Preparing a FACSDiva Experiment

This document is intended to cover the basics of creating an experiment in FACSDiva, performing cytometer setup, recording controls, and recording samples. It is not intended to be a comprehensive treatment of theory and best practices, although both topics will be discussed during the training. For information on flow cytometry in general please contact Flow Cytometry Resource staff.

The windows described below and the drop down menus at the top of the software will be referenced throughout this document.

1. Browser – The browser window allows users to see their experiment outline and is a 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 – There are 6 tabs available in this window. The Parameters tab is the most crucial and will be used during every 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 detector; choosing linear or logarithmic scaling for data display. The other tabs will be discussed briefly during training.
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 rate at which we're collecting events and the number we have collected.
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.

Overview

1. **Creating the outline of your experiment**
2. **Adjusting detector voltages and verifying setup**
3. **Recording samples/experimental controls**
4. **Saving and exporting data**

After checking that the instrument is ready:
1. Log in to the computer via the PPMS System:
   a. This is the same login information you use to log in online for scheduling and training requests.
   b. The lock screen will change and you'll be presented with a screen allowing you to update account info if needed.
   c. Update the account info, or press "OK", and you will be able to access the computer.
2. Log in to the FACSDiva Software:
   a. There is a generic username associated with each cytometer and no password. Just click OK.
   b. If the "CST Mismatch" window opens click on the button that says "Use CST Settings."

The process for creating and running an experiment in the software is broken into 4 major parts outlined below.

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

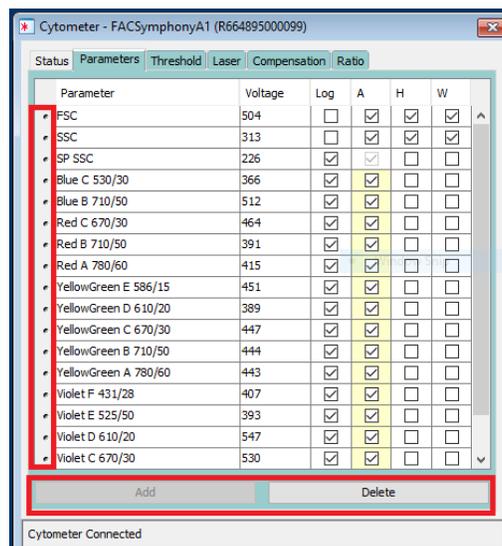1. **Creating the outline of your experiment:**
   1. Add a blank new experiment by clicking on the "New Experiment" button at the top of the browser window.
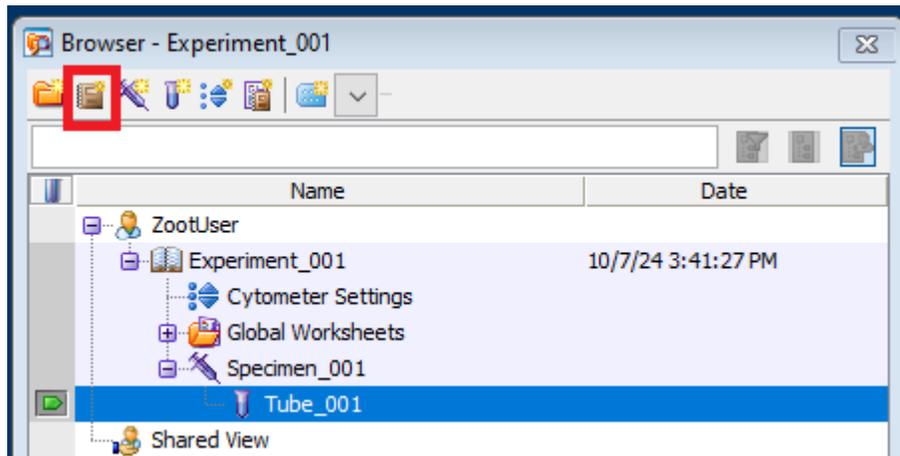
   a. You should name your experiment in the "Browser" window. Single click on "Experiment_001", begin typing (you do not need to wait for a cursor to appear) in the name you choose, and press enter to apply. The name should be unique and identifiable to you.

   2. You must next indicate what detectors you will be using before you can set up your compensation or run your samples:
   a. Click on the small + sign next to the first Specimen included with the blank experiment.
   b. Single click on the pointer (small sideways triangle) to the left of "Tube_001" which will activate the "Cytometer" window. Click on the "Parameters" tab to access the list of detectors (all detectors will be open/on/selected by default.)


   c. To remove detectors from the list you must click on the small point to the left of the name of each one and then click on "Delete" at the bottom of the window.
   d. If you would like to add a detector 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 detectors that you deleted back to the experiment.

