BD FACSDIVA TUTORIAL

BD FACSYMPHONY A5 SE

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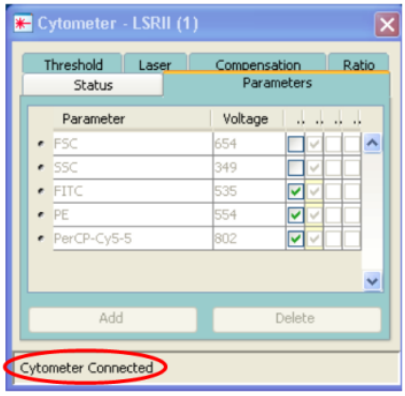
Computer and Software Login



- Turn on the computer.
- Turn on the power to the flow cytometer and the FACSFlow Supply System (FFSS).
- Login on Windows:

  Username: Email *(@medicina.ulisboa.pt* – for internal users)

  Password: Your Agendo´s Password

- Wait for the BD Coherent Connection 4 software to open automatically. Turn on all the lasers (Start All).

Note: allow 30 minutes for the optical system temperature to stabilize. Meanwhile, perform the Cleaning procedure described ahead.

- Start the software: double-click the DIVA shortcut icon on the desktop.
- Login on DIVA using your own login name and password.
- Wait for the cytometer connection and click “Use CST Settings”.

NOTICE: To verify the workstation has successfully connected to the cytometer, check that the Cytometer window displays the message “Cytometer Connected” or “The system is ready” at the bottom of the window. If the message reads “Cytometer Disconnected,” switch the cytometer power OFF, wait 10 seconds, and then switch the power ON. Restart the computer.

Cleaning and Quality Control

(1st user of day on weekdays before 9am, weekends and holidays)

Cleaning

Start up the fluidics:

1. Install a tube with 3 mL of FACSClean on the SIP and close the tube support arm.
2. Press RUN and HIGH on the cytometer fluid control panel. Run for 10 minutes.
3. Remove the tube of FACSClean from the SIP and replace with a tube containing 3 mL of DI water.
4. Run for 10 minutes on RUN HIGH.

Determine whether the fluidics need to be debubbled by priming:

1. Open an experiment to view BD® CS&T Beads (Prime Check) on Shared View.
2. Prepare the CS&T beads according to the technical data sheet provided with the beads or available on the BD Biosciences website (350 µl PBS+1 drop of beads).

3. Press RUN and LOW on the cytometer fluid control panel.
4. Observe the plots for the scatter channel and fluorescent channels. There should be two distinct peaks for each scatter channel and three distinct peaks for each fluorescent channel. The coefficient of variation (CV) for bright peaks should be narrow. If either the CVs are very wide on a detector or no signal is observed, the fluidics need to be primed as described in the following procedure. If everything is ok, move on to running a performance check.

To prime the fluidics:

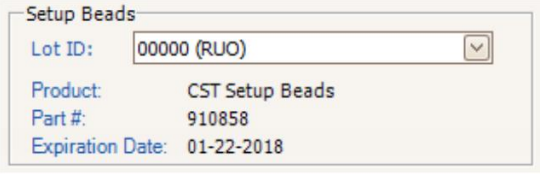
1. Move the tube support arm to the side.
2. Remove the tube from the SIP.
3. Press the PRIME fluid control button to force the fluid out of the flow cell and into the waste container.

   Once drained, the flow cell automatically fills with sheath fluid at a controlled rate to prevent bubble formation or entrapment. The STANDBY button turns amber after completion.
4. When the STANDBY light is on, install a test tube of DI water.
5. Press the RUN fluid control button and run for 10 seconds.
6. Press the PRIME button immediately followed by the RUN button.
7. Repeat step 6 ten times.
8. If more than 2 seconds pass between PRIME and RUN in step 6, restart the procedure.
9. Install a new tube with 1 mL of DI water on the SIP and close the support arm.
10. Leave the cytometer in standby mode.

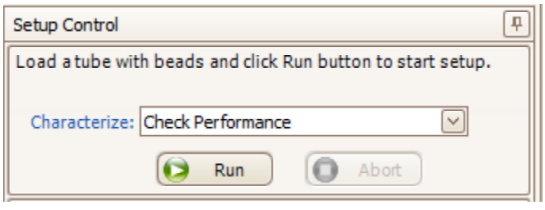
Running a performance check

Procedure to run a performance check:

1. Select **Cytometer > CST**.
2. Verify that the bead lot information under Setup Beads matches the Cytometer Setup and Tracking bead lot.

