

Preparing a FACSDiva Experiment

This document is intended to be a software guide only. It is not intended to teach flow cytometry theory or best practices. That will be discussed during your instrument training, but is not included here. For information on flow cytometry in general please contact the URM Flow Core staff.

Log in to the PPMS System:

- This is the same login information you use to log in online.
- The gray screen will disappear and you will be able to access the computer.

Log in to the FACSDiva Software:

- There is a generic username associated with each cytometer and no password. Just click OK.
- If the “CST Mismatch” window opens click on the button that says “Use CST Settings”.

***If you allow the mouse to hover over icons in the software it will often tell you what that icon is for.**

Setting up your experiment:

1. Add a new experiment by clicking on the “New Experiment” button at the top of the browser window. If you’re accessing a pre-made template, click on “Experiment” from the main pull down menu at the top of the screen. From the list, click on your template of choice and then on OK.
 - a. You should name your experiment in the “Browser” window. Single click on “Experiment_001”, begin typing in the name you choose, and press enter to apply. The name should be unique and identifiable so if we have to search the archives at any point we can find it.
2. You must next indicate what parameters (fluorochromes) you will be using before you can set up your compensation or run your samples:
 - a. First click once on the syringe icon at the top of the browser window to add a new specimen. Next single click on the “+” symbol next to the “Specimen_001” label in the “Browser” window to expand the specimen.
 - b. Single click on the pointer (small sideways triangle) next to “Tube_001” which will activate the “Cytometer” window. Click on the “Parameters” tab to open a default list of parameters.
 - c. The height and width (H and W) measurements should be activated by checking the boxes for FSC and SSC to allow doublet discrimination during analysis.
 - d. To remove parameters from the default list you must click on the small point next to the name of each one and then click on “Delete” at the bottom of the window.
 - e. If you would like to add a parameter back to the experiment after deleting it, you can click the “Add” button at the bottom of the window. Clicking this button will add the parameters that you deleted back to the experiment.
3. You must also organize your sample tubes, and events to record, to complete the experimental setup:
 - a. To add a specimen to your experiment at any time, you can click on the small icon at the top of the “Browser” window that looks like a syringe. Each addition of a specimen comes with the addition of one sample tube. (Use of multiple specimens is not necessary, but is a tool for organization.)
 - b. To add a sample tube to your experiment at any time, you must first select which specimen you would like to add the tube to by clicking on the name of that specimen. Next you click on the small icon that looks like a flow tube at the top of the “Browser” window.
 - c. Name all specimens and tubes by clicking on them (a black rectangle will appear around the current name) and typing in the new name.
4. Label your parameters (fluorochromes) so they can be identified in later analysis. *This step should be completed after the compensation controls have been created for any experiment where compensation will be completed in the DIVA software.*
 - a. Click on “Experiment” from the main pull down menu at the top of the screen, and then on “Experiment Layout”. This opens a new window with the “Labels” tab at the top of the window activated. You should label your parameters here for each tube in your experiment (not including the compensation control tubes the software created for you). It is recommended that you label each parameter with the antibody and fluorochrome you are using.

- b. Next, click on the “Acquisition” tab at the top of the “Experiment Layout” window. In this window you can specify the number of events to record in each tube. *There are other options included in this window that should be explained during your training.*
- c. **For the FACSCanto ONLY:** Make sure that the “Sit Flush” box is checked in the “Acquisition Dashboard” to ensure proper rinsing of the SIP between tubes.

Setting up compensation controls:

1. Click on “Experiment” from the main pull down menu, and then on “Compensation Setup” > “Create Compensation Controls”. A new window will appear which will describe the compensation controls the software will create based on the list of parameters you have chosen. *You should not create your compensation controls until you have completed setting up the parameters in the “Cytometer” window.*
 - a. All the parameters should be labeled “generic” and can be left as such (recommended), or modified (not recommended). *(The labeling of parameters should be discussed in your training.)*
 - b. Click on “OK” to create your compensation controls once you’ve finished labeling. The compensation control tubes will be created in the “Browser” window under a specimen named “Compensation Controls”. The software will also create a tab for each tube in the “Worksheet” window to use during collection.

