Immunophenotyping Flow Cytometry Tutorial

Contents

Experimental Requirements

For immunophenotyping with FITC and PE monoclonal antibody fluorescent conjugates, the research must have:-

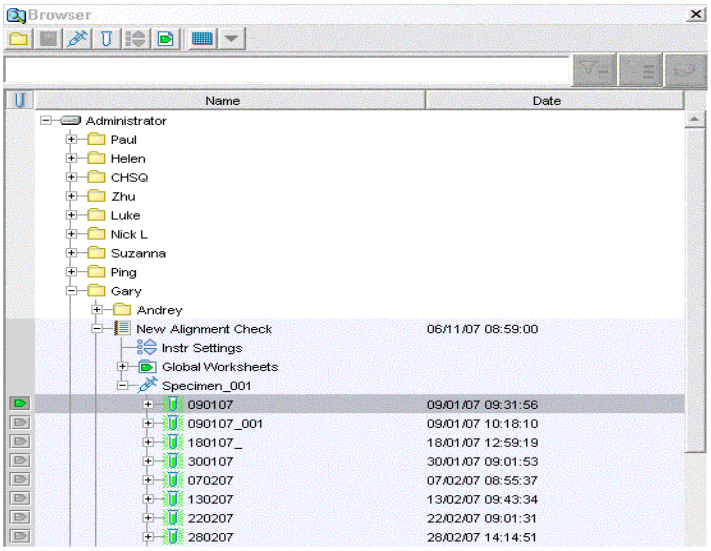
1. Unlabelled cells
2. Single fluorescent conjugate controls containing some negative cells
3. Fluorescent conjugates minus one fluorescence
4. Fluorescent conjugates minus one fluorescence with isotype control

Researchers can also use Becton Dickinson Compensation beads labelled with conjugated antibodies if cell samples do not give large fluorescent signals. This allows the researcher to determine the compensation settings for the different fluorophores used in the actual experiment.

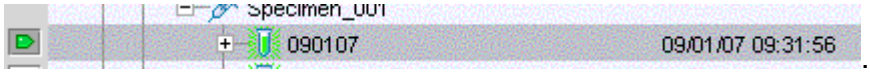
In the example described here the research is using FITC and PE individually labelled beads to demonstrate the process involved in immunophenotyping cells with the same fluorophores.

Data Storage

The user must first create a folder for the experiment in which the data is to be stored, see figure below:-



Main personal folders are first created by clicking on  then single experiments are made via the brown folder icon  , subdivisions of experimental folders are created from the syringe icon  , individual data tubes are created from the test tube icon  , see figure below.

Individual tubes can be renamed by *right clicking* on a tube icon and typing (no two tubes can be named the same). To store data in a tube file the button to the far left has to be activated 

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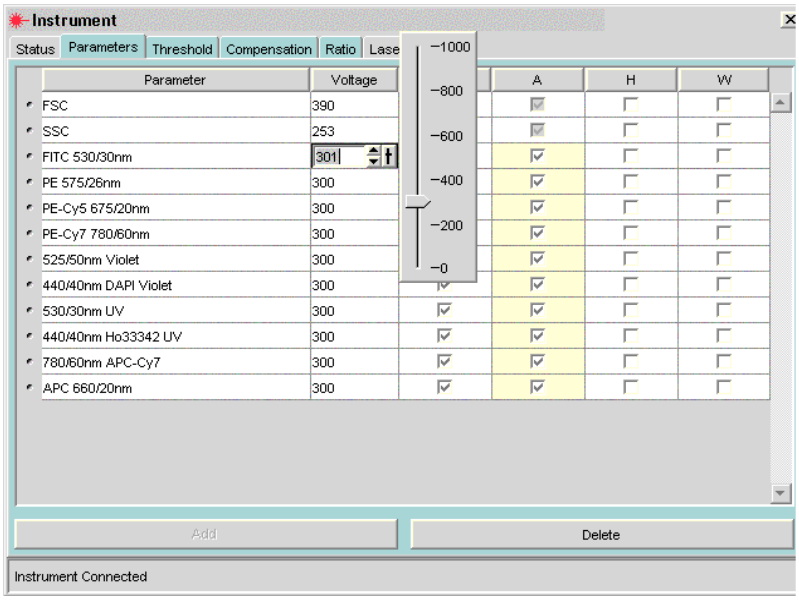
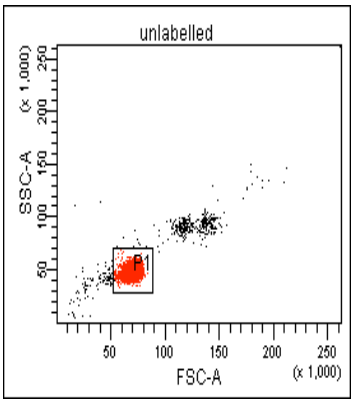
Voltage adjustments

