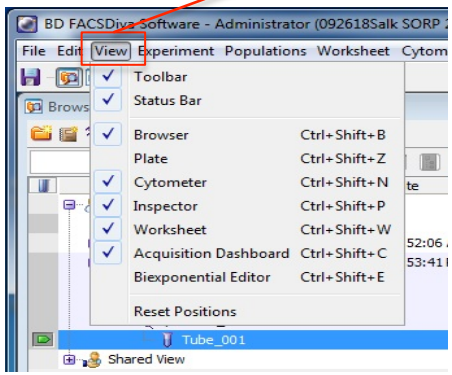
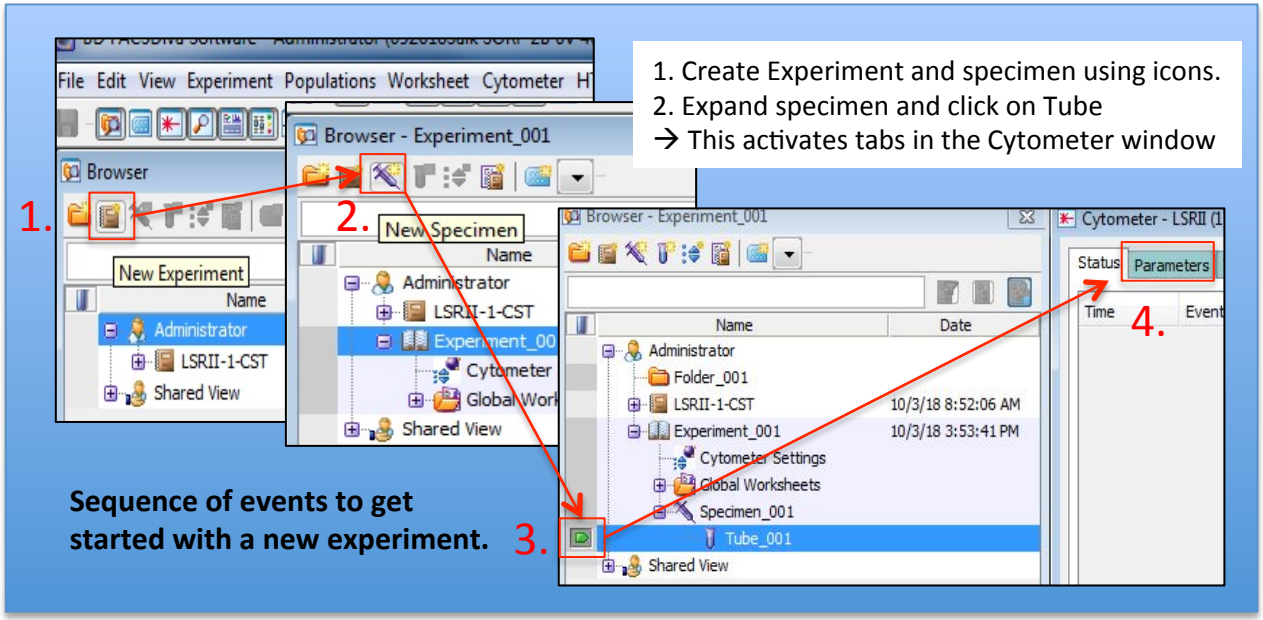
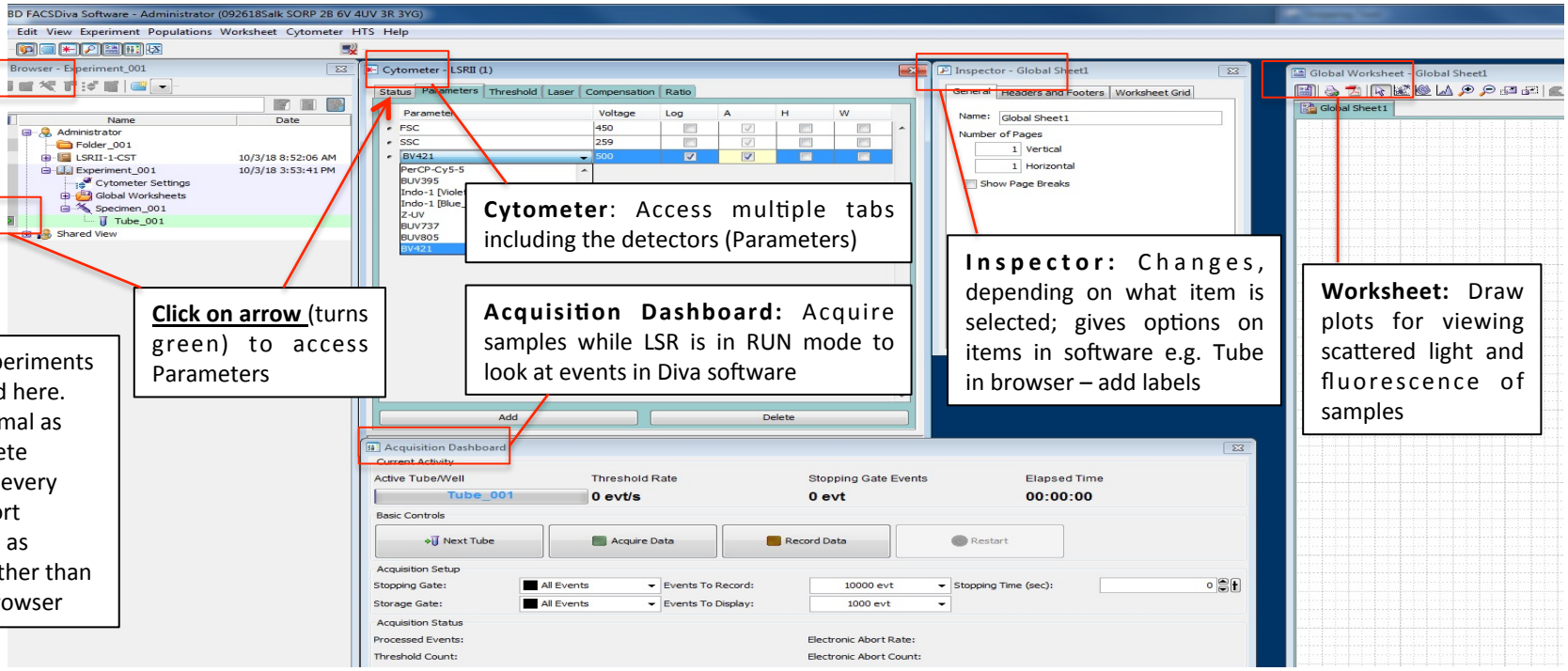
Getting Started

Diva is broken up into windows. These can also be found from the View Menu.