Setting up parameter voltages: Typically voltage settings are adjusted using compensation controls, but if you will not be running compensation then voltage settings can be done with experimental controls. *The theory of fluorescent voltage setting is complicated and beyond the scope of this document but further discussion of this or any topic is encouraged during training or at your convenience.*

1. Click on the pointer next to the unstained control tube under Compensation Controls in the “Browser” window in order to activate it.
 - a. If you do not have compensation controls then you will need to activate an experimental control tube and create graphs in the “Worksheet” window to visualize your data.
2. Adjust Forward scatter (FSC) and Side scatter (SSC) so your population of interest is positioned on the graph where you want it by using the “Voltage” column from “Parameters” tab in the “Cytometer” window.
 - a. Place your unstained control tube on the SIP and click the “Acquire” button in the “Acquisition Dashboard” window. Your events should begin to appear in the “Worksheet” window (be sure the the LSR II is in “Run”. *Do not record your sample until you have adjusted all of your voltages. Do not delete the P1 gate from the FSC/SSC graph for any reason*
 - b. The first adjustment will almost always be to decrease the FSC voltage to move your events from right to left on the graph.
 - i. You can click on the up and down arrows, or hold control on the keyboard while clicking the arrows (the voltage will adjust by 10). You can also type in a number directly or use the slide to adjust the voltage faster.
 - c. If you are using beads for compensation, be aware that the voltages for cells and beads will be different. When you are finished with the steps here, write down the voltages to refer back to.
 - d. Whenever you complete an adjustment to a voltage, pressing restart in the “Acquisition Dashboard” will refresh the data display.
 - e. When your events are on scale in the position you want, adjust the P1 gate in the “Worksheet” window to encompass the entire population of interest (do not delete this gate and redraw it). If you click on the gate to activate it, nodes will appear at all the corners of the gate. Clicking in the nodes will allow you to adjust the gate’s size and shape.
 - f. When the gate is where you want it, right click on the outside edge of the gate and a drop down menu will open. Click on “Apply to all Compensation Controls” and the P1 gate will be copied to each parameter’s worksheet tab. If you need to make adjustments, you can reapply to all as many times as you need. You also do not need to apply to all, but it is built in to be a time saver.
3. Adjust the PMT voltages for each fluorescent parameter.
 - a. Type in the voltage as indicated by the suggested voltages for each channel listed on the side of the instrument and use those voltages as your low end starting point.

- b. Place the first single stained control on the SIP and click the “Acquire” button in the “Acquisition Dashboard” window. *The unstained control pointer should remain activated in the “Browser” window so you can see the fluorescence in all channels.*
- c. There are three things to check:
 - i. Is there fluorescent signal in the primary channel for the fluorochrome.?
 1. If no, there may be an instrument or a sample issue.
 - ii. Is the signal on scale in the primary channel?
 1. If the signal is off scale to the high end, the voltage has to be decreased even if that means you go below the suggested voltage.
 - iii. Is the primary channel the brightest positive signal?
 1. If not, there could be a voltage, staining or instrument issue.
- d. Repeat this process for each single stained compensation control tube.
- e. The process of voltage adjustment for compensation should be covered in detail in your training. This process can be time consuming but is critical to getting accurate and interpretable data.
4. Once all of the voltages have been set you can place the unstained control tube back on the SIP and begin recording data. Click on the “Acquire” button first and allow approximately 4-5 seconds for the flow to stabilize before clicking the “Record” button in the “Acquisition Dashboard” window. A bar will begin to move left to right in the “Acquisition Dashboard” window indicating progress.
5. Place the next tube on the SIP and click on “Next Tube” in the “Acquisition Dashboard” window to switch to the next tube in order. Record the second compensation control.
6. Repeat this process for each single stained compensation control tube.
7. In the “Worksheet” window, scroll through the tabs for each parameter to make sure that the P2 gate is gating the brightest positive peak. The gates can be modified the same as the P1 gate.
8. When all of the positive populations are identified as you would like them, click on “Experiment” from the main drop down menu at the top of the screen, then on “Compensation Setup” > “Calculate Compensation”. The software will calculate the compensation automatically. Common error messages in compensation should be covered in detail in your training.
9. Click “Link and Save” and the compensation will be applied to the experiment and saved so it can be accessed later.