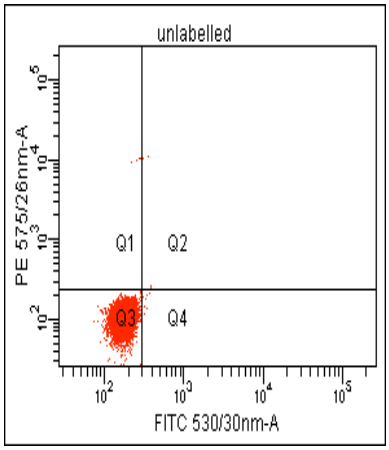
First place a tube of unlabelled cells (or Becton Dickinson Compensation beads) on the instrument and press the **RUN** button on the fluidics control panel. Activate the **Acquire** button on the acquisition control dialogue box in **DIVA Software**. Next adjust the voltages on the **Instrument** panel, see figure below.



First adjust the FSC and SSC parameter voltages to enable gating of cells or the singlet population of beads, see figure below.

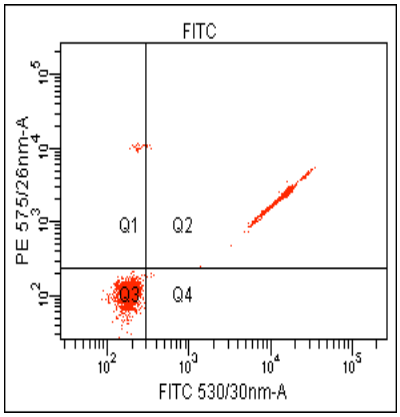


Next adjust Voltages on the FITC 530/30-nm-A and PE 575/26nm-A parameters to place the unlabelled beads or cells towards the origin, see figure below.

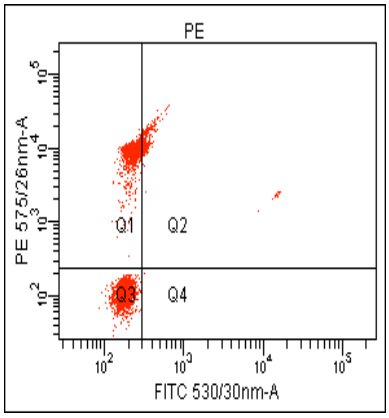


On digital instrumentation it is usual to place the negative events slightly away from the origin as displayed above and not in the origin as is the convention on analogue instrumentation.

Next place a tube FITC labeled cells or a mixture of FITC and unlabelled beads on the flow cytometer and check that no final adjustments to voltage are required to the FITC 530/30nm-A parameter, FITC positive events should be separated from the negative population. BD compensation or ‘Calibrite’ beads give a clear separation from the negative population at approximately log 10^4 on the fluorescence log scale, see figure below.



Next place a tube PE labeled cells or a mixture of PE and unlabelled beads on the flow cytometer and check that no final adjustments to voltage are required to the FITC 575/26nm-A parameter, PE positive events should be separated from the negative population. BD compensation or 'Calibrite' beads give a clear separation from the negative population at approximately log 10^4 on the fluorescence log scale, see figure below.

