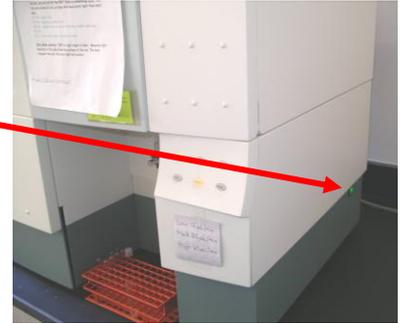


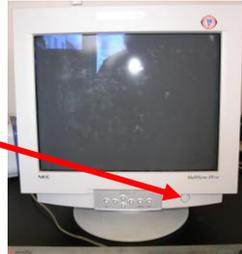
Becton-Dickinson FACSCalibur Flow Cytometer Instructions

Start-up

1. Turn on flow cytometer by pushing **green button** on right side of instrument, toward the back. Give it 30 minutes to warm up before collecting data.



2. Turn on monitor.

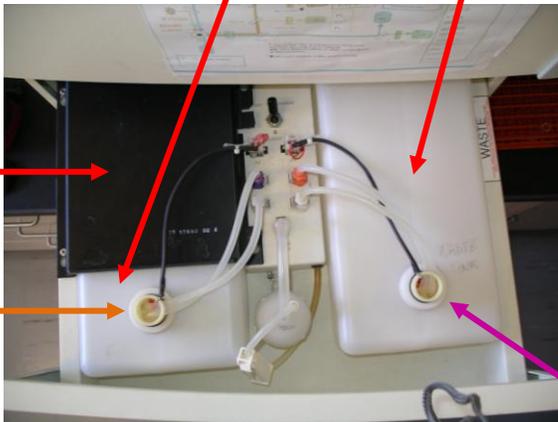
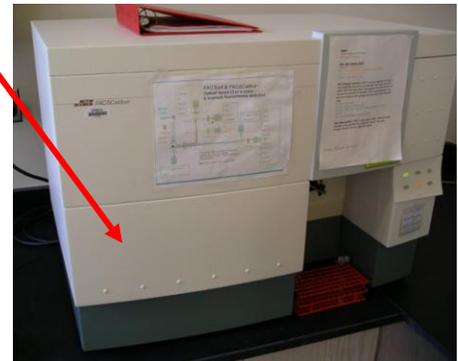


3. Turn on computer.



➤ If cytometer is not already on when computer is turned on, computer will not be able to 'see' the cytometer.

4. Gently pull open drawer forming lower left panel of cytometer to gain access to **sheath fluid tank** and **waste tank**.



5. Flip switch from **Pressurize – Run** position to **Vent – Tank Change** position.



6. If you are analyzing living cells, unscrew top on **waste tank**, remove tank from instrument, and add 200-300 ml of 10% bleach to the **waste tank**. Replace tank and screw top back in.

7. Remove **black metal plate** on top of sheath fluid tank (push forward, then lift off).

Becton-Dickinson FACSCalibur Flow Cytometer Instructions

8. Unscrew top on **sheath fluid tank**, taking care to avoid contaminating parts that get immersed in sheath fluid (lean it upside-down on top of **waste tank** – see photo at top of next page).

9. Remove **sheath fluid tank** from instrument (it should contain some sheath fluid already) and set it on counter. There is a “bump out” on the bottom of tank. Have front of tank sticking off edge of counter to just beyond the bump out so that bottom of tank is parallel with countertop, not inclined.



10. Top off tank by adding sheath fluid up to the black line marked on the front of the tank.

11. Replace tank in instrument and screw top back in.

12. Replace **black metal plate** that sits on top of **sheath fluid tank** (see step 7).



Clean up any spills of sheath fluid immediately, even single drops!

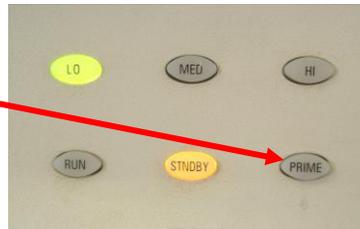
13. Flip switch from **Vent – Tank Change** position back to **Pressurize – Run** position (see step 5).

14. Loosen **clamp** (rotate little wheel to other end of clamp) and allow fluid to flow for just a few seconds. Flick tubing that clamp is on to dislodge any bubbles that can be dislodged. If a bubble won't move, leave it. Then re-tighten clamp again.

