FACS LSR II Compensation Setup – FACSDiva

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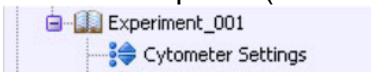
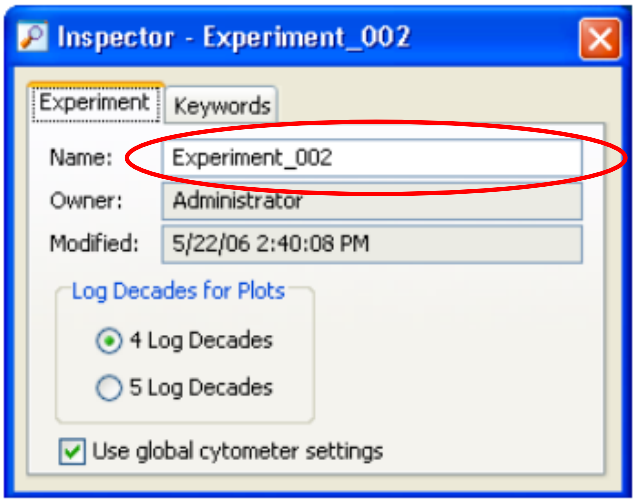
Revised:

This protocol can be used if you plan on compensating your samples using FACSDiva before you collect your samples

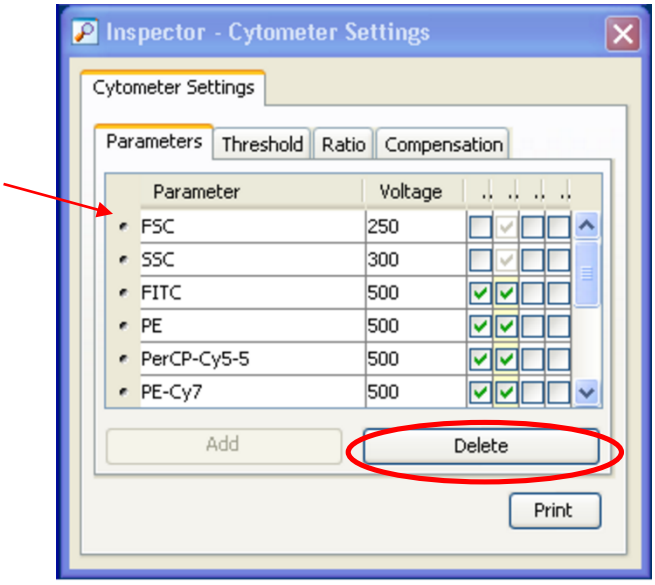
1. Log in
2. Go to “Experiment” → New Experiment
3. On the Side panel (Browser), click Cytometer settings under Exp. 001



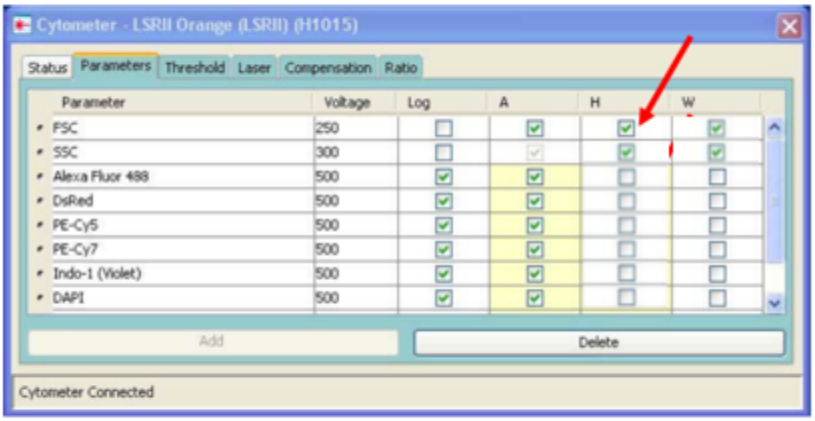
4. Rename “Experiment_001” in the inspector. This will be the name of the folder that all the files are put into



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.

