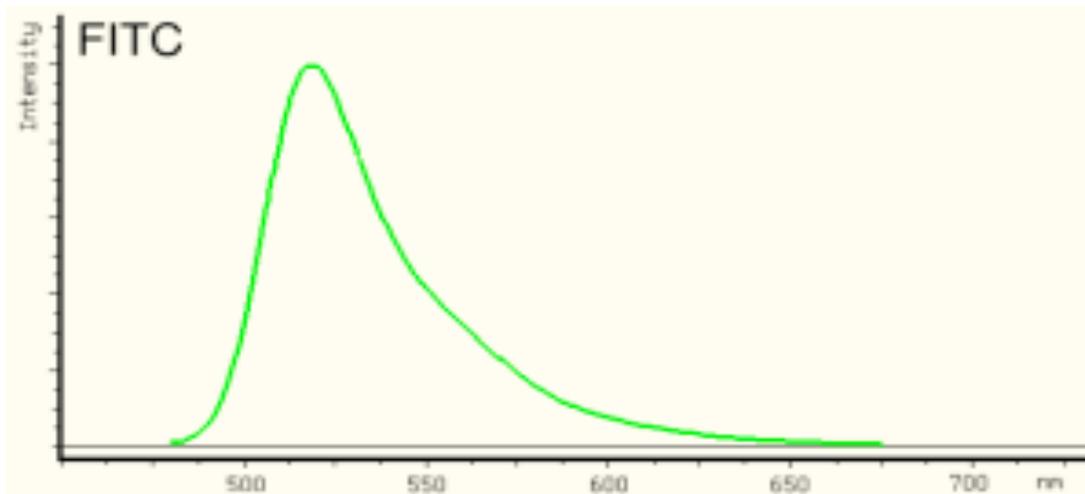


COMPENSATION
MIT Flow Cytometry Core Facility

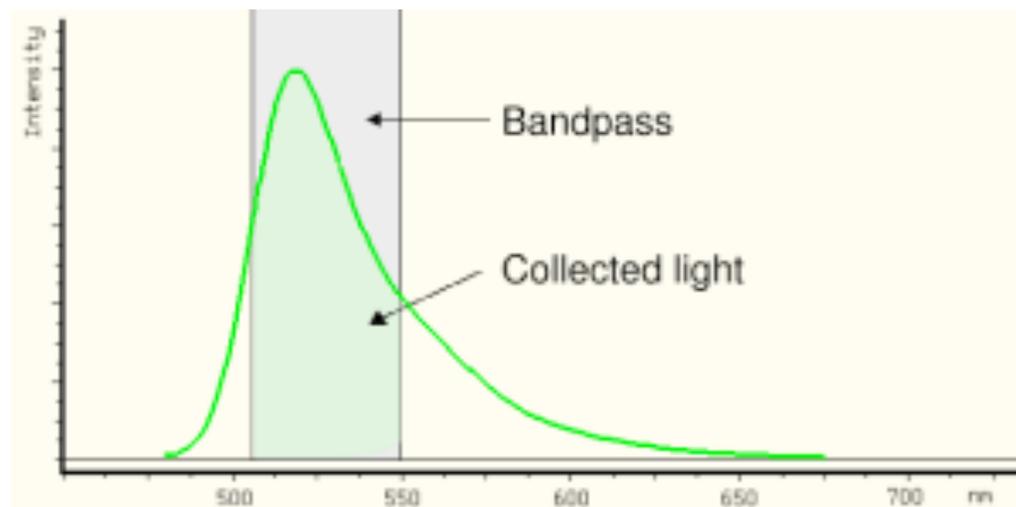
Compensation

- Why do we need compensation?
 - 1) Because the long emission spectrum tail of dyes causes overlap like with the fluorophores FITC and PE.