3. You must also create and organize all additional tubes to complete the outline setup:
    a. Specimens are groups of samples that you can add to your experiment at any time by clicking 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 (the current name will become highlighted) and typing in the new name.

4. Setting up compensation controls: Creating the compensation controls in this way allows the software to create and organize a number of shortcuts and is recommended for everyone using multiple fluorescent labels. If you intend to calculate compensation separately later you do not need to create the compensation controls in this way even though it is recommended, however single stain and unstained controls must still be recorded (as sample tubes.)
    a. Click on the "Experiment" pull down menu at the top of the window, and then on "Compensation Setup" > "Create Compensation Controls". You should not create your compensation controls until you have completed selecting detectors in the "Cytometer" window.
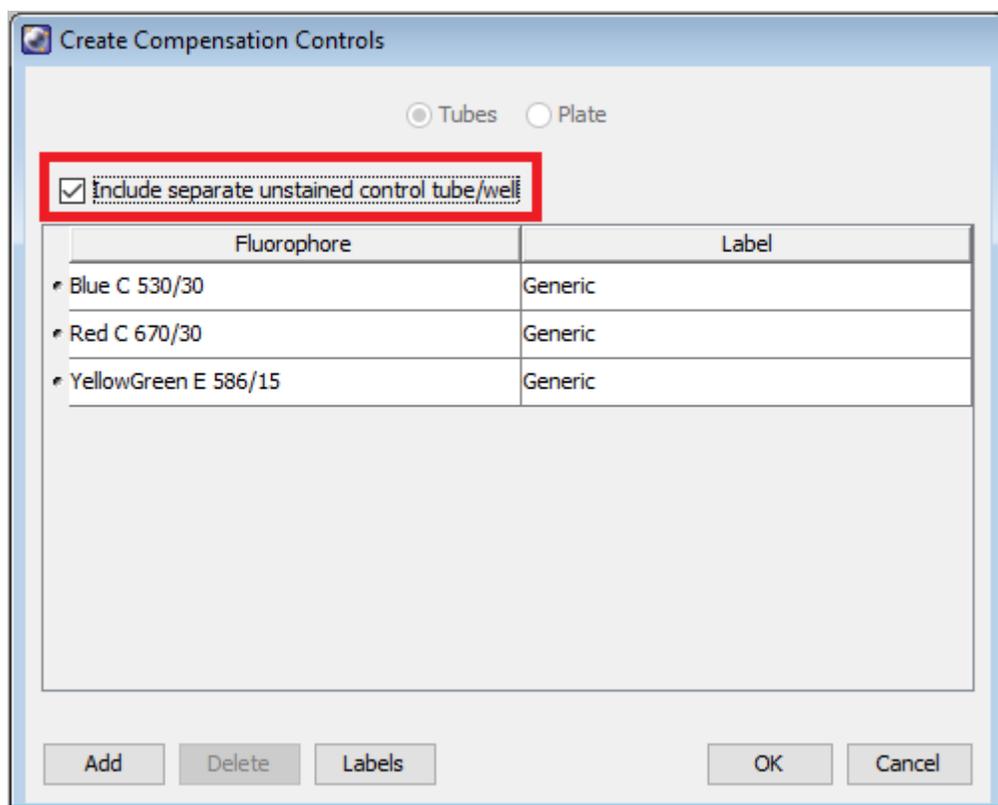

    b. A separate unstained control (also referred to as a 'universal' unstained) is common and the box can usually remain checked. The alternative to a separate unstained control is an internal unstained population (control) present in each single stain control. If using an internal unstained control, the separate unstained control tube/well box can be unchecked.

      i. If using an internal unstained control, an interval gate will need to be made on each single stain control worksheet to indicate the negative population.
c. The compensation controls can be left "Generic" or labeled depending on your preference.
d. 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 "Normal Worksheet" with a tab for each tube to use during review and setting of gates identifying positive and negative signals.