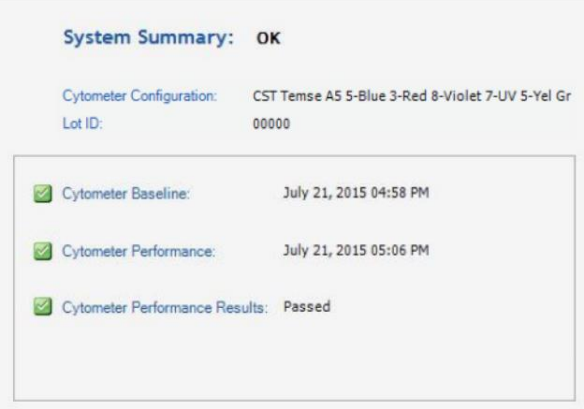


3. Install the CS&T bead tube onto the SIP.
4. In the Setup Control window, select **Check Performance** from the Characterize menu.
5. Click **Run** on the Setup Control window.
6. On the fluidics control panel, ensure that FINE Adj is set to 250, and then press the RUN and LOW buttons. Plots are

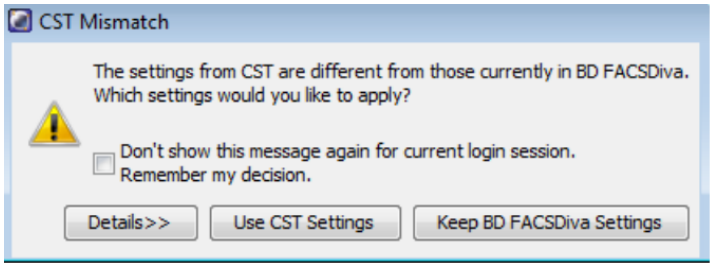


displayed under the Setup tab and the performance check is run. The performance check takes approximately 5 minutes to complete.

7. Once the performance check is complete, click **View Report**.
8. Run a PBS tube to clean the beads.
9. Verify that the cytometer performance passed. In the Setup tab, the cytometer performance results should have a green checkbox displayed and the word Passed next to it.



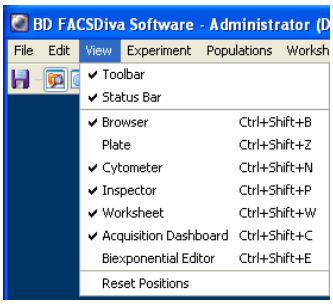
10. Select **File > Exit** to close the CS&T window and return to the BD FACSDiva™ interface. The CST Mismatch dialog opens.



11. Click the Details button to verify which cytometer settings will be updated.
12. Click **Use CST Settings.** By selecting Use CST Settings, the laser delay, area scaling, and other cytometer settings will be updated to the latest settings from the performance check.

Setting Up a Spectral Experiment

Before you start, make sure that you have all the necessary windows open. Go to the "View" menu and make sure the following boxes are checked:

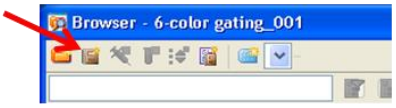


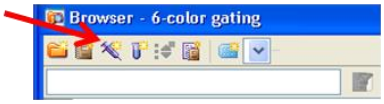
- Browser
- Cytometer
- Inspector
- Worksheet
- Acquisition Dashboard

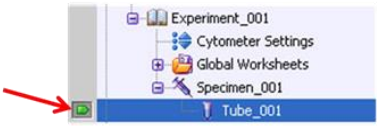
When you add elements or make selections in the **Browser**, the Inspector displays details, properties, and options that correspond to your selection.

Creating a New Experiment

1. Click on the **New Experiment** icon in the browser toolbar and rename it.







   a) To create an experiment based on a saved template, choose **Experiment > New Experiment**. The Experiment Templates dialog appears where you can select your unit and the template.

   b) Click **New Specimen** button to add a specimen and tube to the experiment; click once on the plus sign (+) next to "Specimen_001" to expand it. In the browser, click the icon to the far left of the tube named "Tube_001". The pointer changes to green.

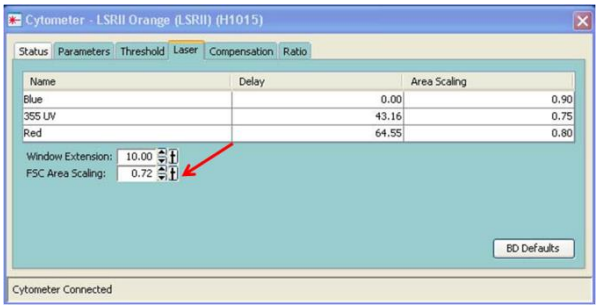
   Note that experiment names cannot contain commas or periods. The experiment modification date is the date of the last data collected.

2. Create a dot plot with FSC vs SSC and adjust the flow cytometer settings as needed for your experiment, including PMT voltage (FSC and SSC) and area scaling.

Adjusting Area Scaling for FSC

If your cells are bigger than lymphocytes you must adjust the area scaling factor for FSC. Area scaling adjusts area measurements to be the same magnitude as height measurements. Create a histogram for each FSC-A, FSC-W, FSC-H parameter. Check area scaling for forward scatter: go

to the “Laser” tab in the “Cytometer” window and adjust the “FSC Area Scaling” value until the population of interest (P1) on the FSC-A, FSC-H and FSC-W Histograms is aligned.

