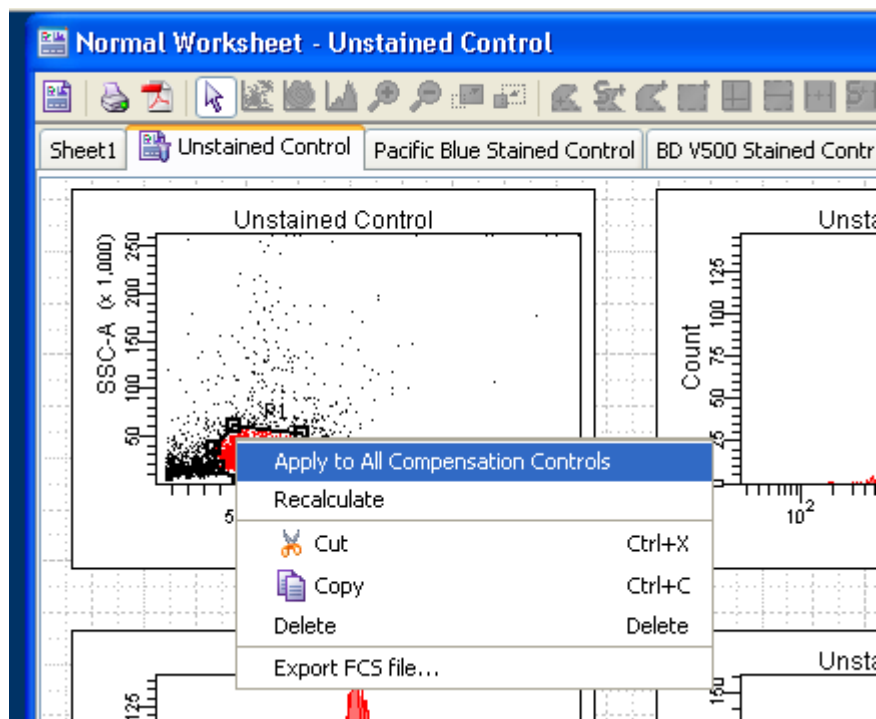
Highlight your adjusted P1 gate, right click, and select Apply to All Compensation Controls.
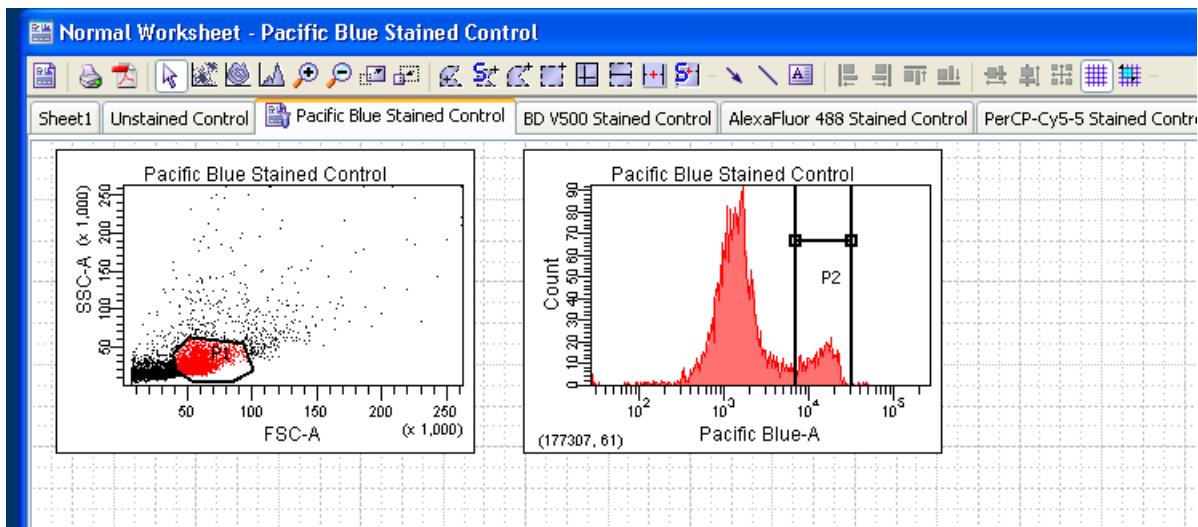
In the Acquisition Dashboard select Next Tube or select the Tube Pointer next to the first single color control sample.
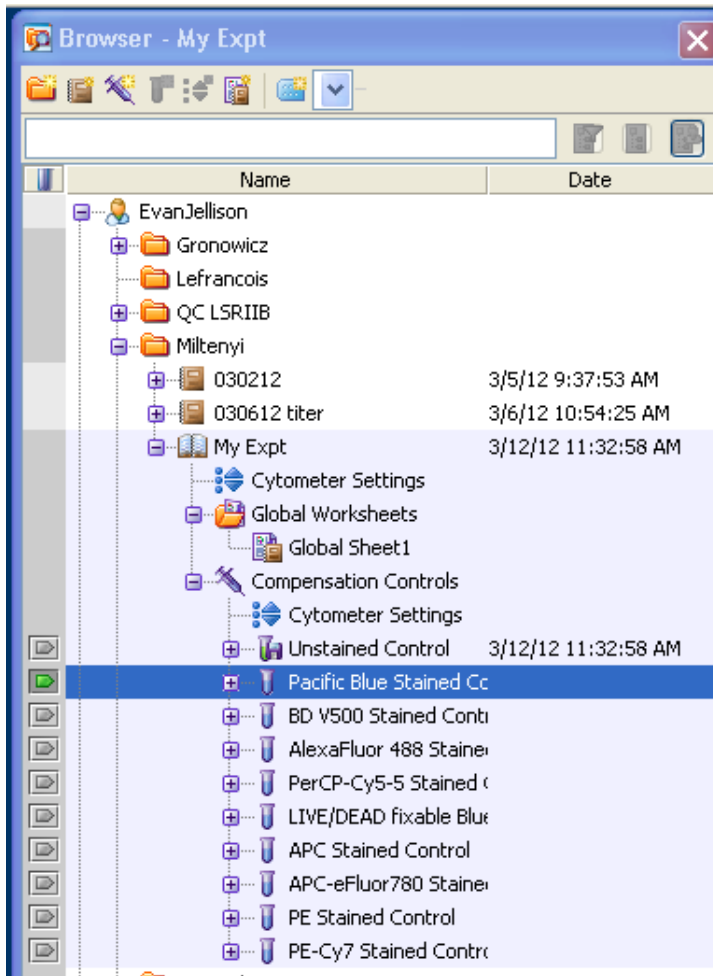
Your Worksheet window will move to the next worksheet (tab) and the P1 gate should be adjusted.

Acquire and Record the single color control that DiVa is asking for (check the Tube Pointer).

The P2 gate will automatically adjust to where DiVa thinks is the positive population. As necessary, manually adjust this gate to encompass the brightest cell population.

Note: You may need to adjust the P1 gate to get positive cells. **If you adjust P1, you should add a P3 negative to the histogram to indicate the location of the negative population for your single color controls.**