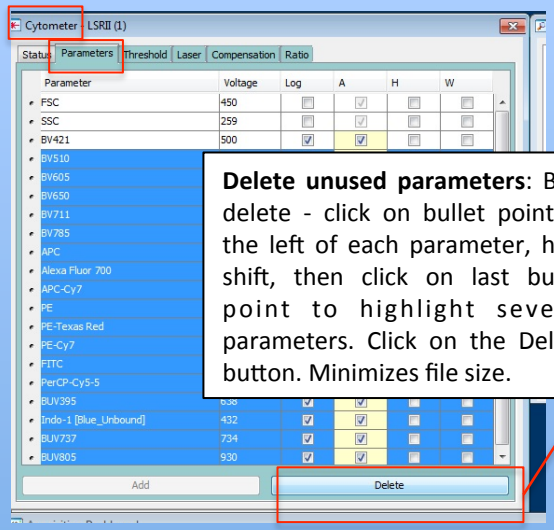




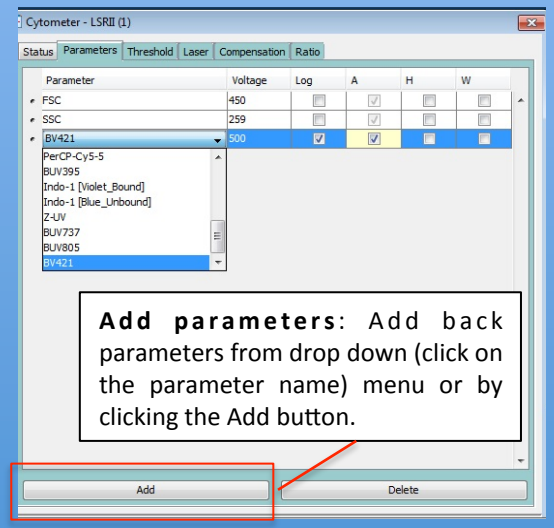


Getting Started

Remove unused parameters and select options such as Width, linear/logarithmic display,

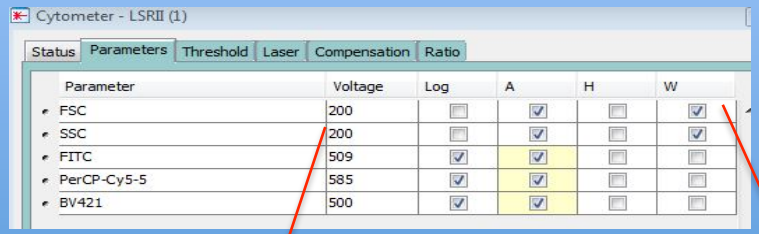


Delete unused parameters: Bulk delete - click on bullet point to the left of each parameter, hold shift, then click on last bullet point to highlight several parameters. Click on the Delete button. Minimizes file size.



Add parameters: Add back parameters from drop down (click on the parameter name) menu or by clicking the Add button.

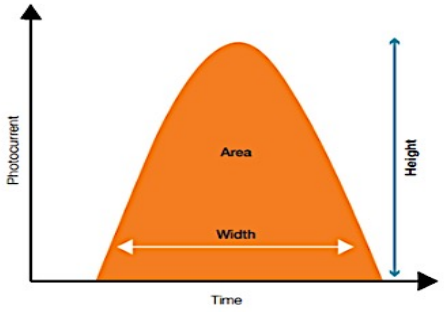
Parameter options include Logarithmic or Linear display (choose linear measurements such as DNA, otherwise leave as logarithmic; begin with FSC and SSC in linear). Measurements are made for every particle for Area (A; total fluorescence), Height (brightest value e.g. fattest part of cell going through laser) and Width (W; analogous to time of flight through lasers).



Cells: Type in 200V for FSC and SSC to start

Exclude aggregates: check the **W** width box for scatter detectors FSC and SSC

Photons are converted to electrical pulses with bell shaped curve. Simplified, it has the shape of a triangle and has the same relationship for Area calculations from the width and height.



Height: The maximum amount of current output by the PMT.

Area: The integral of the pulse.

Width: The time interval during which the pulse occurs.

Signal intensity can be measured by either height or area.

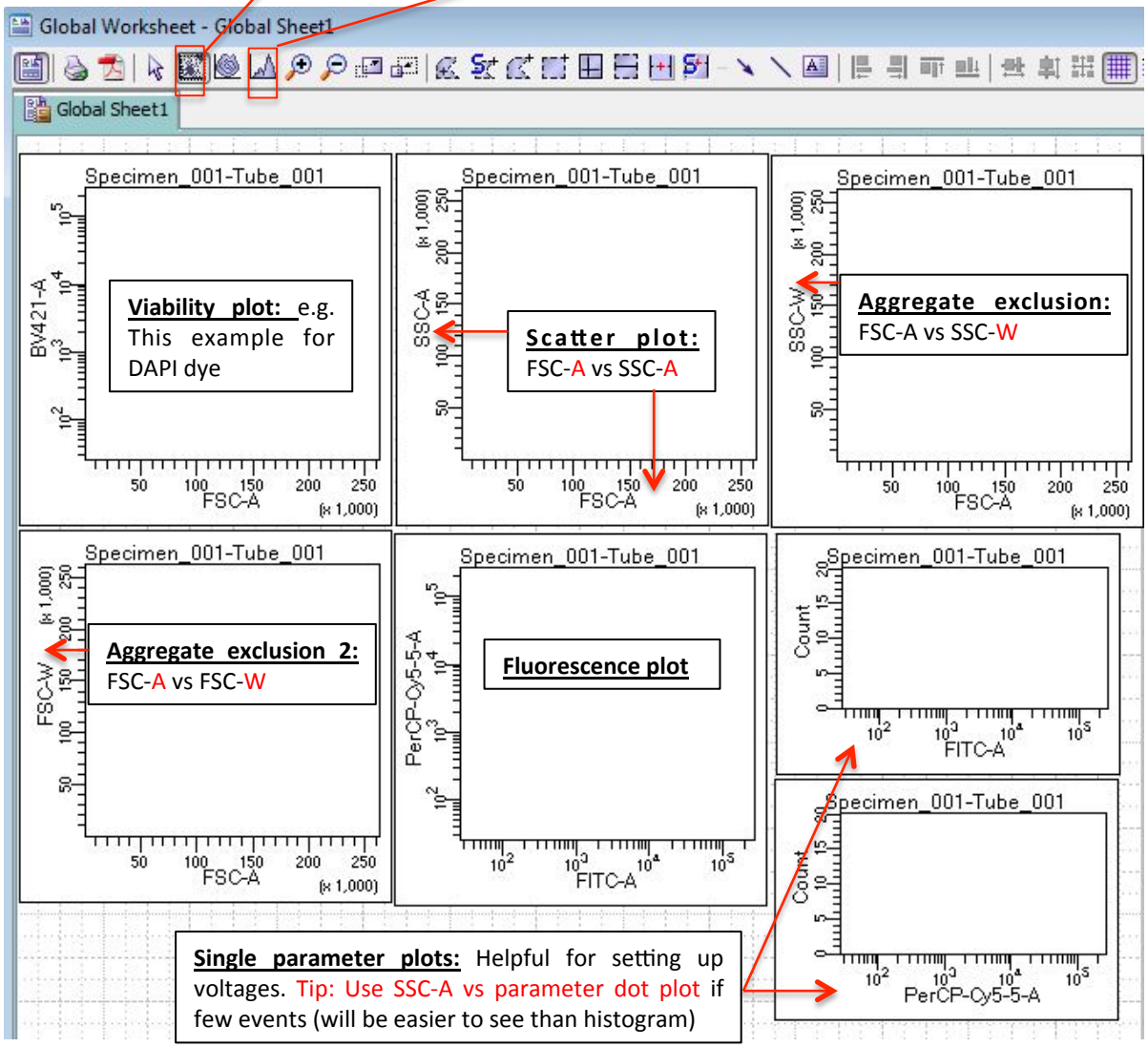
The width parameter measures the **time that the cell spends in the laser.**

Use the Area for the parameter in general, except for excluding aggregates when Width (time of flight) is used to discriminate more than one cell passing through the laser. Use FSC and SSC Width measurements: these are orthogonal to each other; by using two different planes discrimination is more thorough.

