

# FACS LSR II Compensation Setup – FlowJo

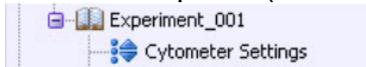
Written by: Laura Johnston

Written: 4/13/16

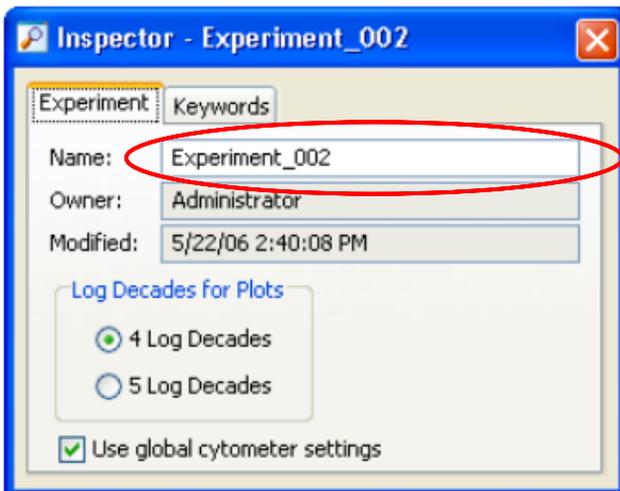
Revised:

This protocol can be used if you plan on compensating your samples using FlowJo

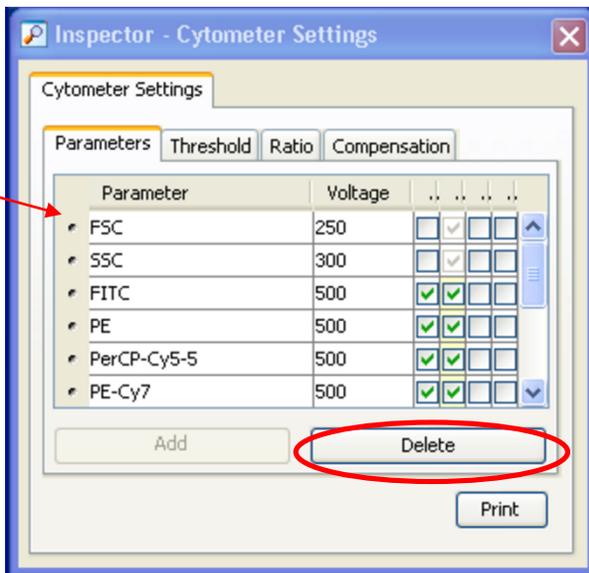
1. Log in
2. Go to “Experiment” → New Experiment
3. On the Side panel (Browser), click Cytometer settings under Exp. 001



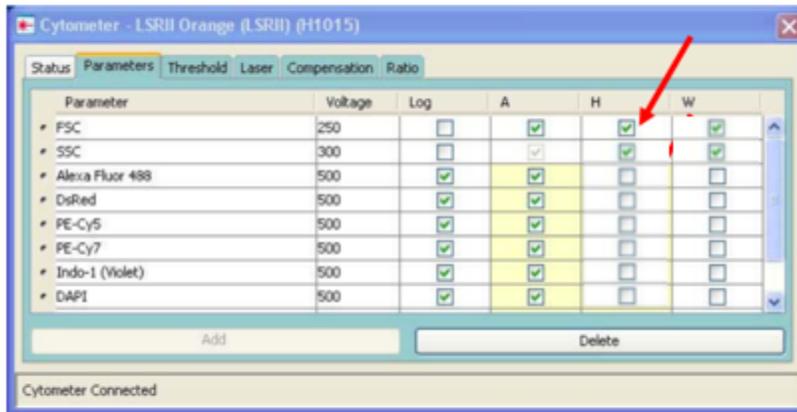
4. Rename “Experiment\_001” in the inspector. This will be the name of the folder that all the files are put into



5. Unclick all the colors NOT being used by clicking on bullet point (far left) and then the delete button. Add colors if needed. Select the fluorophore name to get to a dropdown menu in order to change the color.