Compensation

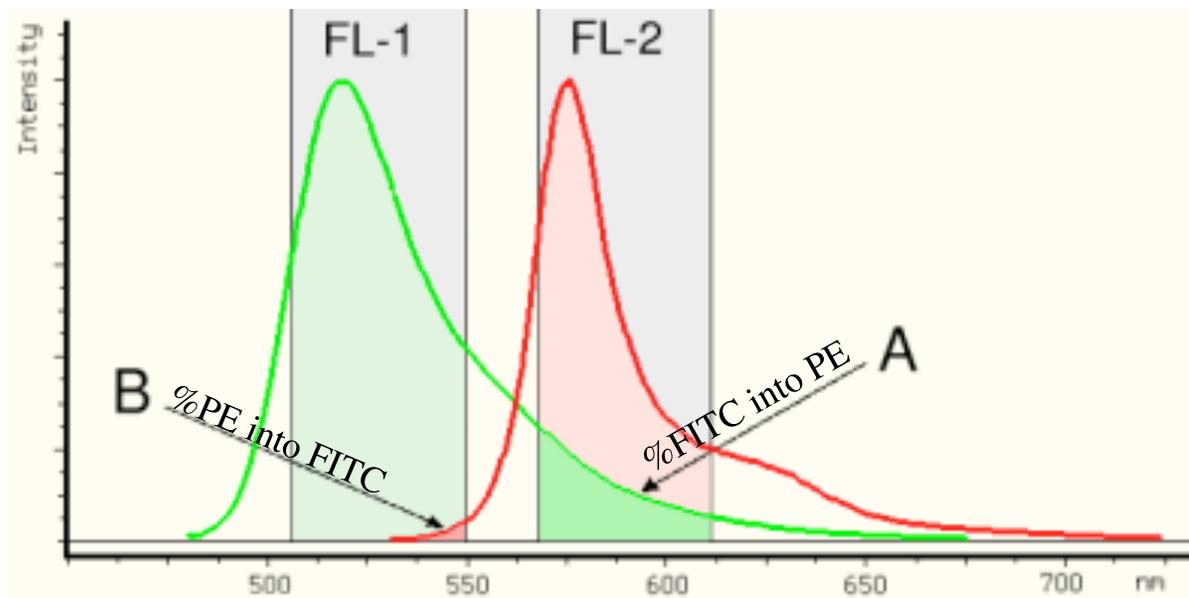
- 2) For sensitivity reasons, we must use broad bandpass and dichroic filters in the cytometer in order to separate fluorescence the emission spectra of dyes from the excitation light source.



Compensation

THE END RESULT IS SPECTRAL OVERLAP

where FITC bleeds into the PE channel and PE bleeds back into FITC.



Compensation

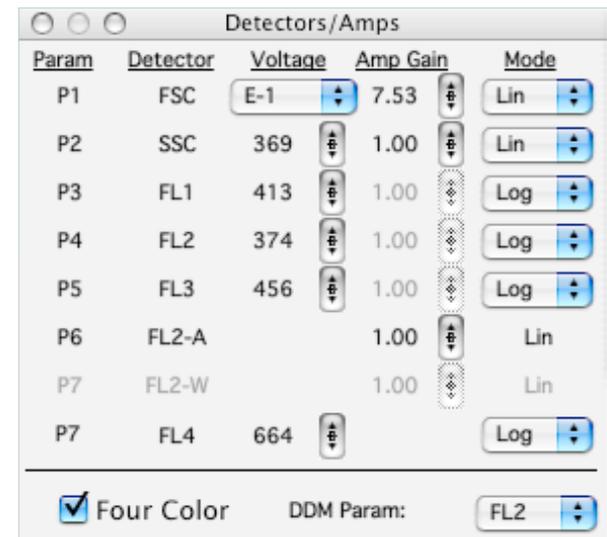
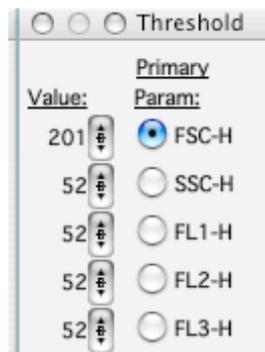
To correct for spectral overlap during multicolor flow cytometry experiments, **color compensation** must be performed.

The goal of color compensation is to correctly quantify each dye with which a particular cell is labeled. This is done by subtracting a portion of one detector's signal from another, leaving only the desired signal.

Compensation

This example will show you how to compensate a FITC and PE experiment using a FacsCalibur flow cytometer.

Open the Detectors/Amps and Threshold windows by selecting them from the **Cytometer** drop down menu in Cell Quest Pro software.