Calculate compensation values by selecting the Experiment dropdown> Compensation Setup> Calculate Compensation…
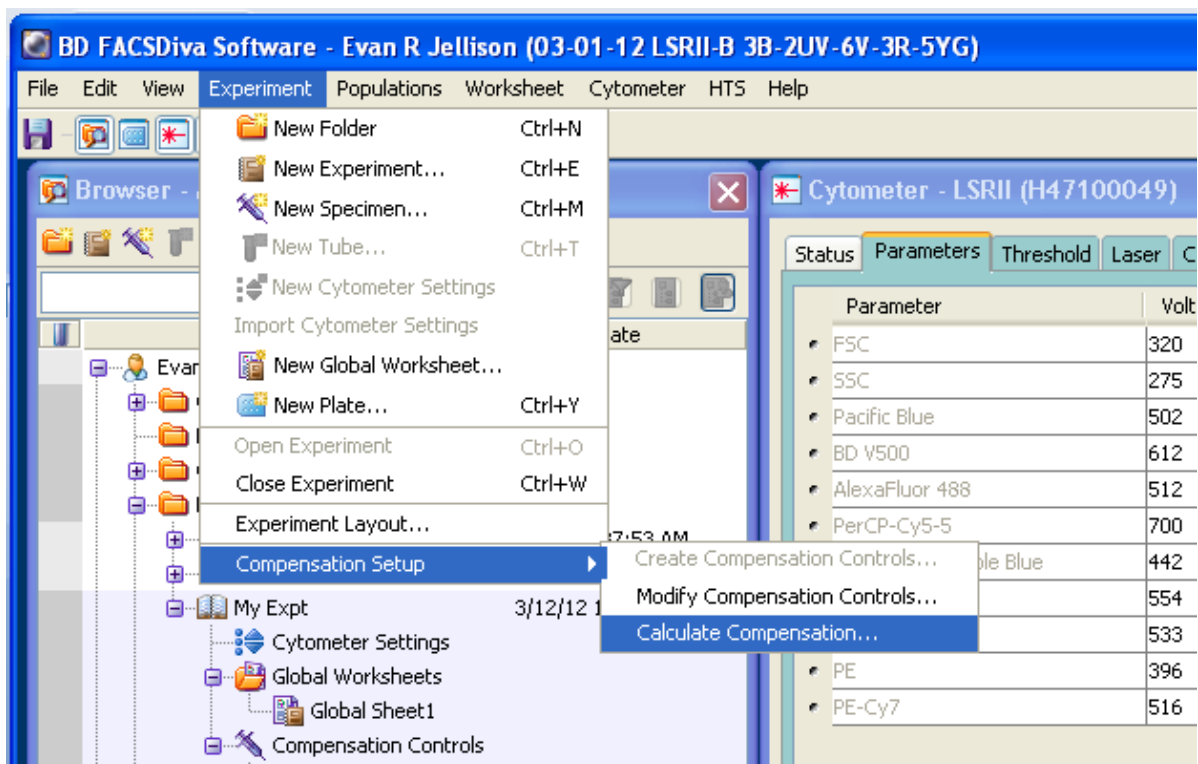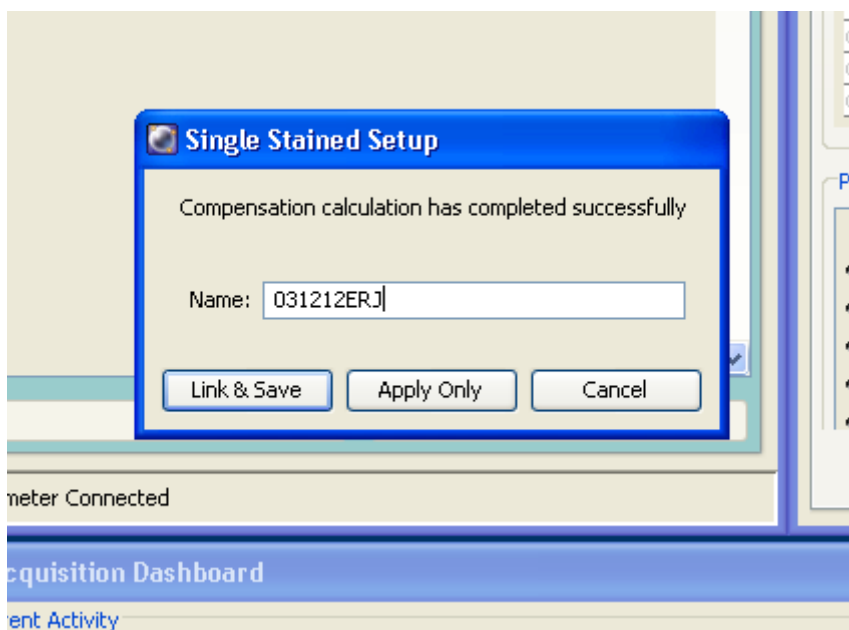

The computer may appear to pause while it calculates.

When presented with the following screen, Compensation has been calculated successfully.
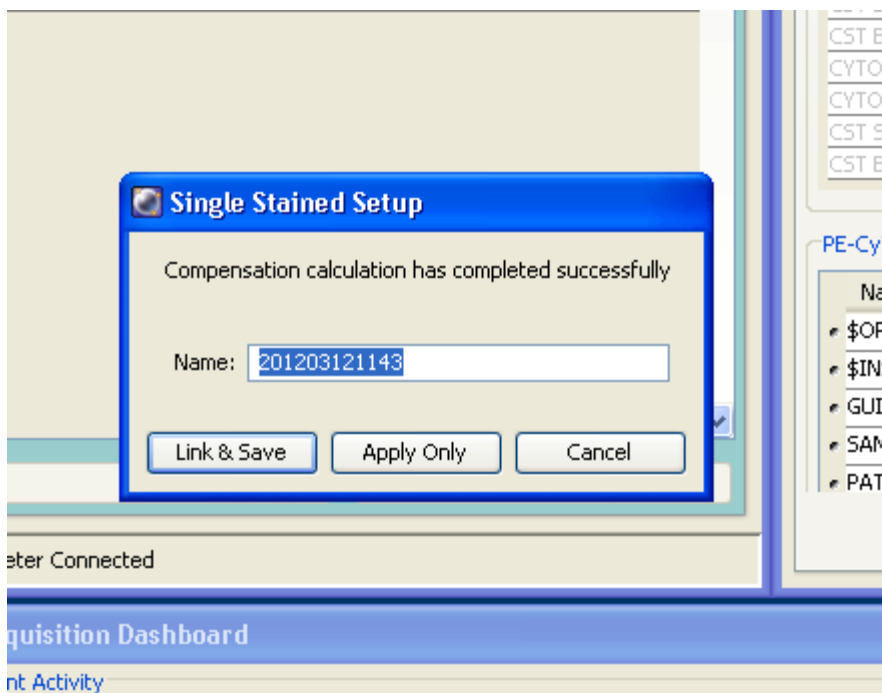
Rename the compensation setup so that you can identify it easily and select Link & Save.