5. Complete annotation of your experiment: Not all steps need to be completed for every experiment but this can greatly improve ability to analyze and organize data if done properly.
a. Click on the "Experiment" pull down menu at the top of the window, and then on "Experiment Layout".
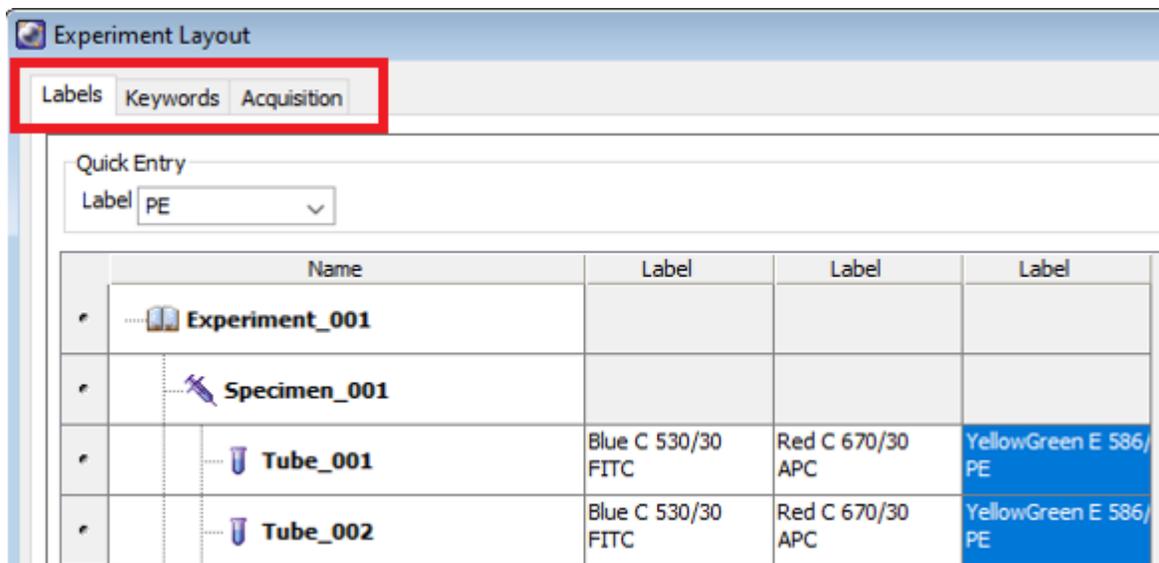
b. In the "Labels" tab that opens you should label your detectors for each tube in your experiment (this does not include the compensation control tubes the software created for you as they were already labeled). It is recommended that you label each detector with the antibody and fluorochrome you are using.
    i. Click on the "Label" column header to highlight the whole column prior to typing.
    ii. If your compensation controls were labeled, the specific name you chose will be included in the drop down menu in the upper left of the "Experiment Layout" window.
c. In the "Acquisition" tab you can specify the number of events to be recorded for each tube in the experiment. Additional items in this window are briefly addressed in the Appendix below.

2. Adjusting detector voltages and verifying set up: 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 basics of fluorescent voltage setting will be introduced here, but further discussion of this or any topic is encouraged during training or at your convenience.*

- The voltages pre-loaded for each detector are calculated by the instrument/software and tend to be set higher than necessary.

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. The graphs needed will be a FSC vs. SSC plot and a histogram for each fluorescent detector.

2. There are no suggestions for Forward scatter (FSC) and Side scatter (SSC) voltages so they will need to be set for each run. Your population of interest can be positioned on the graph where you want it by adjusting the voltage for the FSC or SSC detector from the "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 analyzer 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.

    b. The first adjustment should 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 increments of 10). You can also type in a number directly or use the slider for coarse adjustment.

    c. Once your FSC is close to where you want it you can adjust the SSC voltage. This may go up or down depending on a number of factors which is why you should always adjust FSC first.

    d. If you are using beads for compensation, be aware that the scatter voltages for cells and beads will be different.

