BD FACSDIVA TUTORIAL

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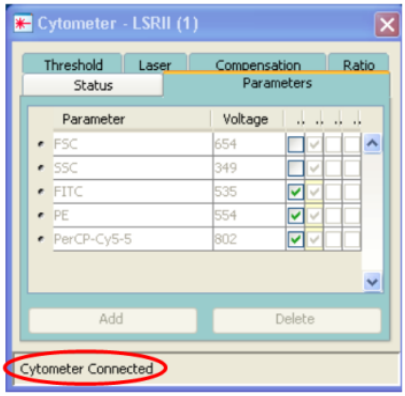
Computer and Software Login



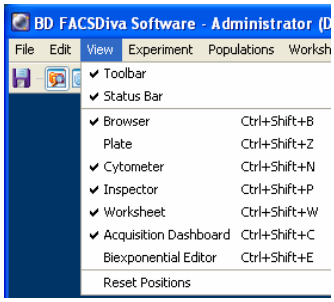
- Login on windows: Username: facsuser
  Password: BDIS
- Start the software: double-click the shortcut icon on the desktop.
- Login on DIVA using your own login name and password.
- Wait for the cytometer connection and click "Use CST Settings".

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.

Setting Up Your Experiment

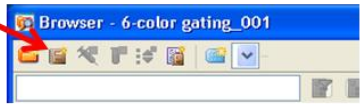
Before you start, make sure that you have all the necessary windows open. Go to the "View" menu and make sure the following boxes are checked:

- Browser
- Cytometer
- Inspector
- Worksheet
- Acquisition Dashboard

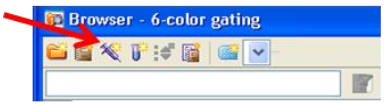


Creating a New Experiment

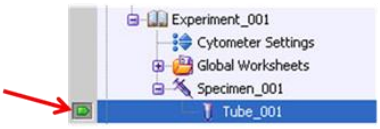
- Click on the "New Experiment" icon in the browser toolbar.
- To create an experiment based on a saved template, choose **Experiment >New Experiment**. The Experiment Templates dialog appears where you can select your unit and the template.



- Click the "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.



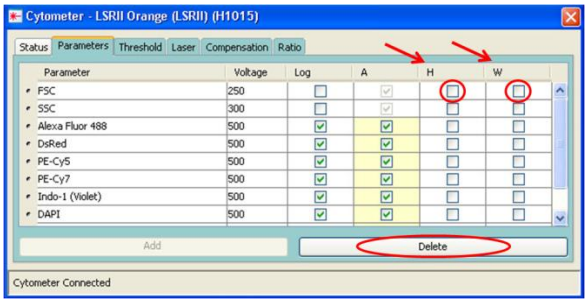
- In the browser, click the icon to the far left of the tube named "Tube_001". The pointer changes to green.



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

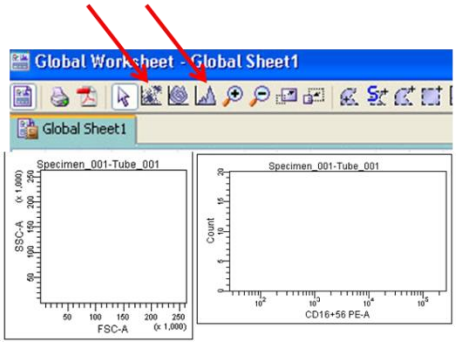
Selecting Parameters

To delete parameters you do not need, click the selection button next to each unneeded parameter. Hold down the Ctrl key to select more than one. When you are finished selecting click Delete. Select H and W for the FSC parameter.



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.



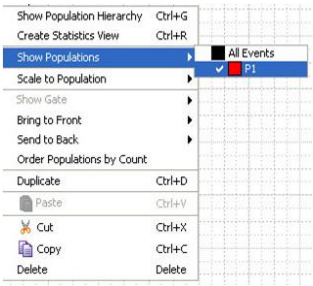
Creating a Gate

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.

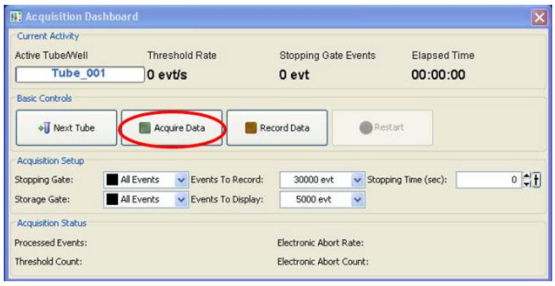
To gate it on the P1 gate, right click inside the FSC-A vs. SSC-A dot plot and in the popup menu select "Show Populations", then "P1".