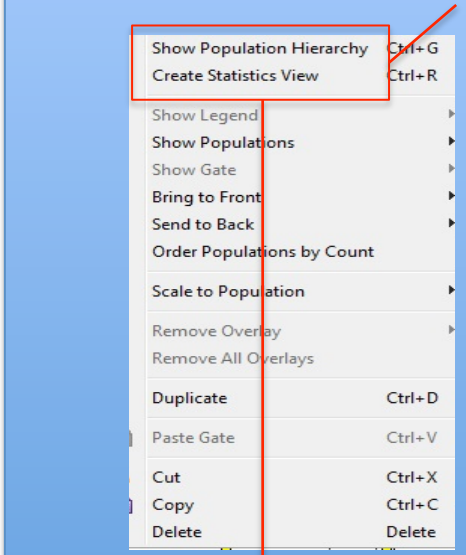
The Worksheet

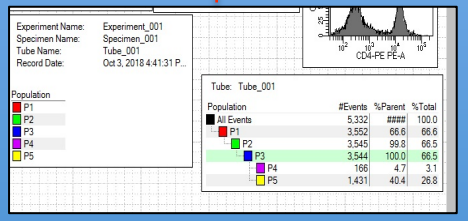
Draw plots using plot icon e.g. dot plot (two parameters) and single parameter histograms

Draw plots: Select plot icon then click once on workspace or drag to create plots of desired size. Create multiple plots quickly by selecting plots (yellow dots appear around plots) → Hold Control, click on edge of a plot and drag to duplicate

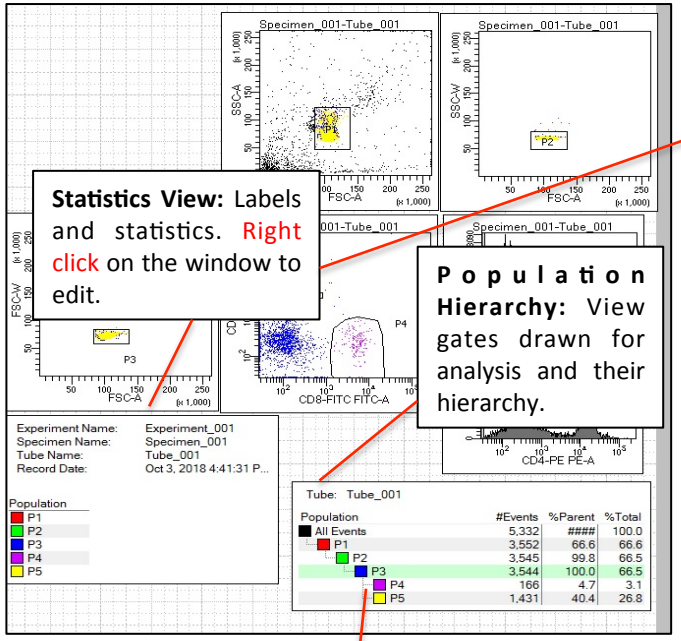


Right click the white space (on the border) of any plot and select Show Population Hierarchy and Create Statistics View. Arrange these on the worksheet





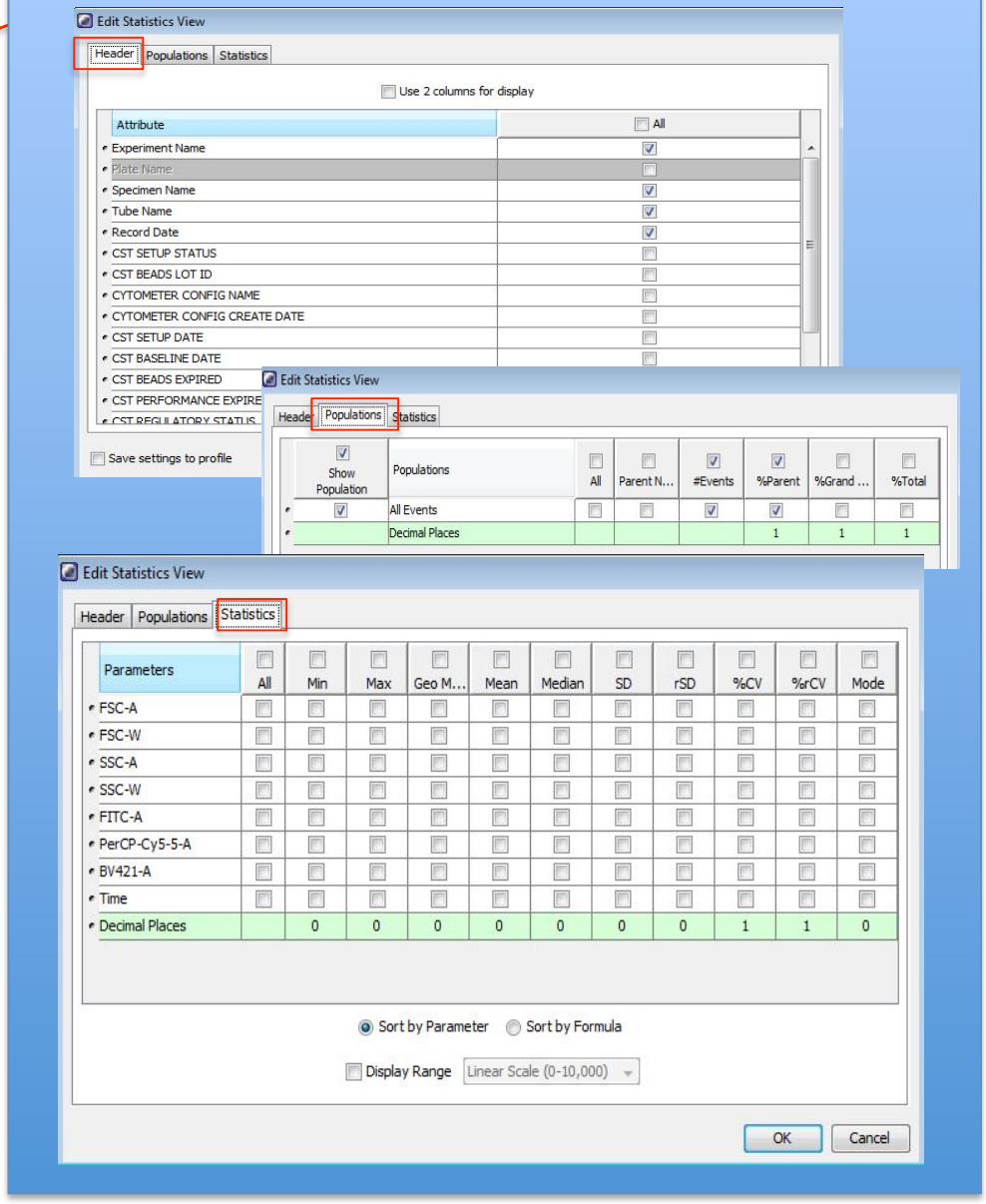
The Population hierarchy and Statistics views



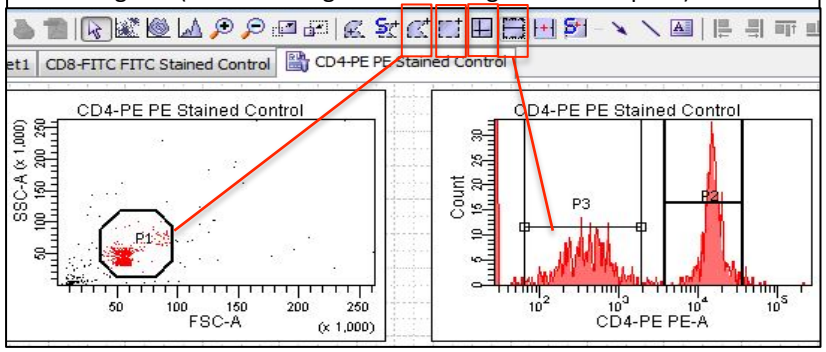
Statistics View: Labels and statistics. Right click on the window to edit.

