



BD FACSDiva 4.1 - TUTORIAL

**TSRI
FLOW CYTOMETRY CORE
FACILITY**

IMPORTANT NOTES BEFORE READING THIS TUTORIAL

- This is a very expensive piece of equipment so PLEASE treat it with respect!
- After you are done using the machine, run 10% bleach for 1-2 minutes and then dH₂O for 1-2 minutes. This will keep the machine running smoothly for other users.
- After you are done using the machine, top off the sheath tank with the sheath fluid provided in the analyzer room (in the big boxes). Also, empty the waste tank if needed, and pour in ~100ml of bleach.
- Because of the increasing popularity of these machines, please **DO NOT STORE YOUR DATA ON THESE MACHINES**. Please delete your experiments from the Browser window; and from the Exported FCS or Experiment Folder. This will keep the computers running smoothly for other users.
- If you are the last person of the day to use this instrument, turn off the machine and the computer. The order in which you do so does not matter for this instrument.

Thank You and May the Flow be with You

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I. Software Login

- Must be set up with Flow Cytometry Core Facility staff
 - Desktop login is **user**, password is **user**
 - Software Login Format: **PnameYourname** (i.e. NemazeeAmanda)
 - All of your experiments will be saved in your own browser, and can not be accessed by other users.
 - You will only be billed while you are logged into the FACSDiva software.

II. Setting up your Experiment

- Your experiments for the day are organized in the Browser window in a hierarchial format:
 - Folder-> Experiment-> Specimen-> Tube
 - See **Figure 1** for an example of how the Browser window should be set up.
- To create a Folder for the day's experiments, highlight your 'hard drive' icon and either select the Folder icon from the top of the Browser window, or right click and select '**New Folder**'
- To create an Experiment, highlight the Folder you just created and either select the Experiment icon, or right click and select '**New Experiment**'.
- You must now create a Specimen. Highlight the Experiment you just created and either select the Specimen icon, or right click and choose '**New Specimen**'.
 - It is a good idea to label your Folder, Experiment, and Specimen as your initial and date (i.e. a050505); but you can label them any way you want.
- A Tube is automatically created under the Specimen. Click on the '+' next to the Specimen and you will see '**Tube_001**'.
 - You will see an **Indicator Arrow** directly to the left of the Tube.
 - **You must click on the arrow so that it turns green in order to proceed. Otherwise you will not see any parameters.**

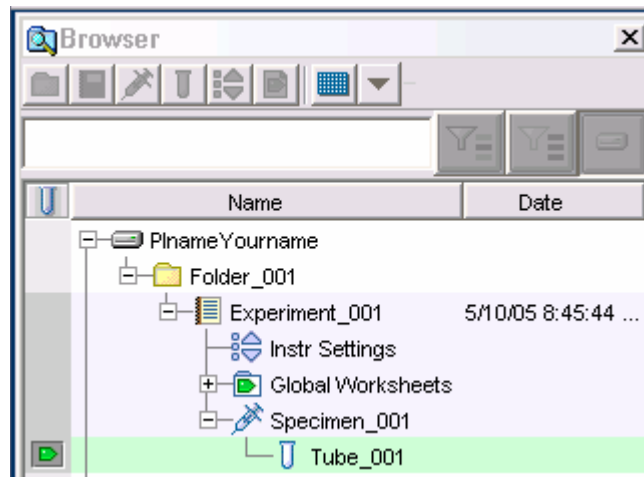


Figure 1

III. Windows

- Go to **View Menu** and make sure all windows are checked and are on the screen.
 - You will not need the 'Plate' window as there is no plate reader attached.
- If you don't see the program on both screens, go to the very right hand edge of the window and drag it to the other screen.
- If you can't find a window, click **Reset Positions** and arrange windows how you like them.

IV. Parameter Selection/Deletion

- Go to the Instrument Window, click on the Parameters Tab; and highlight and delete any parameters you are **NOT** using.
 - **The Indicator Arrow next to the tube must be selected.**
 - This step is very important if you are using Auto-Compensation, and a good idea to keep file sizes down.
- For this example I will be using: FITC, PE, PerCP-Cy5.5, PE-Cy7, APC-Cy7, and APC.
- It is recommended to check the boxes for FSC-W and SSC-W to enable doublet discrimination.
 - If you are using Auto-Compensation these must be checked before you save your controls.
- You may click on certain parameters and change the name of the fluorophore in a particular channel.
 - FITC – may be changed to GFP, CFSE, or Alexa 488
 - PerCP-Cy5.5 – May be changed to PI or PerCP
- See **Figure 2** for an example of how the Instrument Window should look for this 6 color experiment.

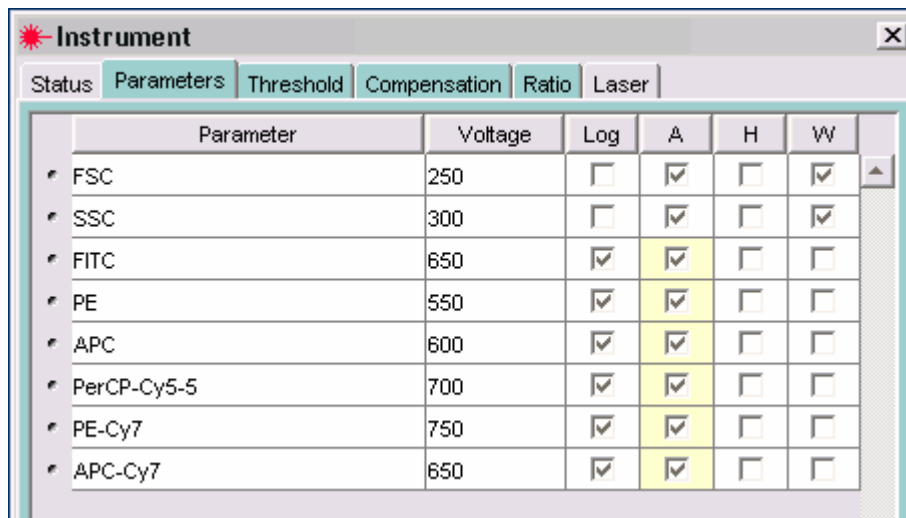


Figure 2

IMPORTANT! DO NOT use the Instrument **Configuration** Window from the Instrument Menu to delete parameters!!!

