

Automated use of Becton Dickinson Compensation Beads



Flow Cytometry Core Facility

Institute of Cell and Molecular Science



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Experimental Requirements

For immunophenotyping cells using multiple fluorophores it is recommended that the FACSDiva software (version 5.0.3 and 6.1.2) automated procedure is followed using Becton Dickinson Compensation Beads rather than cells labelled with a single fluorophores. These types of cell samples often do not give positive and or negative signals for the operator to correctly compensate the fluorophores used. The Becton Dickinson Compensation Beads either bind mouse anti-human antibodies, see link below

<http://www.icms.qmul.ac.uk/flowcytometry/flowcytometry/guides/antimouse.pdf>

or rat anti-mouse antibodies, see link below

<http://www.icms.qmul.ac.uk/flowcytometry/flowcytometry/guides/antirat.pdf>

These kits with two types of beads, one that binds the antibody another that does not, these beads are designed to show a negative and a very bright positive peak of fluorescence from the antibody being used on experimental cells. Thus the user has

there antibody showing separate positive and negative peaks, which might not be true when single antibodies are bound to experimental cells.

Cells should not be used in any circumstances unless using antibodies/dyes/fluorescent proteins that will bind to the BD Compensation beads. The main reason for this is that compensation is computed by the algorithm partly by subtracting the negative population from the positive population and if the negative population is unlabelled cells which have varying degrees of auto-fluorescence the resulting computed compensation matrix will be incorrect. The negative bead population supplied with the BD Compensation kit are known to be absolutely non-fluorescent and given the same reading at all times thus the computed matrix is correct as it is not adversely affected by the auto-fluorescence of unlabelled cells.

The BD Compensation beads also show that the antibody in use if creates a positive and negative peak is fully functional in terms of fluorescence and avidity. This is a useful test of the antibody if the experimental cells express only very low amounts of antigen e.g. stem cell markers.

Fluorochromes

The choice of fluorochromes is relatively complex procedure and Becton Dickinson <http://www.bdbiosciences.com/home/> and Invitrogen <http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-and-Tissue-Analysis/Flow-Cytometry.html> have sites to help chose the correct fluorochromes for your experiment and the instrument you are using.

The ICMS Flow Cytometry Core Facility currently is solely supplied with Becton Dickinson instrumentation; a FACSARIA I cell sorter, LSRII, Canto II and FACScan analysers the fluorochromes that can be used are listed below for each instrument.

The FACSARIA I cell sorter can use a total of 12 fluorochromes, 6 from the Argon Laser, 3 from the Red HeNe and 3 from the violet diode.

The BD LSRII can use a total of 13 fluorochromes, 6 from the Argon Laser, 2 from the Red HeNe, 3 from the violet diode and 2 from the UV laser.

The BD FACSCanto II can use a total of 8 fluorophores, 4 from the Argon laser, 2 from the Red HeNe and 2 from the violet diode.

The BD FACScan can use a total of 3 fluorophores from the Argon laser.

BD FACSAria I

Argon 488nm λ – channel	Red HeNe 633nm λ – channel	Violet Diode 405nm λ – channel
FITC Alexa488 530/30nm	APC Cy5 660/20nm	Pacific Blue V450 Horizon Hoechst 33342 DAPI CFP-YFP FRET 450/40nm
PE PI 575/26nm	APC-Cy5.5 695/40nm	Pacific Orange AmCyan 525/50nm
PE-Texas Red PI Qdot605 610/20nm	APC-Cy7 APC-H7 780/60nm	Qdot605 610/20nm
PE-Cy5 PerCP PI 7-AAD 675/20nm		
PE-Cy5.5 PerCP-Cy5.5 695/40nm		
PE-Cy7 780/60nm		

