Fortessa X-20 training

Instrument Start up

Verify that there is sufficient sheath fluid and that the waste is empty.

Turn on the computer and open the **Tera Term** program (select serial connection for COM1 and say OK) 

Warm up the instrument by turning on the green button located on the right side of the instrument (15 min). The FASC flow supply cart will start automatically.

> **Note: Look to see if the cytometers IP address shows in the Tera Term window**
> **If there is no IP address then hit the enter key and type in the "@" symbol at the prompt and press enter to reboot the cytometer firmware.**
> **If you do not see "[VxWorks]" but an "→" instead, type in the word "reboot" and hit enter to reboot the cytometer.**

Verify that the single tube mode is selected (black toggle switch – right side).

Look to see that there is ~1L buffer in sheath buffer chamber.

Check for bubbles in the sheath filter/lines and that the FACS flow supply cart is on. Bubbles from the sheath filter can be removed by releasing the roller clamp and dispensing some sheath into a beaker while tapping the filter.

- Note: If the alarm on the FACSFlow supply cart indicates low sheath in the middle of an experiment a new sheath container will have to be installed. Ensure the sheath buffer tank on the right side of the cytometer has not run dry. If it has refill it with 1L of sheath and hit the restart button on the FACSFlow supply cart (left of green power button).

Prime the flow cell to remove bubbles. Move the tube arm to one side and remove tube. Then select prime. The system will return to standby once the priming is complete. Repeat priming procedure. After priming return the tube containing 1ml of DI water and put the arm back into place.

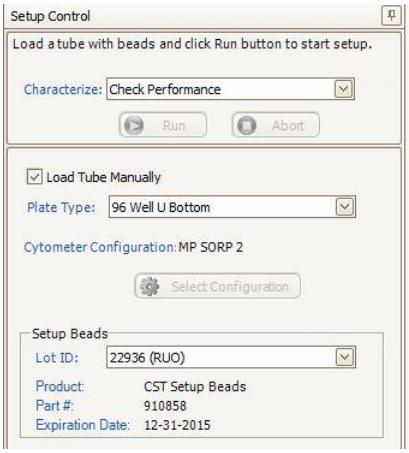
- Note: Debris and bubbles in the flow cell can be indicated by excessive noise while looking at scatter.

Select the low pressure setting and ensure that the fine adjust knob is in mid position (5 turns out from stop).

Turn on the workstation and launch FACS Diva 8. Check status at the bottom right of the screen to see whether the cytometer has connected to the workstation. This will take a few minutes. If it doesn't connect after 5-10 minutes then restart computer and try again. If that fails see *Tera Term procedure* – shut down and relaunch FACS DIVA

Running CS&T - CS&T creates cytometer settings that place beads at MFI target values established from the baseline (Baseline defined by administrator)

Open CS&T under cytometer tab. Under the Setup tab you will see



Ensure configuration is MP SORP 2 (default) and check performance is selected.

Select load tube manually checkbox and appropriate current CS&T lot ID.

Shake the CS&T vial - add 1 drop in 350ml of sheath to the tube - vortex - load tube on cytometer

Verify that the sample flow rate is set to LO and that fine adjust is at mid-point 5 turns from stop).

Change from standby to run on the cytometer and click run.

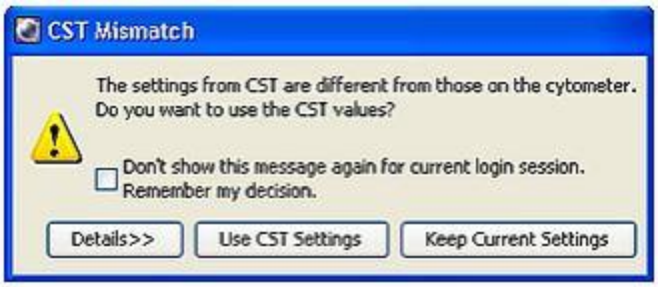
CS&T should run without warnings but if warnings appear try priming the flow cell and rerunning CS&T. You can also clean the flow cell by running bleach and water for 5 min each.

For the report the important thing to look at is the “Bright bead robust CV” for Blue B, Red C, violet F, UV B , YG E detectors which should be less than 6%.

Laser	Detector	Parameter	Target Value	Actual Target Value	% Difference Target Value	Bright Bead % Robust CV	Mid Bead Median Channel	Mid Bead % Robust CV
Blue	FSC	FSC	125000	125447	0	5.96	125474	5.97
Blue	C	SSC	125000	125657	0	3.71	126382	3.64
Blue	B	Alexa Fluor 488	9183	8829	-4	3.8	212	10.32
Blue	A	PerCP-Cy5-5	21931	21406	-3	6.11	664	11.48
Red	C	Alexa Fluor 647	34859	34306	-2	3.55	2145	10.38
Red	B	APC-Alexa 700	26210	25613	-3	3.55	938	7.07
Red	A	APC-Cy7	27868	27387	-2	3.93	914	8.21
Violet	F	BV421	6778	6634	-3	4.1	669	9.51
Violet	E	BV 510	29143	28535	-3	3.43	669	9.51
Violet	D	BV 605	41527	41558	0	4.86	956	28.81
Violet	C	BV 650	40244	40481	0	4.35	1860	13.56
Violet	B	BV 711	18716	18548	-1	5.07	730	12.41
Violet	A	BV785	39315	37704	-5	6.26	848	14.87
UV	B	DAPI	13369	12962	-4	3.74	1481	5.9
UV	A	SIDE POP	30390	29925	-2	9.21	1665	34.53
YG	E	DsRed	23960	23269	-3	3.33	572	15.52
YG	D	PE-CF594	29796	29181	-3	3.5	662	17.14
YG	C	7-AAD	26803	26082	-3	4.06	938	18.35
YG	B	PE-Alexa 700	36347	34974	-4	4.53	1263	12.12
YG	A	PE-Cy7	51902	49942	-4	6.08	1529	13.32

Closing CS&T will re-launch FACSDiva

If a CS&T mismatch dialogue box appears the default will be <use CS&T settings> to update to the new CS&T settings. Do not select <keep current settings> unless you want to keep the expired CS&T settings or you have created a custom settings profile.