V. Acquisition Controls

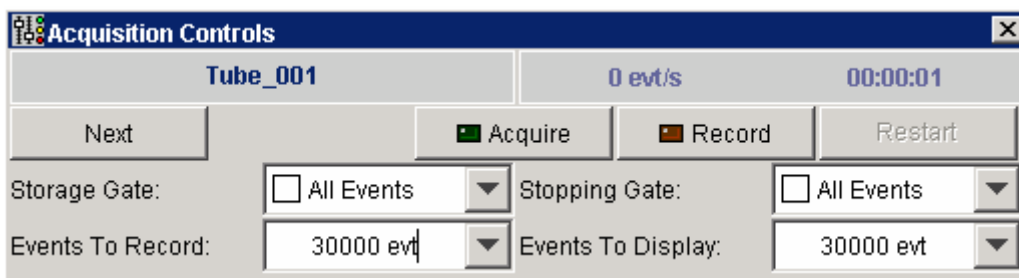


Figure 3

- **Storage Gate:** This gate will determine which cells will be contained in your data file. Usually you will want to leave this gate as 'All Events'. If you select your scatter gate here, everything outside the gate will not be saved. This is useful for keeping the size of your file down if you need to collect a lot of very rare events.
- **Stopping gate:** This gate is used if you would like to collect a finite number of events in one particular gate. For example if your scatter gate (usually P1) is gated on lymphocytes and you would like to collect 30,000 events specific to that gate, you would simply change the stopping gate to P1 and it will collect data on all events until P1 reaches 30,000.
- **Next:** After collecting the desired number of events for Tube_001, click 'Next' to create the 'next' tube.
- **Acquire:** Click here to begin acquisition without recording.
 - Corresponds to a yellow Indicator Arrow in the Browser window
- **Record:** Click here to begin recording the pre determined number of desired events. If you want to stop recording in the middle of the acquisition, but wish to save the data you've already collected, click on **Acquire** to stop acquisition, not Record.
 - Corresponds to an orange Indicator Arrow in the Browser window
- **Restart:** Restarts and refreshes the acquisition. If this is clicked during recording, the recording will start anew.
- **Events to Display:** This determines how many events will be displayed during acquisition.

VI. Auto-Compensation

* IF YOU PREFER TO COMPENSATE YOUR SAMPLES MANUALLY, OR WISH TO USE FLOWJO FOR AUTO-COMPENSATION, YOU CAN SKIP THIS SECTION.

**FOR ANY AUTO-COMPENSATION (FLOWJO OR FACSDiva) YOU WILL NEED AN UNSTAINED CONTROL AND ONE BRIGHT SINGLE COLOR CONTROL (i.e. CD4, B220, CD3, CD8, etc.) FOR EACH FLUOROCHROME.

- Before you proceed make sure you have completed Part IV.

- Go to the Instrument Menu, select Instrument Setup, and choose ‘**Create Compensation Tubes**’. See **Figure 4**.

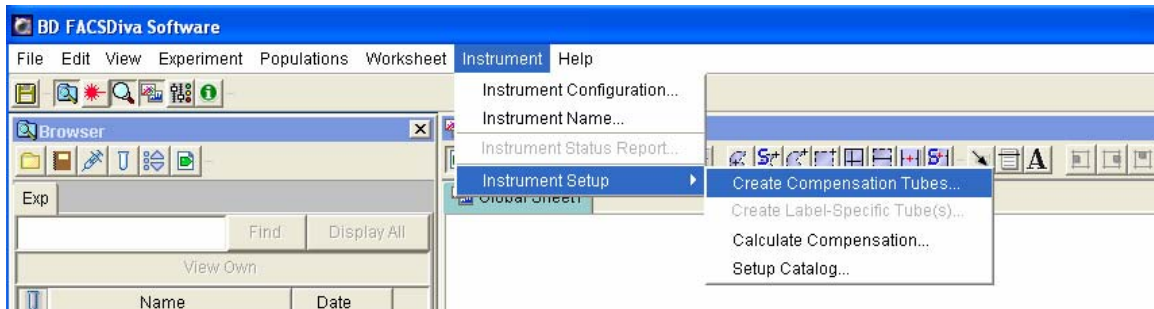


Figure 4

- In the Worksheet Window, preset compensation templates will come up for an unstained control and all your selected fluorophores.
 - These worksheets are different than the Global Worksheets you will use to acquire samples.
 - If you did not delete unused parameters, you will have to start over from the beginning!
- First put on your unstained control and set the FSC and SSC voltages to appropriate values.
 - You will need to move the P1 gate to your cell population on the scatter plot to find your cells. Right click P1 and select ‘**Apply to all Compensation Tubes**’.
- Now it is time to set your fluorescence voltages. Run through all your set up tubes **without** saving them and make sure everything is on scale.
 - You can adjust the voltages in the Instrument Window (Parameter Tab). Ideally, set all your voltages to the recommended voltages (posted on the machine and located in **Section XV, Figure 14**) and only lower them if the positive signal is off scale. Placing the negative population in the first decade is not the appropriate method.
 - If you have titered your antibodies, you should be able to see a negative peak and a positive peak separated by at least a decade.
- Once you have your voltages set to where you want them, it is time to save a file for each set up tube.
 - Note: once you apply compensation, you will not be able to change your fluorescence voltages; however, you may change FSC and SSC voltages.
 - To save a file, click on the Indicator Arrow next to the appropriate tube.
 - This will apply the appropriate template for each control.
 - Do not just click on the tab at the top of the worksheet; make sure the arrow is lit up in the Browser next to the tube you want to record.
 - The default number of events to record is 5000 and is sufficient for calculating compensation.