BD LSR II

Argon 488nm λ – channel	Red HeNe 633nm λ – channel	Violet Diode 405nm λ – channel	UV 350-60nm λ – channel
FITC Alexa488 530/30nm	APC Cy5 660/20nm	Pacific Blue V450 Horizon Hoechst 33342 DAPI CFP-YFP FRET 450/40nm	Hoechst 33342 DAPI Indo-1 440/40nm
PE PI 575/26nm	APC-Cy7 APC-H7 780/60nm	Pacific Orange AmCyan 525/50nm	Indo-1 530/30nm
PE-Texas Red PI Qdot605 610/20nm		Qdot605 610/20nm	
PE-Cy5 PerCP PI 7-AAD 675/20nm			
PE-Cy5.5 PerCP-Cy5.5 695/40nm			
PE-Cy7 780/60nm			

BD FACSCanto II

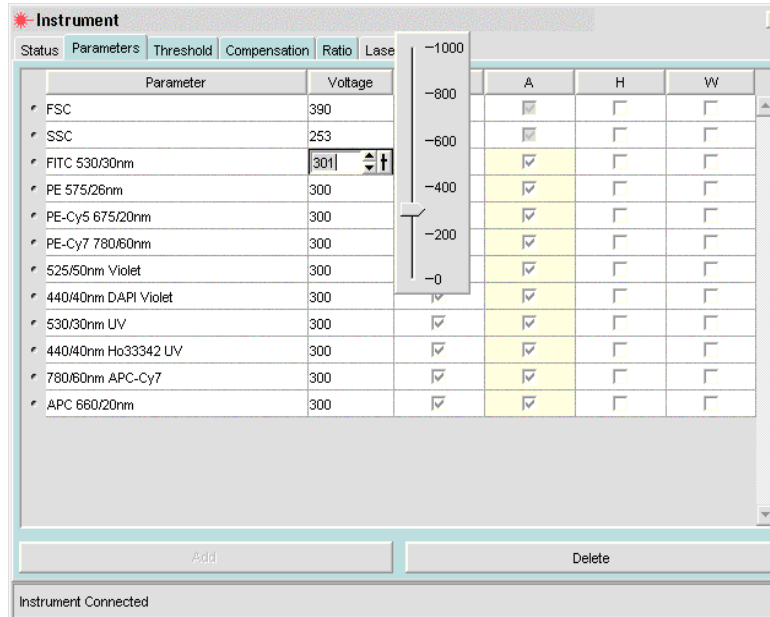
Argon 488nm λ – channel	Red HeNe 633nm λ – channel	Violet Diode 405nm λ – channel
FITC Alexa488 530/30nm	APC Cy5 660/20nm	Pacific Blue V450 Horizon Hoechst 33342 DAPI CFP-YFP FRET 450/50nm
PE PI 575/26nm	APC-Cy7 APC-H7 780/60nm	Pacific Orange AmCyan 510/50nm
PE-Cy5 PerCP PE-Cy5.5 PerCP-Cy5.5 PI 7-AAD 650LP		
PE-Cy7 780/60nm		

BD FACScan

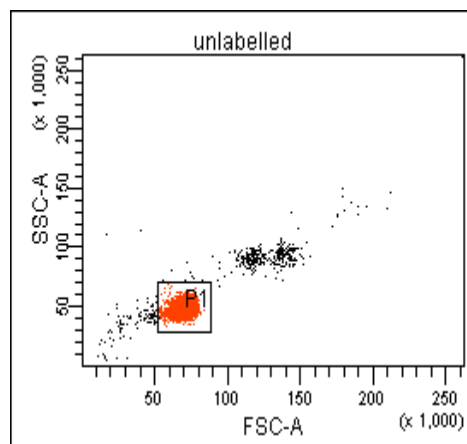
Argon 488nm λ - channel
FL-1 FITC Alexa488 530-30nm
FL-2 PE PI 575/25nm
FL-3 PE-Cy5 PerCP PE-Cy5.5 PerCP-Cy5.5 PE-Cy7 PI 7-AAD 650LP

