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 \[\text{folder}\] then single experiments are made via the brown folder icon \[\text{folder}\], subdivisions of experimental folders are created from the syringe icon \[\text{syringe}\], individual data tubes are created from the test tube icon \[\text{test tube}\], 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.
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.

![Graph showing PE and FITC channels](image)

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.
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.

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).

<table>
<thead>
<tr>
<th>Population</th>
<th>%Parent</th>
<th>Median 580/530nm-A</th>
<th>Median 575/520nm-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Events</td>
<td>100</td>
<td>16,113</td>
<td>920</td>
</tr>
<tr>
<td>P1</td>
<td>92.2</td>
<td>16,089</td>
<td>902</td>
</tr>
<tr>
<td>Q1</td>
<td>0.2</td>
<td>223</td>
<td>0.0</td>
</tr>
<tr>
<td>Q2</td>
<td>0.4</td>
<td>15,302</td>
<td>855</td>
</tr>
<tr>
<td>Q3</td>
<td>19.3</td>
<td>178</td>
<td>60</td>
</tr>
<tr>
<td>Q4</td>
<td>0.1</td>
<td>365</td>
<td>149</td>
</tr>
</tbody>
</table>

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.
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.
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.

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.

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.