Sample Recording: The compensation is automatically applied to all tubes **recorded after** it is calculated.

1. Click on the small button at the top left of the “Worksheet” window to open a Global Sheet. This sheet allows you to create analysis graphs once and apply them to each sample tube automatically. The graphing and gating tools are the buttons across the top of the “Worksheet” window.
2. Graphs and analysis can be created and modified at any point during the sample run. *Data analysis is another complex topic that is beyond the scope of this document, but discussion of this topic is encouraged during training or separately.*
3. Click on the pointer next to your first sample tube to activate that tube. Fluorescent voltages are fixed at this point and can not be changed, however, FSC and SSC voltages can be modified if needed.
4. Begin acquiring the sample by clicking on the “acquire” button in the “Acquisition Dashboard” window.
5. After a couple seconds of acquiring, you can click on the “Record” button in the “Acquisition Dashboard” window to begin recording and saving your sample.
6. Repeat this process for each specimen tube.

Finishing the Experiment: When you have recorded all of your samples the experiment is done and needs to be exported for analysis in another program.

1. Right click on the name of your experiment in the “Browser” window to open a drop down menu. Click on “Export” at the bottom of the list, and then on “Experiment”.
2. This should open another window with a pathway to your experiments folder on the D: drive. The pathway should read D:\BD Export\Experiment\“current year” \“current month”. Click “OK” and the files will be exported. These files are archived on the flow core server and can be used to reopen your experiment in FACSDiva software if needed. *The Experiment and FCS file pathways should never be*

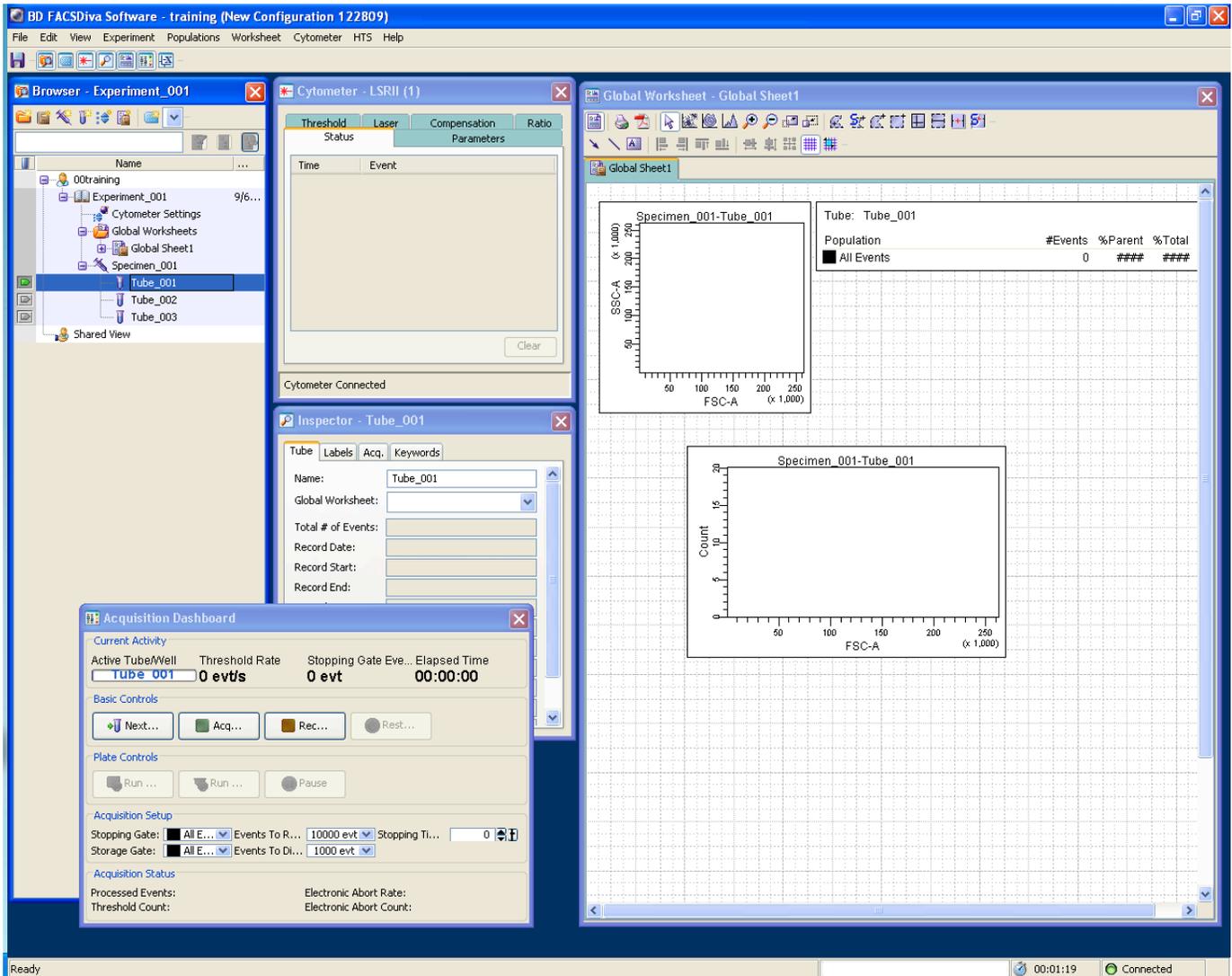
changed, however, it does happen occasionally so needs to be double checked. If they are incorrect please reset them to make sure your data is archived properly.