Open BD Coherent software 

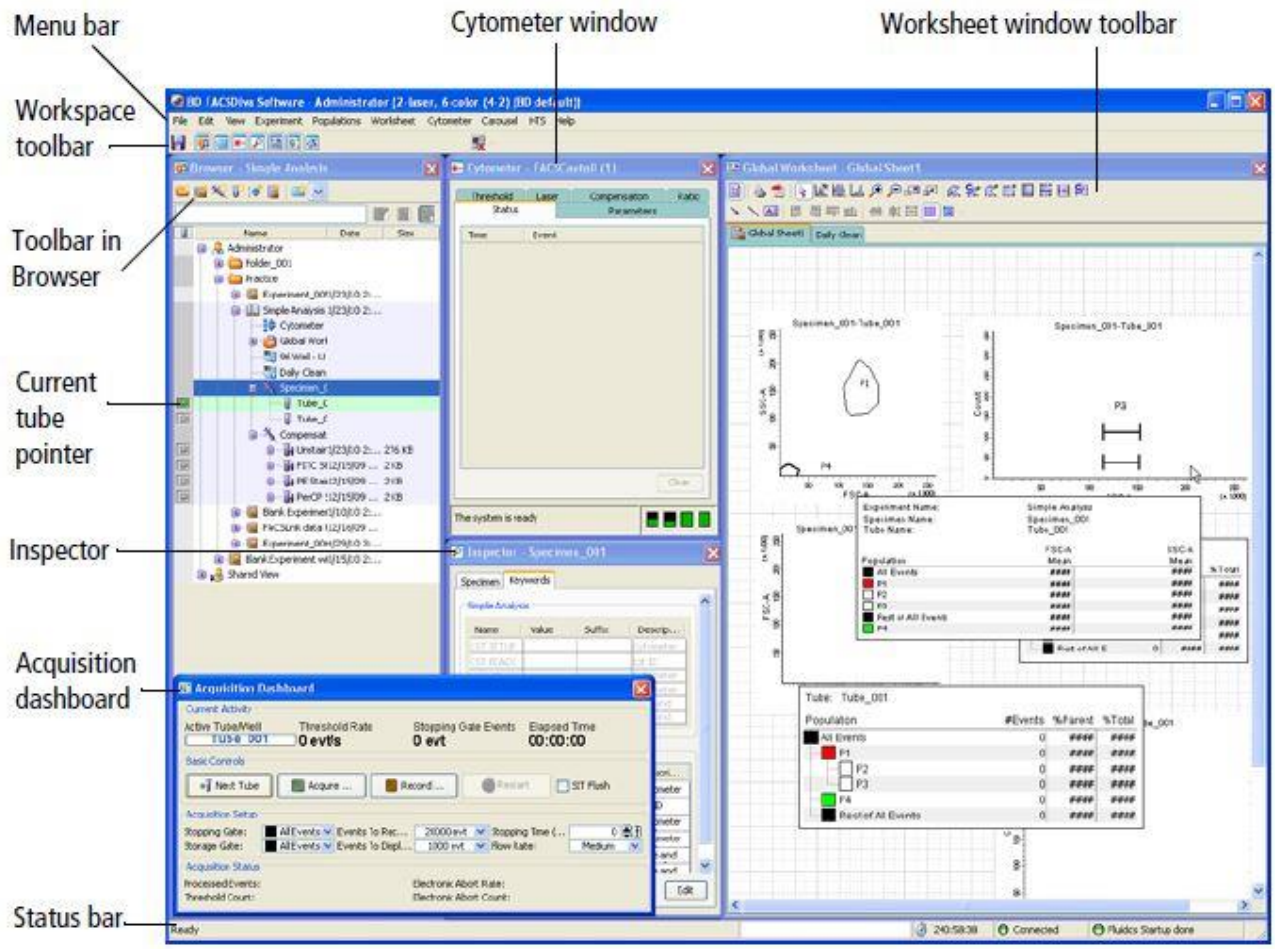
This will allow you to turn off lasers not in use. Lasers have a defined lifespan and are very expensive. Turning them off while not in use will dramatically reduce expenses!

To turn the lasers off simply click on the stop button for the respective laser. In this example the UV and violet lasers have been turned off (red dial at zero instead of 100%)

You can also use this software if your signal is off scale. Simply reduce laser power as necessary to put the signal on scale.

Getting started

Workspace



Workspace Toolbar

Icon	Button Name	Description
	Save	Saves the current experiment to the database. Experiments are also saved when you close an experiment or quit the software.
	Browser	Hides or shows the Browser. See Browser on page 43.

	Plate	Hides or shows the Plate window. This button appears only if your cytometer is compatible with the BD™ High Throughput Sampler (HTS) option.
	Cytometer	Hides or shows the Cytometer window. See Cytometer Controls on page 108.
	Inspector	Hides or shows the Inspector. See Inspector on page 42.
	Worksheet	Hides or shows the Worksheet window. See Worksheets on page 156.
	Acquisition Controls	Hides or shows the Acquisition Dashboard. See Acquisition Dashboard on page 117.
	Biexponential Editor	Hides or shows the Biexponential Editor. See Working with the Biexponential Editor on page 203.

- To see the cytometer controls for changing PMT voltages there needs to be an open experiment and tube selected
- To adjust the workspace to use the second monitor select the minimize button at the top right and then drake the DIVA workspace to cover the second monitor. Then drag the worksheet and cytometer settings over to the right monitor.