Voltage Adjustments

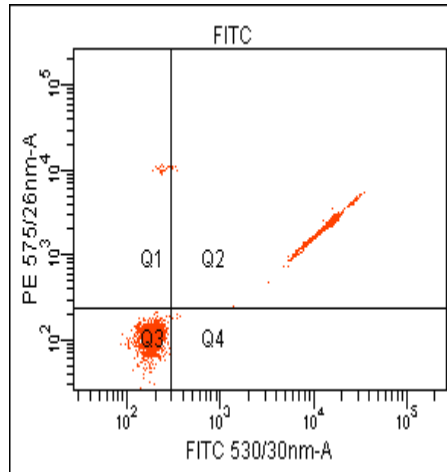
First place a tube of labelled BD Compensation Beads on the instrument and adjust the voltage on the **Instrument panel** if needed, this will be the case on the LSR II. On the Canto II this is usually not necessary due to the CS&T software will automatically preset the voltage to run, FITC, PE, PE-Cy5/PE-Cy5.5/PE-Cy7, APC/Cy5, APC-Cy7, Pacific Blue and AmCyan/Pacific Orange. See voltage control dialogue box display below.



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



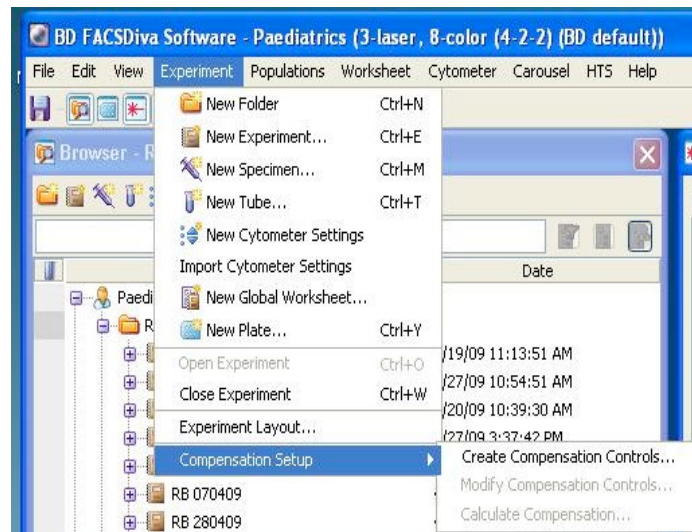
Next place a tube of the first labelled BD Compensation beads, and if necessary adjust the voltage so that the fluorescent peak is at 10^4 , see figure below. On digital instrumentation it is the convention not to have the negative population in the origin.



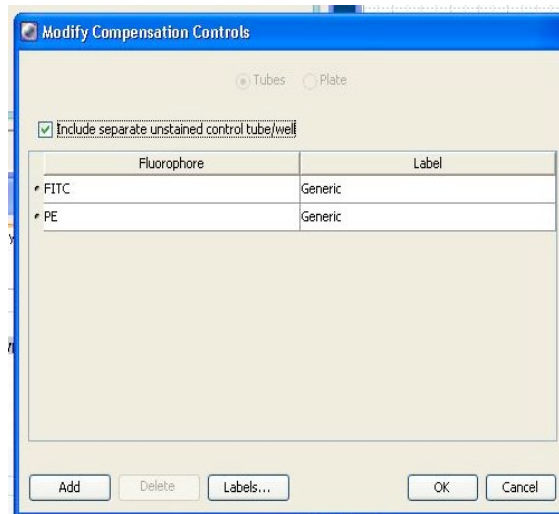
This procedure is repeated for all fluorophores to be used in the experiment.