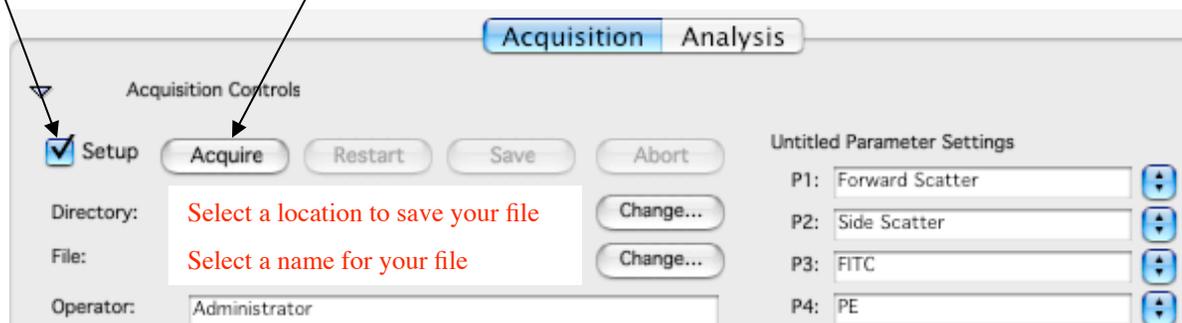


Compensation

Loading your negative control

Put the cytometer on run mode and put your negative control on the SIP

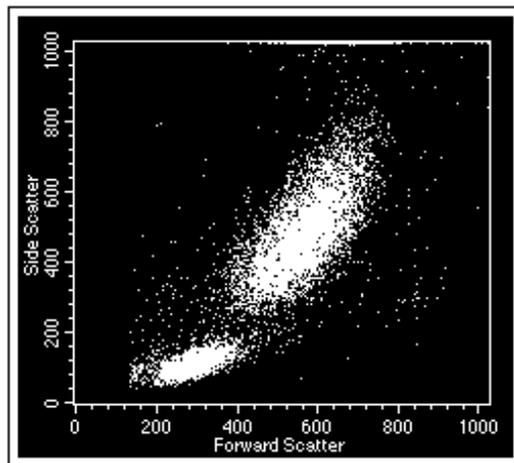
While the negative control is running under setup mode press acquire and make the following changes



Compensation

Make sure the population of interest is clear visible by making adjustment to the FSC/ SSC.

Adjust the forward and side scatter by sliding the bar to bring the population of interest toward the center of the plot.



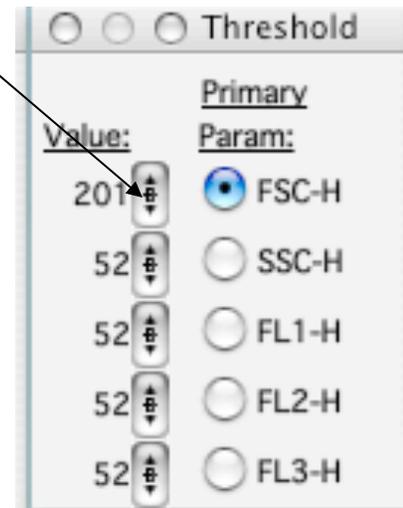
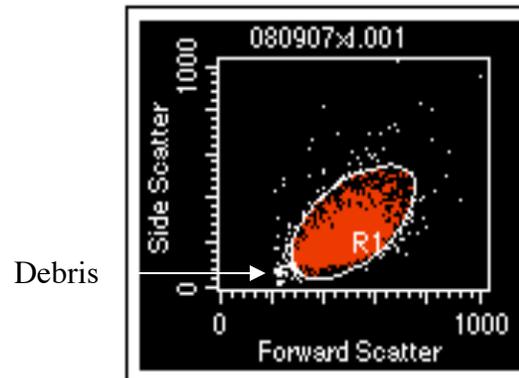
Param	Detector	Voltage	Amp Gain	Mode
P1	FSC	E-1	7.53	Lin
P2	SSC	369	1.00	Lin
P3	FL1	413	1.00	Log
P4	FL2	374	1.00	Log
P5	FL3	456	1.00	Log
P6	FL2-A		1.00	Lin
P7	FL2-W		1.00	Lin
P7	FL4	664		Log

Four Color DDM Param: FL2

Compensation

Remove debris by using threshold.

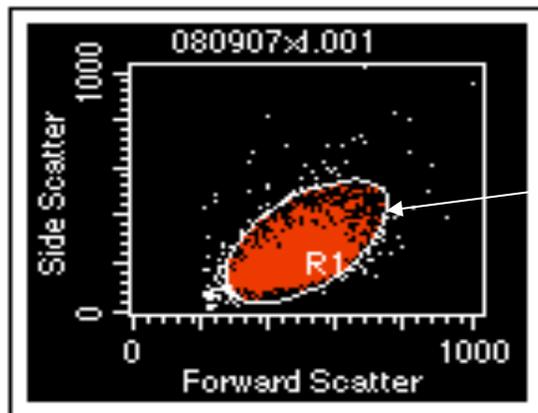
Adjust the threshold value by sliding the bar on the threshold window enough to remove most of the debris but not all of it. It is a good idea to have some debris on scale to make sure you are not thresholding out your population of interest.



