

# APPLICATION INFORMATION

## Flow Cytometry

### 3-COLOR COMPENSATION

Raquel Cabana,\* Mark Cheatham, Jay Enten, Yong Song, Michael Thomas,\* and Brendan S. Yee  
 Beckman Coulter, Inc., Miami FL 33196  
 \*NPE Systems, Pembroke Pines FL 33027

#### Introduction

When analyzing cells or particles stained with multiple dyes using the flow cytometer, it is well known that the emission spectra of the dyes can overlap. In this case, the observed signal in a fluorescence channel is a concatenation of the overlapping spectral responses of the dyes. Color compensation is the process used to subtract the overlapping emission spectra so as to report the values of each dye independently. Without color compensation, the results produced in multicolor flow cytometric experiments may be difficult to analyze.<sup>1,2</sup>

This application note is intended to demonstrate the method used by the Cell Lab Quanta SC Flow Cytometer to compensate the data for the emission spectral overlap.

#### How Color Compensation is performed by the Quanta SC Flow Cytometer System

The goal of compensation is to correctly quantify each dye with which a particular cell is labeled. To accomplish this, it is necessary to know the measured amount of each color, how much each dye overlaps, and then apply the appropriate mathematical formula to adjust the data to the correct value.

The Cell Lab Quanta SC software does the necessary calculations based on a matrix of values obtained by running single color controls. The matrix is inverted to remove the effects of multiple interactions between colors.

The experimental set up in Figure 1 shows how

the software automatically determines the correct amount of compensation one color at a time.

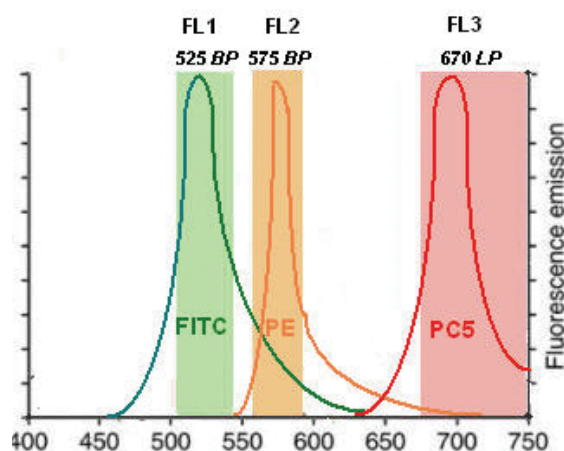
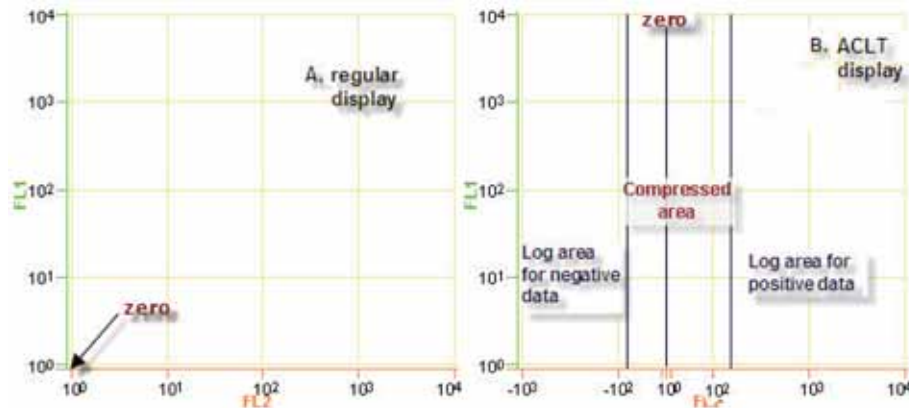


Figure 1: Wavelength (nm)

While color compensation is performed, it may be useful to adjust the axes and scaling of the plot to display the data in an easy-to-view format. The Axial Compressed Log Transformation (ACLT) display (Figure 2) makes it easier to tell if the correct amount of compensation has been applied to a single color control by showing the data above and below the zero level. ACLT shows a log scale to the left and right of the zero and a compressed area near the zero. The amount of compression can be varied either by the system or the user.

Compensation Settings can be saved and recalled in the Cell Lab Quanta SC software for repeated experiments.



**Figure 2.** FL1 vs FL2 regular display is shown in A, the arrow points to the  $10^0$  channel. The ACLT display is shown in B, the  $10^0$  was re-positioned so that values  $< 10^0$  can be seen. The amount of compression is adjustable and determines the location of the negatives log, compressed and positive log areas on the graph.

## Experimental Setup

Below are some important facts to keep in mind when attempting to apply compensation to an experiment.