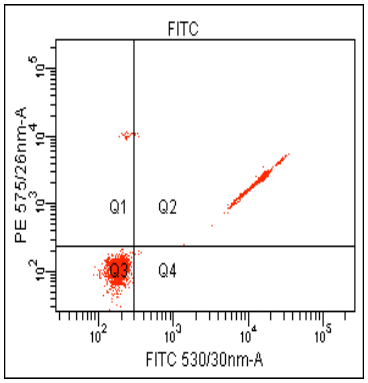


The user should then not change the voltage settings on the  **Instrument** panel, as subsequent **Compensation** will not be valid if instrument settings for fluorescence channels are different for the three samples used in this tutorial (not FSC and SSC). The User should then **Record** data from all three tubes included in this study, 1) unlabelled beads, 2) FITC+unlabelled beads and 3) PE+unlabelled beads.

Compensation

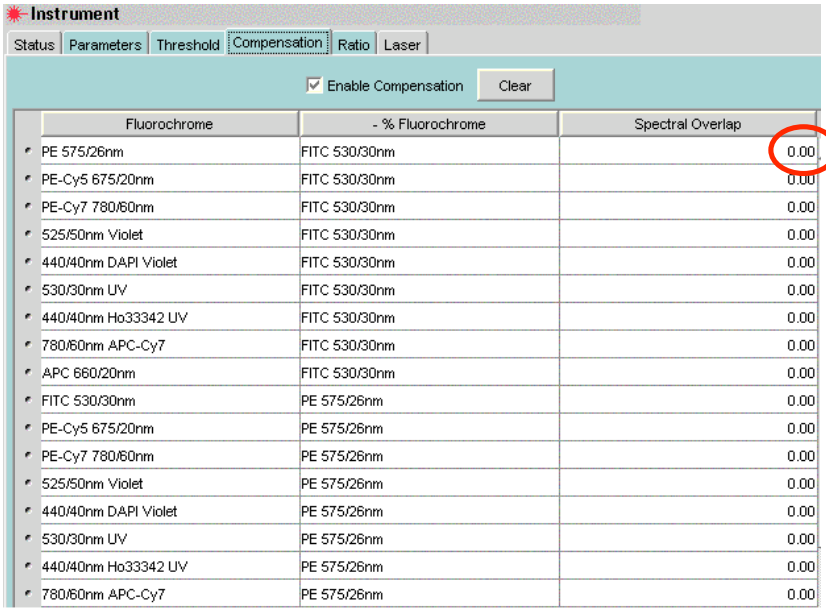
Once the voltages are set for each of the fluorochromes, the data from each of the tubes can be analysed and stored. The compensation can then be applied to the stored data, if using analogue instrumentation the data must be compensated before the data is analyzed and stored *e.g.* FACScan.

Users may find it easier to start the compensation process by starting on the fluorophores with the lowest wavelength *e.g.* in this case FITC. After gating on the single bead population on a FSC vs SSC dot-plot, the user should display the FITC+blank beads data file in a plot of FITC vs PE in this particular case, see figure below.



Population	%Parent	FITC 530/30nm-A Median	PE 575/26nm-A Median
All Events	####	16,113	2,538
P1	92.2	16,086	2,533
Q1	0.2	228	10,316
Q2	83.5	16,302	2,571
Q3	16.3	178	109
Q4	0.0	329	184