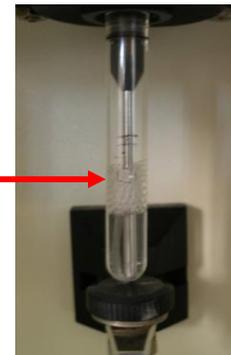
➤ **If the clamp is not re-tightened, all of the sheath fluid will flow into the waste tank after a short time.**



15. Push **PRIME** button. You should see bubbling within the tube of water left on the instrument, as well as movement of fluid into the **waste tank**.



16. Repeat step 15 two more times. A bubble usually remains in this tube. That's fine.



17. Close drawer containing **sheath fluid tank** and **waste tank**.

18. Double-click icon.



Acquire
Acquisition & Storage Parameter Description Custom Keywords Counters
Edit Reagent List... Edit Panels...
Quantitative Calibration
Connect to Cytometer ⌘B

Becton-Dickinson FACSCalibur Flow Cytometer Instructions

19. Under **Acquire**, click **Connect to Cytometer**. An **Acquisition Control** window will open. Leave it open throughout your session.



Basic Data Acquisition

Note: A **CellQuest User's Guide** PDF is on the computer desktop (feel free to copy it onto a USB drive if you want to browse through it at your leisure) and contains much more information on the software than will be discussed here. The following is just an outline of some steps often taken in a typical initial acquisition.

20. Under **Acquire**, click **Acquisition & Storage** (see diagram on previous page). In window that opens, enter number of events (i.e. cells usually) that you wish to count during an acquisition (10,000 is typical).

21. Click **Parameters Saved**.

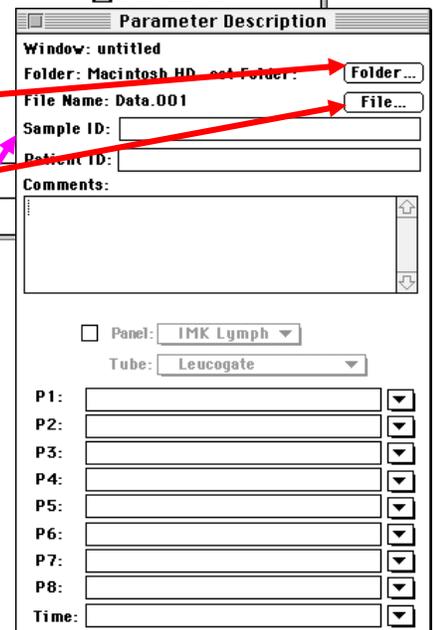
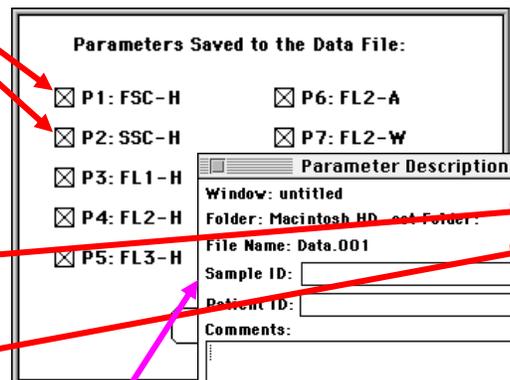
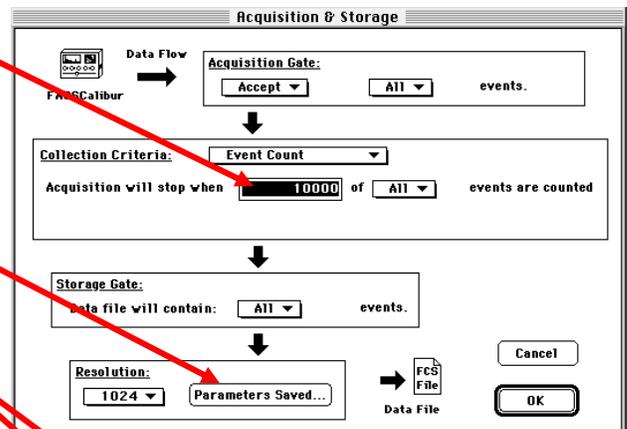
22. In window that opens, check off only parameters you wish to save from an acquisition. You will probably always want to save forward scatter (FSC) and side scatter (SSC) data; thus, **P1** and **P2** will be checked.