3. Again, right click on the name of your experiment to open a drop down menu. Click on “Export” at the bottom of the list, and then on “FCS”. When a window pops up, click “OK.”
4. This should open a window with a pathway to the FCS folder in the D: drive. The pathway should read D:\BD Export\FCS. Click on “Save” and the files will be exported.
5. Minimize the FACSDiva software to go back to the desktop of the computer and double click on Shortcut to FCS icon to check to be sure your files were properly exported.
6. With the FCS file folder open, click on the Shortcut to Transfer icon on the desktop. This will open the transfer pipeline section of the flow core server. With the transfer pipeline folder open, you can then drag and drop your FCS files to your PI’s folder on the pipeline. To check that your files transferred properly, you can drag and drop your folder again. You should get a pop-up that says “Confirm Folder Replace” and you can click either “no” or “cancel”.
7. After making sure your files are saved on the hard drive and transferred to the pipeline you can maximize the FACSDiva software again. Right click on the experiment name and click on delete to remove the experiment from the software. This is critical because too many active experiments in the software can cause the software to crash and cause loss of data.
8. Log out of the FACSDiva software by clicking on the File menu and selecting “Log Out”.
9. The cleaning procedure must be completed after every run and is posted on each instrument as well as being described in your training.

Log out of the PPMS System:

- Click on the small “Log Out” button at the bottom of the screen.
- The gray screen will appear and you will no longer be able to access the computer.

Thank You.



1. Browser – The browser window allows users to see their experiments and place where you visually organize your experiment and can name the sections. The browser is where you select and move the pointer from tube to tube in order to acquire and record events in the proper tube.
2. Cytometer – Using this window you can directly control how the instrument operates for any experiment. The list of things that can be controlled in this window under the Parameters tab include: Turning off and on the detectors within the instrument; setting the voltages for each parameter; choosing linear or logarithmic scaling for data display; turning on the height and width measurements for collection.
3. Inspector – This window allows control of the software based on what we have highlighted, be it a tube, specimen, plot, gate, or anything else you click on. It is important to note that with Cytometer Settings highlighted, this window can look very similar to the Cytometer window (Only the Cytometer window should be used to change voltages and detectors).
4. Acquisition Dashboard – The acquisition dashboard is where we tell the cytometer to acquire data, record data, refresh the plots, change events to record or display, and change the stopping and storage gates. It also tells us the number of events we have run.
5. Worksheet – The worksheet window is for data analysis and can be either Global or Normal worksheets. Global worksheets are universal and provide the same gating and analysis to any tube that is selected with the pointer. Normal worksheets provide a set of plots for each tube. Usually compensation is done on normal worksheets while samples are collected while looking at a global worksheet.