- After all setup tubes have been collected, go through the positive controls. The P1 interval gate will automatically adjust to your positive population. You may manipulate it to better fit your population if you wish.
- After all positive populations have been defined, go back to the Instrument Menu, select Instrument Setup, and choose ‘**Calculate Compensation**’. See **Figure 5**.

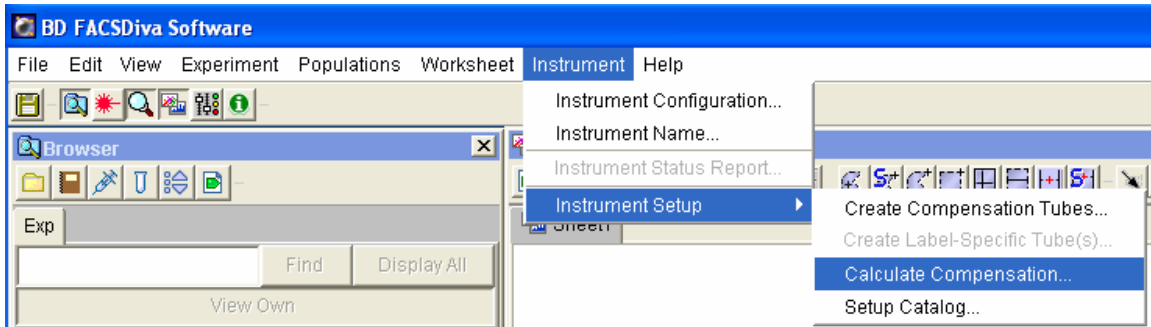


Figure 5

- The software will ask you to save the compensation matrix, use the same name as your experiment.
- Select **Tube_001** by clicking on the arrow (arrow should be green, implying that no acquisition is taking place).
- Double click Global Sheet in the Browser Window, or click on the arrow on the top left of the Worksheet Window above the Tab that says ‘**Sheet 1**’.
 - This arrow toggles between your Global Worksheets and the Compensation Worksheets.
 - **DO NOT Acquire on Sheet 1 of Compensation Worksheets!**
- Go to the Instrument Window and click the Compensation Tab, all of your compensation values will be there.
 - Compensation Values will not show up until you click on the Indicator Arrow next to Tube_001 from the original Specimen.
 - Make sure ‘**Enable Compensation**’ is checked in the Instrument Window.

VII. Setting up your Global Worksheet

- To create your Global Worksheet for your experiment, first double click on Global Sheet1 to get a blank template.
 - If you wish to make multiple templates within the same experiment, right click on Global Worksheet in the Browser Window and select ‘**New Global Worksheet**’. You may also rename these worksheets if you like.
- Select the Dot Plot icon from the Tool Pallet above the template.
- Draw a Dot Plot to a size of your liking
 - Either, click on the template and drag to the desired size, or click once for a preset dot plot size.
 - You may resize the plot by grabbing any of the vertices and dragging.

- **SHORTCUT:** Hold down **CONTROL** and click on the white border of the plot and drag it elsewhere on the template.
 - Repeat as necessary
- Define all the parameters on your dot plots by clicking on the parameter in the white border and selecting from the list.

VIII. Labeling Your Parameters and Tubes

- To label your parameters, highlight your tube, go to the Inspector Window, and click on the **Labels Tab**. See **Figure 6**.

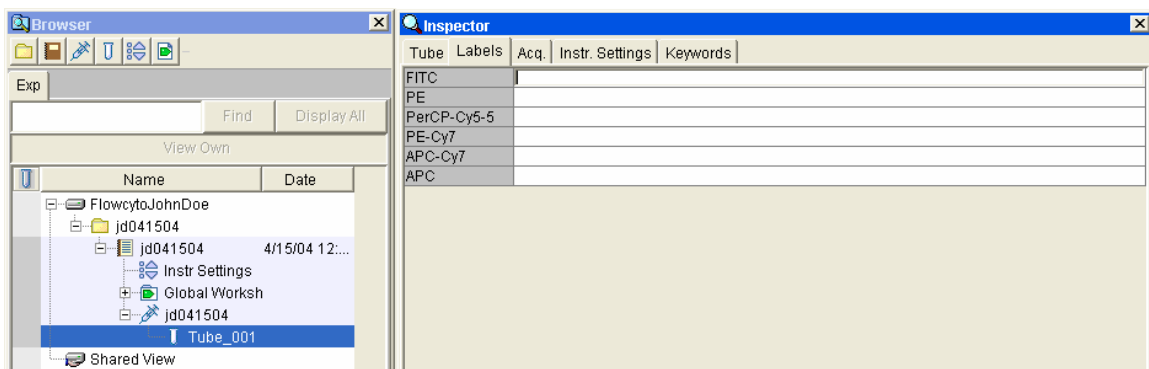


Figure 6

- Note: The software does not allow decimal points in the labels.
- To label your tube anything other than Tube_001, right click on the tube and select rename.
 - The next tube will be named whatever you renamed the previous tube followed by _001.
 - If you leave the default name, Tube_001, the next tube will be named Tube_002, then Tube_003, etc.

IX. Saving and Retrieving Analysis Templates

- After you have created a Global Worksheet for your experiment, you may save it as an Analysis Template.
 - First, make sure the template is how you wish to save it.
 - Next, in the Browser Window, expand where it says '**Global Worksheets**'.
 - Right click on the Global Sheet you wish to save and go down on the menu to **Export** and choose '**Analysis Template**'.
 - A dialog will come up asking you to name your template.
 - This template will be accessible to all users.
 - Click on '**Lock Template**' to ensure that your template cannot be altered by other users.

- There will potentially be several templates to choose from when you want to retrieve this template, so name it something you will remember, and put your name on it.
- To retrieve your Analysis Template for a new experiment, expand ‘**Global Worksheets**’, right click on ‘**Global Sheet1**’, and choose ‘**Apply Analysis Template**’.
 - Choose your template from the list in the dialog and your saved template will be retrieved.