You will also check off the fluorescence detectors on which your fluorescence(s) of interest will be detected (**FL1**, **FL2**, **FL3**). After making your selections, click **OK** here and on **Acquisition & Storage** window.

23. Under **Acquire** (again), click **Parameter Description**. Leave **Parameter Description** window open throughout session.

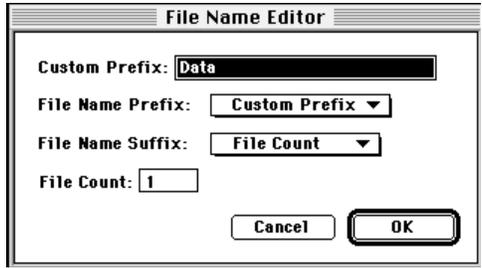
24. Click **Folder** button. Navigate to a destination folder in which you will store data files and select it.

25. Click **File** button. In **File Name Editor** window, provide a prefix that stored files will start with (e.g. the date). Click **OK**.



Becton-Dickinson FACSCalibur Flow Cytometer Instructions

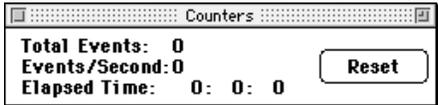
26. Enter a **Sample ID** name for each acquisition if you wish.



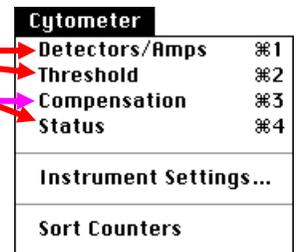
27. Provide axis labels for plots of your data by clicking pull-down lists next to parameters you are collecting. For example, forward scatter data is always Parameter 1 (P1), so if you want the forward scatter axis to be labeled “Forward Scatter”, click here

for the P1 pull-down list and select **Forward Scatter**. If a desired label is not on the list, you can just type it in once the appropriate parameter box is highlighted.

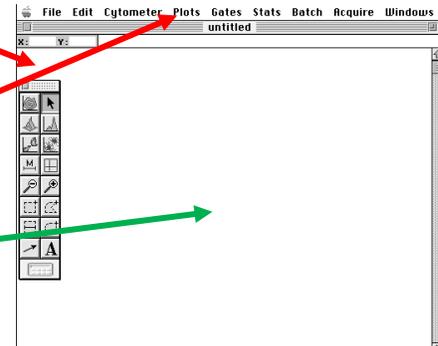
28. Under **Acquire** (yet again), click **Counters**. Leave this window open throughout your session. It will show numbers of events (e.g. cells) analyzed during an acquisition.



29. Under **Cytometer**, click **Detectors/Amps**, **Threshold**, and **Status**. Leave these windows open throughout your session. If you are working with 2 or more fluorophores and will be collecting data on fluorescence detectors (FL) 1 and 2, or FL 2 and 3, or FL 3 and 4, then also open **Compensation** window.



30. On what CellQuest calls the Experiment Document, create one or more dot plots/density plots (plot two parameters) or histograms (one parameter) in order to see data from your cells during data acquisition. Plots are created either by 1) using pull-down menu under **Plots** and selecting the desired plot type or 2) by clicking appropriate button on Tool Palette and then doing a click-drag-release in empty area of Experiment Document.

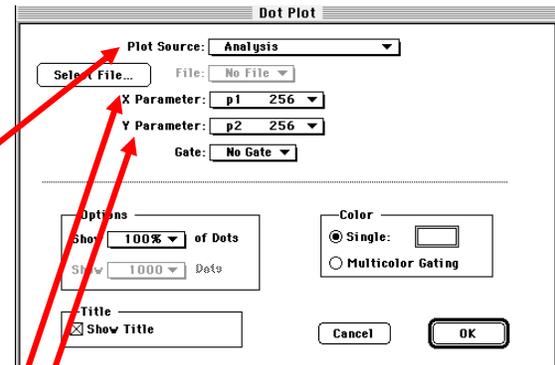


Histogram
Density Plot
Dot Plot



You can avoid having to create the same plots multiple times by creating a template of plots that can be re-used as often as you like. See section 2.6 in CellQuest User’s Guide PDF.