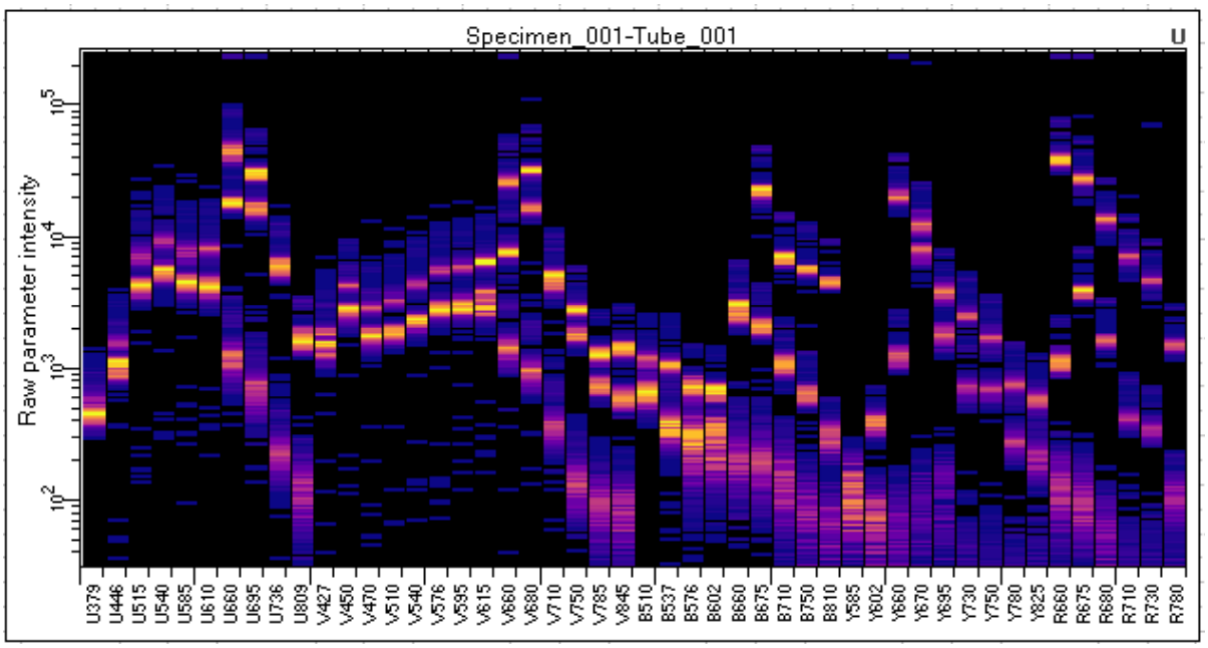


3. Create a spectral plot using the multicolor tube to ensure that the signal across all detectors is on-scale, not just the signal for the primary detector for each fluorochrome.

   Note: In addition to the dot and density plots, contour plots, and histogram plots for single-parameter data used in compensation cytometry, the BD FACSymphony™ A5 SE flow cytometer features the ability to create spectral plots, where the plot shows combined color-coded histograms of all raw detector values. The icon on the worksheet toolbar for creating a spectral plot is outlined in red in the following illustration:

   The detector names displayed along the x-axis combine the first character of the laser color with the center value of the filter bandpass range for that detector.

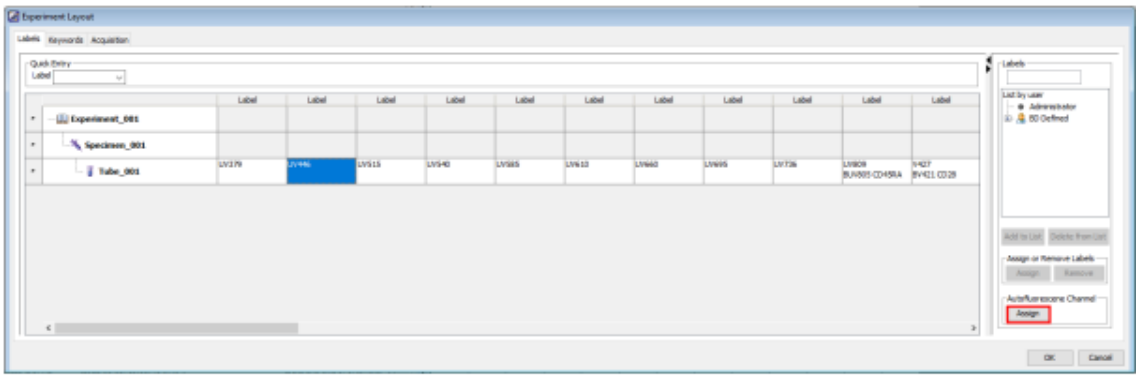
   The plot supports the display of specific populations or combinations of populations, and displays live data during acquisition. Density color schemes or color by population can be modified in the inspector window when the plot is selected. The spectral plot does not support zoom, biexponential scaling, or gate drawing.



4. If everything is on scale:
    a) No changes on the PMTs voltages are needed and experiments **can be performed with standardization** between experiments and equipment's.
    b) The PMTs voltages for each experiment are updated every day with the quality control.