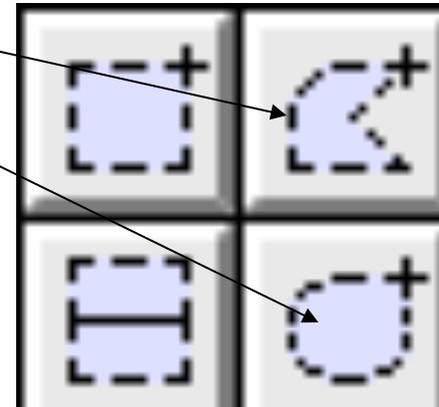
Compensation

Select your population of interest by creating a gate around that population

Draw a gate, which will allow you to remove unwanted debris and aggregates, around the population of interest by selecting a circle or a polygon from the tool template.



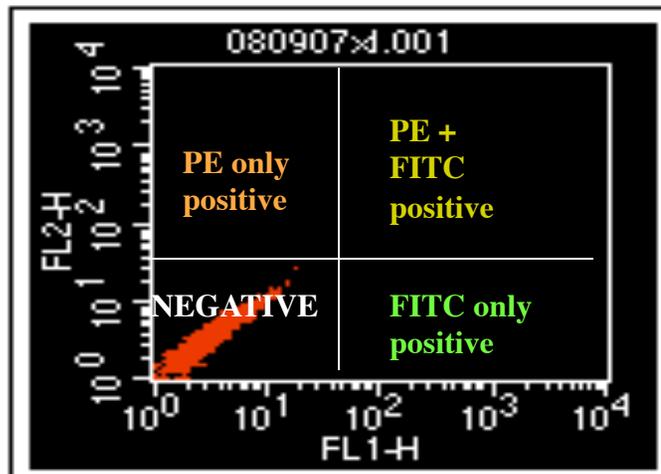
REGION R1



Compensation

Adjust the voltage on the FITC and PE detectors to put the negative cells in the center of the first log.

Adjust the FL1 and FL2 voltage by sliding the bar up or down to bring the population in the middle of the first log.



The screenshot shows the 'Detectors/Amps' window with a table of detector parameters. The 'Voltage' column has sliders for each detector. Arrows from the text above point to the sliders for FL1 and FL2. The 'Mode' column has dropdown menus for each detector. At the bottom, there is a 'Four Color' checkbox and a 'DDM Param:' dropdown menu set to 'FL2'.

Param	Detector	Voltage	Amp Gain	Mode
P1	FSC	E-1	7.53	Lin
P2	SSC	369	1.00	Lin
P3	FL1	413	1.00	Log
P4	FL2	374	1.00	Log
P5	FL3	456	1.00	Log
P6	FL2-A		1.00	Lin
P7	FL2-W		1.00	Lin
P7	FL4	664		Log

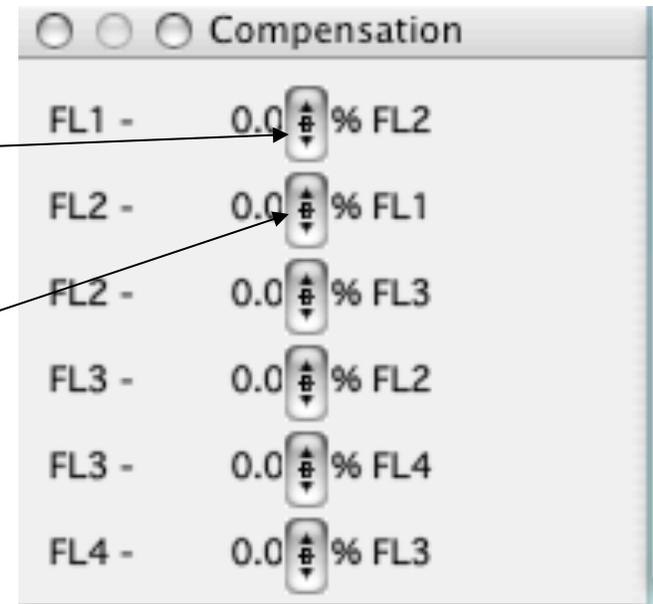
Compensation

You are now done with the negative control and ready to move on to the first single stained control

Open the compensation window by selecting it from the **Cytometer** drop down menu.