Notice the gold chain that appears by cytometer settings. This indicates that the compensation has been successfully linked to your experiment. Congratulations!
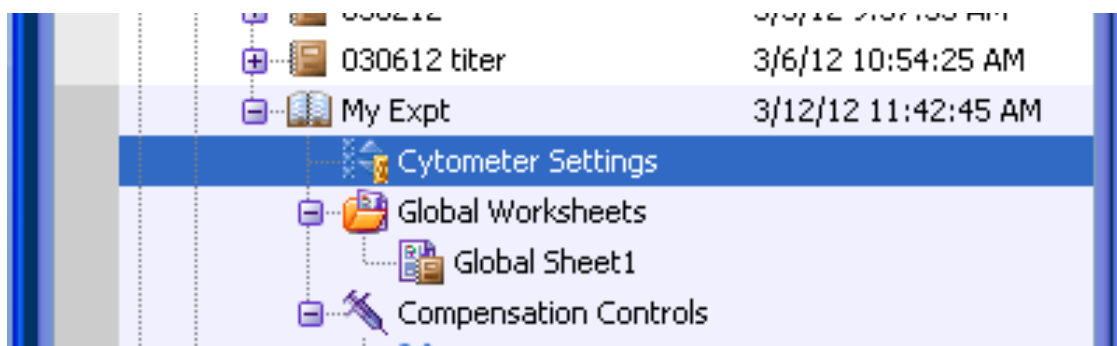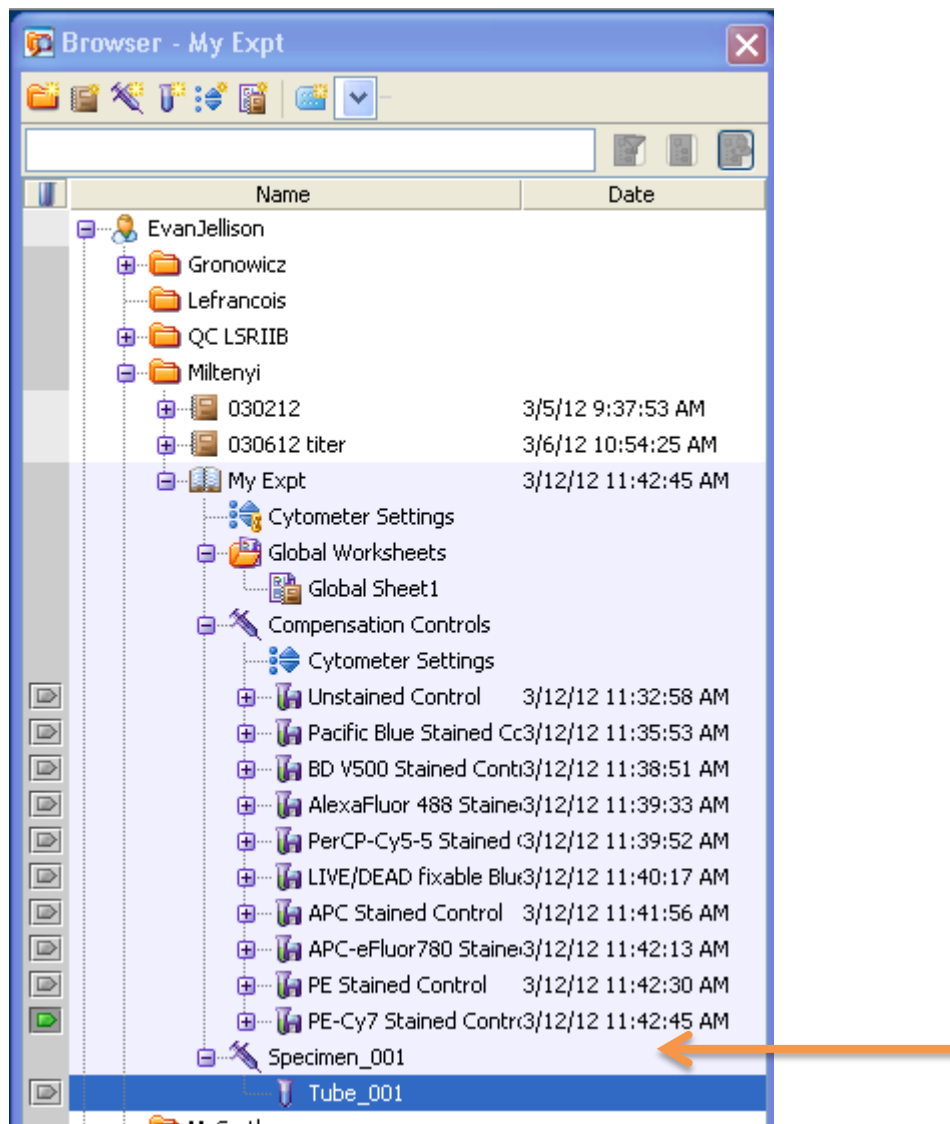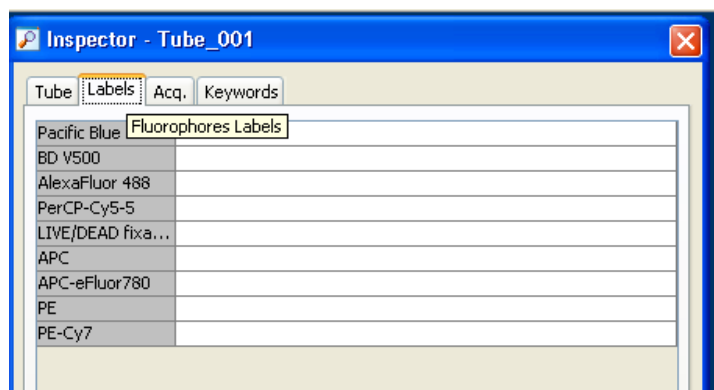
Collecting Experimental Sampes:

Add a new specimen by clicking the syringe icon or by navigating to the Experiment dropdown> New specimen and select Blank Panel.

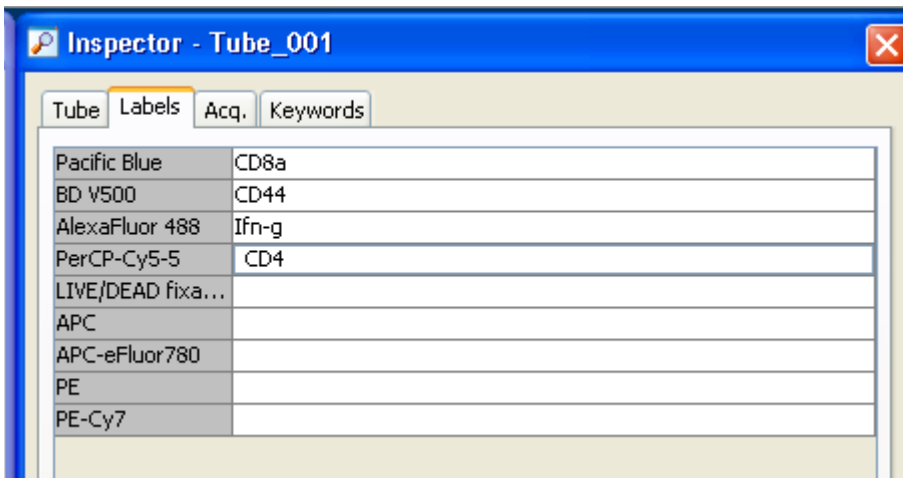A new Specimen and Tube will appear in the browser.

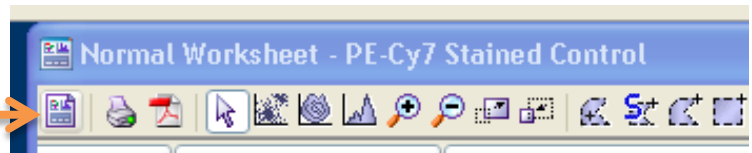Highlight the tube and, in the Inspector window, select the Label tab.

Label your antigens on this screen. *Press enter after typing the label.

**DiVa advanced:** labeling can be done globally using Experiment> Experiment Layout...

Switch to the Global Worksheet by selecting the worksheet toggle in the upper left corner of the worksheet window.