    If some channels are out of scale, antibody titration is required. If not possible:
    a) Adjust the PMTs voltages as needed for your experiment. Ensure that the signal across all detectors is on-scale, not just the signal for the primary detector for each fluorochrome.
    b) The PMTs voltages for each experiment are fixed and **standardization between experiments and equipment's is not possible**.

5. Check the unstained to identify the channel with more autofluorescence.
6. Check all the single colors and confirm if the primary channel is the expected one (important for compensation workflow).
7. Navigate to **Experiment > Experiment Layout** and assign parameter labels for each reagent to an individual primary detector. Typically, both the dye and the antibody names are added to the label, as shown in the following example. To add an autofluorescence control to your experiment, select a paramater label associated with a channel with high autofluorescence, typically one of the lower wavelengths of the UV or Violet laser. Click the **Assign** button in the Autofluorescence Channel section of the Labels panel to add AutoF to the selected parameter label. Click Ok.

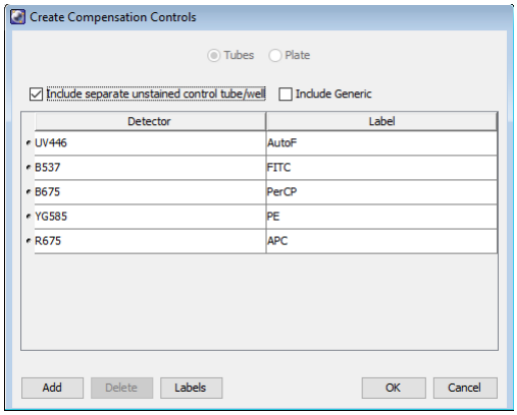


Note: The "AutoF" label is case-sensitive. The Autofluorescence control is only supported in spectral workflows, not compensation workflows. **Calculate Compensation cannot be performed if an AutoF label is assigned.** AutoF should only be assigned to a single channel

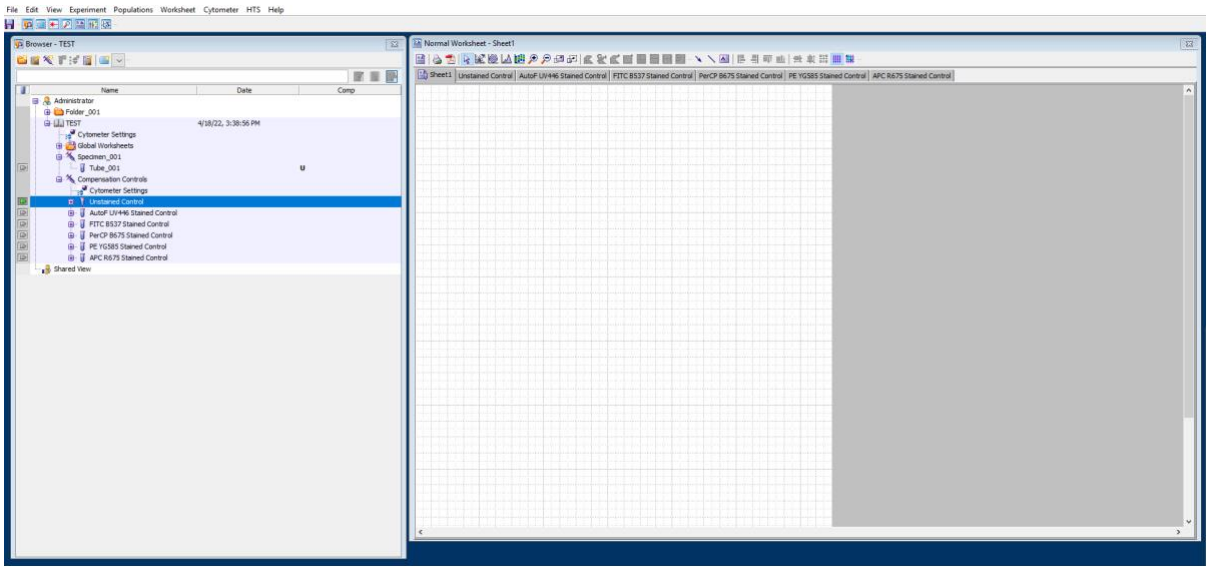
within an experiment setup. Calculate Spectral Unmixing cannot be performed if more than one channel has AutoF assigned.

Creating a Spectral Unmixing matrix

1. Select **Experiment > Compensation Setup > Create Compensation Controls** from the main menu.

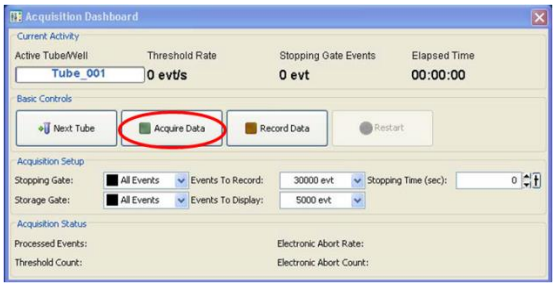


2. Leave the checkbox “Include Generic” in its default (deselected) state.
3. Click **OK** to create the controls. This adds the controls to the experiment Browser window, under Compensation Controls, and creates a tab for each control on the worksheet. If you assigned AutoF to a detector in the experiment layout, an AutoF control tube will display here too, as shown in the following example.