X. Gating

- Gating works on a hierarchial platform so there’s no need to make combined gates like in CellQuest.
- First draw a scatter gate, usually a FSC vs SSC plot or PI vs FSC plot.
- To format your next plot, generally FSC-A vs FSC-W which depicts doublet/aggregate discrimination, click anywhere on the white border of the plot of interest so that the plot is highlighted. Next, go to the Inspector window and click on P1, or you can right click on the plot and highlight ‘**Show Populations**’ and select P1. The plot will now be formatted to your scatter gate.
- The next step is to gate out doublets. Draw a gate around the major horizontal population. This will be P2.
- Usually you will want to format all your other plots to these two gates. To do this, highlight any other plots (you can highlight multiple plots by holding down Control) and click on the box next to P2 in the Inspector window, or right click on the plot, highlight show populations, and select P2.
- Keep adding gates as needed.
- To see your population hierarchy, select ‘**Show Population Hierarchy**’ from either the Populations Menu, or you may right click any plot on the right border and select from that menu.
- To re-label your gates as lymphocytes, live cells, etc., simply click on the gate on the Population Hierarchy Window and type the gate’s new name.
- To change a gate’s default color, double click on the colored square next to the gate in the Population Hierarchy Window.
- An example of one gating strategy is shown in **Figure 8**.

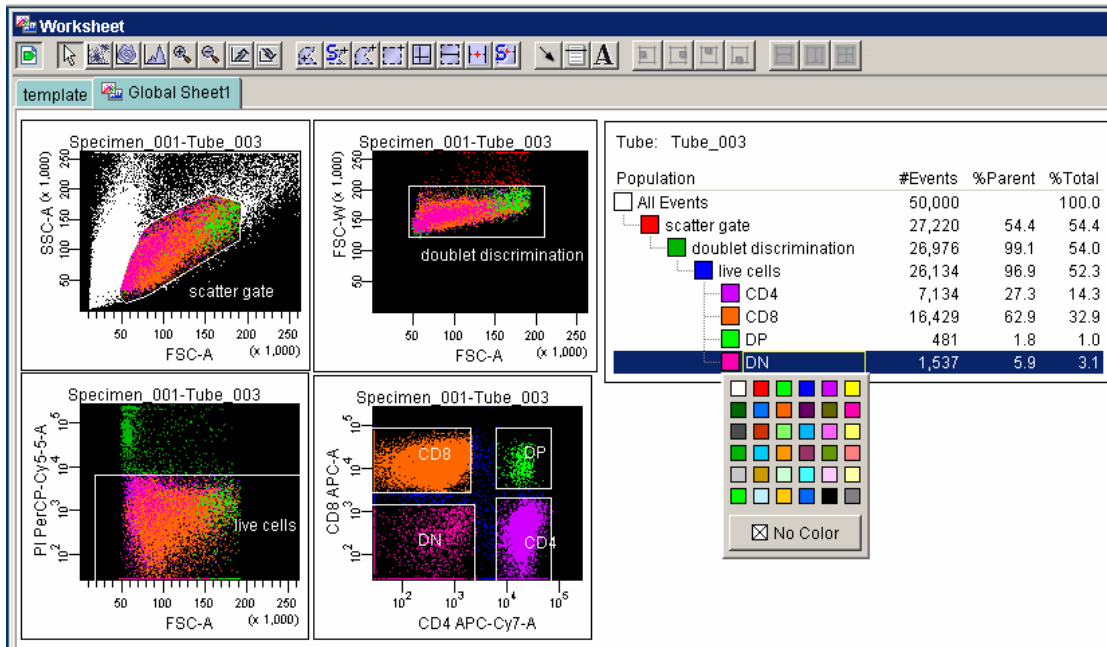


Figure 8

- The statistics given in the Population Hierarchy Window are: The number of events being displayed for each gate, the percentage of the parent gate (i.e. the live cells gate is the parent gate of the three bottom gates), and the percentage of the total events being displayed.
- For Mean, Median, Etc., right click any plot and select Create Statistics View. To edit the statistics shown, right click the on the Statistics Window and select Edit Statistics View.

XI. Exporting Your Data

- To view your data in other software (i.e. FlowJo), you must first export it before you burn it to a CD or transfer it to a disk or removable USB storage device.
- To export your data, first close your experiment by double clicking on the experiment or right clicking and selecting Close Experiment.
- Next, make sure your experiment is highlighted and go to the File Menu. Go down to Export, and export as FCS. See **Figure 9**.

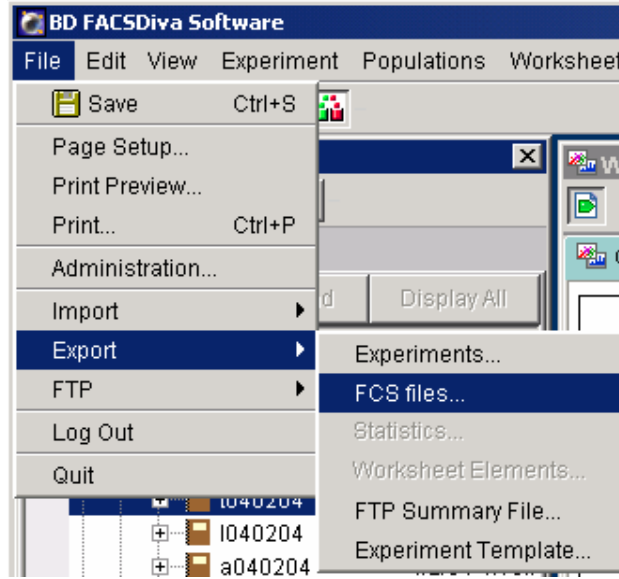


Figure 9

- The default FCS format is FCS 3.0. This format is for data analysis in FlowJo. Leave all values as linear, the software will convert them back to log.
- After files have been exported to **D:\BDExport\FCS**, you may close the software. This will effectively log you out.
- To find your exported files, double click **'My Computer'**, select the D:\ drive, select BDExport; then select FCS. Your files will be in this directory.
- The best way to archive your data is by exporting as **'Experiments'**.
 - This method of exporting will enable you to bring up your gates and instrument settings if you wanted to repeat the same experiment.
 - This will not allow you to analyze your data in FlowJo; however you may bring up your experiment to reanalyze in FACSDiva software.
- **Please delete your experiments from the hard drive when you are finished.**
 - This will keep the computer running smoothly for other users.
 - If you wish to leave an Experiment in your Browser window to use as a template for future experiments, right click your experiment and choose **'Duplicate Without Data'**.
 - This will recreate your experiment with the same settings, tubes, labels, and Global Worksheets without the data.