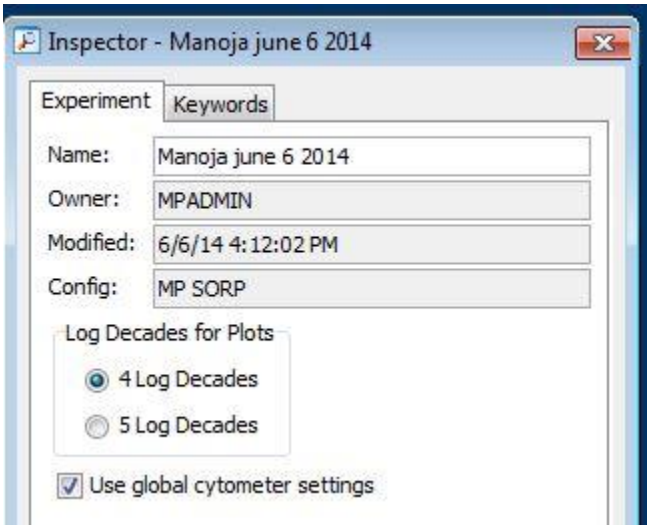
Browser

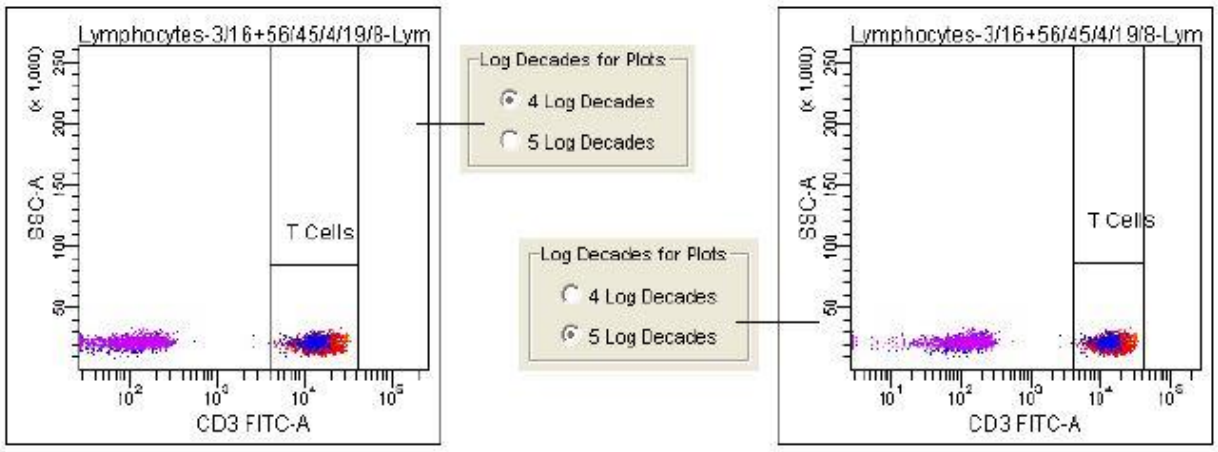
Organizes experiments into a hierarchical view with folders and experiments added as necessary (right click to rename). Experiments can be duplicated without data and used as a template which retains the cytometer but not compensation settings. Alternatively experiments can be exported as a template to be imported later.

	New Folder		Adds a new folder as a child of the selected user or folder.
	New Experiment (icon)		Adds a new experiment (based on the blank experiment template) as a child of the selected user or folder. Note that this is slightly different behavior from selecting Experiment > New Experiment from the menu. See Adding Experiments on page 50.
	New Specimen		Adds a new specimen as a child of the selected experiment.
	New Tube		Adds a new tube as a child of the selected specimen.
	New Cytometer Settings		Adds new tube-specific cytometer settings as a child of the selected tube or new specimen-specific cytometer settings element as a child of the selected specimen.
	New Global Worksheet		Adds a new global worksheet as a child of the selected experiment.

Open and close experiments by double clicking to access cytometer settings and define specimens.

Experiment Inspector



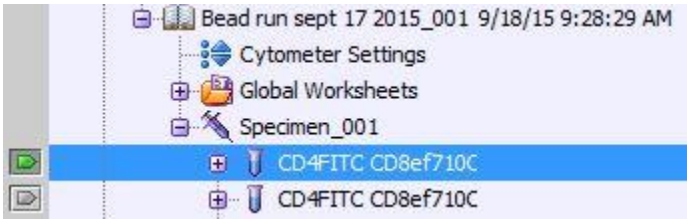


Four-log plots display values from 26–262,143. Five-log plots display values from 2.6–262,143. Thus, the first log decade ranges from 2.6–26 or 26–262, depending on the selected scale.

- Moving from 4 to 5 log decades changes scaling at the low end and not at the high end. Use it to spread out and visualize very dim signals. Changing scales will not help if the signal intensity is off scale!
- As a default the “use global cytometer settings” is selected to synchronize any tube and specimen cytometer cyto-settings changed during an experiment. Uncheck it if you want separate specimen or tube specific cyto-settings.