4. If you have a separate tube for your unstained control, record the unstained control as follows:
   a) Select the **Unstained Control** tab on the worksheet.
   b) In the experiment Browser, set the run pointer on **Unstained Control**.

c) On the Acquisition Dashboard, click **Acquire Data.**



d) Adjust FSC and SSC voltages so you can see the population of interest in the center of the FSC-A vs. SSC-A dot plot.

e) Move the P1 gate to cover the events of interest.

f) (Optional) Right-click the gate and select **Apply to All Compensation Controls** from the context menu.

g) In the Acquisition Dashboard, click **Record Data**. The acquisition and recording automatically stops when the number of events in the Events to Record list box is reached (default value is 5000 events, we suggest to acquire 30000 events).

5. Record each of the single-color controls as follows:

a) Load the single-color control tube. If you are using an autofluorescence control in your experiment, record your autofluorescence tube using your unstained cell sample.

b) Select the tab for the single-color control on the worksheet.

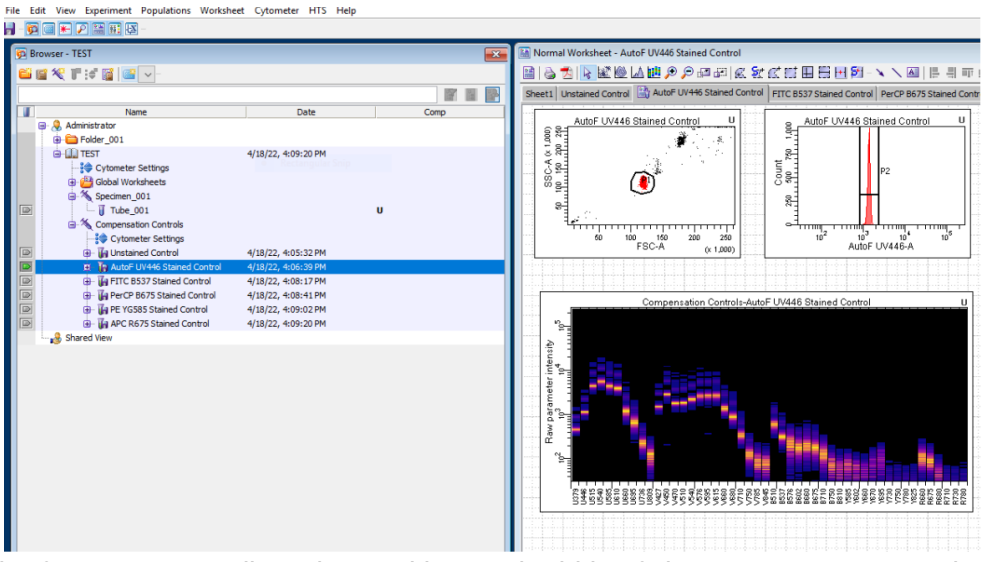
c) In the experiment Browser, set the run pointer on the corresponding control.

d) On the Acquisition Dashboard, click **Acquire Data**.

e) If necessary, move the P1 gate on the left dot plot to include the events of interest.

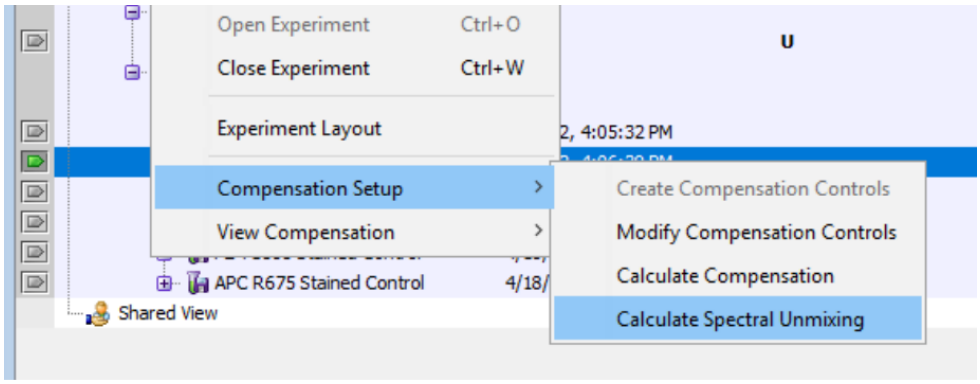
f) In the Acquisition Dashboard, click **Record Data**.

g) When the recording completes, remove the control tube.

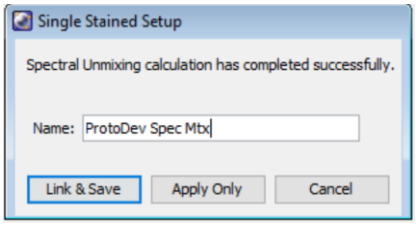


h) If necessary, adjust the position and width of the P2 gate to span the positive population on the histogram plot.

6. Select **Experiment > Compensation Setup > Calculate Spectral Unmixing**.



The screen displays the Single Stained Setup dialog.