XII. Transferring Data to a Storage Medium

- Now that you have exported your files, you may close the software, thus logging yourself out.
- Insert a blank CD-R (provided at no cost by the Core Facility) or your removable USB storage device.
- To find your files, double click on **'My Computer'**, select the D:\ drive, select BDExport, and select FCS. Your files have been exported to this folder.
- To transfer your files to a removable USB storage device, simply drag and drop your files to the removable drive (usually G:\ drive)

- To safely remove the device, click on its icon in the bottom right corner of the screen.
- A dialogue will appear telling you that you may safely remove the device.
- To burn you files to a CD-R, open the recording software '**HP Record Now**', select Make a Data CD, follow the dialogue and drag and drop your files onto the CD-R.

XIII. Fluidics

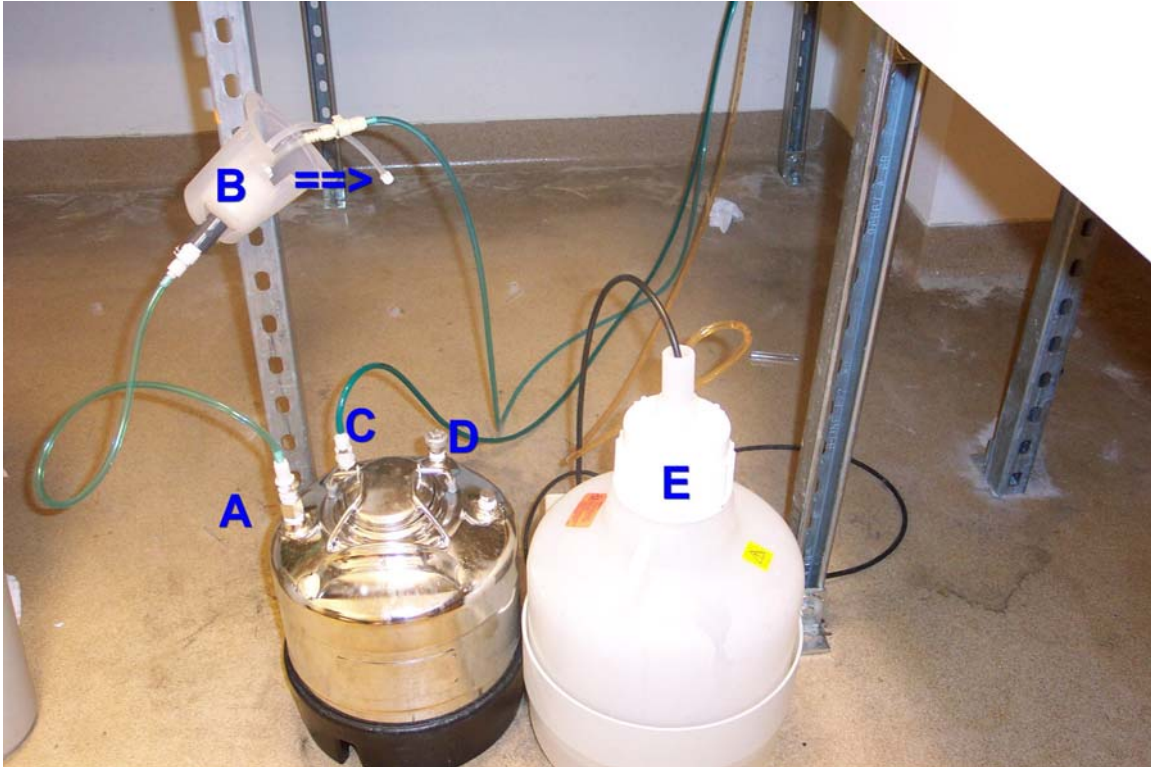


Figure 10

- **A: Sheath Line** – Carries the sheath fluid from the pressurized tank to the cytometer. Unfasten this when refilling the tank.
- **B: Sheath Filter** – Filters the sheath fluid. If the system has been run dry, you may need to purge air out of this filter by removing the white cap (indicated by the arrow).
- **C: Pressure Line** – Pressurizes the Sheath Tank. Unfasten this when refilling the tank.
- **D: Pressure Release Valve** – Pull this valve up to release the pressure when refilling the tank. Will not open when pressurized.
- **E: Waste Tank** – Not pressurized. An alarm will sound when the waste tank is full, unscrew the white cap to stop the alarm and empty. Waste goes down sink in main analyzer room. Be sure to add ~100 ml of bleach to the tank after emptying.

XIV. New Features

- ***Bisexponential Display:***

- The new version of this software allows you to view your data on a biexponential scale. This is a linear/logarithmic hybrid scale that allows you to see your data more linearly toward (and below) zero, and logarithmically in the higher channels.
- The scalar transformation does not affect your data in any way. It simply allows you to see events that are usually seen piled up on the axes.
- To transform the scale on any plot, highlight the plot by clicking on the border. Then go to the Inspector Window, select the 'Plot' tab and you may select to transform the X-axis, Y-axis, or both.
- Two examples of compensated data on both the traditional logarithmic scale (top) and the biexponential scale (bottom) are shown in **Figure 11**.