Setting Up Voltages Based on an Unstained/Negative Control

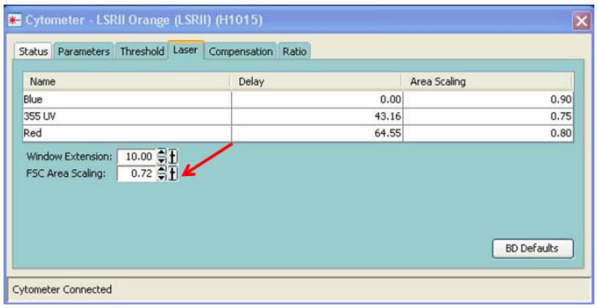
Your first tube should be an unstained control. Make sure that the pointer on the far left from the tube name is activated.

Place the tube on the Sample Injection Port (SIP). Press the "Low" and the "Run" buttons on the instrument. Click the "Acquire Data" button on the "Acquisition Dashboard".



Adjust the voltages so you can see the population of interest in the center of the FSC-A vs. SSC-A dot plot. Adjust the voltages so you are able to see both the ascending and descending slopes of the peak for each fluorescent parameter.

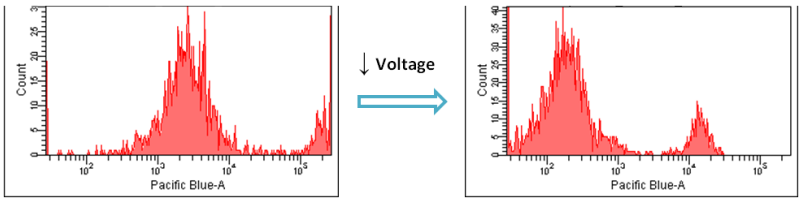
Adjusting Area Scaling for FSC



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.

Checking the Single Color Tubes

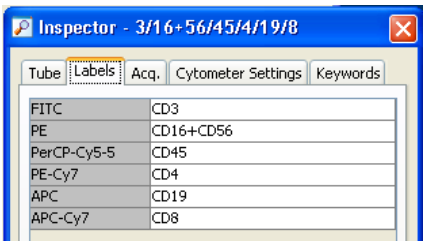
Create a histogram for each single color. Check each one to insure all the data that you are going to collect will fall within the scale. If the positive population appears out of scale you must decresase the parameter voltage. Make sure that the single-stained color tube that you are acquiring is not brighter for other parameter than the one that is on the tube.



Recording your data

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.

If you wish, in the **"Inspector"** window you can check the **"Tube"** tab to name the tube and to view certain keywords and settings saved with recorded data. Use the **"Labels"** tab to enter parameter labels for each fluorochrome. Labels are displayed on plot axes and in statistics views.

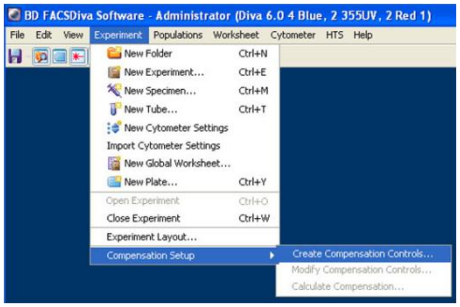


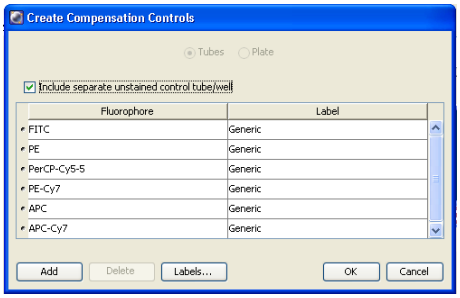
Acquiring Compensation Controls and Calculating Compensation

FACSDiva software has a feature that allows you to calculate compensations automatically. To use this feature all your single color tubes must have a good positive peak (as previous histogram shows). If you don't have nice positive peaks you should calculate the compensation manually.

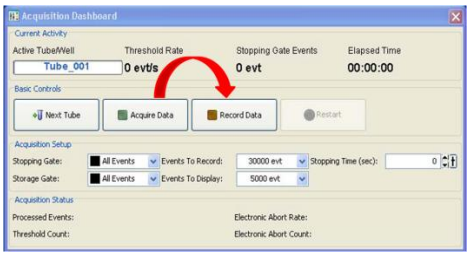
Calculating Compensation Automatically

The Compensation Setup feature in BD FACSDiva software is designed to automatically calculate spectral overlap values for an experiment. Select "**Compensation Setup**" from the "**Experiment**" menu; then select "**Create Compensation Controls**".