This bar allows you to remove spill over of PE into FITC

This bar allows you to remove spill over of FITC into PE



Compensation

Compensating the FITC Tube

Still under the Setup Mode, load the FITC single color control in the cytometer then press Acquire. Your sample will look like the data on the left. Adjust the slide on the FL2- %FL1 to remove the % of FITC spill over in the PE channel. Try not to over compensate, pay attention to the mean of the negative and the FITC positive, they should be the same.

FL1 - 0.0 % FL2

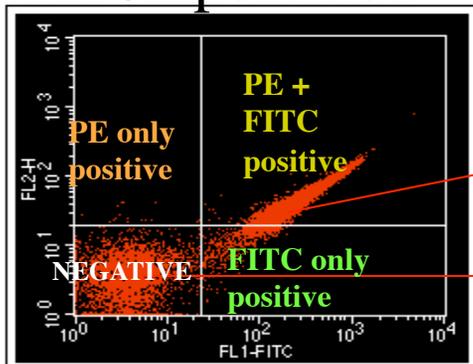
FL2 - 0.0 % FL1

We went from 0.0% to 26.0%

FL2 - 26.0 % FL1

compensated

uncompensated

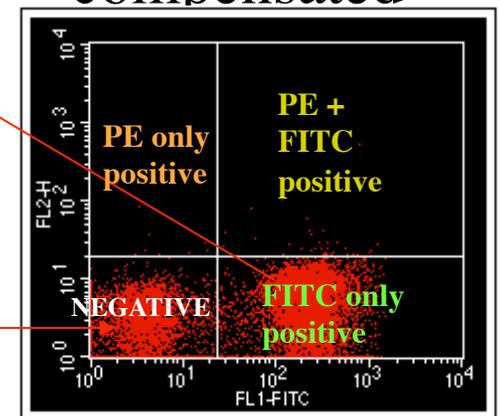


Y mean = 65

Y mean = 5

Y mean = 5

Y mean = 5

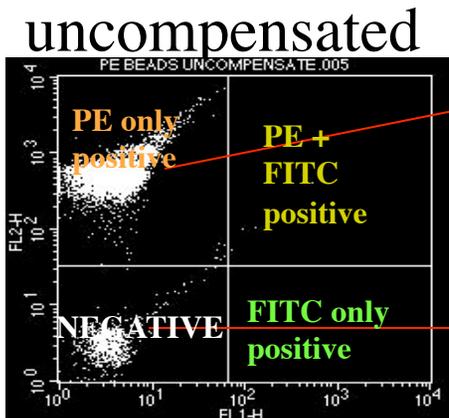


Compensation

You are now done with the FITC tube and ready to compensate your PE control

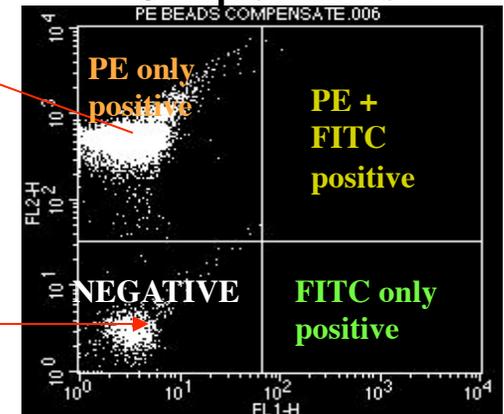
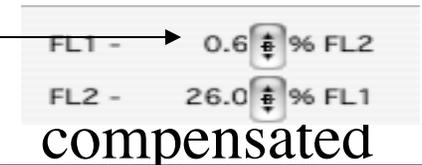
Still under Setup Mode, load the PE single control in the cytometer then press Acquire. Your sample will look like the data on the left. Adjust the slide on the FL1- %FL2 to remove the % of PE spill over in the FITC channel. Try not to over compensate, pay attention to the mean of the negative and the PE positive, they should be the same.

We went from 0.0% to 0.6%



X mean = 9

X mean = 5



X mean = 5

X mean = 5

Compensation

THE FITC AND PE COMPENSATION IS DONE.

YOU ARE NOW READY TO RECORD DATA AND SAVE
YOUR INSTRUMENT SETTING.

TO SAVE YOUR INSTRUMENT SETTING:

GO TO CYTOMETER MENU
THEN SELECT INSTRUMENT
SETTING. LOCATE YOUR
DIRECTORY AND PRESS SAVE
THEN PRESS DONE.