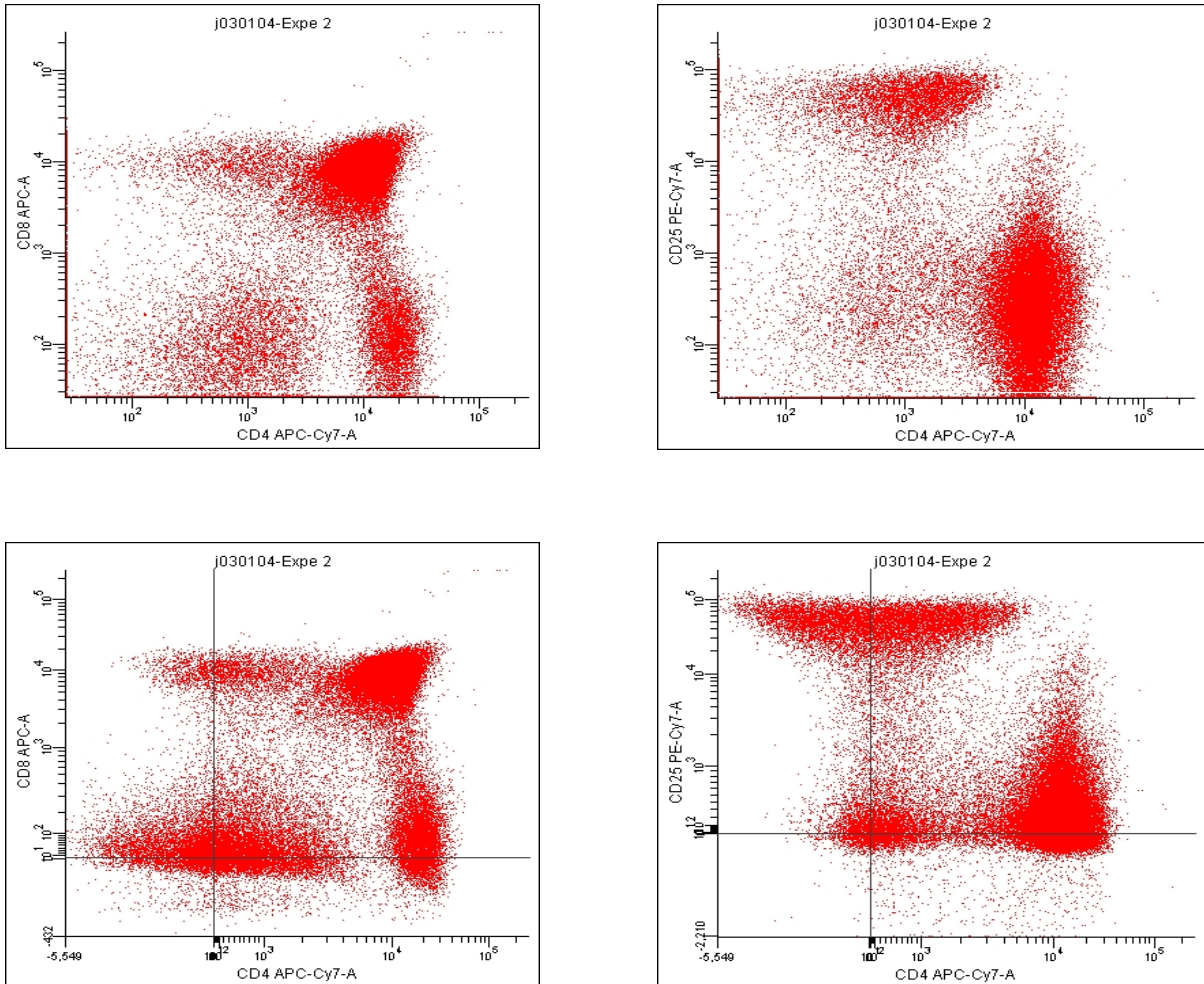


Figure 11

- **Hinged Quadrant Gates:**
 - Quadrant gates in the new version of FACSDiva software are hinged, allowing you to manipulate them to better suit your populations.
 - Aside from moving the position of the quadrant intersection, you may manipulate the quadrants in one of two ways:
 - You can grab the pivot point, where the quadrant gate intersects the border of the plot, and drag it to a new location to form non-rectilinear quadrants.
 - You can also grab the offset handles, in the middle of the quadrant boundary, and drag it to fit your population of interest.
 - To return the quadrant to its original rectilinear form, hold down the **'SHIFT'** key and click anywhere on the quadrant boundary.
 - See **Figure 12** for an example of normal, pivot point, and offset quadrants.