Automated Software

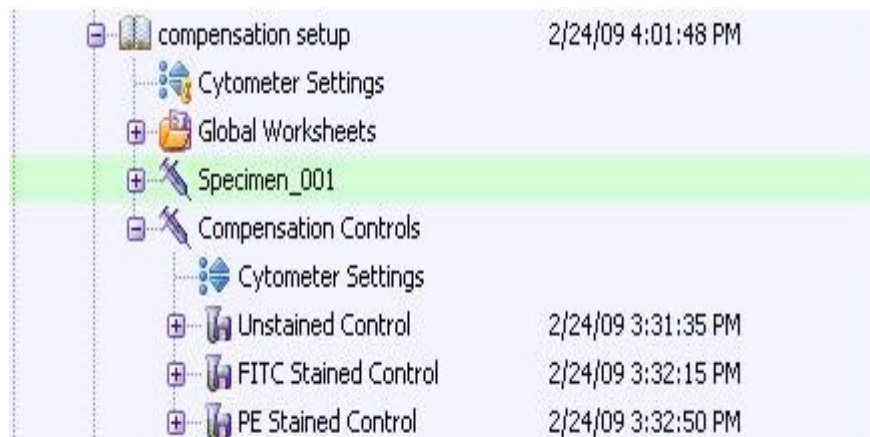
Once the voltages for each fluorophores are set the investigator can then setup the automated compensation matrix. This is done by *clicking* on **Experiment** (when a data folder is open), chose **Compensation Setup** and then *click* on **Create Compensation Controls**



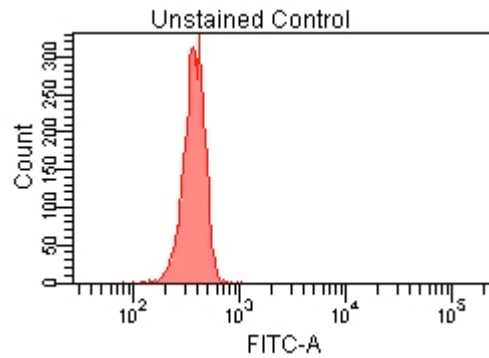
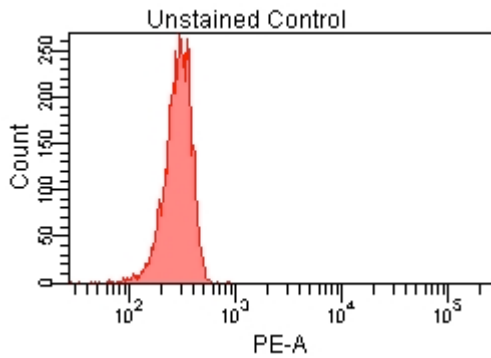
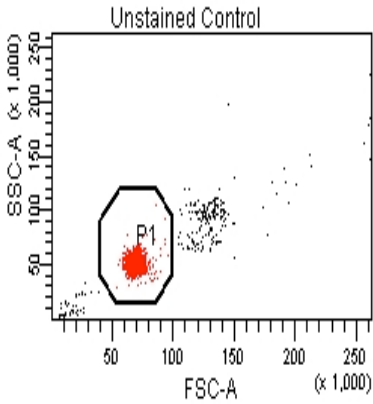
There is then a choice to choose the parameters, see figure below



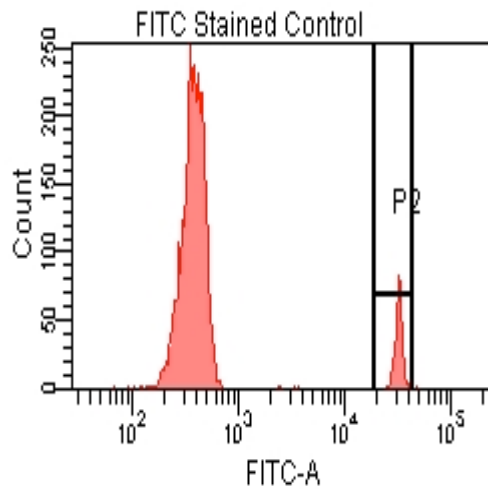
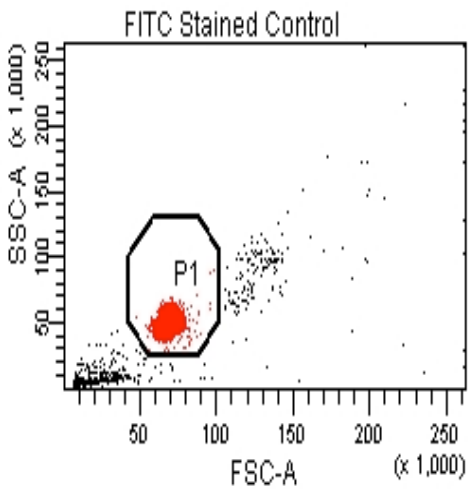
The software then creates a separate **Specimen** folder in the **Experiment** folder labelled **Compensation controls** and there will be a tube for each fluorophores chosen, see figure below, showing unlabelled, FITC and PE compensation tubes.