7. Enter a name for the setup and click **Link & Save** to link the compensation setup to the cytometer settings and to save the settings to the Compensation Settings catalog.

Note: To view the saved settings, select menu item **Cytometer > Catalogs** and select the Compensation Setup tab. The Type column on this tab classifies settings created using Calculate Spectral Unmixing as **Spectral** and settings created using Calculate Compensation as **Compensated.**

Recording and analyzing spectral data

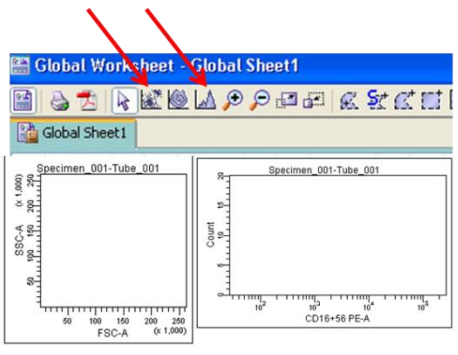
In the Global Worksheet, with the specimen tube selected, create more plots, gates, and statistics in addition to those created when initially setting up the experiment.

Selecting Parameters

Select H and W for the FSC parameter and/or SSC for doublet exclusion.

Creating a Dot Plot and Histogram

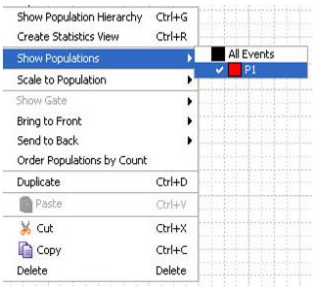
Create a FSC-A vs. SSC-A plot on the global worksheet. Select the “Dot Plot” icon on the Worksheet toolbar and click once in the upper left corner of the worksheet field. A default size plot is drawn. Create a histogram for each fluorescent parameter, selecting “Histogram” icon on the Worksheet toolbar and click on the worksheet field.



Creating a Gate

Create a gate by selecting the "Polygonal Gate" button and drawing it in the dot plot. Be sure you finish your gate at the same point you started drawing it. This gate (P1) will let you gate on a single cell population and exclude aggregates, dead cells and debris.

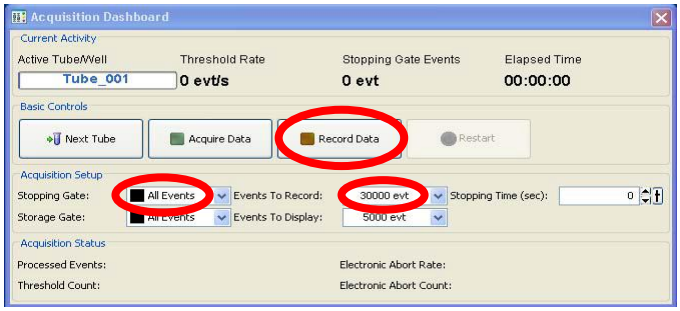
Selecting Parameters for Dot Plot Axes



Change the parameters on your dot plot by left clicking on them and selecting the one you need in the pop-up menu.
To gate it on the P1 gate, right click inside the FSC-A vs. SSC-A dot plot and in the popup menu select "Show Populations", then "P1".

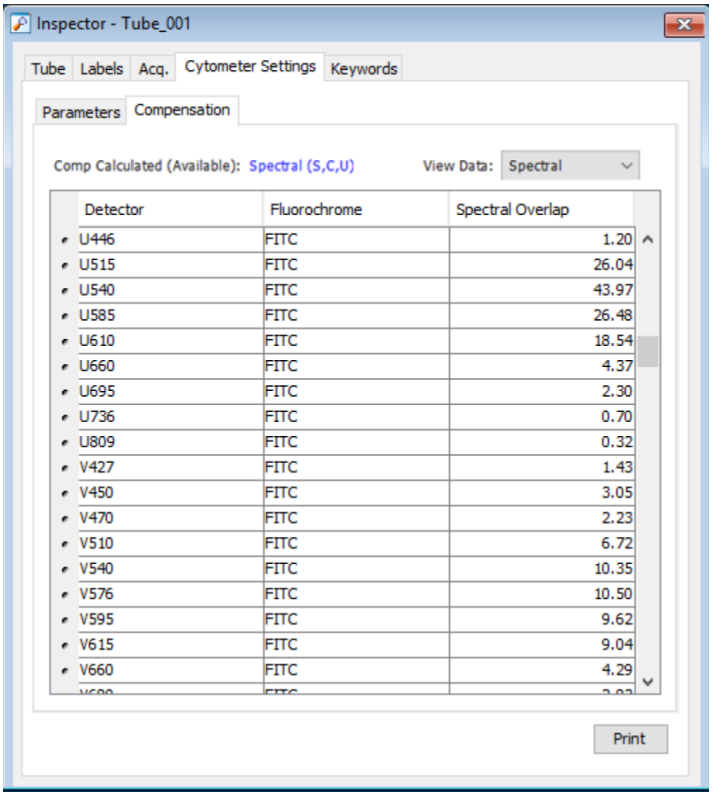
1. Record sample data using the initial tube.