Worksheet Toggle button ⟶

Create plots on the worksheet by clicking the desired plot button on the worksheet toolbar and clicking anywhere on the worksheet. Custom-sized plot can be created by clicking and dragging on the worksheet. Double-clicking a plot button makes it sticky, which will make a plot each time you click in the worksheet. To "unstick", single click on the plot button again or click on the arrow button.
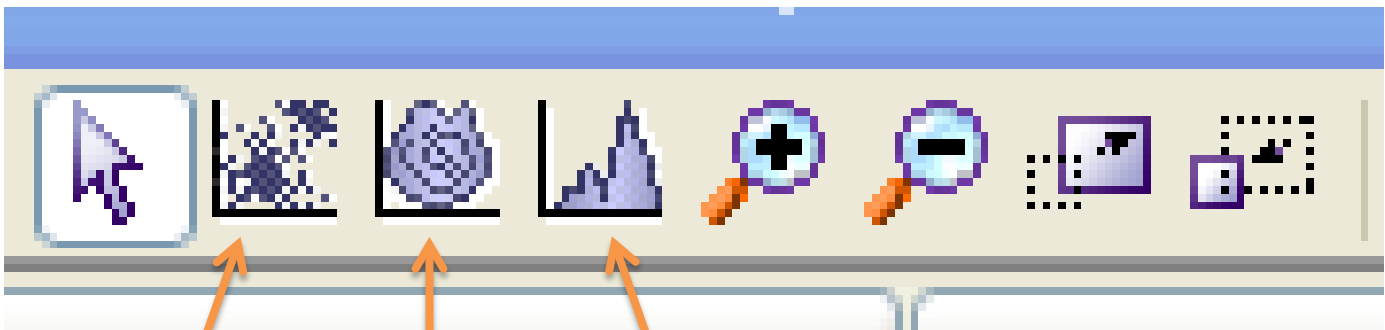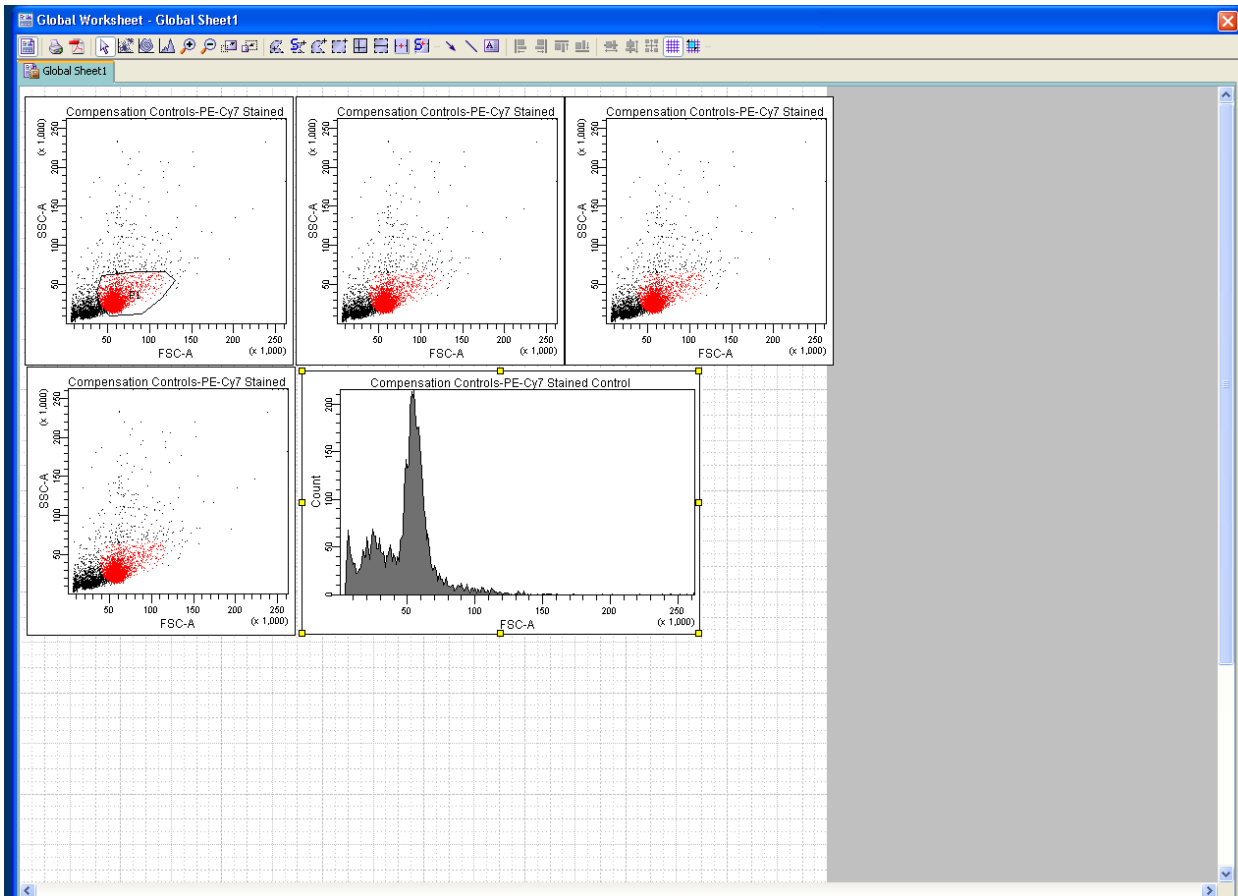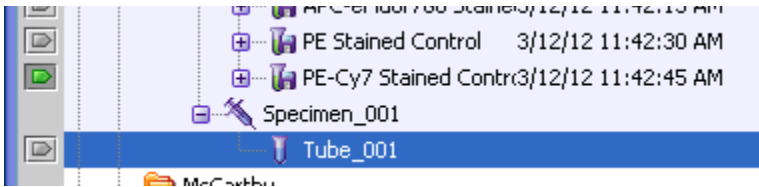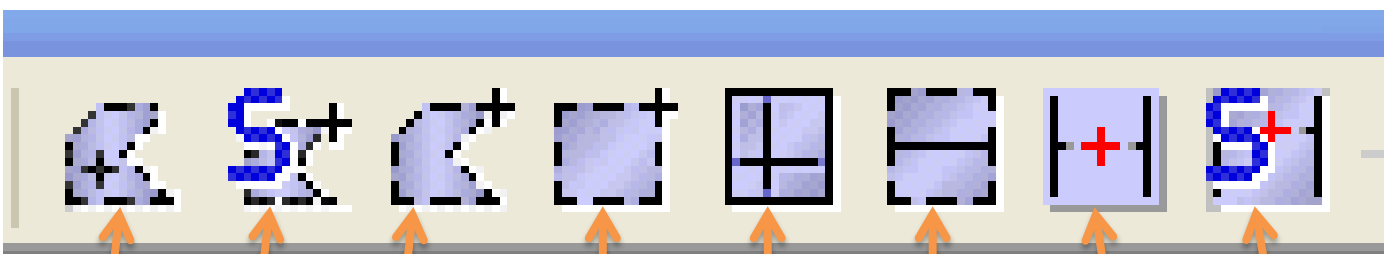

Dot plot   Contour/Density plot   Histogram

Note: If the tube pointer is still pointed at your last single color control, the plots you create will show the events from the single color control. You may elect to use this to do the initial gating for your experiment. See example below.

Create gates using the gate buttons on the Worksheet toolbar.

Auto-polygon   Polygon   Quadrant   Auto-Interval

Snap to polygon   Rectangle   Interval   Snap to Interval

Auto gates will attempt to surround the most dense population of cells that is selected. **Snap-to** gates do the same but will adjust from one sample to the next in order to find the most dense population. Use these with caution.