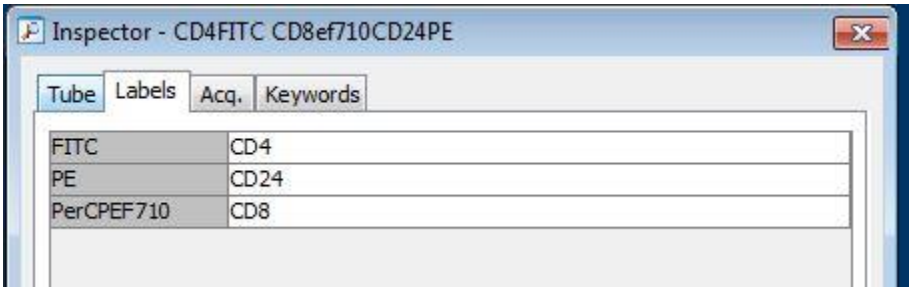
Tube inspector

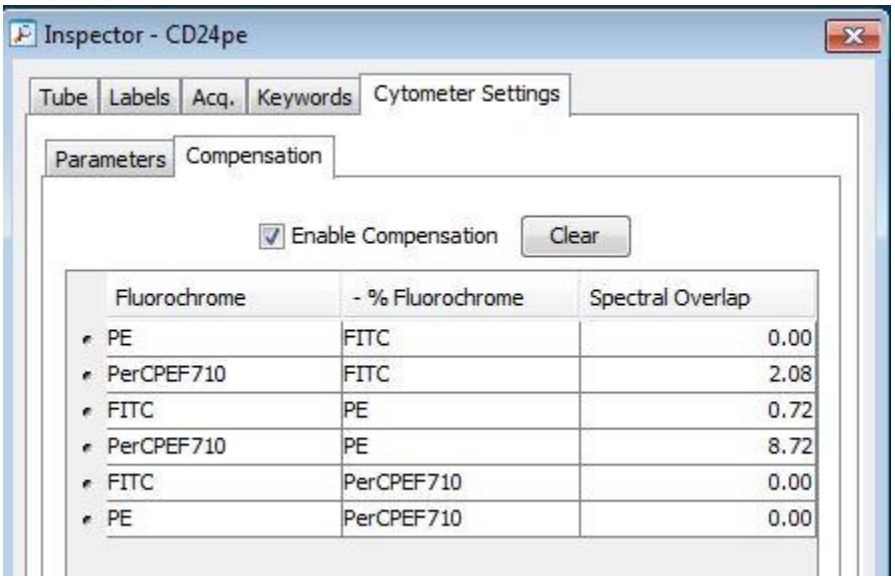
When a tube is selected by clicking on the tube pointer it turns green.



Under the view menu a tube inspector window can be opened. Here the tube can be renamed, fluorochrome labels added, cytometer settings viewed/changed and acquisition parameters changed. Compensation can also be altered manually under the tube inspector.

- Note when using DIVA offline a plot icon is used instead of the tube pointer to indicate recorded data. If selected this data will be loaded into analysis templates on the worksheets.





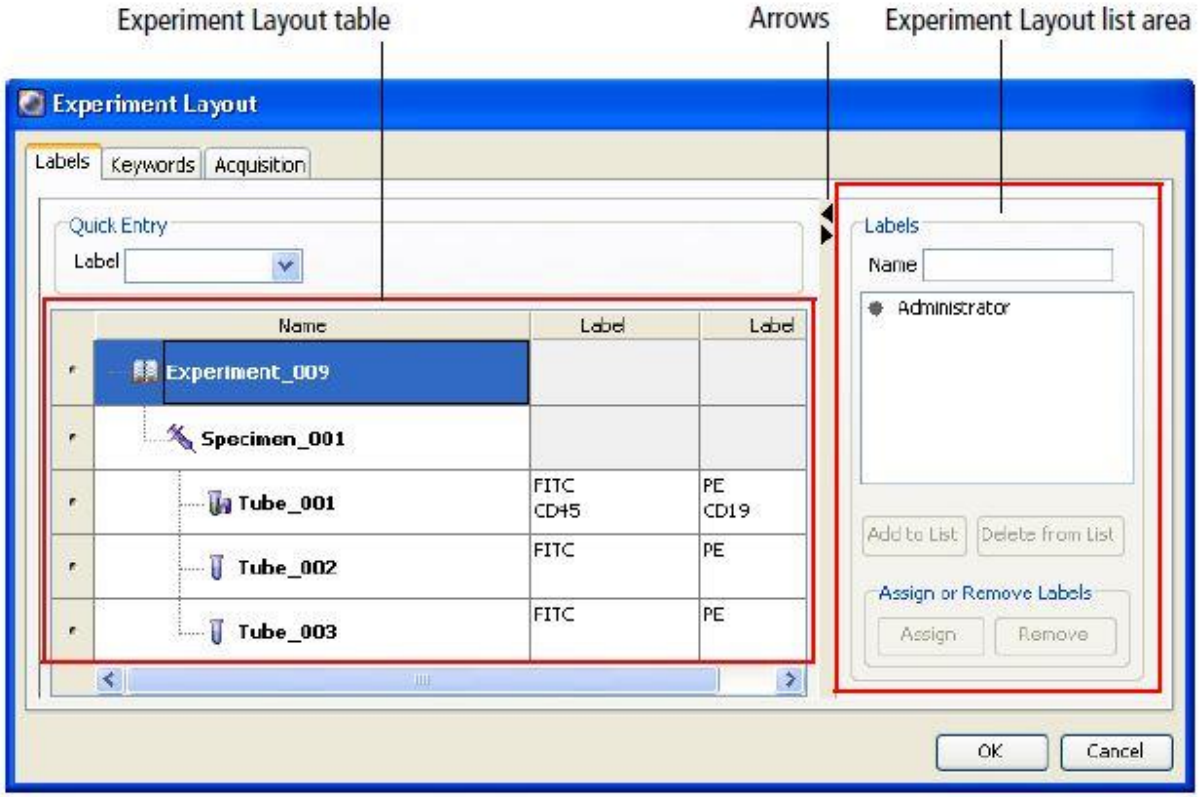
Acquisition dashboard

In the acquisition dashboard a sample can be acquired to make adjustments to cytosettings and then later recorded. Stopping and storage gates can be set along with event numbers or time parameters. Selecting next tube will add a new tube to the acquisition. Acquisition gates can be set at the tube inspector level as well.