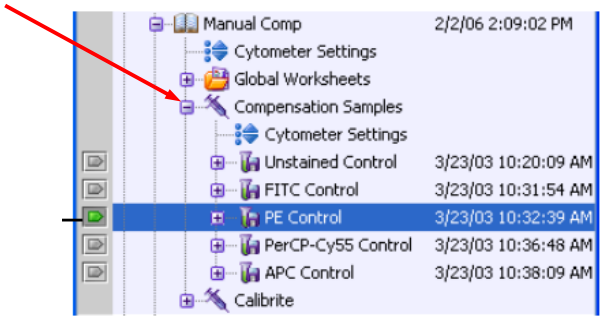


6. In the cytometer parameters, click the checkboxes under H and W for FSC and SSC to collect cell height and width data (identify singlets vs doublets).

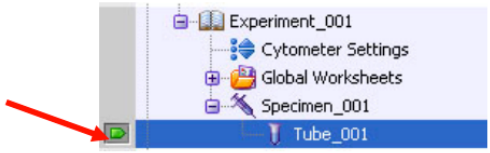


For compensation:

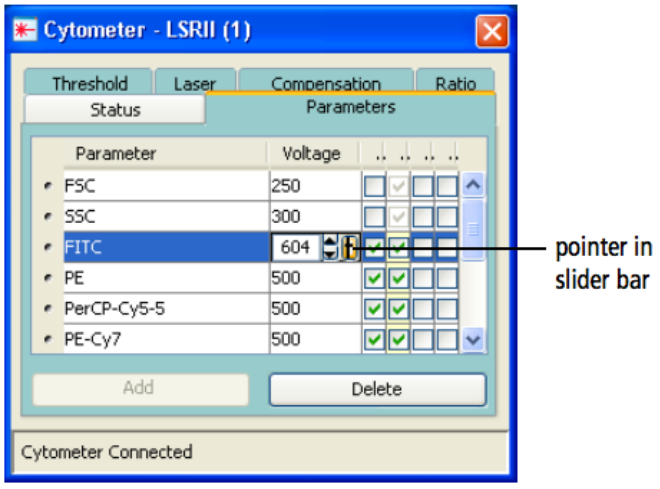
7. Under “Experiment” in the Menu Bar → Compensation setup → create compensation controls
8. Label the fluorophores with their corresponding antibody name or leave fluorophores as “generic” → select OK
9. In the browser, expand compensation samples



10. Click “green” arrow to the left of the unstained control tube in the browser to select the tube



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



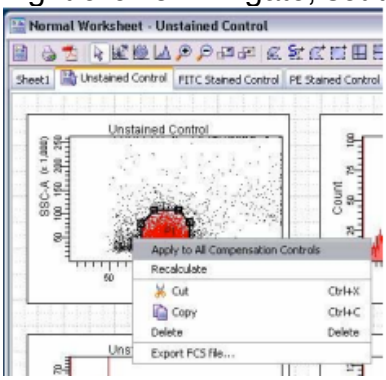
14. Set P1 gate in FSC vs SSC around cells/beads of interest
15. If you are starting a new panel, lower the voltages to all of the peaks are between 0 and 10^2
16. Stop acquiring data
17. Remove unstained cells
18. NOTE: you will have to run all of your single stain tubes 3 times
    a. First to adjust voltages
    b. Second to make sure everything looks good – not touching voltage
    c. Third to collect events
19. Put one of your single stain tubes on the machine and click acquire
20. 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 then have cells beyond 10^5.
21. Stop acquiring
22. Repeat steps 19-21 for the single fluorophores – only acquire – DO NOT RECORD YET. You can leave the green arrow on the any tube.
23. 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.
24. 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!!

To collect data for compensation

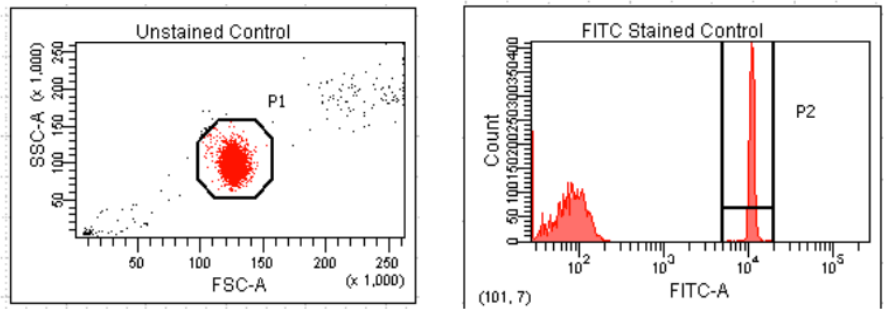
25. Once all voltages have been set, record samples (instead of acquire), matching the appropriate sample to the compensation tubes
26. Put in unstained control