Change the parameters on the plots by clicking the label on the plot and selecting the desired parameter…
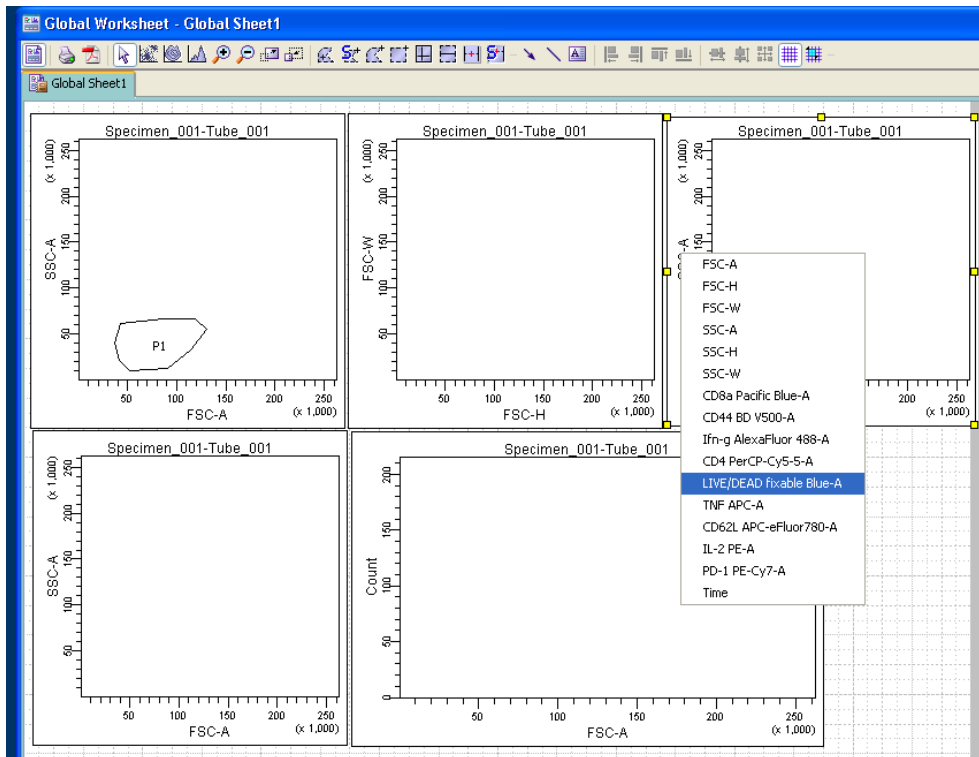
or by selecting the plot and changing the drop down menus in the Inspector Window's Plot tab.
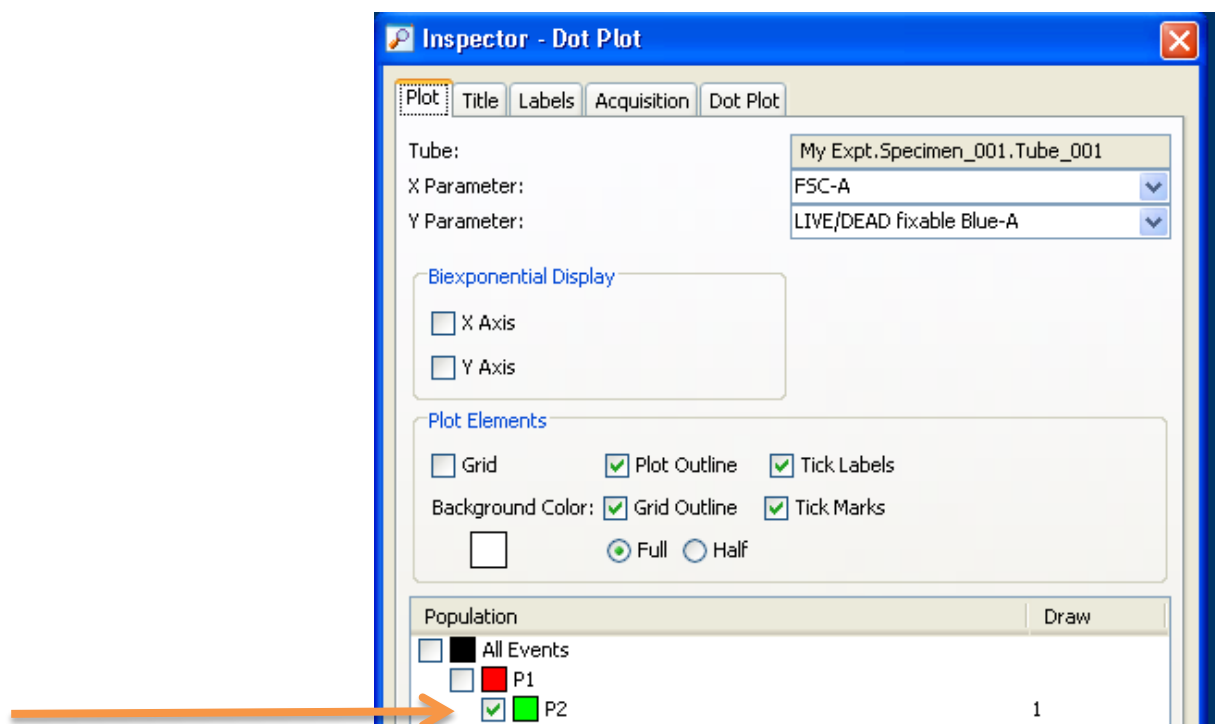
Some parameters may not be available until you move the Tube Pointer to the first experimental tube.
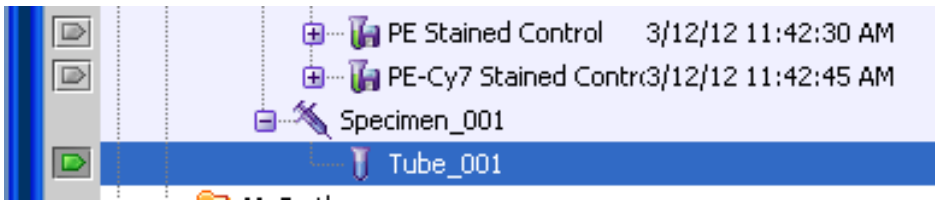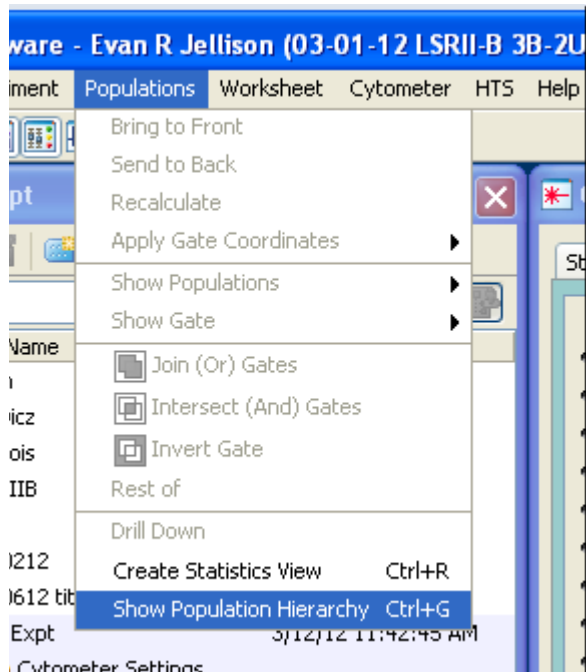

To make a plot show only gated events either right-click the plot and navigate to Show Populations…
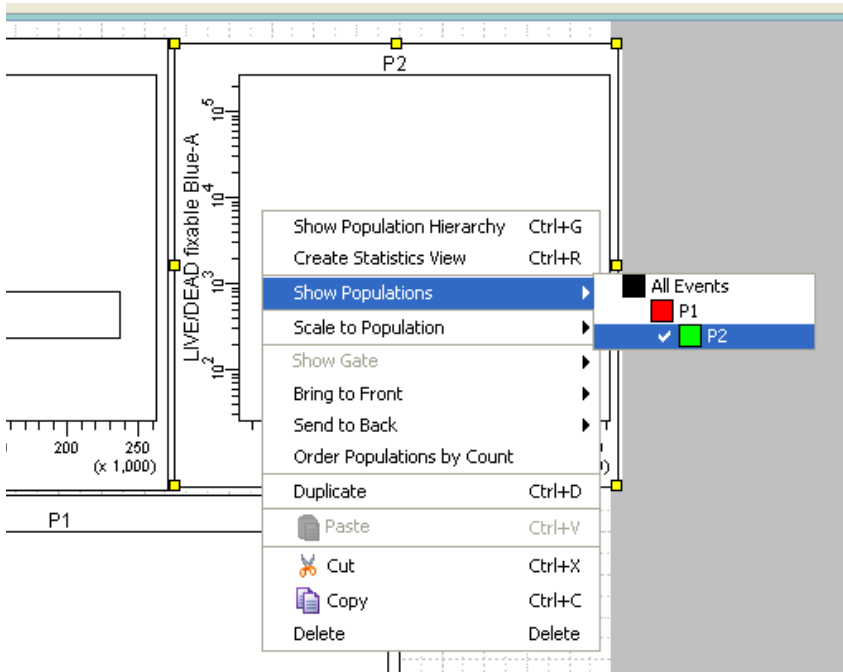

or select the plot and check the appropriate population in the Plot tab in the Inspector window.