    e. Whenever you complete an adjustment to a voltage, pressing restart in the "Acquisition Dashboard" will refresh the data display. A similar effect can be accomplished in the "Acquisition Dashboard" by setting the "Events to Display" to a low value so the data refreshes more quickly as you run.
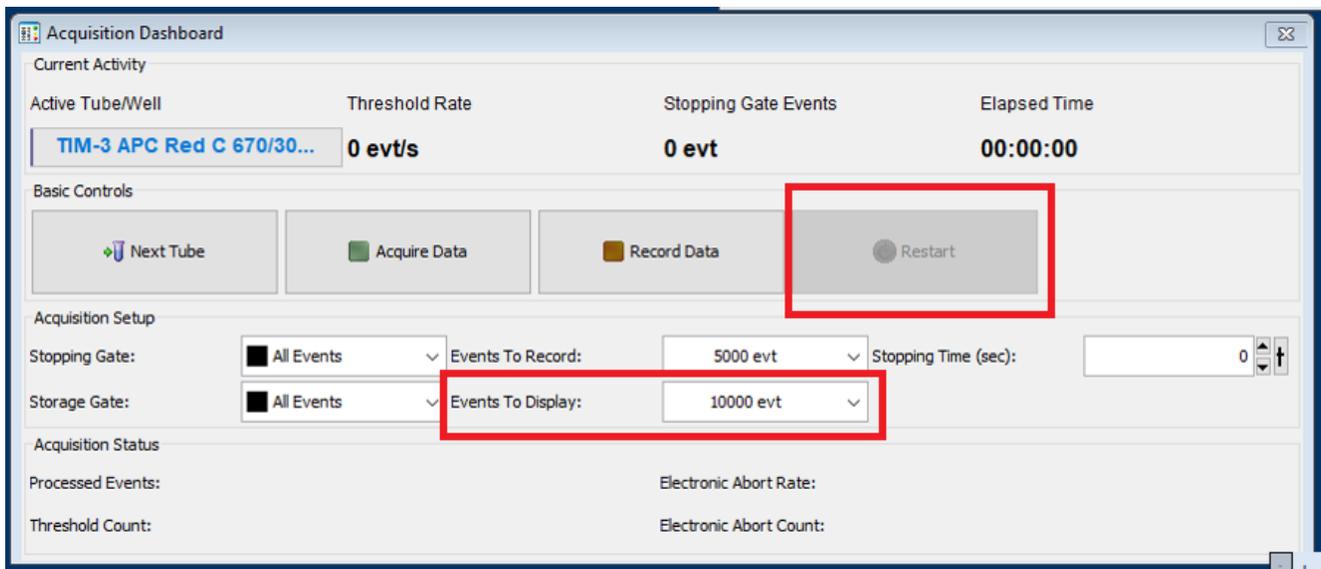
f. When your events are on scale in the position you want, adjust the P1 gate in the "Worksheet" window to encompass the entire population of interest (do not delete this gate and redraw it.) If you click on the gate to activate it, nodes will appear at all the corners of the gate. Clicking in the nodes will allow you to adjust the gate's size and shape.

g. 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 control'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. Check each fluorescent detector and adjust the voltage if necessary.

    a. 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.*

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    b. There are three things to check:
        i. Is there fluorescent signal in the primary detector 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. Are there any unexpected overlaps into the secondary detectors?
            1. This could be difficult to diagnose and you are encouraged to reach out to the FCR staff in real time if you encounter something you're unclear on.
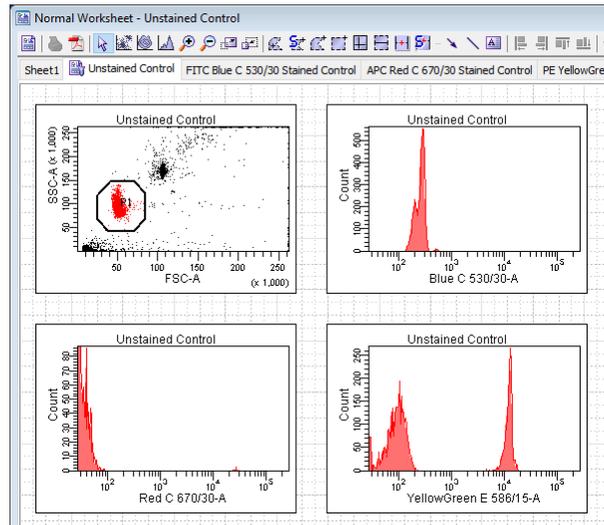
    c. Repeat this process for each single stained compensation control tube.
    d. The process of voltage adjustment for compensation should be covered in detail in your training. This process can be time consuming but is critical to getting accurate and interpretable data.
4. Once all of the voltages 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 progress bar will begin to move left to right in the "Acquisition Dashboard" window.
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 control to make sure that the P2 gate is gating the brightest positive peak. The gates can be modified manually as needed. *For compensation you always want to gate on the brightest positive signal, not necessarily all of the positive signal*.
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.

Bonus: Compensation Warning Messages
1. PMT voltage is not consistent among compensation tubes: This indicates that one or more of your compensation controls were recorded using fluorescent detector voltage values that do not match (FSC and SSC are not fluorescent detectors and will not cause this warning message if different between controls.) You will need to identify which controls have inconsistent detector voltage values and re-record them using the desired set of voltage values that matches the rest of the controls.
    Compensation controls and samples should be recorded using the same set of fluorescent voltages.