When your settings are done you should be ready to start acquiring your samples. First you need to define the number of events you want to record – you can do so in **"Events To Record"** by setting the number you wish. In **"Stopping Gate"** you have the option to choose one gate, and the acquisition will only stop when the number of events is reached in that specific gate. The **"Storage Gate"** should always be set to 'All Events'. Press **"Record Data"** when you want to start to record your acquisition.



2. To acquire and record more tubes, click **Next Tube** and/or **Duplicate the Specimen**. Tubes must have the same parameter labels as was set up in the Spectral Unmixing algorithm by the Create Compensation Controls menu command.

When the software calculates spectral unmixing, it also calculates compensation values (as if you had selected **Experiment > Compensation Setup > Calculate Compensation**). You can switch between spectral unmixed and traditional compensation values, by toggling between Spectral and Compensated in the dropdown menu, to compare the plot data in each case. By default, when you select spectral unmixing, the Spectral settings are selected in the dropdown menu on the Compensation panel of the Inspector window as shown in the following example:



In any case, do no adjust any of these matrix values (neither in compensated or spectral mode). If afterwards you want to adjust the matrix, you should do it in the spectrally unmixed fcs files (you create a blank identity matrix, edit the comp interactions from zero to a different value and apply it to your files)

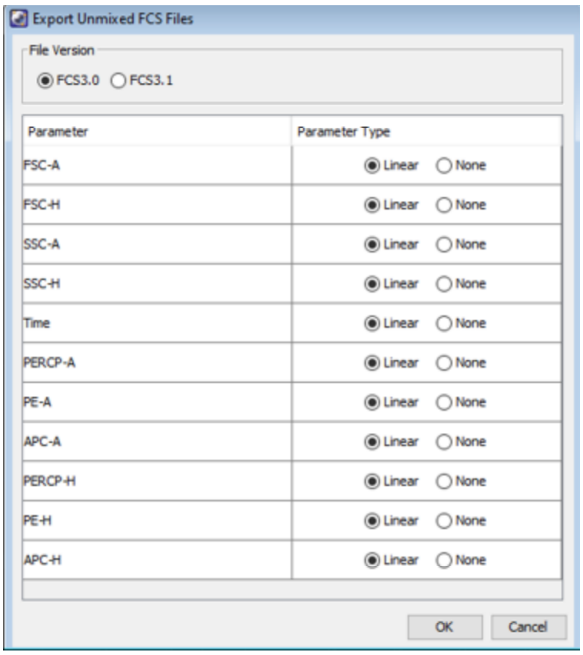
Exporting your data

After you finish recording your last sample, you can now export your data.

Always export your data as:

- **Unmixed fcs files** – Removes all secondary detector channels and only writes the spectrally unmixed/compensated data to the primary detectors for each stain reagent. Smaller files, fewer detectors, easy to analyze. And you can edit the compensation interactions by creating a blank identity matrix, adjusting the values and applying it to your files.
    - Right-click in the selected files or Experiment to export > **Export > Unmixed FCS Files**.

      Note: When exporting data to an FCS file, the exported data will reflect the setting applied in the dropdown control on the Compensation panel. Must select Spectral to proceed and export as unmixed file. The software displays an **Export Unmixed FCS Files** dialog, similar to the following:



- **Experiment** – Always export the experiment where you have created your settings. In case you want to overwrite any single-color and/or redo the unmixing.
    - Right-click in the Experiment to export > **Export > Experiment**.

- **FCS files** - Exports all 48 parameters acquired and writes the raw data + compensation matrix calculated on the instrument. You will keep your raw data and, if needed, use it to recompensate from scratch in FlowJo.
    - Right-click in the selected files or Experiment to export > **Export > FCS Files**.

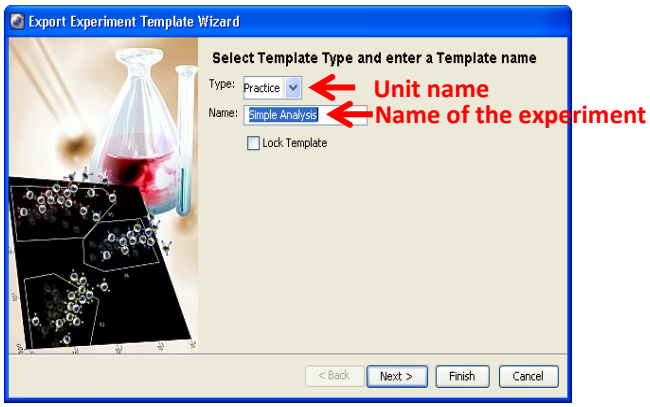
Templates:

- If you have standardized settings - Export your **Panel Template** to the computer. Right click in the **Specimen > Export > Panel Template** >On "Type" select your Lab. Click "**Finish**". Your Panel Template is now saved.

  Templates are stored outside the Browser to simplify the Browser display.