- Choose dyes that minimize the spectral overlap in the collection area of the filters being used.
- Use cells stained with a single color as controls to determine the amount of compensation. Studies have shown that setting up flow cytometry systems with cells stained with single color immunofluorescent markers is the optimal method for calculation of the color compensation required for the experiment.<sup>(3,4)</sup> Other methods using mixtures of colored beads may not be as satisfactory as they do not represent real cells stained with real antibody-based fluorochromes.
- Applying too much PMT voltage to one of the detectors may make it difficult to compensate out overlap into that detector.
- Only the fluorochromes used in the experiment should be used for color compensation setup.
- Compensation settings are tied to the sample preparation and instrument configuration. Any adjustment to the gain, PMT voltage, cells, dyes, or filters require that the amount of compensation be re-determined for the experiment.

## Sample Compensation Experiment

### 3 color compensation for FITC, PE and PC5

When running immunofluorescence experiments it is essential that color compensation is properly set to subsequently determine how many cells are

positive and negative for each monoclonal antibody. Other dye combinations also require color compensation. Their utilization follows a similar path to the one shown below.

The fluorochromes FITC, PE and PC5 are the dyes of choice for use with the default optical configuration of the Cell Lab Quanta SC (525BP filter in FL1, 575 BP filter in FL2 and 670 LP filter in FL3).

## Reagents

CYTO-COMP™ Cell Kit PN 6607023

CD45-FITC 100 tests IM0782\*

CD45-PE 100 tests IM2078\*

CD45-PC5 100 tests IM2653\*

Isotypes: MABs IgG1-FITC/IgG1-PE/IgG1-PC5 (CYTO-STAT® triCHROME™ PN 6607054 or OptiClone® PN 1672)\*\*

CYTO-COMP™ Cell Kit is a preparation of lyophilized human lymphocytes and reconstitution buffer used in conjunction with monoclonal antibodies to establish and monitor color compensation settings for multicolor analysis.

MABs against the Leukocyte Common Antigen (CD45) are preferred because CD45 has the highest expression, which produces a high intensity signal in all the fluorescent detectors.

### Sample Preparation (Single Color Compensation)

- Reconstitute the CYTO-COMP™ Cell Kit following the instructions on the package insert.
- Prepare five tubes as follows:

\*Sold in the United States as an "Analyte Specific Reagent. Analytical and Performance characteristics are not established." Sold in other geographies "For Research Only. Not for use in diagnostic procedures."

\*\*For Research Use Only. Not for use in diagnostic procedures.

- 1) Negative Control, in this particular case, a tri-color Isotype control to adjust the PMT voltages to place the negative populations in the first decade.
- 2) FITC CD45 only stained cells
- 3) PE CD45 only stained cells
- 4) PC5 CD45 only stained cells
- 5) Compensation Verification Control consisting of cells stained with tri-color MABs with different specificity, used to verify compensation.

- Add into different tubes 20  $\mu$ L of the tri-color isotype, MABs CD45 FITC, PE, PC5, and the selected tri-color verification control (i.e. CD45-FITC, CD4-PE, and CD3-PC5).
- Add 100  $\mu$ L of reconstituted cells to each vial.
- Vortex and incubate for 15 min. in the dark at RT.
- Add 1 mL of PBS
- Analyze within 1 hour.

## Instrument Setup

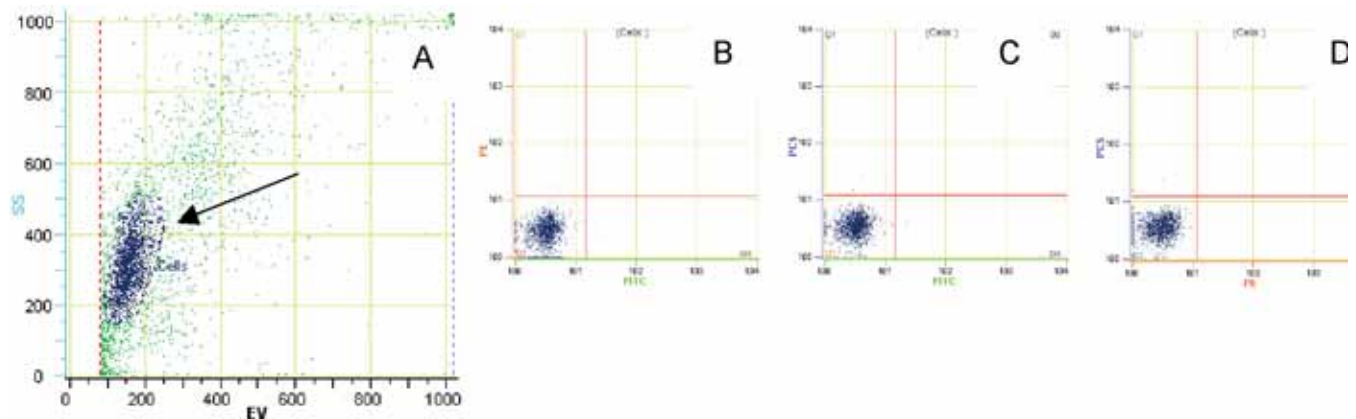
Ensure a proper system start up has been performed and the instrument is meeting expectation of HPCVs and channel means using Flow-Check™

Fluorospheres, as described in the Cell Lab Quanta SC Instructions for Use, Section 8.5.