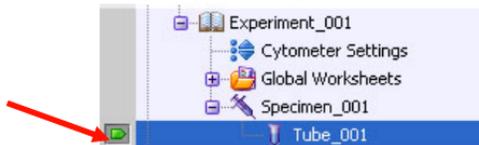


6. In the cytometer parameters, click the checkboxes under H and W for FSC and SSC to collect cell height and width data (identify singlets vs doublets).

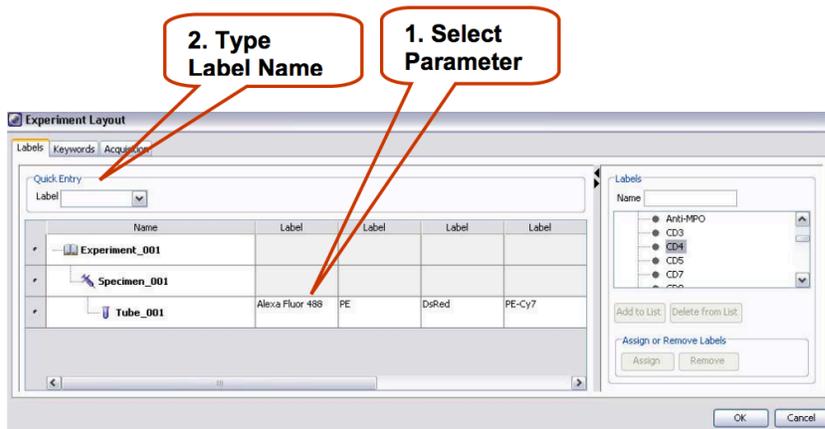


For compensation:

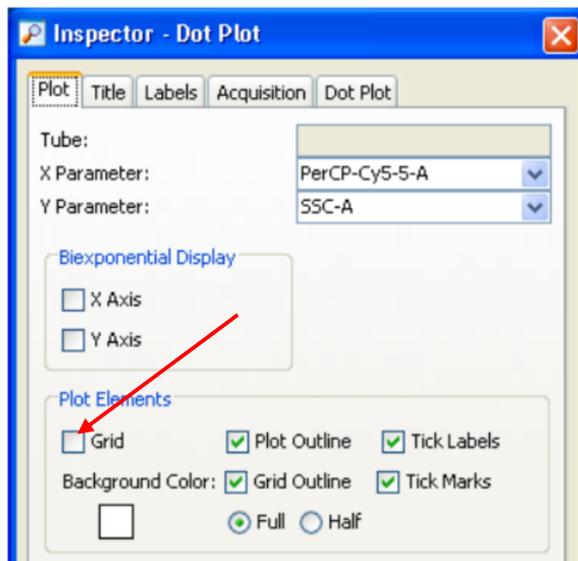
1. Go to Experiment in Menu Bar → New Specimen
2. Rename Specimen in the inspector window
  - a. Each file will contain this label. E.g., files will be named “Specimen\_Tube\_001”, “Specimen\_Tube\_002”, etc. Therefore, I like to label this “beads” or “comp”
3. In browser, expand Specimen and click green arrow to select Tube\_001