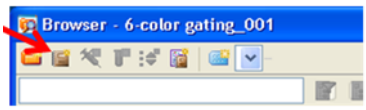
- If you don't have standardized settings - export your **Experiment Template** to the computer. Right click in the **Experiment > Export > Experiment Template** >On "Type" select your Lab. Click "**Finish**". Your Experiment Template is now saved.

  Templates are stored outside the Browser to simplify the Browser display.

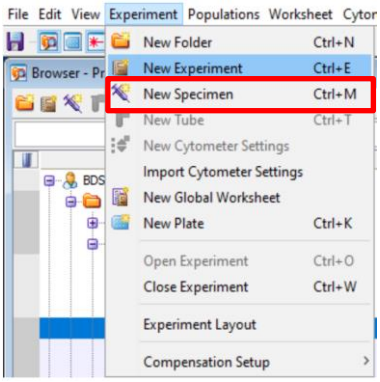


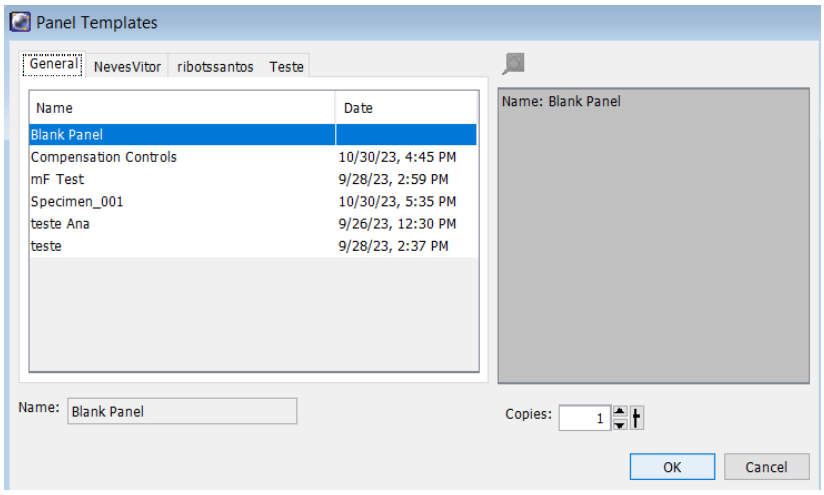
Repeating a spectral experiment

- If you have standardized settings: In the Browser, select a location to create the new experiment. **Open a New experiment**.



From the menu bar, select Experiment > **New Specimen**. The Panel Templates dialog is displayed.

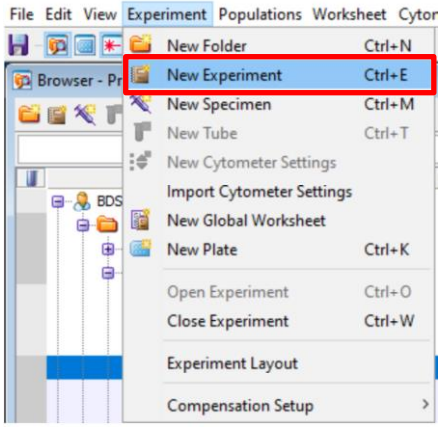


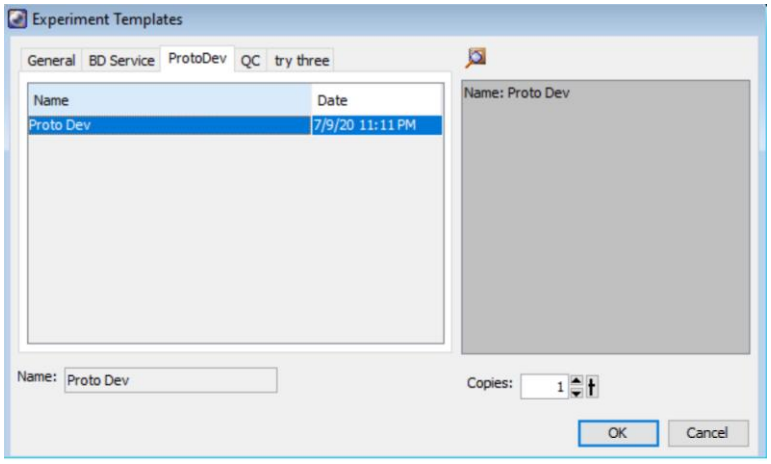


In the tab containing the experiment template that you want to load, select the template and click **OK**. Adjust FSC, SSC and Threshold accordingly. Confirm the parameters and labels are correct for the assay that you want to repeat. Record and analyze the first tube. Copy the recorded tube cytometer settings and paste to the Experiment cytometers settings so that the correct settings are applied to all the experiment and following tubes.

- If you don't have standardized settings: In the Browser, select a location to create the new experiment.

From the menu bar, select Experiment > **New Experiment**. The Experiment Templates dialog is displayed





In the tab containing the experiment template that you want to load, select the template and click **OK**.

Adjust FSC, SSC and Threshold accordingly.

Confirm the parameters and labels are correct for the assay that you want to repeat.

Record and analyze spectral data as described in Recording and analyzing spectral data.