31. On [Dot, Density, Histogram] Plot window that opens, select **Acquisition**→**Analysis** option under **Plot Source**,

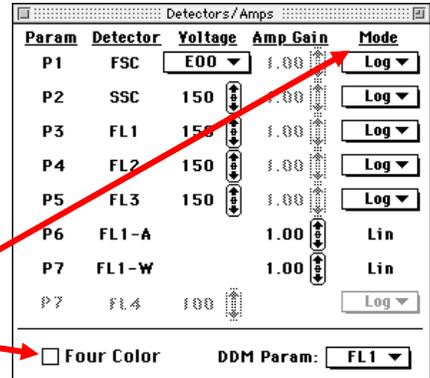


Becton-Dickinson FACSCalibur Flow Cytometer Instructions

and select the desired parameter for **X Parameter** (and for **Y Parameter** for dot and density plots).

Click **OK**.

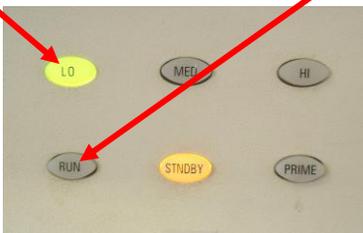
32. Most often a linear scale is used for plot axes displaying forward-scatter (FSC) and side-scatter (SSC) data, while a log scale is used for plot axes displaying fluorescence intensities. But there are many exceptions, such as in cell cycle analysis studies in which fluorescence is monitored on a linear scale to distinguish different ploidy states. Linear and log options for different parameters can be selected in the **Detectors/Amps** window in the **Mode** column. If collecting data on **FL 4**, click **Four Color** box to turn on second laser.



33. Vortex or flick tube containing your sample to resuspend particles (e.g. cells). A concentration of around 10^6 particles/ml is generally ideal. Remove old tube currently on cytometer by moving arm below it to the side and pulling down on old tube. Put new tube containing your sample in its place and move arm back to its original position, now under your new tube.

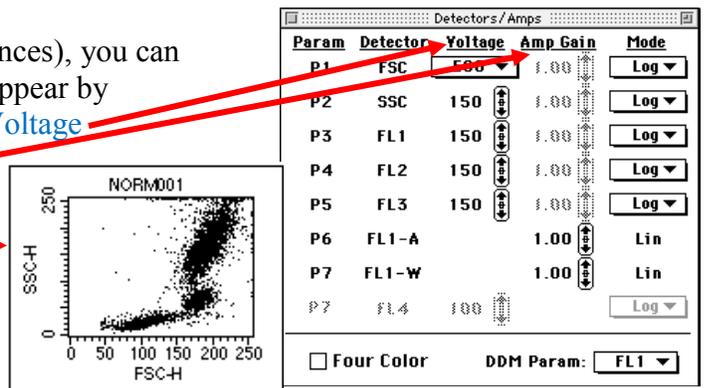


34. With **Setup** box checked in **Acquisition Control** window and **LO** flow rate selected, push **RUN** button, then click **Acquire**.



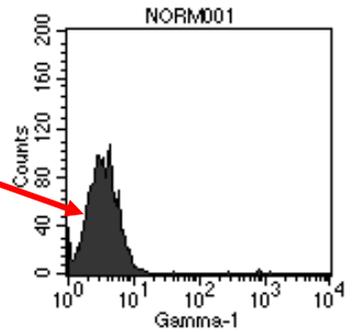
When **Setup** box is checked, data will appear in plots, but these will not be saved. This is so that you can see if any adjustments to acquisition conditions need to be made before you uncheck **Setup** box and collect the data that will be saved.

35. For the various parameters (FSC, SSC, fluorescences), you can make adjustments to where on the plot the data appear by making changes in **Detectors/Amps** window to **Voltage** and, if a linear scale is being used, **Amp Gain**. In this way, at least most data of interest should be made to occur on-scale. For example,