Exporting experiments and FCS files

Once an FCS file is created it is stored to <D:BDExport\FCS>. FCS files can be exported as FCS 2.0 (10 bit or 1024 channels resolution), 3.0 (18 bit or 262,144) or 3.1 (18bit). FCS 3.1 is the most up to date file format but if you are using older analysis software versions you may need to use FCS3.0. FSC 2.0 is a very old file format used with Cell Quest!

Experiment Layout (access under Experiment tab)



Used to assign tube labels and acquisition criteria for each tube in an experiment. You can create and assign your own custom labels or use the ones supplied.

Run an experiment

Setup

In the browser menu add a new experiment (right click to rename it <your name_training>)

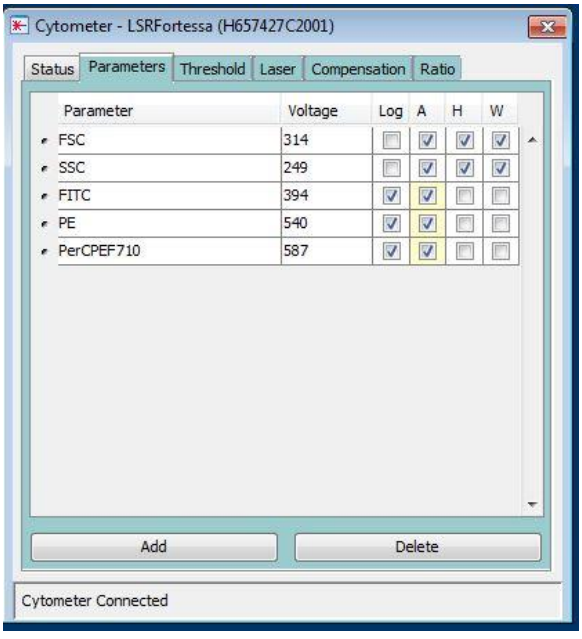
Open this experiment and add a specimen which will automatically add Tube 001 rename it <bead mix>.

Click on the arrow in front of the <bead mix> tube (grey > green means tube is selected)

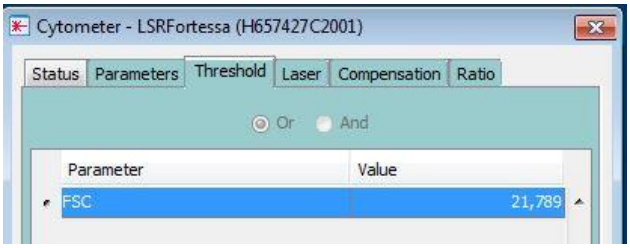
Once a tube is selected the cytometer settings can be accessed (click on cyto-settings icon in workspace toolbar) 

Highlight and delete the parameters not in use. For this experiment we will use (FSC A, W ,H lin, (SSC A, W ,H lin), (FITC A log), (PE A log) and (PerCP EF-710 A log).

Note CS&T was used to set optimal voltages for the detectors in use. Do not adjust voltages unless the signal is off scale (have the antibodies been tittered?). If the cells are very different than immune cells such as very large cells then it may be necessary to optimize voltage settings using a special procedure (See administrator for help).

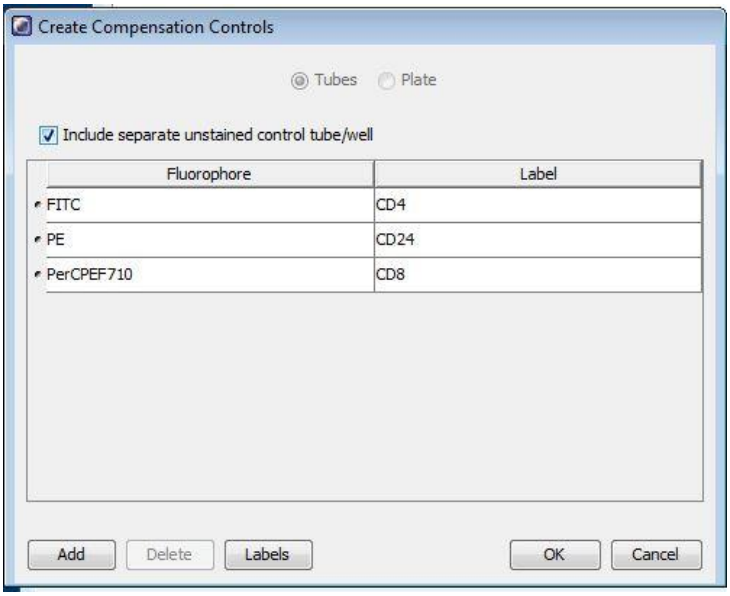


Set threshold around 15,000 – varies with cell type



Add compensation controls (under experiment tab)

Add fluorchromes used for compensation and check box to include separate unstained tube.