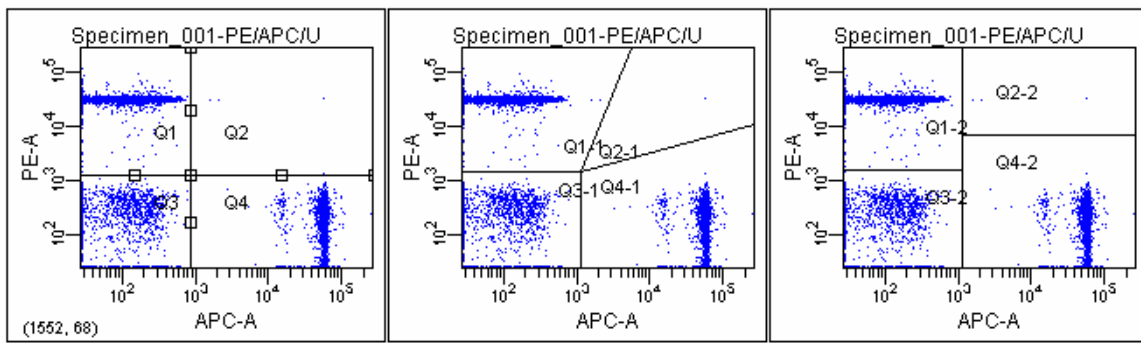


Figure 12

- **Copy/Paste Worksheet Elements:**
 - You may now copy and paste plots and statistics from your Global Worksheet directly to other applications (such as Word or PowerPoint).
- **Experiment Layout**
 - The Experiment Layout dialog box is used to create parameter (fluorophore) labels, keywords, or enter acquisition criteria for each Tube in your Experiment.
 - This is an excellent tool if you have multiple different labels for each fluorophore, and is the best substitute for the **'Panel'** function in BD-CellQuest.
 - To use this function, first delete parameters you will not be using. Then, create all of the Specimens and Tubes that you will need for your Experiment in the Browser Window. The easiest way to do this is by clicking on the Specimen or Tube icon for as many as you need.
 - After you have everything set up in the Browser Window, select **'Experiment Layout'** from the Experiment drop down menu. This will bring up the Experiment Layout dialog.

- Once in the dialog, you can add parameter labels, keywords, or acquisition criteria.
 - To label your parameters, first click on the Parameter Tab. Next, click on the appropriate square on the grid, and type in the label (i.e. CD4, CD8) in the top left corner. You may select multiple squares by holding down **CONTROL** and clicking on them.
 - To change the acquisition criteria for your Tubes, click on the Acquisition Tab, select one or multiple Tubes, and change the value in the top left corner.
 - Keywords are more useful in a clinical setting.
- **Figure 13** shows an example of the Experiment Layout dialog for a sample experiment with two Specimens with five Tubes in each Specimen. Note that the experiment contains Fluorescence Minus One (FMO) controls.

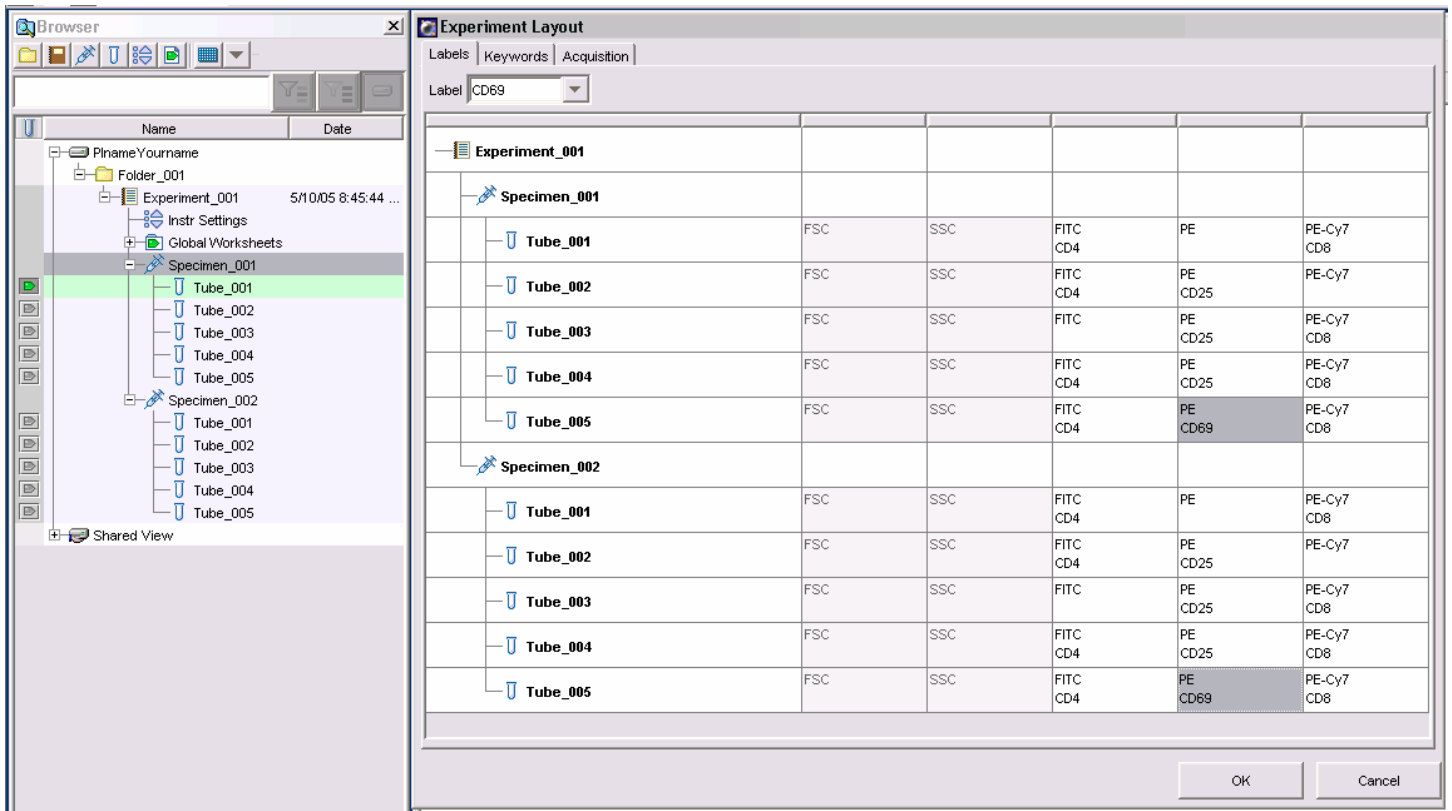


Figure 13

Background FMO (Fluorescence Minus One) Controls:

- In multi-color flow cytometry, the absolute background in a given channel, is a function of all the channels that spillover and require compensation in the channel of interest. Extremely bright fluorophores can add to the level of background in a corrected channel that will limit the sensitivity in that channel. The proper way to evaluate the background is to have a control that is all the other stains that you will be using, except for the color detected in that channel, hence the name "**Fluorescence Minus One Control**" (FMOC). This control is a must if you are trying to evaluate very dim populations in multi-color space. See **Figure 13** for an example of how to set up an experiment with FMO controls.