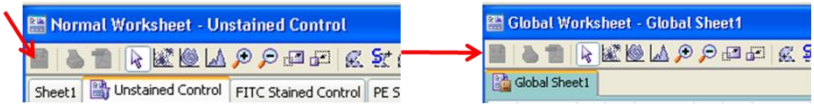
Notice: In the Create Compensation Controls dialog, you can add only parameters that are listed in the Parameters tab. To change to another fluorochrome for any parameter, edit cytometer settings in the Cytometer window or Inspector before you create compensation controls.



Click once on the plus sign (+) next to the **Compensation Controls Specimen** to expand it. Select "**Unstained Control**" by clicking on the icon to the left of the tube name. Place the tube with the unstained sample on the Sample Injection Port (SIP). Click the "**Acquire Data**" button, wait a few seconds until the threshold rate is stable, and then click the "**Record Data**" button on the Acquisition Dashboard. After you are done recording, adjust the P1 gate o include your population of interest. Then you may right click on the P1 gate and select "Apply to All Compensation Controls".

Record data for all tubes in the Compensation Control Specimen. Adjust P1 and P2 (positive events) gates as needed.

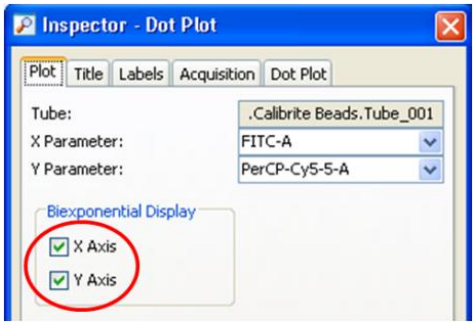
Navigate to **"File" > "Experiment"**, then **"Compensation Setup"**, and then select **"Calculate Compensation"**. The computer will calculate compensation for all parameters. In the window that appears select **"Link & Save.** Switch back to **Global Worksheet** by clicking on the top left icon. Now you are ready to collect data for your samples.



Notice that the pointer on the far left of the tube for which you are about to record the data should be selected.

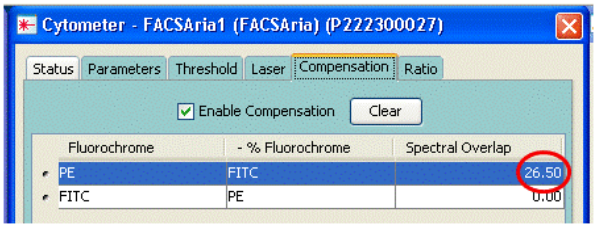
Calculating Compensation Manually

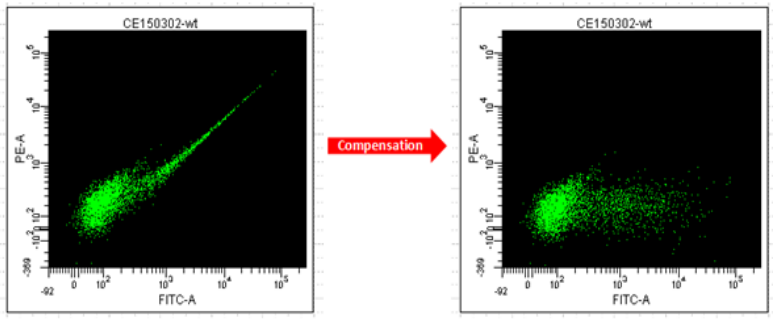
- To determine settings, the means for each fluorescence positive population are compared to the means for its negative population.
- This section describes how to determine spillover coefficients for the FITC fluorochrome. The same procedure is used for the remaining fluorochromes in the experiment.



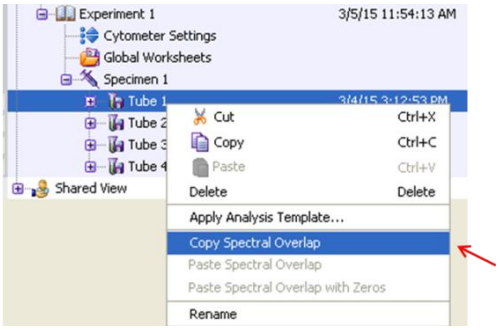
- Create a dot plot and change the plot axes to display the appropriate fluorochromes (e.g. FITC vs PE). Use biexponential display to view populations with negative events: click on the Plot Inspector, select the Biexponential Display checkboxes for the X and Y axes.