On the acquistion dashboard:

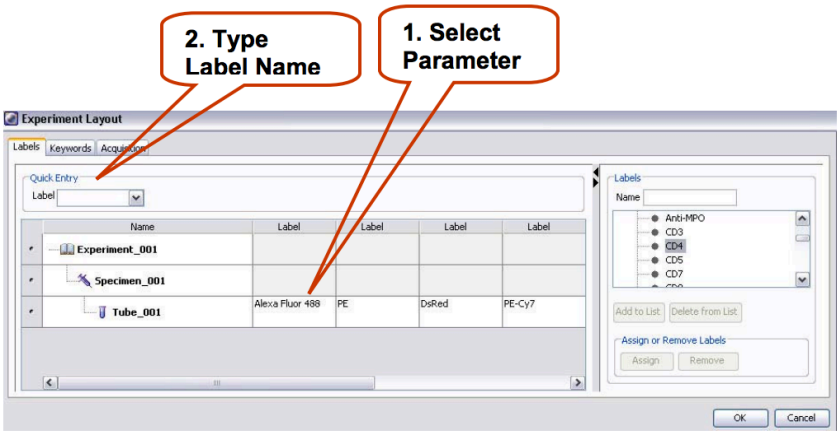
27. Select at least 3,000-10,000 events for controls for collection
28. Right click on P1 gate, set to apply to all compensation controls



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
31. Adjust P2 gates to fit the positive cells on the histograms



32. After all fluorophore data is collected, select Experiment in Menu Bar→ Compensation Setup→ calculate compensation → link and save
33. Under "Experiment" in the Menu Bar → Experiment layout
34. Label the fluorophores for the corresponding antibody



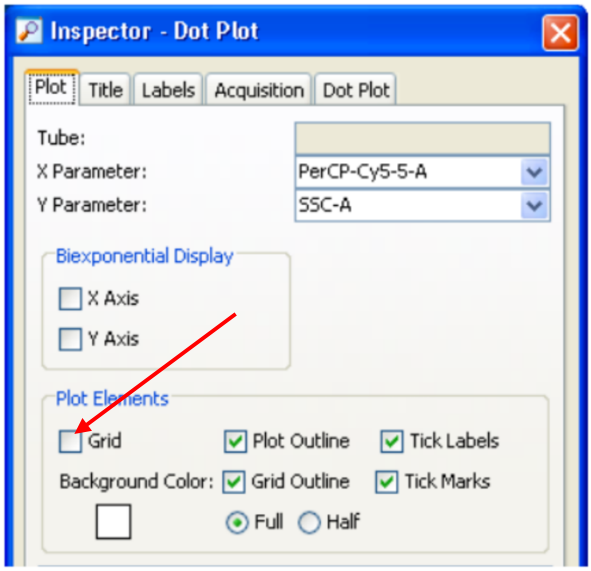
OPTIONAL: Check that your compensation is correct

35. If you want to make sure that the calculated compensation is correct, you can run your single stains as samples (which compensation is applied to)
36. Create graphs in the global worksheet: one for each color
    a. Set the x axis so that each color is represented
    b. Set the y axis for all graphs to your first color
37. Collect the first single stain and make sure that the sample is compensated correctly (the dots should be in a verticle line)
38. Switch to the second single stain and change the y axis of all graphs to the second color. Leave the x axis the same as before.
39. Repeat for all colors

Run Sample Tubes

40. Go to Experiment in Menu Bar → New Specimen
41. 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 put the date and sometimes cell type (BALF, spleen) in the specimen label. Do not put any specific sample name (WT1).
42. In browser, expand Specimen and click green arrow to select Tube_001, click "ok" for all colors in the pop up windows
43. 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.
44. 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



45. 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)
46. Put in sample tube, and click aquire
47. Make sure your sample is running less than 3000 events/sec
48. Click record
49. When the machine automatically stops (or you can manually stop by clicking stop acquiring), go to the next tube and repeat….
50. To quit: Run bleach and then water tubes for 3-5 minutes each (instructions on the door)
51. To export samples, select the entire experiment (the brown notebook icon that is open), then select File→ export→ FCS files
52. File→ logout