To know the parent/child relationships of gates, open the population hierarchy by navigating to the Populations dropdown and selecting Show Population Hierarchy.
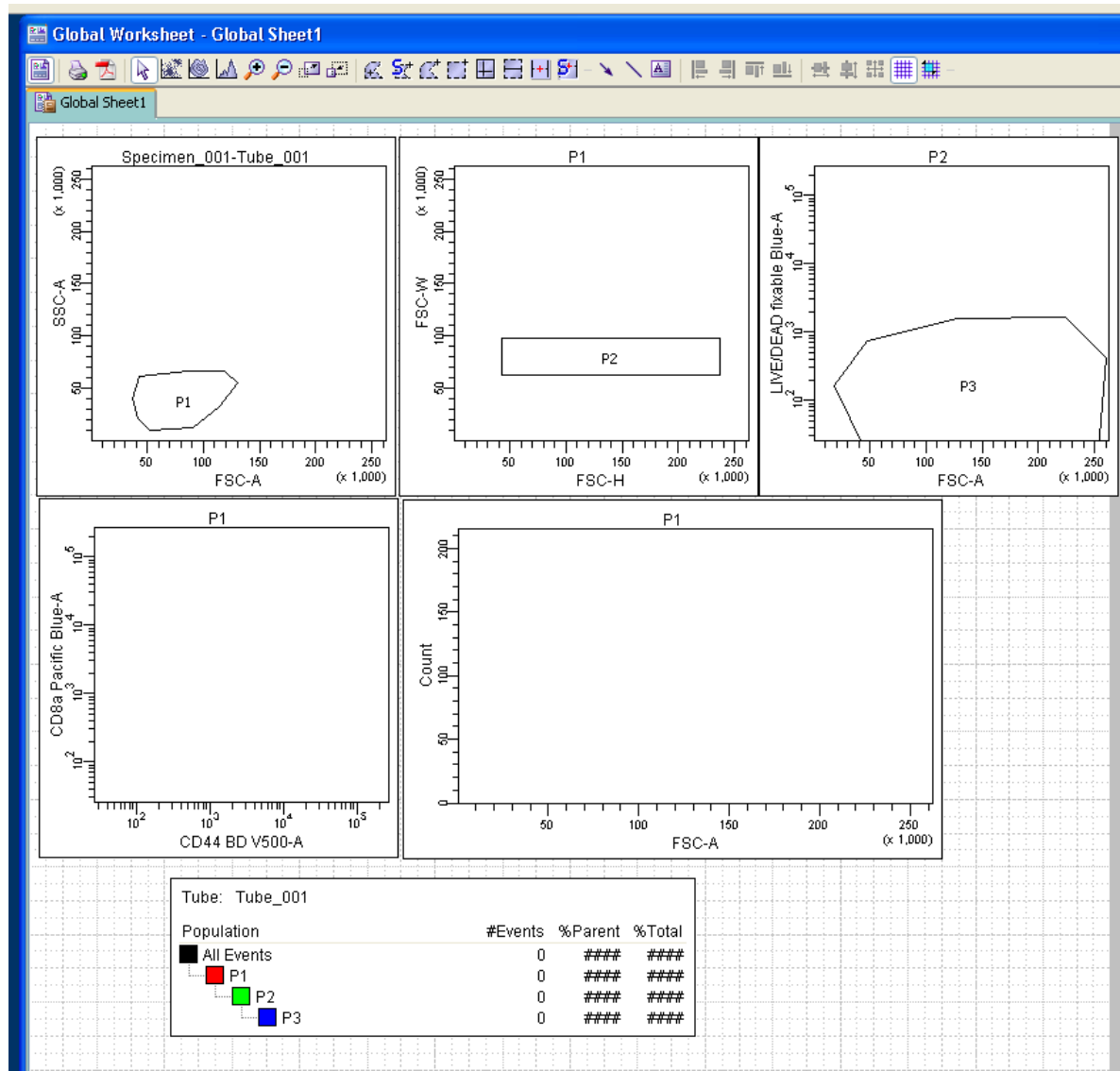

The population hierarchy tells us that P3 is a child of P2, which is a child of P1, which is a child of all events.
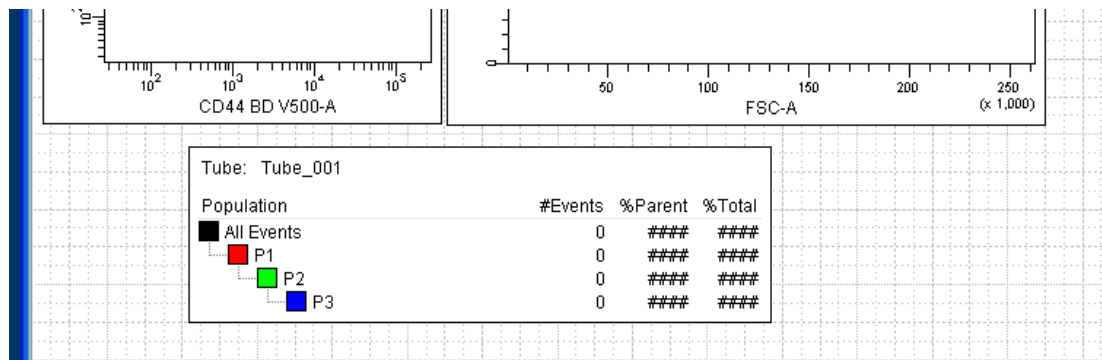
Plot titles can be changed to indicate the gate that is being shown by selecting the plot and selecting Populations in the Title tab of the Inspector Window.
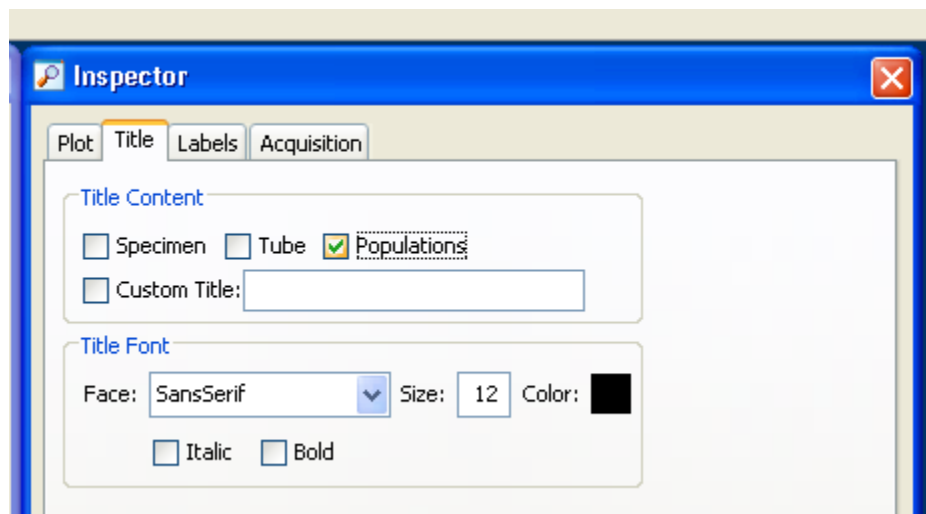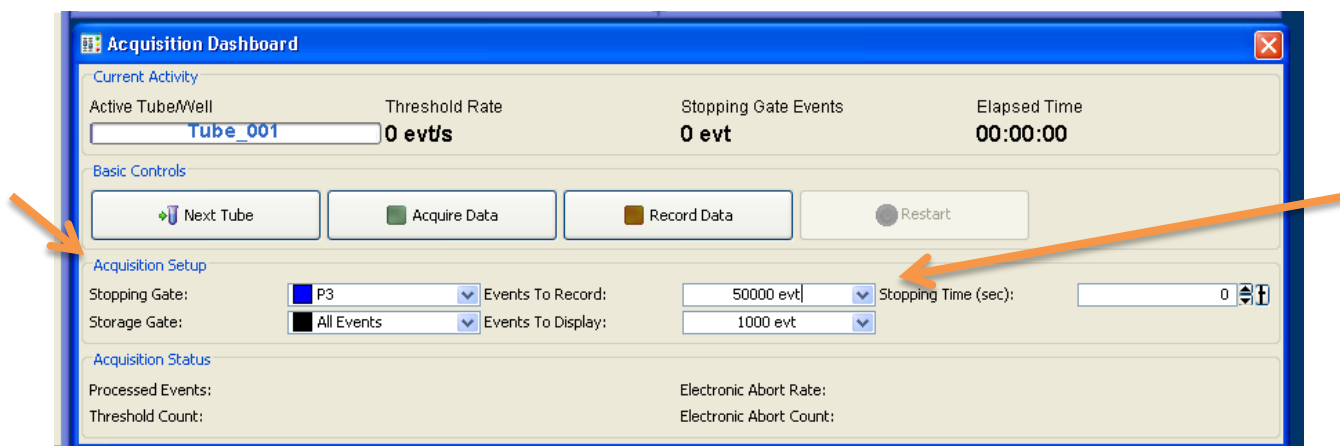
Below is an example of a finished Global worksheet.