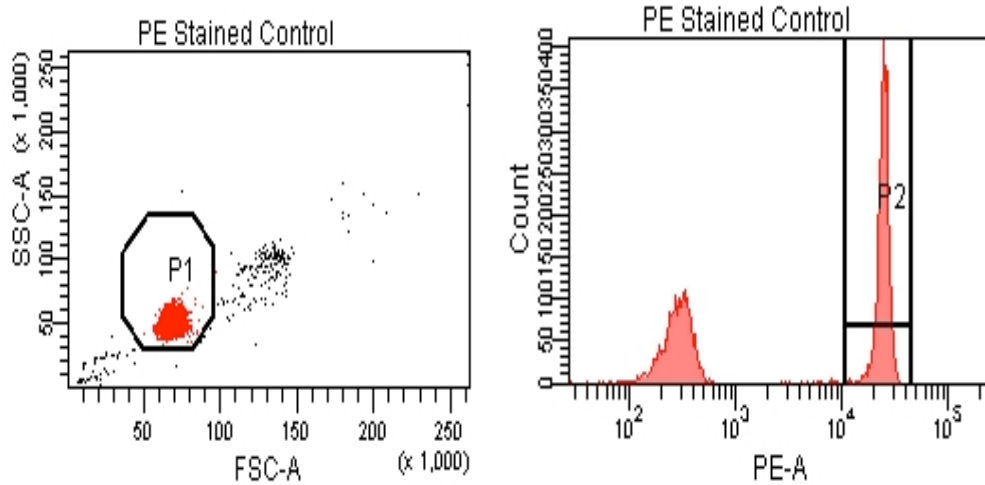
Each of the compensation tubes are then collected following the list generated in **Compensation Controls**. The unlabelled tube will be displayed automatically as shown below.



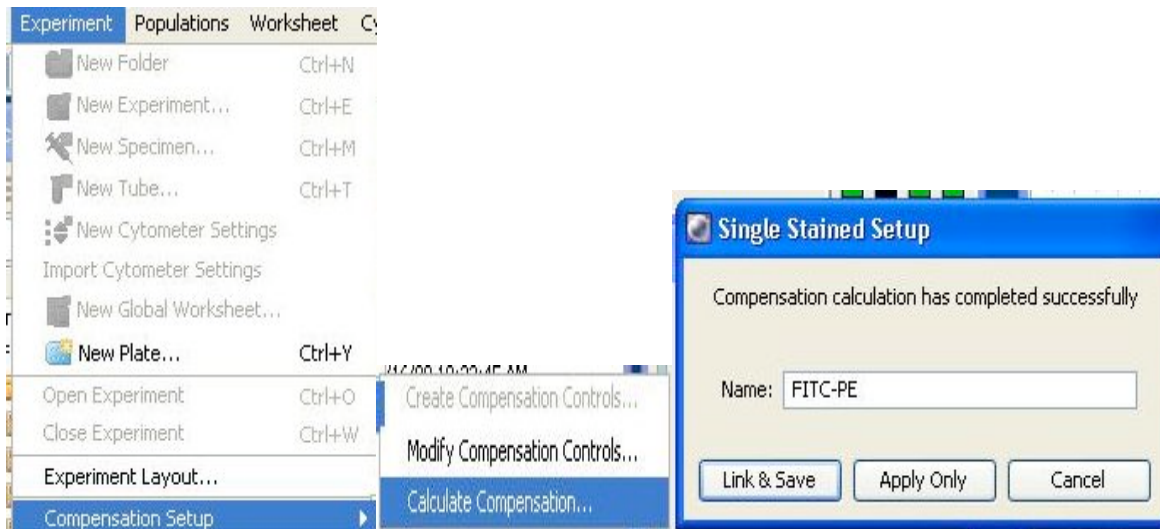
The **FITC** and **PE** compensation controls are then collected next showing the display for **FITC**, see figure below.