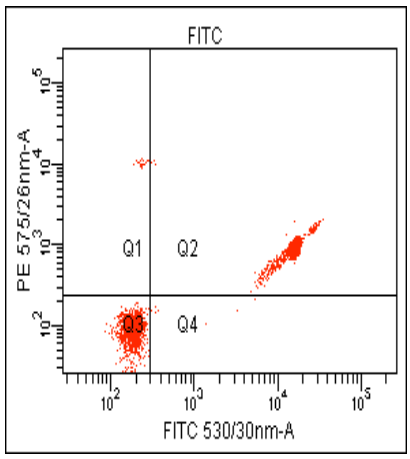
Quadrant analysis of this uncompensated data shows the negative beads in the origin (Q3) with the FITC beads in the double positive quadrant (Q2). A statistics display should be changed to display Median channel number for FITC and PE channels, the Median channel number for FITC beads reads 2,571 off the PE axis, showing that the FITC beads are displaying a spectral overlap (or bleed through) with PE. The user should then apply compensation on the PE – FITC option highlighted in the compensation matrix in the figure shown below.



Fluorochrome	- % Fluorochrome	Spectral Overlap
PE 575/26nm	FITC 530/30nm	0.00
PE-Cy5 675/20nm	FITC 530/30nm	0.00
PE-Cy7 780/60nm	FITC 530/30nm	0.00
525/50nm Violet	FITC 530/30nm	0.00
440/40nm DAPI Violet	FITC 530/30nm	0.00
530/30nm UV	FITC 530/30nm	0.00
440/40nm Ho33342 UV	FITC 530/30nm	0.00
780/60nm APC-Cy7	FITC 530/30nm	0.00
APC 660/20nm	FITC 530/30nm	0.00
FITC 530/30nm	PE 575/26nm	0.00
PE-Cy5 675/20nm	PE 575/26nm	0.00
PE-Cy7 780/60nm	PE 575/26nm	0.00
525/50nm Violet	PE 575/26nm	0.00
440/40nm DAPI Violet	PE 575/26nm	0.00
530/30nm UV	PE 575/26nm	0.00
440/40nm Ho33342 UV	PE 575/26nm	0.00
780/60nm APC-Cy7	PE 575/26nm	0.00

The median channel number is used to allow for data that is not normally distributed, Mean analysis is only applicable for data that is normally distributed. The convention is to apply enough compensation to the FITC signal so that the statistical analysis

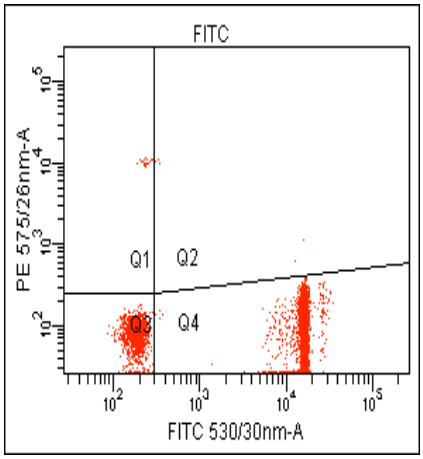
indicates that the FITC signal and negative signal show the same value for their peak heights on the y-axis (PE channel in this case).



Population	%Parent	FITC 530/30nm-A Median	PE 575/26nm-A Median
All Events	####	16,113	920
P1	92.2	16,086	917
Q1	0.2	228	10,296
Q2	83.4	16,302	933
Q3	16.3	178	90
Q4	0.1	365	149

Applying 10% compensation in the PE-FITC option in the compensation matrix moves the FITC beads in Q2 to a lower position giving a Median fluorescence value of 10,296. The negative population has moved slightly lower to 90 (from 109).

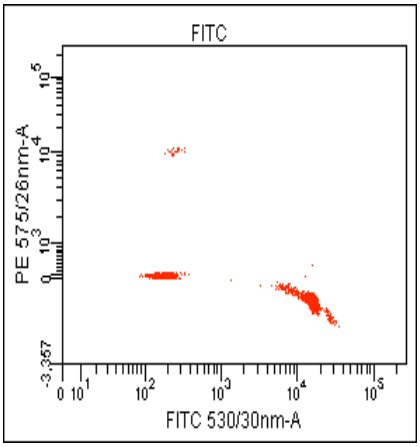
Applying 15.28% compensation in the PE-FITC option in the compensation matrix moves the FITC beads in Q2 to a lower position giving a Median fluorescence value of 79. The negative population has moved slightly lower to 80, see figure below.