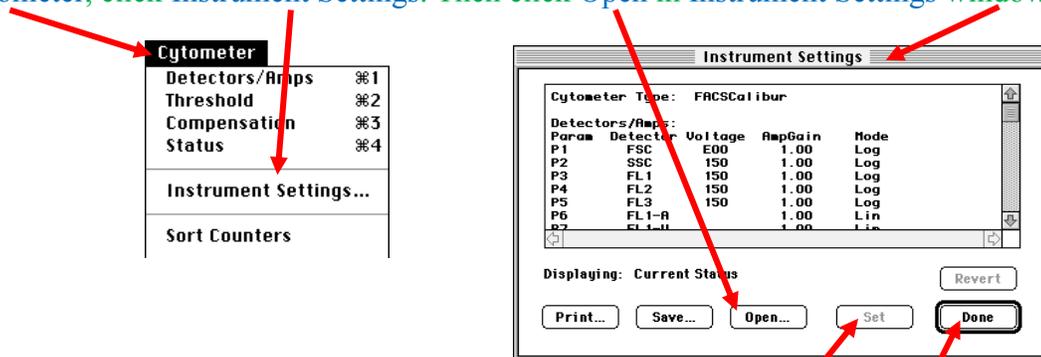
Becton-Dickinson FACSCalibur Flow Cytometer Instructions

Once **Voltage** and **Amp Gain** are set for your negative control (with the bulk of particles [e.g. cells] positioned between 10^0 and 10^1 on log scale axis of fluorescence intensity), do not change them when subsequently analyzing your experimental samples.



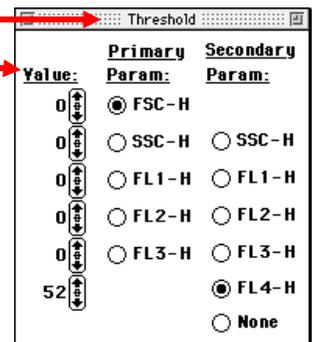
Voltage and **Amp Gain** settings do not have to be established from scratch each day if you are analyzing similar types of samples. You can load the settings from a previously saved analysis prior to running a new sample so that at most only small adjustments will need to be made to **Voltage** and **Amp Gain**. To do this, see following page.

Under **Cytometer**, click **Instrument Settings**. Then click **Open** in **Instrument Settings** window and



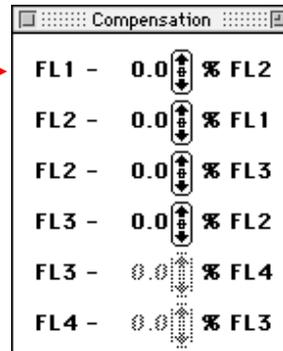
navigate to and select the desired previously saved file. Then click **Set**. Once the settings from this saved file are loaded, click **Done**.

36. Thresholds for up to two parameters can be set in **Threshold** window. Any data point with a measured value below the set threshold **Value** is not counted as an “event” (e.g. cell) and not saved. For example, a threshold is often set for FSC so that small bits of cellular debris in a sample are not counted as cells. In such an instance, a threshold **Value** around 50 is often used.



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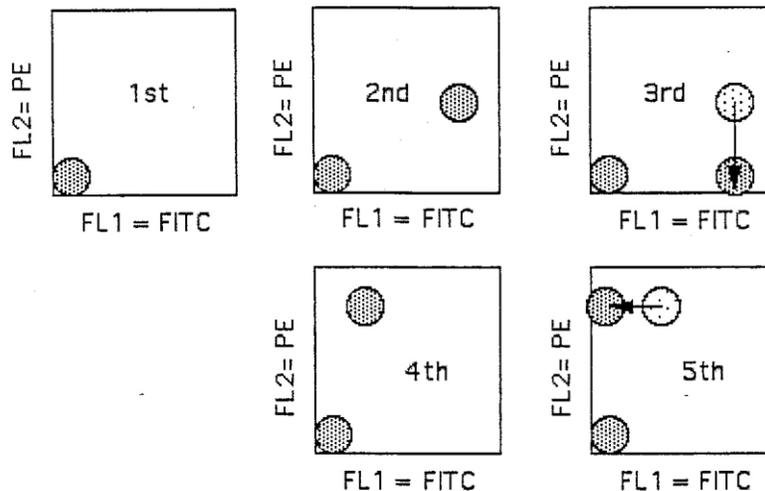
37. If measuring fluorescence from two or more fluorophores with overlapping emission spectra (step 29), you will need to set compensation values in **Compensation** window. This is accomplished using the procedure on the following page (taken from Dartmouth's Flow Cytometry facility).