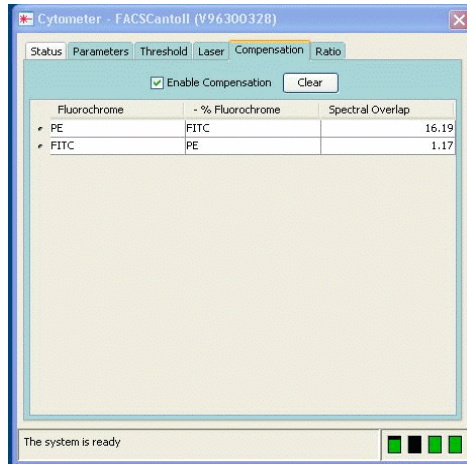
PE compensation control, see figure below



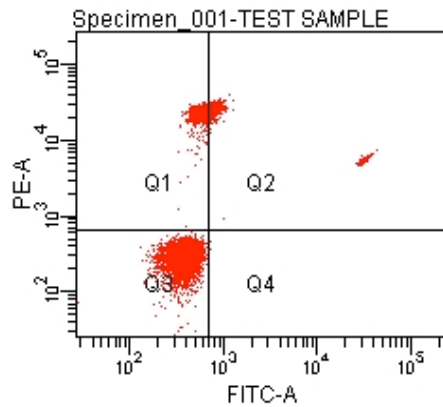
The compensation is then **Calculated** via **Experiment** and **Compensation Setup** followed by **Calculate Compensation** and save under **Link & Save** option.



The compensation matrix under the test samples will then show the calculated percentages in the **Compensation Matrix** dialogue box, see figure below.

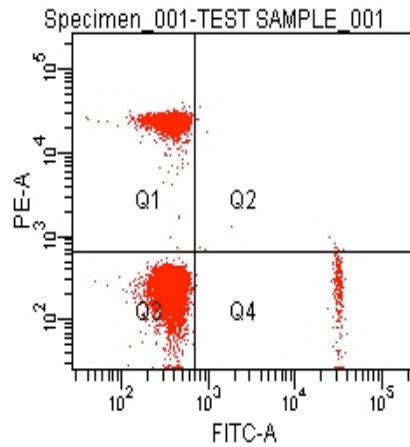


The test samples can now be collected, uncompensated files are shown below.



Population	#Events	%Parent	FITC-A Median	PE-A Median
P1	9,159	91.6	448	373
Q1	2,031	22.2	598	24,347
Q2	1,695	18.5	760	25,061
Q3	5,430	59.3	370	290
Q4	3	0.0	690	367

Automated compensated test samples are shown below,



Population	#Events	%Parent	FITC-A Median	PE-A Median
All Events	10,000	###	362	307
P1	9,118	91.2	362	306
Q1	3,610	39.6	361	24,520
Q2	15	0.2	1,767	825
Q3	5,259	57.7	358	226
Q4	234	2.6	32,134	219

The FITC compensation from the PE is checked by comparing the Median channel values of the FITC to blank beads or the values shown in quadrants Q3 and Q4 on the PE-A axis should be similar. Likewise to check the PE subtraction from FITC Median channel values are compared in quadrants Q1 and Q3 on the FITC-A axis.