Population Hierarchy: View gates drawn for analysis and their hierarchy.

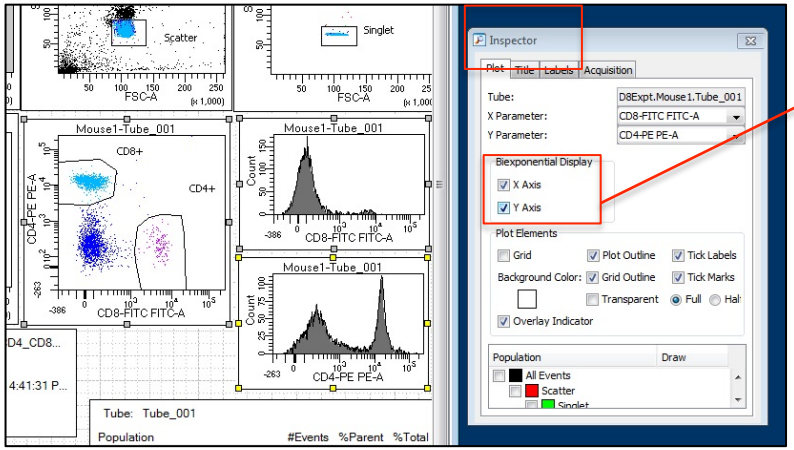
Edit Statistics View: Different tabs with multiple options



Forming the gating (Population) Hierarchy: Regions of interest can be defined with the gating tool icons. After drawing the first region of interest (a "gate"), right click on the next plot and select Show Populations → choose that gate, before drawing the next gate. This will form a hierarchy. **Left to right below:** Polygon (left click to drop "points" and click twice to close the gate), square (click and drag), quadrant (click on the plot center to drop onto plot), and histogram interval gates (click and drag from left to right around a peak)

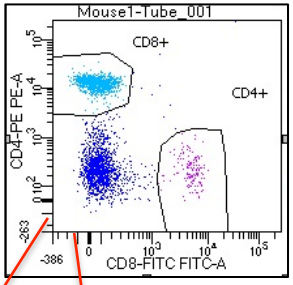


The Biexponential Display



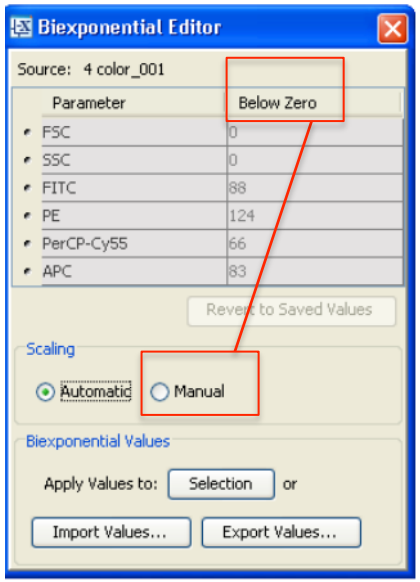
Turn on Biexponential Display for fluorescence plots: This allows events on the axes to be viewed (adds channels below zero). This is purely a display function and is helpful for viewing the whole population. **Click on plots (hold shift to select several) → in the inspector, check the Biexponential Display box for x and y axis.**

Biexponential Editor



Each parameter can be adjusted

Visualization tool for adjusting amount of extra channels around axes : Open from View Menu. More channels = more "white space". Can be adjusted for each parameter axes to aid display, if data not appropriately scaled by Diva. Set Scaling to Manual and add desired # channels to suit sample display.



The Acquisition Dashboard

Next Tube: Create new empty tube for recording in Browser. No need to record or create new empty tubes when setting up, recommend using one tube (OK to swap and run different samples to make voltage adjustments).

Stopping Time: Indicate regions of interest as stopping criteria once gates are drawn on plots

Acquire: Start showing data from tube if instrument is in RUN mode. Do not need to stop acquiring to change tubes when setting up

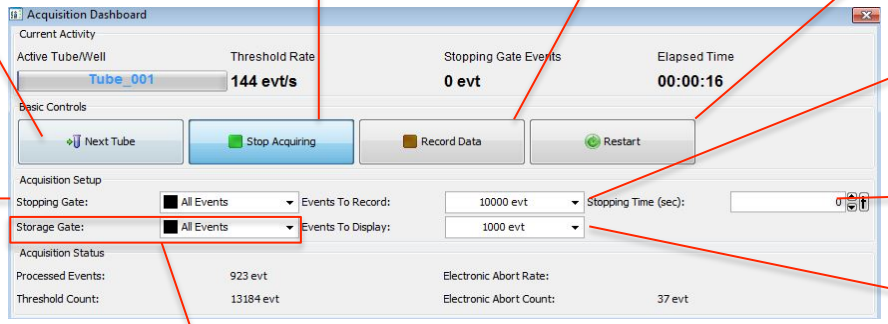
Record data: Will stop according to designated collection criteria below

Record data: Refreshes view (event accumulation) in software

Stopping Time: Specify # events to record of stopping gate (at least 10K relevant events, or at least 100 for "rare event" analyses.

Stopping Time: Set time as a stopping criteria

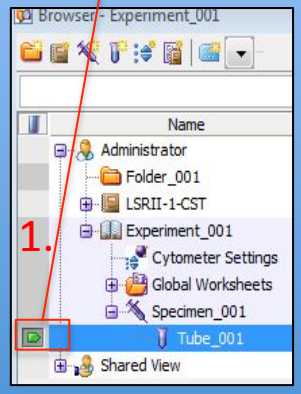
Events to Display: Less = faster refresh rate on software. Set low when recording for faster processing



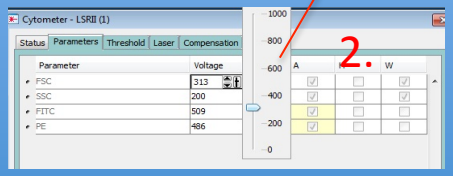
Storage Gate: DO NOT CHANGE THIS ONE BY ACCIDENT

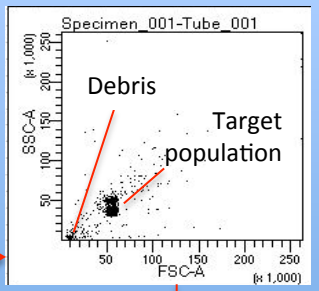
Setting up detector voltages:

Place cytometer in RUN, the select an empty tube in the browser (click on the arrow). Click Acquire on the Acquisition Dashboard. Arrow should turn green. Events should populate the plots.

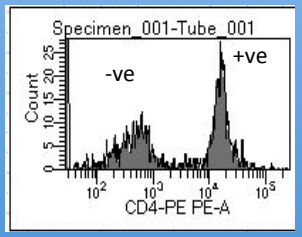


Adjust the FSC and SSC detector voltage to bring samples on scale into the middle of the plot (look at plots and make changes with the slider, typing, or in increments of 10 units by holding Control and using the arrows.