Label Specific Compensation Controls:

- Tandem dyes such as PE-Cy7, PerCP-Cy5.5, and APC-Cy7 tend to differ slightly from batch to batch. For example CD4 PE-Cy7 and CD8 PE-Cy7, even if bought from the same company, may be slightly different in emission wavelength, and effectively not be the same color.
- To account for this phenomenon, the FACSDiva 4.1 allows for you to make compensation controls for different markers of the same dye.
- After you choose ‘Create Compensation Tubes’, a dialog will come up allowing you to add or delete compensation tubes. If you have different labels for PE-Cy7 for example on different panels of staining, you may add them here. See **Figure 14**.
- This will bring up separate compensation control tubes for each label of the tandem dye you are using.

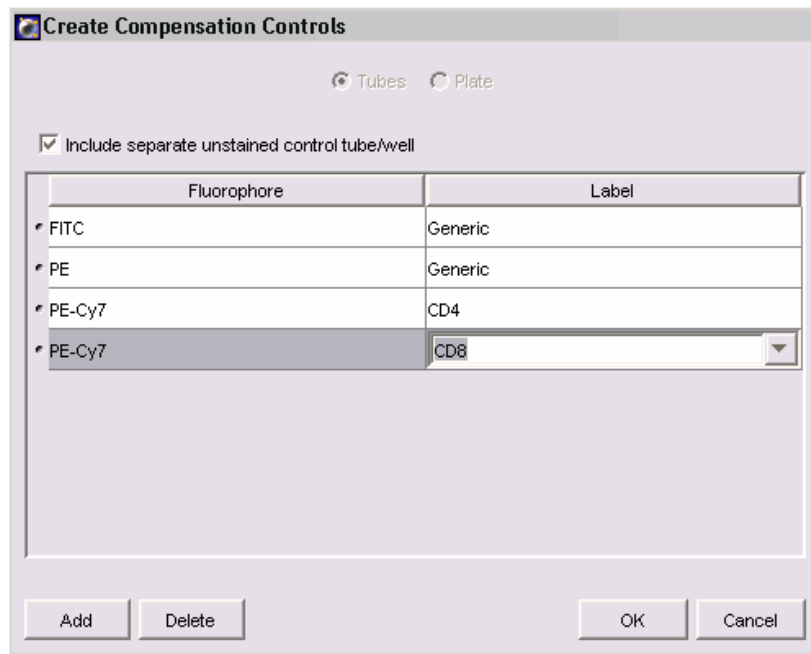
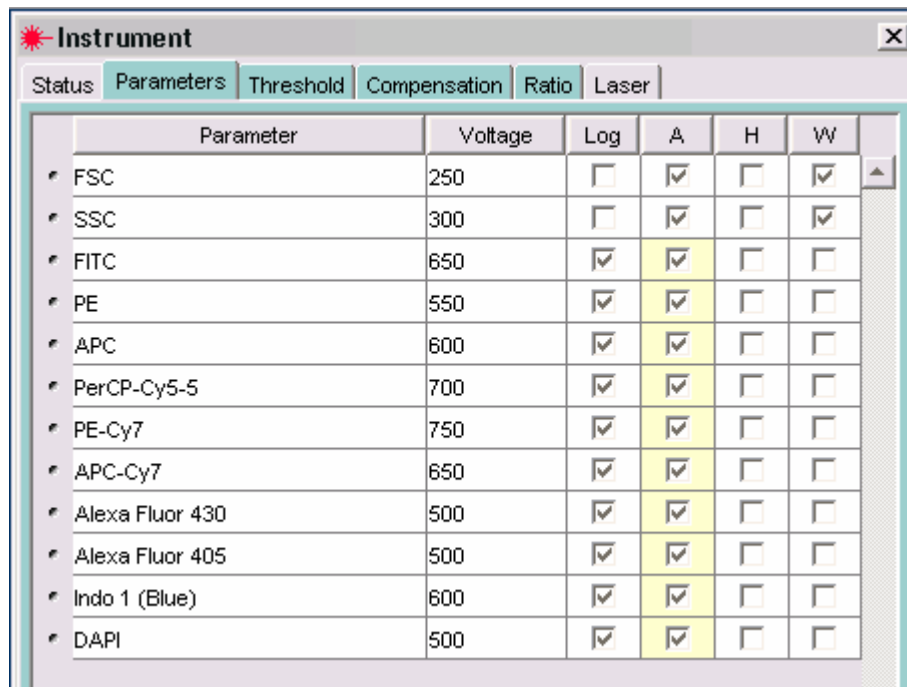


Figure 14

XV. Optimal Voltages

- We have determined the optimal voltages for each fluorescence parameter for achieving the optimal signal to noise ratio using low fluorescence particles.
- PMT voltage settings greater than those listed in **Figure 14** do not improve the sensitivity of the experiment.
- PMT voltage settings lower than those listed do not provide the optimal signal to noise ratio that allows for the maximal separation and sensitivity.
- Use these values as your minimal voltage settings, and lower the voltages only to move very bright events on scale.
- Ideally you should titer you antibodies so that you see the best separation at these settings.



The screenshot shows the 'Instrument' software interface with the 'Parameters' tab selected. A table lists the optimal voltage settings for various fluorescence parameters. The 'Log' column contains checkboxes, and the 'A', 'H', and 'W' columns contain checkboxes. The 'A' column for FITC, PE, APC, PerCP-Cy5-5, PE-Cy7, Alexa Fluor 430, Alexa Fluor 405, and Indo 1 (Blue) is highlighted in yellow.

Parameter	Voltage	Log	A	H	W
FSC	250	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
SSC	300	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
FITC	650	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
PE	550	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
APC	600	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
PerCP-Cy5-5	700	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
PE-Cy7	750	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
APC-Cy7	650	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Alexa Fluor 430	500	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Alexa Fluor 405	500	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Indo 1 (Blue)	600	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
DAPI	500	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Figure 14