Becton-Dickinson FACSCalibur Flow Cytometer Instructions

COMPENSATION: SETTING UP THE CYTOMETER FOR DUAL (OR MORE) COLOR ANALYSIS

1. Use control-stained (i.e. negative) cells and change the PMT detector voltages until these cells are in the lower left corner of the dot plot (1st figure below).
2. Use a 50:50 mix of control (i.e. negative) cells mixed with your brightest FITC-positive cells (stained with your brightest FITC antibody as well as with a PE-conjugated negative control if you are using one). See 2nd figure below.
3. Change the FL2-FL1 compensation until the negative and FITC-positive cells are "parallel" on the dual color plot horizontal axis (assuming that you are using a dot plot with FITC on the horizontal axis and PE on the vertical axis). Technically, you want the median FL2 values of the two distributions to be at the same intensities. See 3rd figure below.
4. Use a 50:50 mix of control (i.e. negative) cells mixed with your brightest PE-positive cells (stained with your brightest PE antibody as well as with a FITC-conjugated negative control). See 5th figure below.
5. Change the FL1-FL2 compensation until the negative and PE-positive cells are "parallel" on the dual color plot vertical axis (with the median values on the FL1 scale identical). See 5th figure below.



6. If you are using three colors, you should repeat this procedure with all combinations, that is, look at green-stained cells in both the green vs orange and green vs red plots; look at orange-stained cells in both the orange vs green and orange vs red plots; and look at red-stained cells in both the red vs orange and red vs green plots. Adjust the relevant compensations so that the mixed positive and negative cells give "parallel" patterns in all these combinations.
7. Save these settings on a disk so that you don't have to go through all this trouble each time you do a similar experiment.
8. Do NOT change the detector voltages after you have adjusted the compensation. Different compensation levels are necessary for different voltages. For this reason, the standard procedure is to adjust the detector voltages first in order to put the negative cells where you want them with respect to each color and THEN to adjust the compensations.

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38. With **RUN** and **Acquire** selected (see step 34) and sample being drawn into cytometer, note in **Counters** window (see step 28) the number of **Events/Second**. This number will fluctuate as sample flows by laser(s), but should ideally be **around 500 events/sec**. If it is substantially lower than 500, push **MED** flow rate or, if still too low, **HI** flow rate button (see step 34), though you are better off if you can find a way to concentrate your samples so that only the **LO** flow rate is needed. If it is much higher than 500 when on **LO** flow rate, dilute sample.
39. When ready to collect data that will be saved, in **Acquisition Control** window (see step 34) click **Abort** and then uncheck **Setup** box. Now when you click **Acquire**, data will be collected until the number of events you selected in step 20 is reached. The data will be automatically saved, with the file name having the prefix you entered in step 25. Once the number of desired events is reached, push **STNDBY** button (see step 34) to stop sample being drawn into cytometer.
40. After analyzing all samples, you should take your data with you on a USB drive in case our old Mac crashes.

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Shut-down

41. Fill a tube about half to two-thirds full of 10% bleach. Put it on cytometer and leave arm positioned to the side as shown in step 33 for 1 minute. Then move arm under tube as shown in step 15, push HI flow rate button and RUN button (see step 34), and allow bleach solution to be drawn into cytometer for 5 minutes. Then push STNDBY button (step 34) and remove tube.
42. Repeat step 41, except using diluted Jet-Dry solution.
43. Repeat step 41, except using distilled water. Leave a tube containing distilled water on cytometer until the next time the cytometer is used.

44. Things to do during the cleaning periods in steps 41-43:
 - 1) under Acquire (see step 19), click Disconnect from Cytometer.
 - 2) under File, click Quit.
 - 3) under Special, click Shut Down.



- 4) Turn off monitor (see step 2).
 - 5) Sign in on Dell computer closest to door for room W118. This is done by double-clicking Instrument Usage icon on computer desktop. On Enter Approach File Password window that appears, do not enter a password; just click OK. Under Student Use column, click New Flow Cytometer Entry. Under Customer Name, select person paying for cytometer usage from the pull-down list. Enter No. of Hours cytometer was used and No. of Samples in the appropriate boxes. Click red Menu button. Under File, click Exit Approach.
45. Remove waste tank from cytometer (see step 6), empty contents in sink, rinse inside of waste tank with distilled water, and replace waste tank in cytometer. If working with a toxic or hazardous fluorophore, contents of waste tank should instead be transferred to a waste disposal container (supplied by user) for disposal by EH&S. Consult MSDS for your fluorophore for information on its toxicity.
 46. From the final time the STNDBY button (see step 34) is pushed, wait 5 minutes and then turn off power to cytometer (green button; see step 1).