FSC and SSC voltages should place events on scale into the middle of the plot (look at plots and make changes with the slider, typing, or in increments of 10 units by holding Control and using the arrows. Place populations in an appropriate position on the plot where a gate can be drawn to discriminate from debris.



3.

Check unstained and also labeled single color samples: The order is up to you however you will need to ensure brightest samples are on scale (i.e. if accumulating on the top end of the plot to reduce voltage) and that when comparing to the negative they are indeed positively labeled.

Setting up fluorescence voltages:

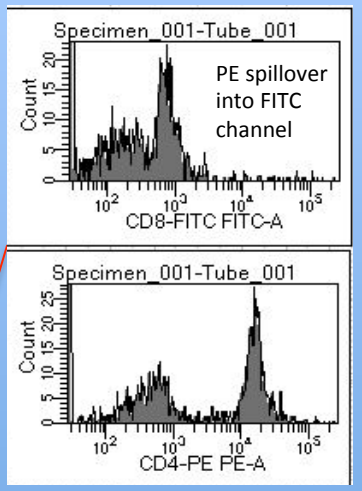
If using more than one fluor, compensation corrections for fluorescence spill-over may be needed to account for photons from fluors that overlap spectrally and are picked up by detectors other than their own.

This can be calculated if single stained controls are recorded, and can be stained cells, or compensation beads (antibody capture beads). Negative events are needed as well as a bright population at least as bright as the sample. Excessively bright controls that cannot be run at the ideal sample voltage may compromise sample resolution (e.g. for antibodies these should be titrated lower).

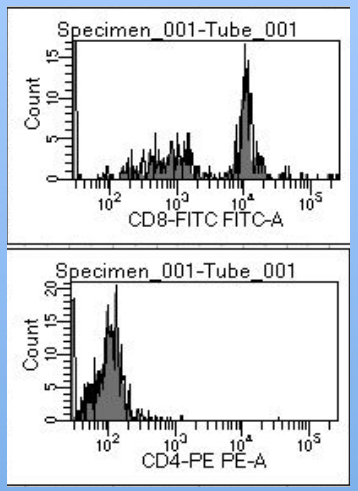
This can be calculated if single stained controls are recorded, and can be stained cells, or compensation beads (antibody capture beads), or a mixture. Negative events are needed as well as a bright population at least as bright as the sample. Excessively bright controls that cannot be run at the ideal sample voltage may compromise sample resolution (e.g. for antibodies these need to be titrated lower, or bring several concentrations in the first run).

1. **Check stains and adjust detector voltages:** Check each single color control ensuring that the positive population is brightest in it's own channel. Adjust detector voltages if needed. Increase voltage, or decrease spill over channel voltage (checking that the spillover channel single stain has sufficient resolution if large changes are made).

PE single stain (uncompensated)



FITC single stain (uncompensated)

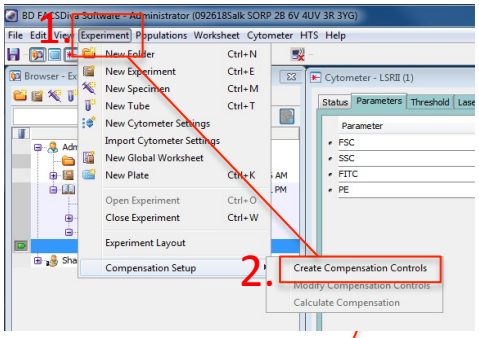


In this example the PE single stain is significantly brighter in PE channel than FITC and there is minimal FITC to PE spillover (this is tied to the instrument configuration and optics); some fluors will be very close and need to be checked carefully.

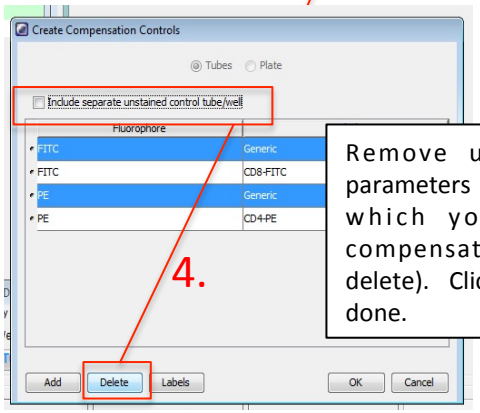
2. **Diva software compensation:** Once all single stains are acceptable, move on to calculating software compensation.

Calculating compensation

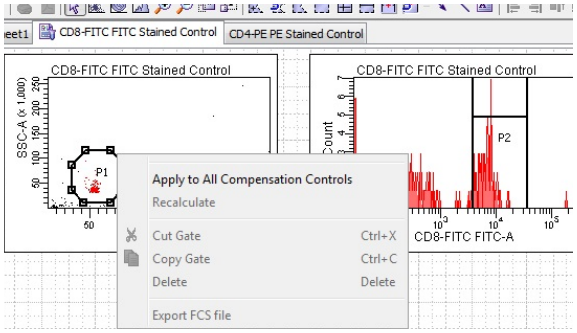
From the Experiment Menu→
Create Compensation Controls



3. Indicate if you are running a universal unstained tube

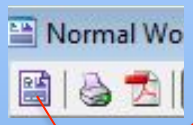


4. Remove unnecessary parameters or those for which you are not compensating (select, delete). Click OK when done.

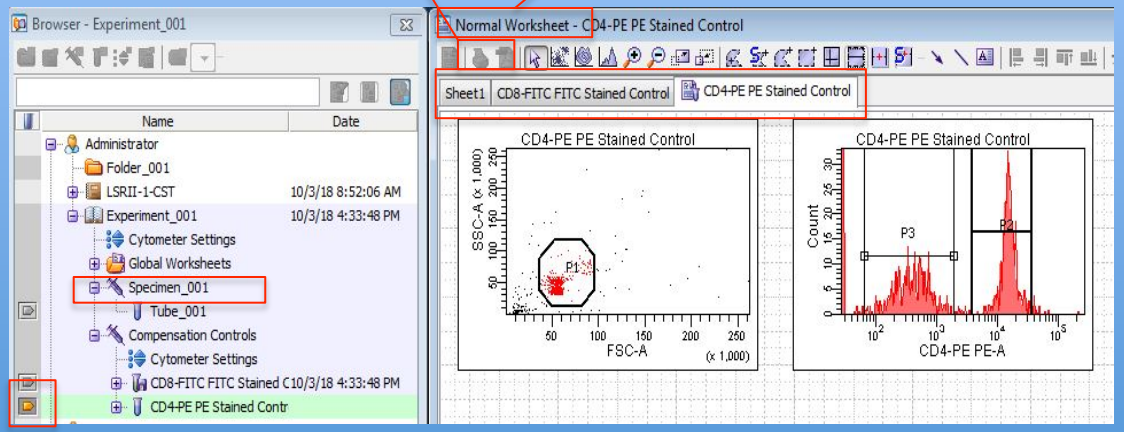


5. A separate specimen named Compensation Controls will be created. The worksheet will switch to a tube specific Global View

Icon to toggle between Global vs Normal. TOP LEFT of Worksheet

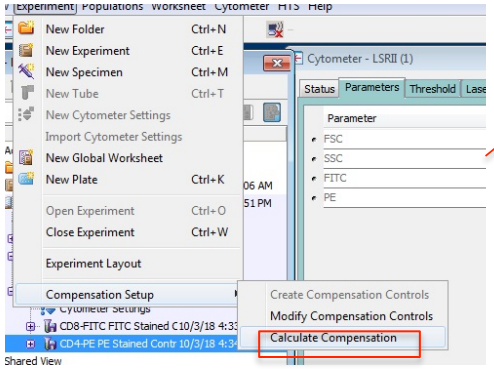


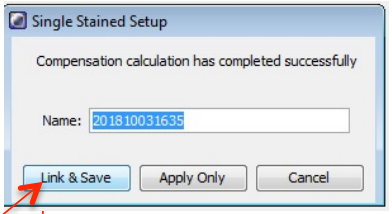
Worksheet type: Global vs Normal (tube specific).