The automated compensation matrix is generated by multiple cross checking the matrix every time a compensation tube is collected this is not possible to carry out in a manual fashion as they are just checked once for each fluorophores. For example checking all the FITC subtractions from all the fluorophores, this matrix is then pasted onto the PE compensation tubes then the PE subtractions against all the other fluorophores are checked. Some changes to the compensation matrix later may well affect FITC and these will not be checked if a manual approach is used making the compensation matrix less accurate than it should be. Having said that all automated compensation matrices should be checked as is the case above the Median fluorescent channels for Q3 and Q4 for FITC subtraction from PE is slightly incorrect (228 versus 219) and this can then be corrected during a manual check of the automatically generated matrix.

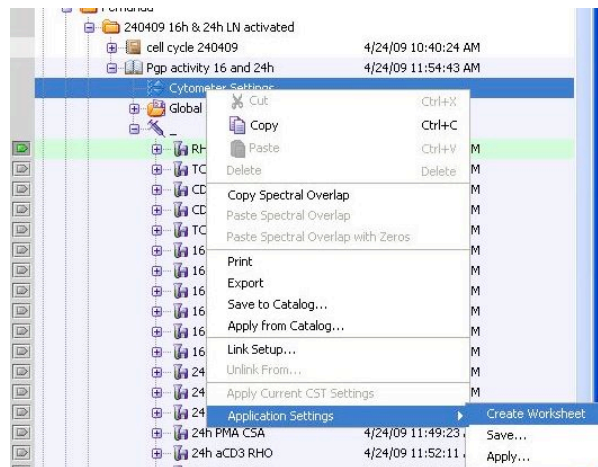
Cytometer Setup & Tracking or CS&T

The Becton Dickinson Cytometer Setup and Tracking software allows the definition of a baseline performance of the digital cytometer. This is done by running CS&T tracking beads daily which are made up of 3 bead populations negative, intermediate and bright fluorescence from which the median fluorescence intensity (MFI) and percent robust CV (%CV) are recorded for the 3 bead populations on all detectors, including forward and side scatter parameters. The software algorithms analyse linearity of signals, detector efficiency (Qr), optical background (Br), electronic noise, area scaling factors and laser delays. The software then adjusts PMT voltages for fluorophores at the wavelengths the set for each detector e.g. 530/30nm for FITC. The software then presets all the PMTs and FSC diode which are suitable for organic fluorophores. The Forward and Side Scatter parameters have to be changed when running cells as the detectors are set for 3um latex beads. When using fluorescent dyes, proteins or Qdots the CS&T preset voltages may well not be suitable and will require adjustment and **Automated Compensation** will need to be calculated from experimental cells.

Once the baseline is set (until the next service) daily performance checks should be run every time the instrument is used. All QC parameters are then plotted against time and are flagged if these go outside the preset range. The software will adjust voltages so that the CS&T beads always fall in the same channel. This high level of reproducibility allows the user if they have used the **Automated BD Compensation** system to use the same compensation matrix without the need for using single colour controls every time an experiment is carried out, see section below for more information.

CS&T linked data folders

This application allows the investigator to link the instrument settings and the Automated BD Compensation bead generated compensation matrix to subsequent experiments.



If CS&T performance checks are carried out daily the user can link the adjustments made to PMT voltages etc to their experimental file.

This can be done by right *clicking* on the **Cytometer Settings** subsection under the **Experimental Folder** move the cursor to **Application Settings** and select **Save**, see figure above. Now any changes in instrument performance will be detected by the **CS&T Tracking** system and will validate any compensation matrix linked to the CS&T settings. Users should test new lots or changes of stocks of directly conjugated antibodies (or old antibodies) with the BD Compensation beads and adjust the compensation matrix in an automated fashion accordingly.