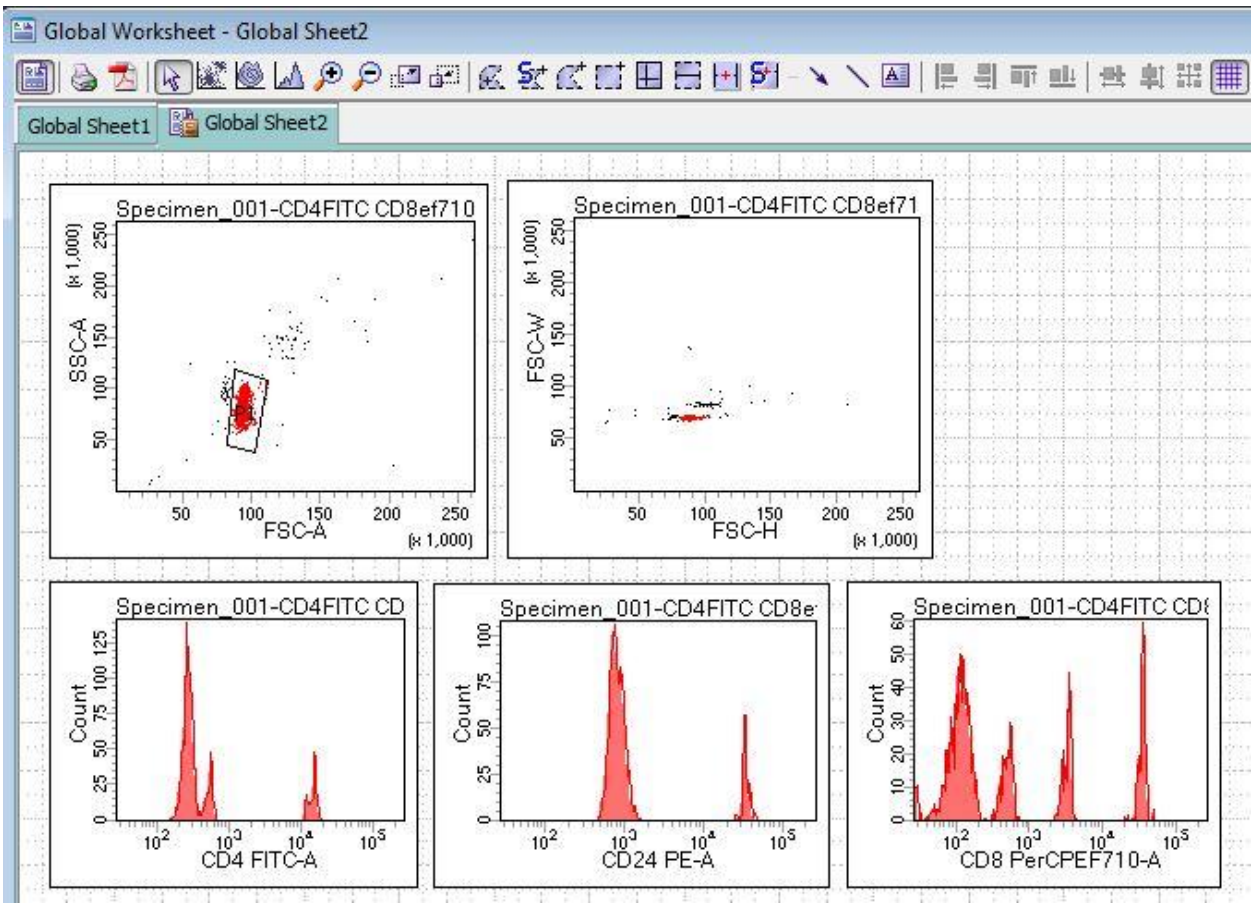
Acquisition dashboard

Set the events to record at 10,000 and the events to display at 1000.

Check to see that all of the fluorochromes are on scale.

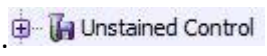
On the global worksheet create a SSC vs. FSC scatter plot, a FSC W vs. FSC H pot for doublet discrimination and histograms for FITC, PE and PerCPef710.

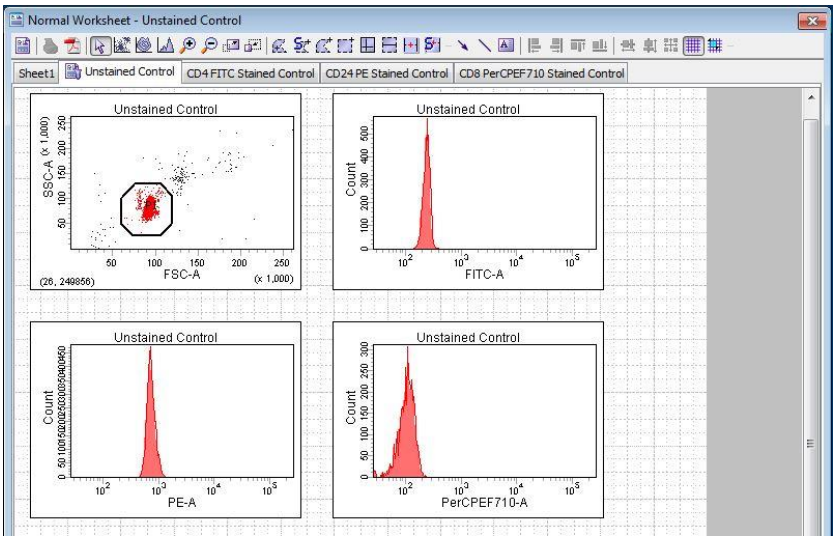
Activate the tube pointer in front of bead mix tube and start acquisition. Adjust the P1 gate to encompass the bead population and show P1 on subsequent plots. Note the bead aggregates I the FSC W vs. FSC A plots as well as the multiple peaks in the histograms due to spillover from other fluorochromes! Adjust detector voltages if signals are off scale.