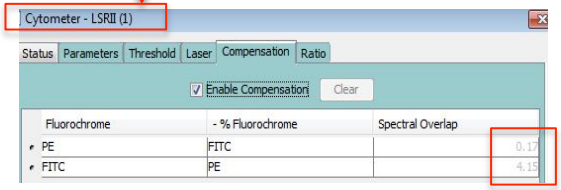
Record each single color into the appropriate tube in the Browser

6. From the Experiment Menu→ Calculate Compensation. Link and Select Link and Save





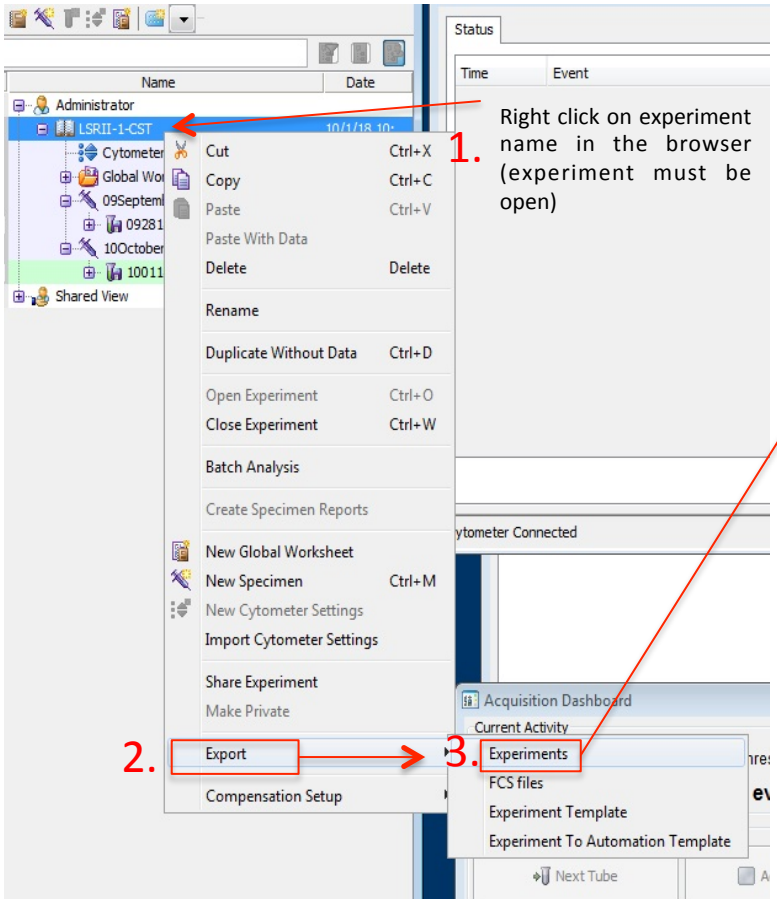
7. Compensation tab: compensation values (Cytometer window), will be present for tubes in Browser.



Exporting data and template (e.g. to back up to Filer server) *2 step process:

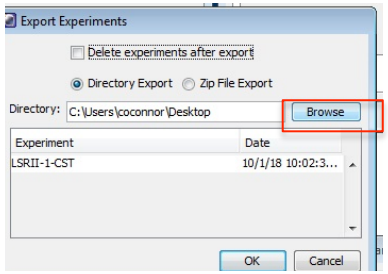
1. Choose Export → Experiments

Export Experiments = data (.fcs files) as well as entire experiment template (.xml file)

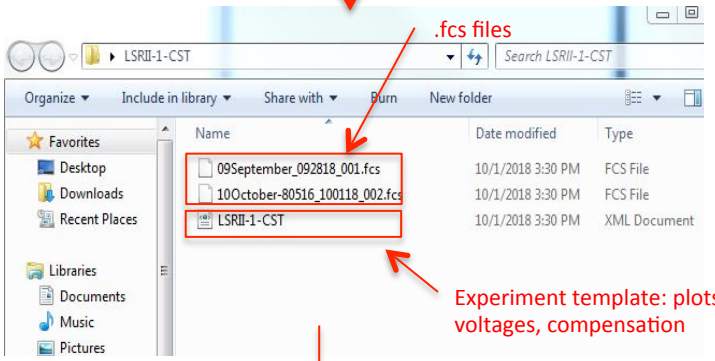


Right click on experiment name in the browser (experiment must be open)

4. Click Browse button to select designation (recommend Desktop for large file transfers followed by Cut and Paste to your Filer share)



Exported Experiment folder contains template as well as .fcs files



5. Repeat export process and choose to export FCS files instead of Experiment. Browse and choose the desktop → This will locate the folder already created in the Export Experiment step. Choose to "replace" the FCS files : This will overwrite the FCS files with ones which can be read properly in Flowjo.

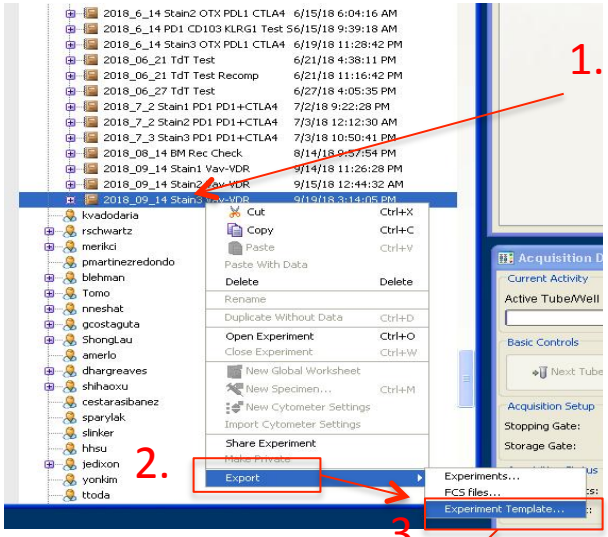
6. Cut and paste your Experiment folder (has the template and the correctly formatted FCS files) from desktop to Filer if needed. Mapping to your Lab Share on Filer server: Right click on My Computer→ Select Map Network drive and type in the path:

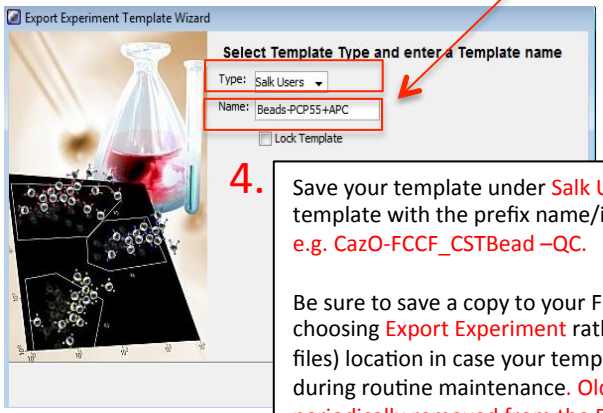
\\filer.ad.salk.edu\lab code → e.g. \\filer.ad.salk.edu\fccf

Exporting Experiment template for use in Diva:

Choose Export → Experiment Template

Templates contain full experiment without data: plots, voltages, compensation - templates are accessed from the Experiment menu



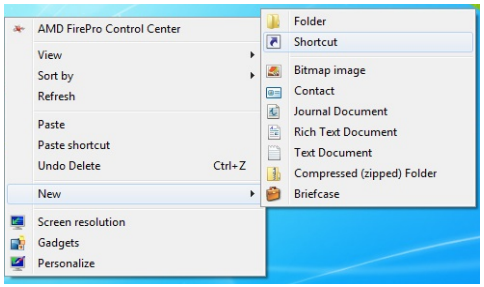


Save your template under Salk Users and label your template with the prefix name/initials and lab prefix: e.g. CazO-FCCF_CSTBead –QC.