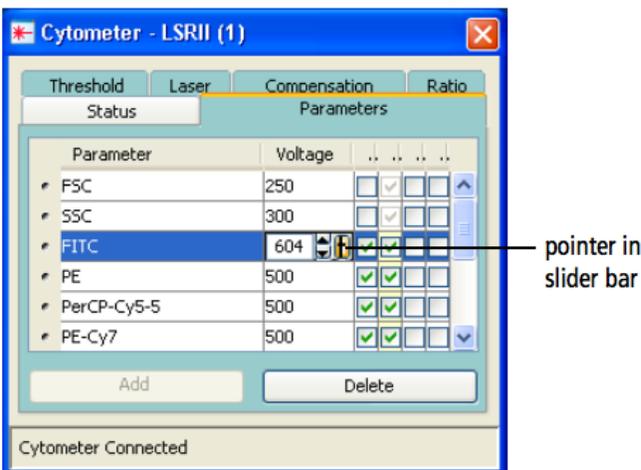
4. In Inspector, under Tube tab, label tube name – create one for each of your single stains
  - a. If you have both beads and cells, make sure you collect both unstained beads and unstained cells
5. Additionally, create a new specimen tube for your samples
  - a. I like to put the date and sometimes cell type (BALF, spleen) in the specimen label. Do not put any specific sample name (WT1).
6. Create tubes for all of your samples and FMOs
7. In Inspector, under Tube tab, label tube name – Sample #
  - a. If your samples are numbered, maintain the “\_001”. Each time you click “next tube” the number will automatically increase. If your mice are ear tagged 4625-4634, label the tube “4\_625” to save yourself the trouble of naming each sample.
  - b. Enter in number of events to collect. If you want, you can also enter a time to stop acquiring and the machine will automatically stop recording (either events or time, whichever comes first)
  - c. Click next tube to create more tubes
8. Under “Experiment” in the Menu Bar → Experiment layout
9. Label the fluorophores for the corresponding antibody



10. On right screen, on normal worksheet, click on global worksheet (far left icon)
  - a. Open/create histograms for each fluorophore and any dot plots you want
  - b. Once you make the histogram, select it and click the “grid” checkbox in the inspector window



11. Put in unstained control cells/beads and make sure the machine is set to low and run
12. Click Acquire data.
13. Look at the FSC/SSC and adjust voltages accordingly
  - To move FSC the L: lower voltage, R: increase voltage
  - To move SSC up: increase voltage, down: decrease voltage



14. Set P1 gate in FSC vs SSC around cells/beads of interest
  - a. This will clean up the histograms
15. Right click each histogram and select Show Populations → P1
16. If you are starting a new panel, lower the voltages to all of the peaks are between 0 and  $10^2$
17. Stop acquiring data
18. Remove unstained cells
19. NOTE: you will have to run all of your single stain tubes 3 times
  - a. First to adjust voltages
  - b. Second to make sure everything looks good – not touching voltage
  - c. Third to collect events
20. Put one of your single stain tubes on the machine and click acquire
21. Adjust the voltage so that the color of your single stain has the highest peak (aka if you are looking at the PE single stain, the PE peak should be the highest – ideally one log higher, but at minimum, PE peak should not overlap with any other peak)
  - a. Ideally, all cells/beads should fit in the window. However, it is better to have cells on the x or y axes than have cells beyond  $10^5$ .
22. Stop acquiring
23. Repeat steps 20-22 for the single fluorophores – only acquire – DO NOT RECORD YET. You can leave the green arrow on the any tube.
24. After all of the voltages have been adjusted, acquire each tube one more time without touching the voltages – DO NOT RECORD YET. Check that the single stain is still the highest peak.
25. Quickly acquire a few events with one of your fully stained samples and make sure that there aren't too many events on the far right axis
  - a. Don't forget to change the FSC and SSC before and after if you're switching between beads and cells!!
  - b. You may also need to change your P1 gate, or make a second larger gate and select each histogram to show P2 so that you can see all of the cells

*To collect data for compensation*

26. Once all voltages have been set, record samples (instead of acquire), matching the appropriate sample to the compensation tubes
  27. Put in unstained control
  28. On the acquisition dashboard: select at least 3,000-10,000 events for controls for collection
  29. Click "Record data" in the acquisition dashboard
  30. Once done, move onto next positive control sample by clicking "next tube" and "record data"
- Note – pay attention to which fluorophore is selected to determine which sample to run next

*Run Sample Tubes*

31. Put in sample tube, and click acquire
32. Make sure your sample is running less than 3000 events/sec
33. Click record
34. When the machine automatically stops (or you can manually stop by clicking stop acquiring), go to the next tube and repeat....
35. To quit: Run bleach and then water tubes for 3-5 minutes each (instructions on the door)
36. To export samples, select the entire experiment (the brown notebook icon that is open), then select File → export → FCS files
37. File → logout