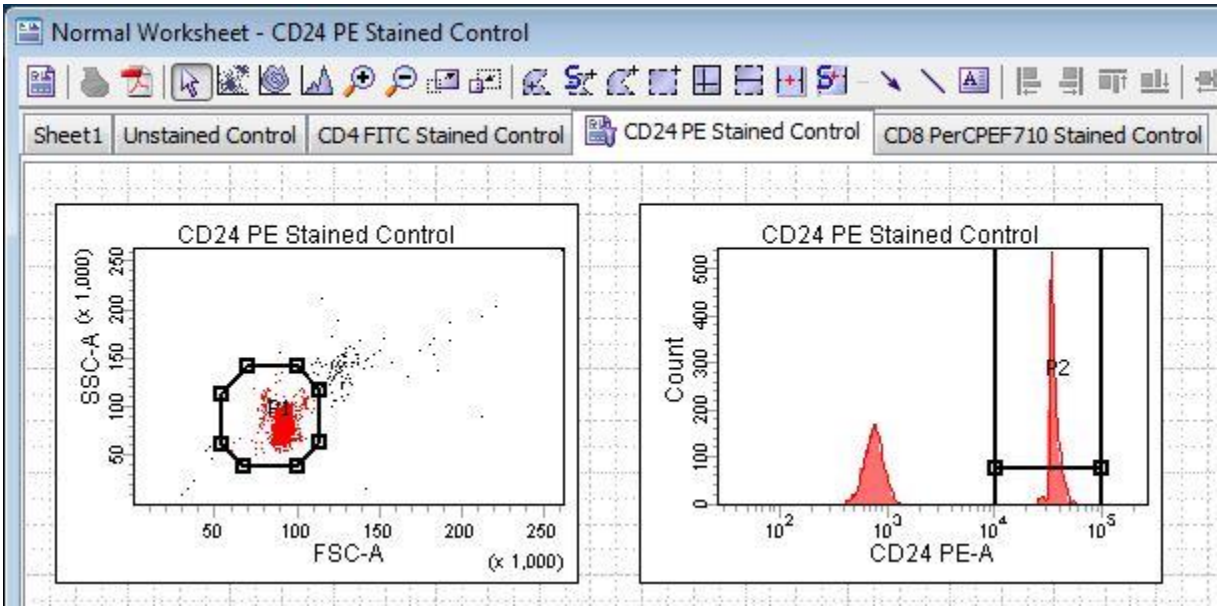
Perform Compensation

Activate the tube pointer in front of unstained tube control and start acquisition.

A normal worksheet appears showing a histogram of the parameter being measured. Adjust the FWD and SSC gates over to cover the population and record. Note you can right click to apply to all compensation controls if all samples have the same scatter properties. Hit record and a disk will appear next to the tube one completed. 

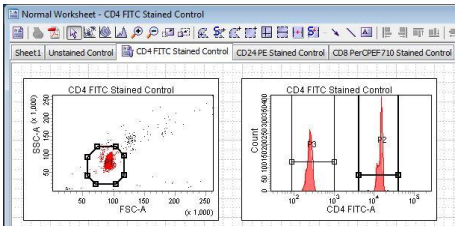


For the FITC, PE and PerCPef710 single stain bead controls. Hit acquire and move the P2 interval gate to encompass the positive population and record 10,000 events. Adjust the voltage only if necessary and hit record.



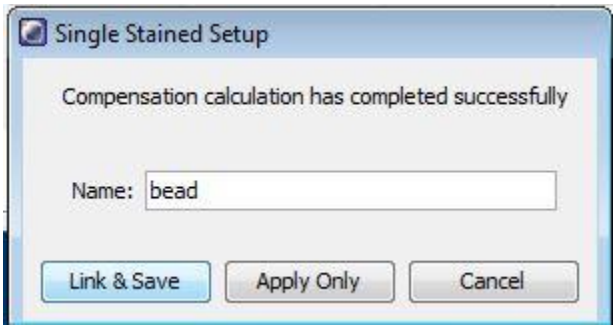
- Note: If you were using a mixture of beads and cells for single stained controls then you need to include a P3 gate on the negative population in the cell control to ensure the compensation uses the correct negative (not the unstained bead control!)

E.g.

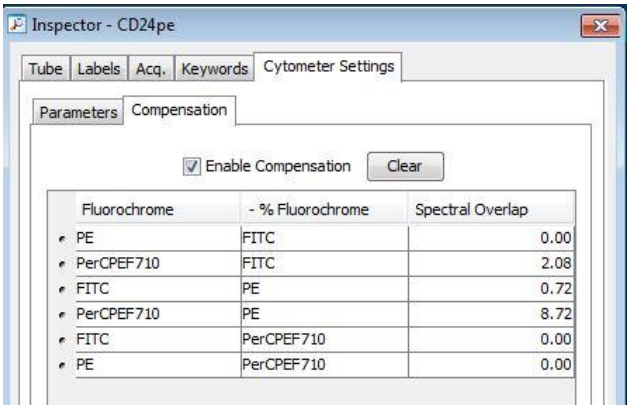


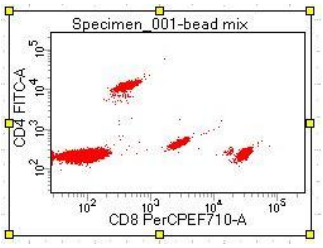
Calculate compensation

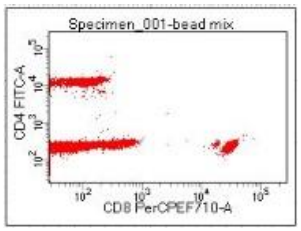
Under compensation set-up select “create compensation”. After it calculates the spillover matrix a dialogue box will open and ask whether you want to “link & save” or “apply only”. Choosing link & save will save the spillover matrix to a data base which can be used if the same experiment is run again (remember to use the experiment name when naming it!). Choosing “apply only” will apply the spillover matrix to the current experiment. If the spillover matrix shows warnings you can adjust the interval gates for the comp tubes and recalculate. You can also change voltages, rerun and overwrite the comp tubes and recalculate.



You can highlight a tube in the tube inspector and toggle back and forth on the “enable compensation” checkbox to see what the samples or single stained controls look like uncompensated and compensated. Note spillover from other flourochromes (2.08% of FITC signal shows up in the PerCPef 710 detector) 







Collecting the Sample

Create a global worksheet

SSC vs FSC, FCS W vs FCS H (doublet discrimination), FITC his, PE His, ef-710 his, FITC vs ef-710 his. In the tube inspector under labels you can add custom labels to parameters.