Be sure to save a copy to your Filer server (e.g. choosing Export Experiment rather than Export FCS files) location in case your template is removed during routine maintenance. Old templates will be periodically removed from the D drive.

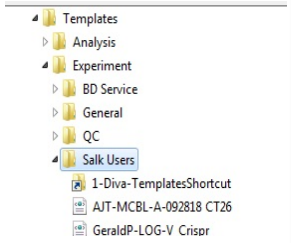
Tip: Create a shortcut on your desktop to the D drive "Salk Users" folder to easily add templates from Filer – ask core staff if needed

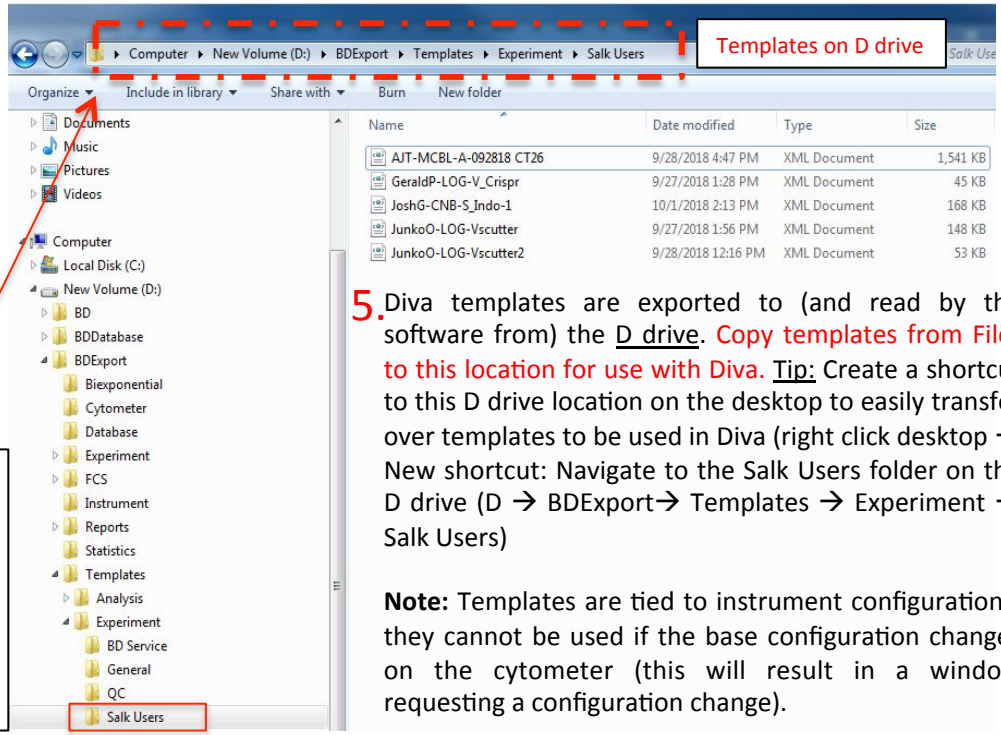
A. Right click→ Shortcut



C. Label shortcut eg " Templates Shortcut" and select Finish

B. Browse to the Salk User folder in the D Drive BD Export folder





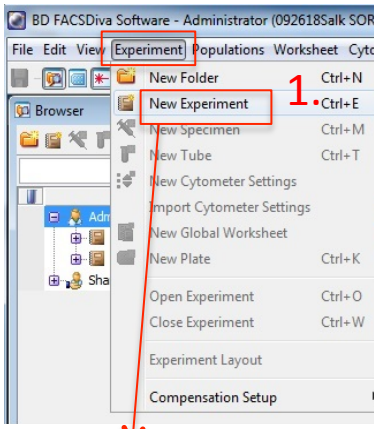
5. Diva templates are exported to (and read by the software from) the D drive. Copy templates from Filer to this location for use with Diva. Tip: Create a shortcut to this D drive location on the desktop to easily transfer over templates to be used in Diva (right click desktop → New shortcut: Navigate to the Salk Users folder on the D drive (D → BDExport→ Templates → Experiment → Salk Users)

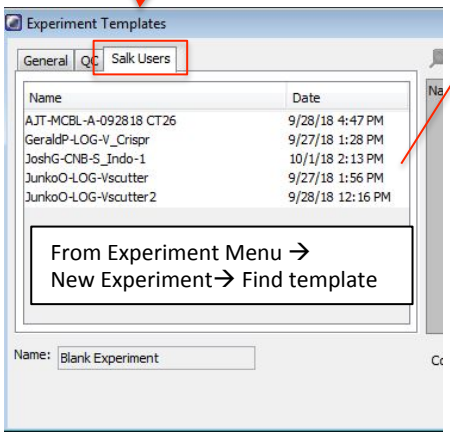
Note: Templates are tied to instrument configurations; they cannot be used if the base configuration changes on the cytometer (this will result in a window requesting a configuration change).

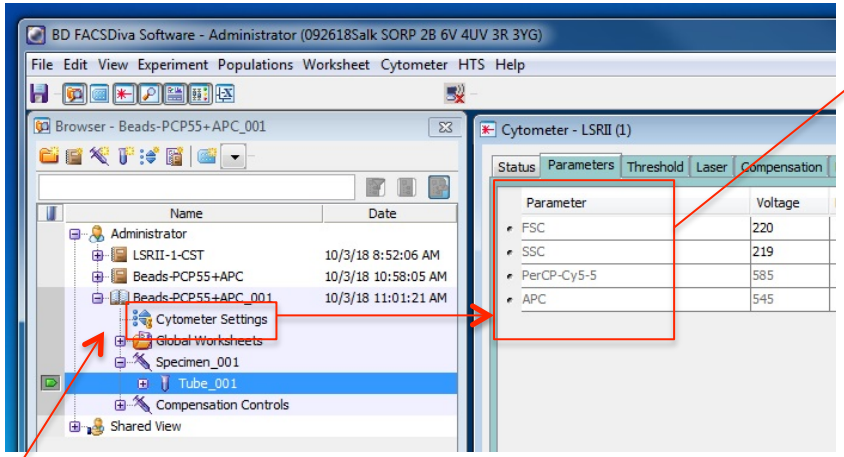
Using Experiment templates:

Experiment Menu → Select template → Empty experiment loads into browser

Changes can be made if template is "unlinked" first (this does not delete compensation), e.g. parameters can be added, or voltages adjusted . Note: Compensation controls (calculate new comp) may need to be run if changes are made.