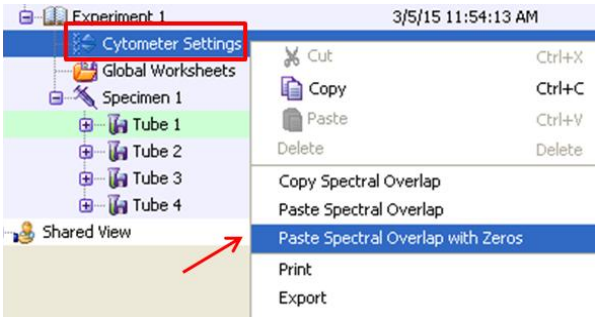
  On the **Cytometer** window select the **Compensation** tab. Increase the spectral overlap value until you see the positive population correctly compensated on your FITC vs PE dot plot.



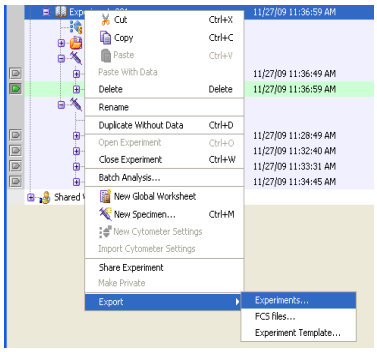


- After you finish compensating you need to apply these compensation values to the settings of your experiment. Right click one of your compensated tubes and select **Copy Spectral Overlap**. Then, right click **Cytometer Settings** (under your experiment title) and click **Paste Spectral Overlap with Zeros**.





Exporting your data



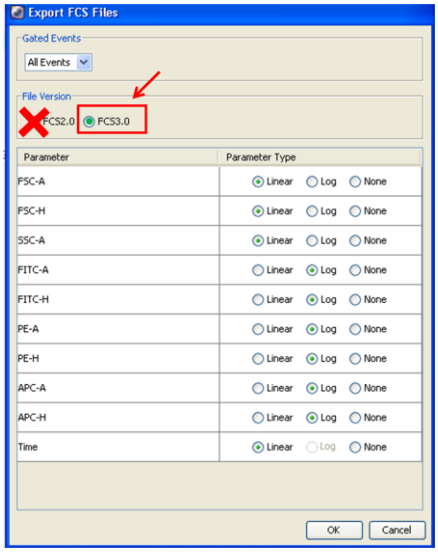
After you finish recording your last sample, you can now export your data. Right click on the name of the experiment you wish to export and select "**Export"**. There are three options: export experiment; export FCS files; or export experiment template.

Experiment

When you use the **"Export experiment"** option FACSDiva will create a folder named after the title of your experiment, and place the .fcs files in there along with the .xml file that contains the map of the experiment. The whole experiment including worksheets, gates, samples and specimens is saved.

Select **"Export" > "Experiment"** and in the window that appears click the **"Browse"** button. Select your destination folder, press OK and then click **"Export"** to save your files.

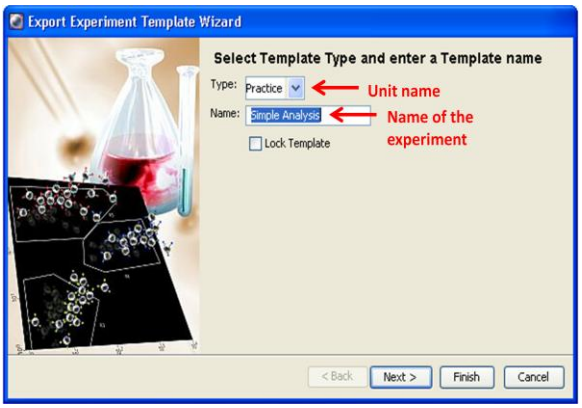
FCS files



Use this option if you plan to analyze your acquired data later using analyzing other software (such as FlowJo). By default, FACSDiva exports the .fcs files into a folder named after the title of your experiment. The compensation, if applied in FACSDiva, is stored in the .fcs files and can be edited or stripped during further data analysis. No information on gating is recorded. When ready to analyze the data, add the files to the FlowJo workspace or create a New Experiment in FACSDiva and use the "import FCS files" option.

Select **"Export" > "FCS files"**. In the window that appears make sure you select the **"FCS 3.0"** option and press OK. You do not need to change any of the other parameters. In the next window click **"Browse"**, select your destination folder and press "**Save"**.

Experiment template



Use this option if you have acquired some data and need to repeat a similar experiment in the future. Experiment templates include specimens, tubes, cytometer settings, labels and worksheets, but do not include the recorded data. Any experiment can be saved as a template.

Select **"Export" > "Experiment Template"**. The **Export Experiment Template Wizard** appears. For the template **type** select your Unit. Click "**Finish**". Your Experiment Template is now saved.

Templates are stored outside the Browser to simplify the Browser display. To open an experiment template you need to create a new experiment using the menu command: click **"File" > "New Experiment"**. The Experiment Template dialog appears. Choose the tab corresponding to your unit and select the desired template. Click OK.