DiVa advanced: If you plan to repeat this experiment or use this Global Worksheet again, this is the point at which you export the Acquisition Template.

Adjust the numbers of events and stopping gate in the acquisition setup on the Aquisition Dashboard.

Stopping Gate: Defines what events will stop recording

Storage Gate: Events that will be written to the hard drive. *safest to leave as All Events

Events To Record: How many Stopping Gate Events will you need?

Events To Display: The number of events that appear in the Worksheet Window. A lower number will refresh the Worksheet Window faster.

Stopping Time (sec): Stop recording after this many seconds. (0 = off or don't stop based on time)

Relabel your Specimen and Tube in the Inspector or right-click> rename. **Hint:** If your tube name ends in "_001", when you select Next Tube it will automatically be called "Tube name_002".
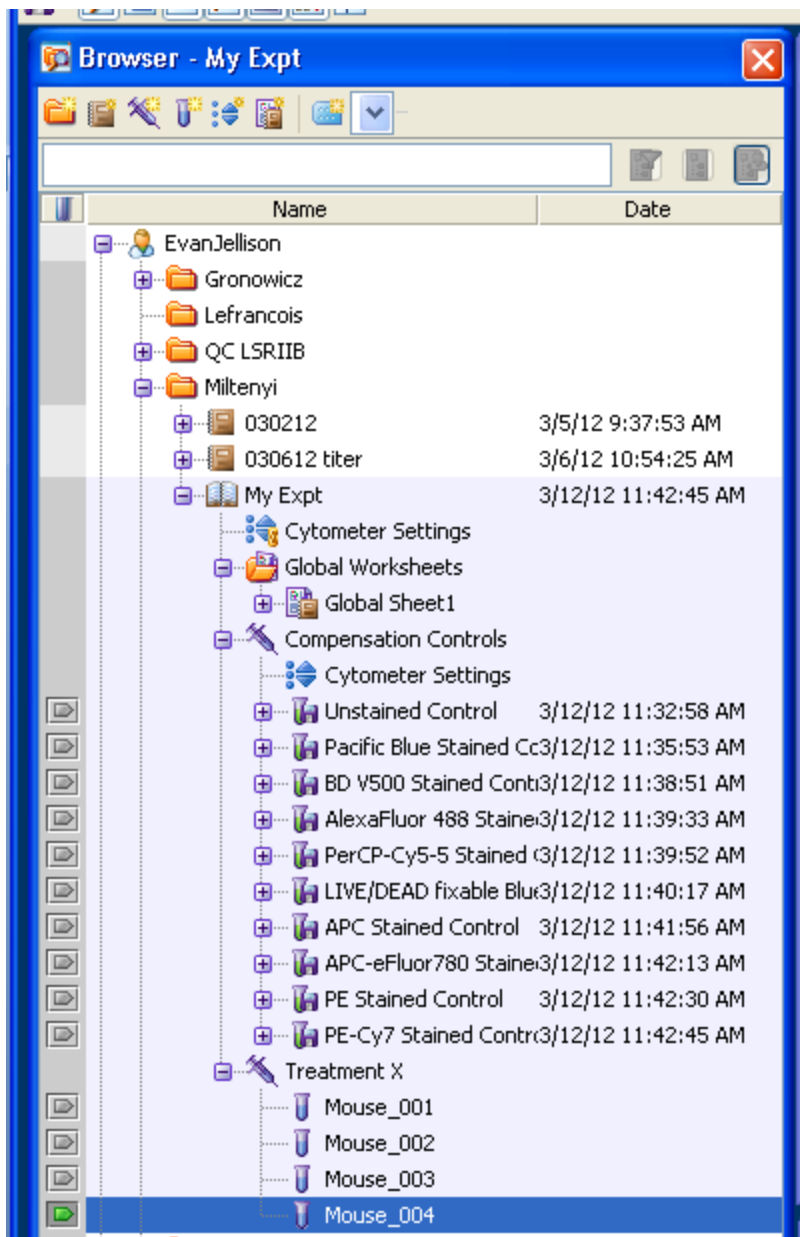
For faster processing between samples, click Next Tube until you have the correct number of tubes and move the Tube Pointer back to the first tube.
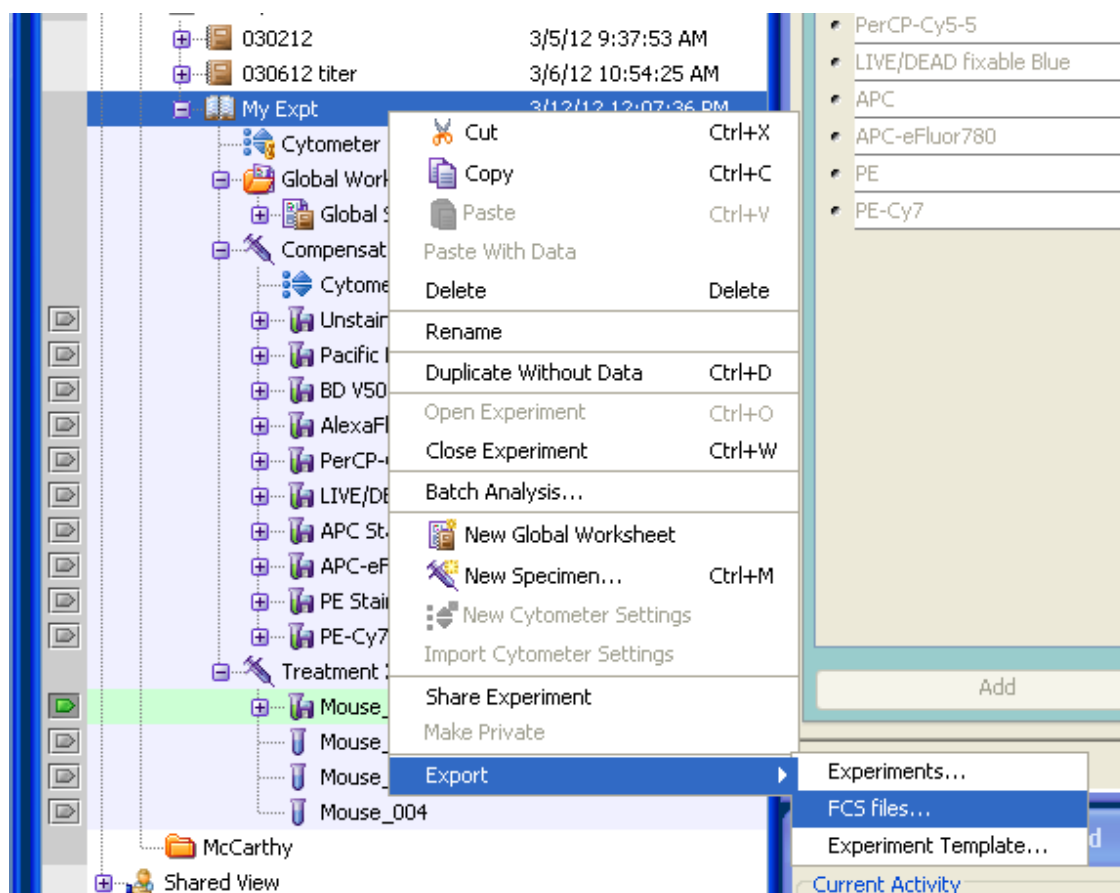
Acquire your first experimental tube. Adjust the flow rate using Low, Med, and High on the cytometer. When the event rate stabilizes (keep below 10,000 evt/s), click Record in the Acquisition Dashboard.
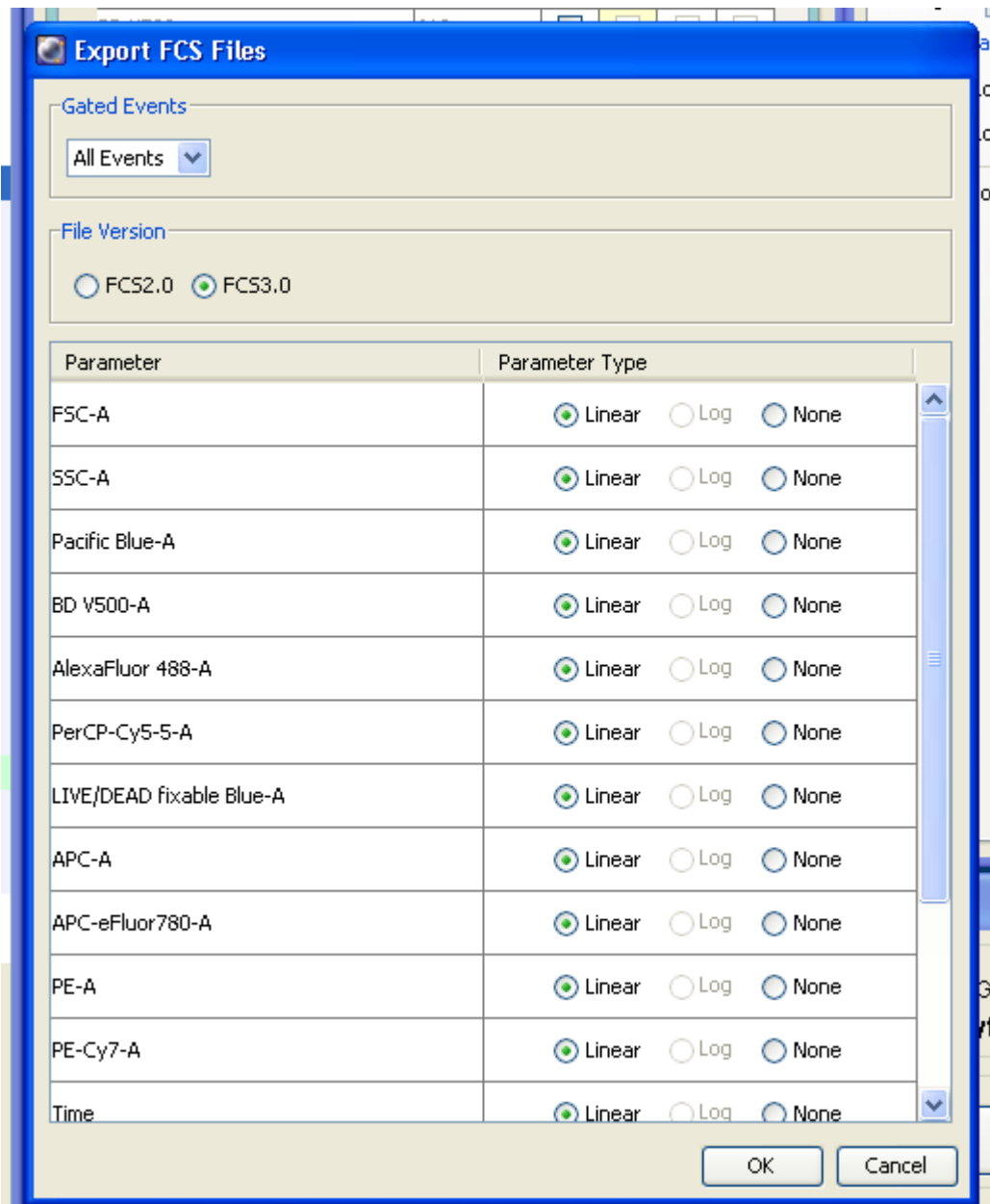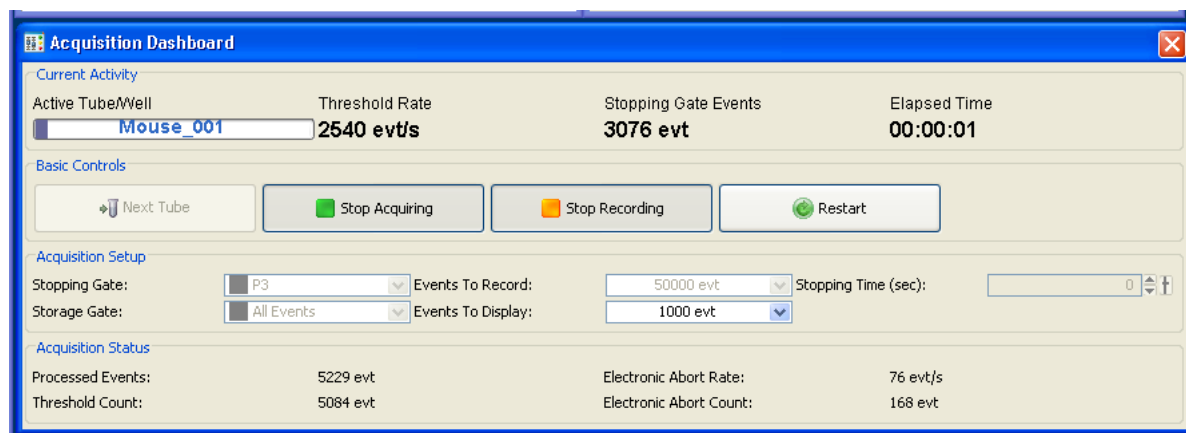