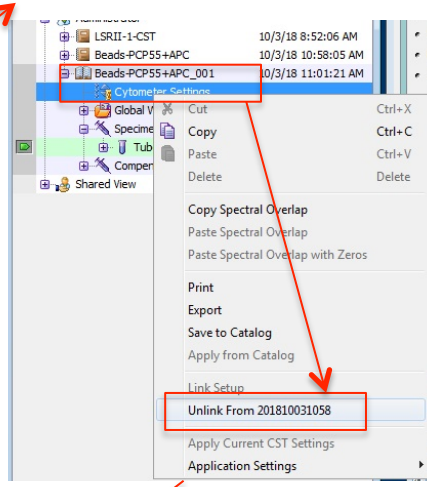


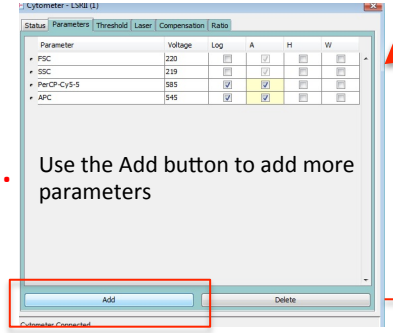




2. Empty experiment will load into browser. Yellow icon on Cytometer Settings means settings cannot be adjusted ("linked" to a catalog). Detector voltages will be greyed out.

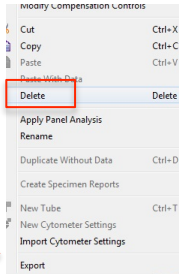
3. To enable changes (then re-run comps), unlink settings: Right click on Experiment and Select Unlink





4. Use the Add button to add more parameters

5. Delete the compensation controls specimen, and create new ones to record files calculate new compensation

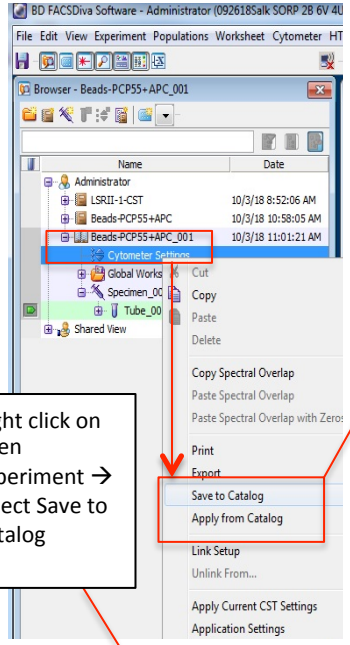


Saving/applying settings from an experiment to a catalog (compensation and voltages):

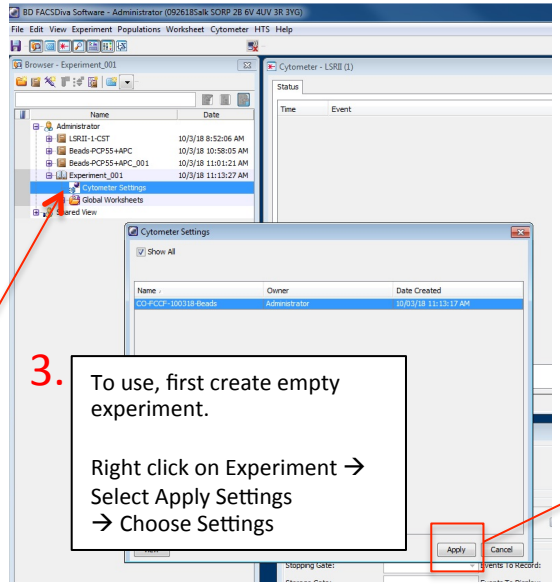
Open experiment → Right click on experiment in Browser→ Select Save to Catalog

Save settings with prefix: Name, Lab Code, Date → e.g. CO-FCCF-100218-Beads
This will allow the template to be backed up to a Lab Share if needed.

1.



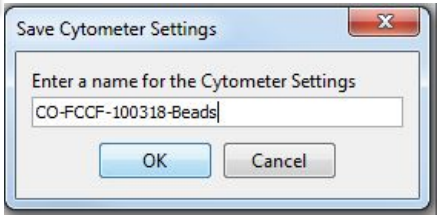
Right click on open Experiment → Select Save to Catalog

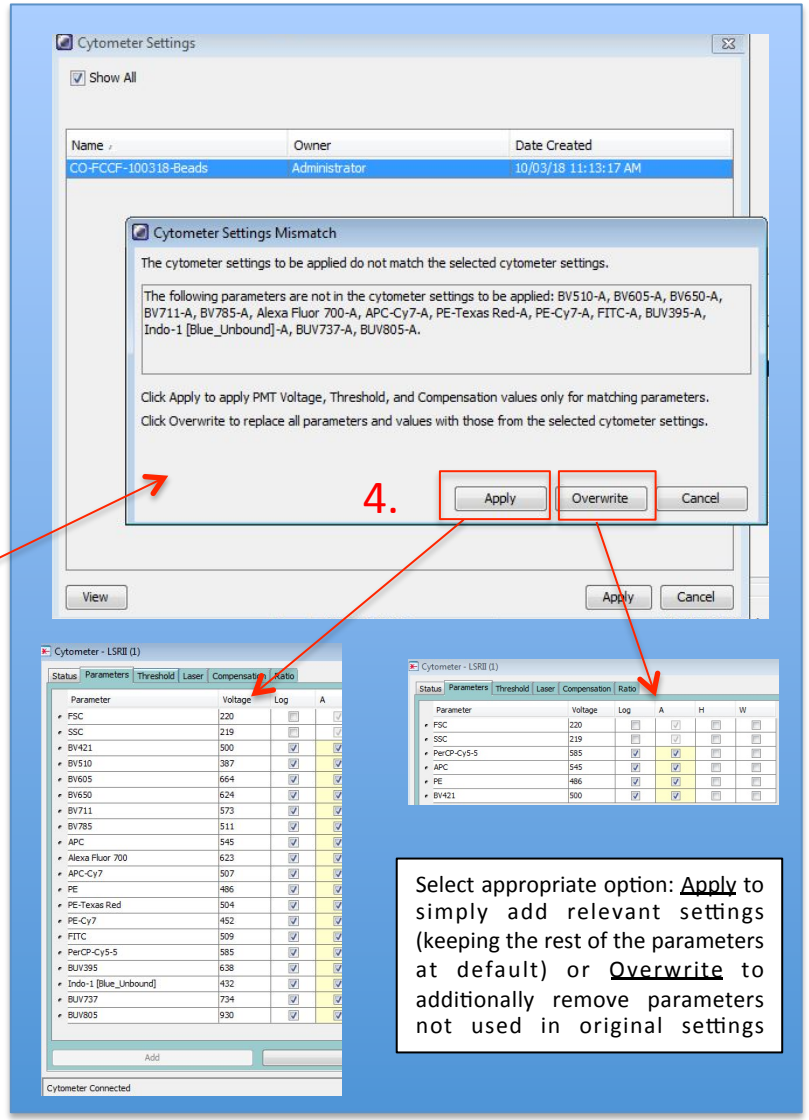


3. To use, first create empty experiment.

Right click on Experiment → Select Apply Settings → Choose Settings

2. Label Settings with Name, Lab, Date etc. (in case we need to back it up for you





Select appropriate option: Apply to simply add relevant settings (keeping the rest of the parameters at default) or Overwrite to additionally remove parameters not used in original settings