Population	%Parent	FITC 530/30nm-A Median	PE 575/26nm-A Median
All Events	####	16,113	82
P1	92.2	16,086	80
Q1	0.2	231	10,498
Q2	0.0	6,482	5,630
Q3	16.3	178	81
Q4	83.4	16,302	79

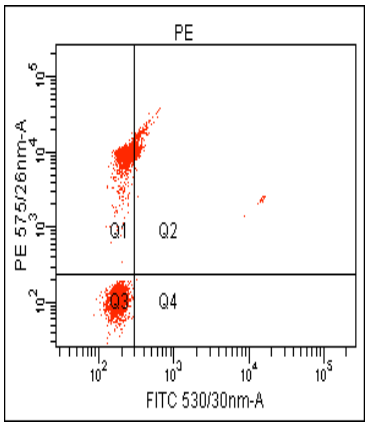
Thus the FITC beads are now correctly compensated against PE beads, the user may notice that the horizontal quadrant has moved to an angle to analyse the data correctly. The user must bear in mind that the statistics displayed is based on the position of the quadrants, so a useful tip is to move the quadrants around whilst adjusting the compensation to verify the compensation values.

The user should be aware that over compensation of a sample can give incorrect results. The figure below shows the FITC+blank data with 20% compensation applied to PE-FITC. The Q4 (FITC) quadrant reads -687, the data below zero can be observed by activating the bi-exponential option in the **Inspector** dialogue box.



Population	%Parent	FITC 530/30nm-A Median	PE 575/26nm-A Median
All Events	####	16,113	-667
P1	92.2	16,086	-664
Q1	0.2	228	10,275
Q2	0.1	318	10,674
Q3	16.3	178	73
Q4	83.4	16,302	-687

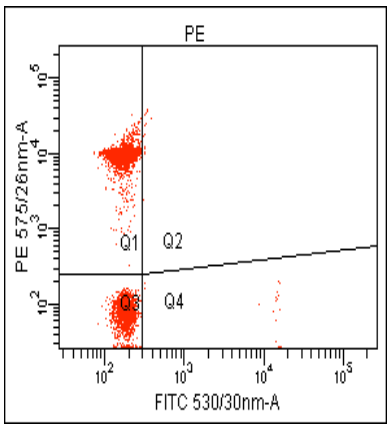
The same procedure is used to compensate PE from the FITC channel. The PE+Blank data file shows a slight spectral overlap with the FITC channel, see figure below.



Population	%Parent	FITC 530/30nm-A Median	PE 575/26nm-A Median
All Events	####	239	9,955
P1	98.0	239	9,966
Q1	71.2	244	10,198
Q2	8.6	387	10,823
Q3	20.2	176	107
Q4	0.0	310	216

When comparing a positive sample to blanks on the y-axis the user should compare statistics on the x-axis. In this case for the peak height of the PE and blank populations to be the same they have to be directly above each other *e.g.* quadrants Q1 and Q3. Currently the median values for the PE and blank populations read 244 and 310.

Applying 0.75% compensation to the FITC – PE option in the compensation matrix gives correct compensation for the PE spectral overlap into the FITC channel, see figure below.



Population	%Parent	FITC 530/30nm-A Median	PE 575/26nm-A Median
All Events	####	173	9,929
P1	98.0	173	9,941
Q1	79.5	172	10,239
Q2	0.1	313	23,709
Q3	20.2	175	79
Q4	0.2	14,899	102

The median channel values for Q1 and Q4 now read 172 and 174 respectively.

All data can now be compensated with the same compensation matrix by use of the copy and paste spectral overlap function in the data browser, this is activated by *right clicking* on a data file. Please note that spectral profiles cannot be copied to empty data tubes.