2. Not Enough Events in P1 or P2: This indicates that you have too few events in the P1 or P2 gate for one or more of the compensation controls. The software will cite which controls do not have enough events in which gate. You will need to record more events, or change the position of the gate. Be cautious if changing the gate position because you should never include very dim or negative events in your positive (P2) gate.

3. Compensation Overlap Warning: This indicates that the signal in a secondary detector is brighter than the signal in a fluorochromes primary detector, it does not necessarily indicate a problem with your experiment. If you are unclear on the cause of the overlap please contact the FCR staff in real time to discuss. If you understand the cause this message can be ignored and compensation values can be calculated and applied. Spectral overlap/spillover can be a product of a number of things beyond the scope of this document but can and should be discussed separately as needed.

4. Completed Successfully: This indicates that compensation values were calculated, and all values are under 100%. This **does not** indicate that your compensation values are correct, only that they were able to be calculated based on the data provided.

3. Recording Samples/experimental controls: 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 the 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.
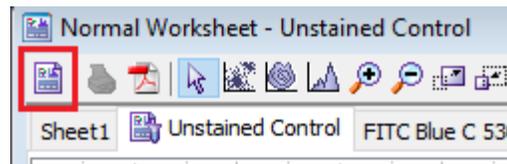
2. *Graphs and analysis can be created and modified at any point during the sample run. Data analysis is another complex topic that is beyond the scope of this document, but discussion of this topic is encouraged during training or separately.*

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.

4. Saving and exporting data: 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 by the FCR and can be used to reopen your experiment in FACSDiva software if needed. The Experiment pathway should never be changed, however, it does happen occasionally so needs to be double checked. If it is incorrect please reset it to make sure your data is archived properly.
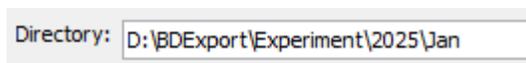
3. Again, right click on the name of your experiment to open a drop down menu. Click on "Export" at the bottom of the list, and then on "FCS". When the next window pops up, click "OK."

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. The FCS file pathway should never be changed, however, it does happen occasionally so needs to be double checked.
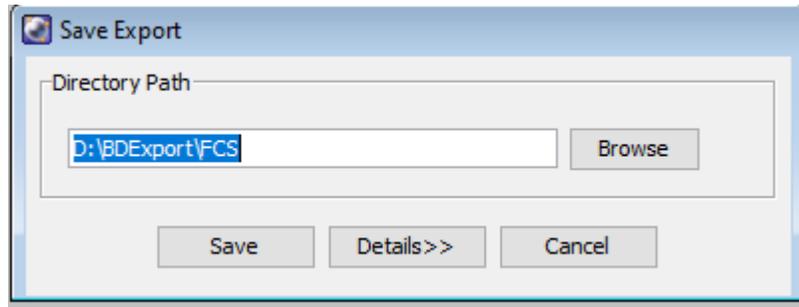
5. Minimize the FACSDiva software to go back to the desktop of the computer and double click on Shortcut to FCS icon to check to be sure your files were properly exported.
6. With the FCS file folder open, click on the Shortcut to Transfer icon on the desktop. This will open the transfer pipeline section 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.
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 and PPMS to complete your session.

Thank You.

Appendix

- **Templates**: If you're accessing a pre-made experiment or data analysis worksheet 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.

- **Keywords**: In the Experiment Layout window, click on the "Keywords" tab to include experiment specific keywords. The software provides two additional default keywords for use, but additional keywords can be created for large or long term studies as needed. *Keywords are a useful tool for organizing or sorting large experiments or projects. Further support regarding this topic will be offered upon request.*

- **Acquisition** (Experiment Layout):
  - Stopping Gate: Only the gated events will be counted toward the total events to record though all events above threshold are saved. Allows for normalization of event count based on a certain important gate.
  - Storage Gate: Only the gated events are saved in the file. All events that are not within the storage gate are discarded by the software.

- **Voltage/detector optimization**: The FCR performs a voltage titration on all of its conventional cytometers twice a year using a fluorescent standard. This voltage titration is used to identify the suggested 'low end' voltage for each detector on each instrument and these voltage values are posted at each conventional instrument. This is intended to be used as a guideline for users performing voltage adjustment and provide a 'low starting point voltage'. Voltages above those posted that result in positive fluorescence signals on scale will likely result in usable data. If you are interested in "optimizing" your detector voltages for a given project/panel please consult the FCR staff.