Recording will stop automatically when the Events To Record have been collected (or the Stopping Time has passed) but your sample will continue to be drawn as long as it remains on the SIP. If the Events to Record has not been achieved and there is no sample left to run, promptly remove your tube and move on. Some samples can be "good to the last drop" by adding saline to the bottom of an empty or nearly empty tube. **Never leave an empty tube on the SIP with the arm engaged.** This will force bubbles into the flow cell.

Select Next Tube in the Acquisition Dashboard to move to the next sample or use the Tube Pointer to select the tube. Record as above.

Once all of your tubes have been collected, export your data as FCS files. Highlight the Experiment name in the Browser window, right-click, and select Export> FCS Files…
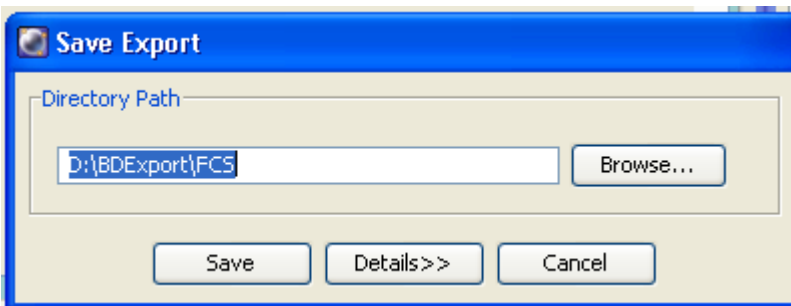
Selct the type of FCS file required (generally this should be 3.0) and click the OK button.

Export the FCS files to the D:/BDExport/FCS folder or to your attached portable drive. We recommend exporting to D:/BDExport/FCS and then copying to your drive. **We do <u>NOT</u> recommend that you export directly to Network Drives or Servers.**

When the Export FCS File(s) progress bar reaches 100%, copy the FCS files to your own personal drive. We encourage you to delete your own data from our computers, however we will **universally** delete the data after 2 weeks. Data should be cleared from both the Browser Window in FACS DiVa and the BD Export folder.

Log off FACS DiVa.

Clean and Prepare for the next user:

Put a tube with ~3ml (~half tube) BLEACH (squeeze bottle) on the SIP and run in HIGH with the arm to the side for 1 minute. This step is crucial to decontaminate the instrument.

Run the same tube of BLEACH in HIGH for 3 minutes with the arm underneath the tube. This step is crucial to clean the flow cell.

Repeat #1 and #2 with dWATER. This step clears the instrument of BLEACH and prevents your cells from popping in the future.

Leave ~1mL of dWATER on the SIP and hit STANDBY.

Be Kind: Check sheath/waste and refill/empty as necessary.

If you finish after 6 PM on a normal weekday or <u>any</u> time on the weekend and you do not have visible or telephone confirmation from the next scheduled user, switch <u>OFF</u> the instrument.

Shutdown (Last user of the day):

Quit FACS DiVa.

Exit the OPSL Laser control software.

Turn off the cytometer by pressing the green power button.

Turn off the fluidics pump on LSR II-B.

Weekday: Log off Windows XP

Friday/Weekend: Shutdown Windows XP.