- Note the default pulse parameter collected is Area but it can be changed to height or width if these parameters were selected in the cytosettings.
- FCS files are exported in linear format which allows adjustment in the spillover matrix post acquisition.
- You can create multiple global work sheets under the experiment tab or by clicking the icon in the browser menu with different analysis templates and toggle between them.

Select the tube pointer for the bead mix tube

- Note if the tube pointer is selected you can view the spillover matrix in the tube inspector and make manual adjustments during acquisition or on previously recorded data. Normally it is better to do this post acquisition.

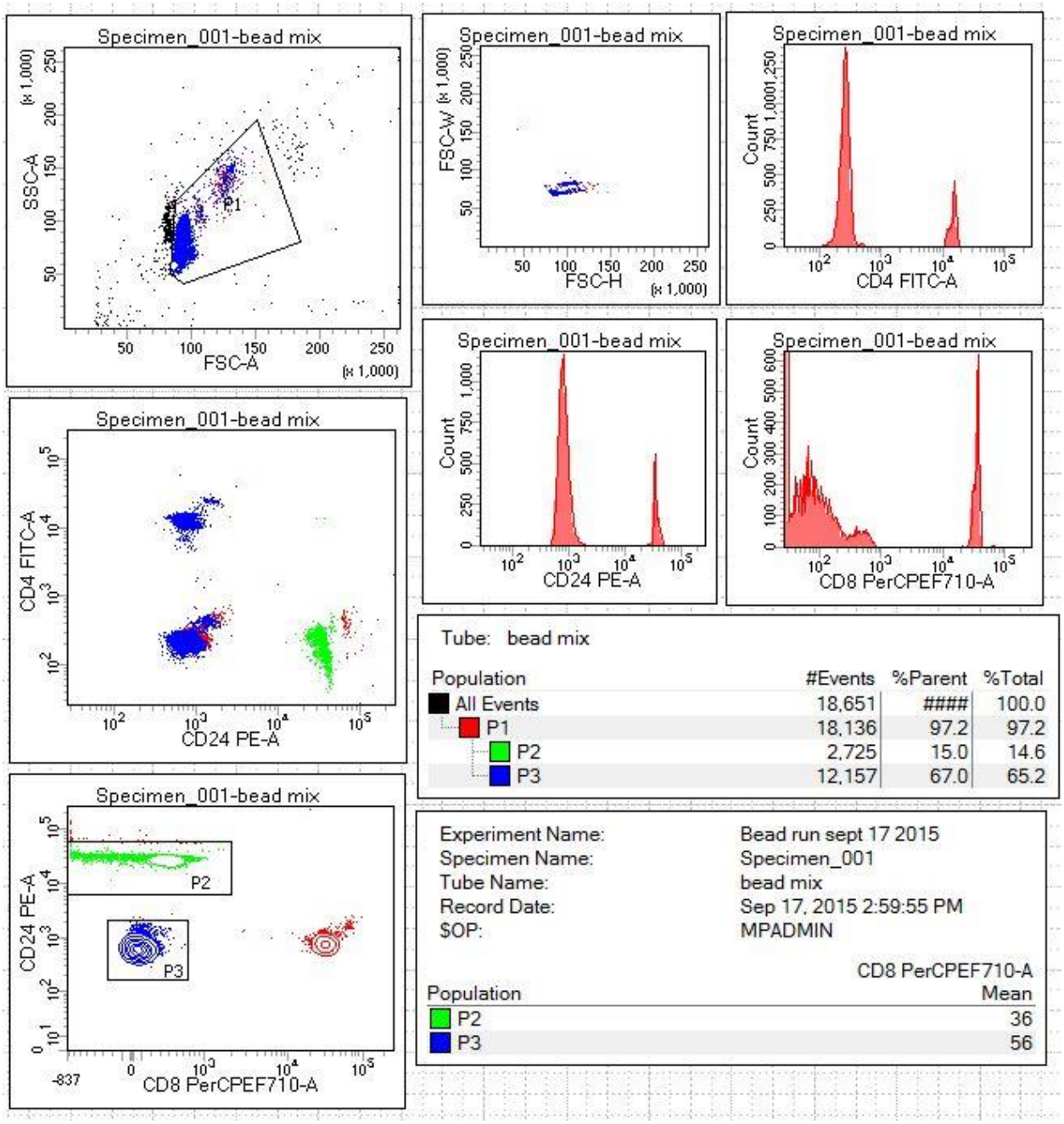
Select the acquisition events to record to 50,000 and events to display to 2000.

Acquire and record sample.

Data analysis

Draw a gate around the cells in the SSC vs FSC plot (P1), In FSC W vs FSC H plot right click and select “show population P1” and draw a gate around the single cells (P2). For the other plots select “show population P2”

- Note : You can select a histogram and open the histogram inspector under the view tab to change the scaling between counts and percentage.
- Note: You can select a dot plot and open the plot inspector to change the scaling to bi-exponential



Right click on any plot to “show population hierarchy”. You can relabel populations by clicking on the population in the hierarchy and you can change the population color by clicking on the colored square.

Right click on plot to create a statistics view which can be edited.

This global worksheet can be saved as an analysis template by highlighting the global worksheet in the browser – right clicking and exporting as an analysis template. To import highlight global worksheet – right click and choose apply analysis template.

Application Settings

If an experiment is to be repeated with the same cells and stains you can save the application settings. These settings will include parameters, detector voltages, thresholds, and area scaling but not spillover values (compensation set-up needs to be re-run).

At the experiment level right click on cytometer settings, scroll down to application settings and save. To apply application settings highlight cytometer settings, right click and select apply under" application settings".

Application settings save the PMT voltages for your experiment relative to the CS&T PMT voltages. As CS&T voltages are adjusted to hit certain MFI targets then experimental voltages will be adjusted accordingly to ensure that population MFI's remain consistent between experiments.