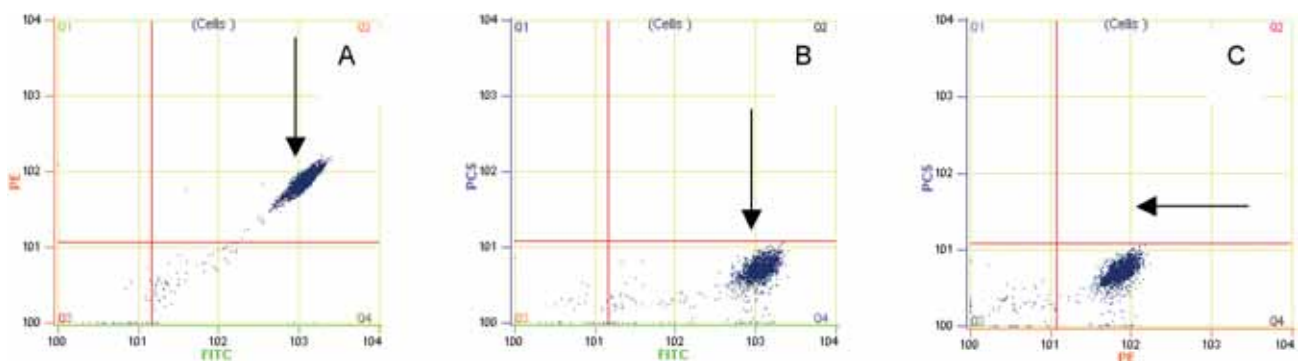
For a full description of instrument Color Compensation, please refer to the Instructions for Use, section 7.8.

### Brief description

- Load the Compensation Protocol for your application. In the Compensation Settings window, “Display Compensated Data” should be unchecked at this time.
  - 1) Run the Negative Control. Adjust or redraw the region around the lymphocyte population. Select Regions and set the lymphocyte region as the Gating Region. Set the LLD just to the left of the lymphocytes, thus excluding the cellular debris from the acquisition. (Figure 3.A)
  - 2) Select the Lymphs region as the gate for each of the following plots: FL1 log/FL2 Log, FL2 Log/FL3 log and FL3 log/FL1 Log. Adjust the PMT voltages in order to have all the negatives in the first decade and adjust the quadrants. (Figure 3)

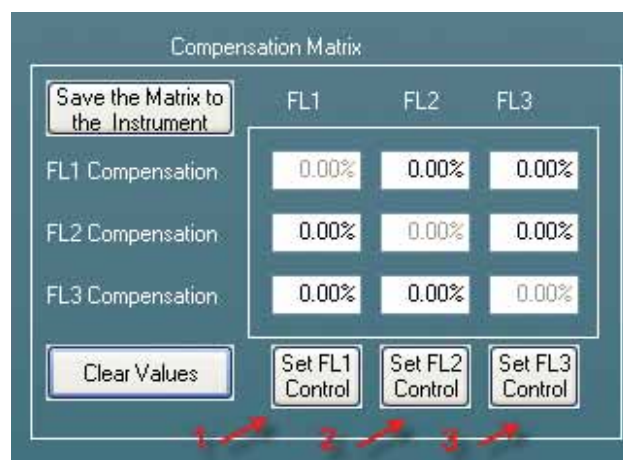


**Figure 3.** The arrow points at the lymphocyte population in A; the region created is selected as gating region. With the Negative Control the gains are adjusted to place unstained population on the first decade (B, C & D)



**Figure 4.** Run each of the single stained samples beginning with FITC

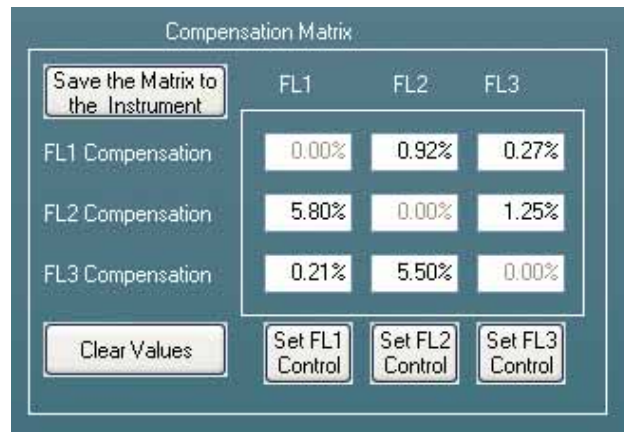
- 3) Run each of the single stained samples (tubes 2, 3 and 4) one at a time. Begin with the FITC stained sample as is shown in Figure 4 A, B & C.
- 4) In the software Compensation Settings window, for the FITC stained sample, press “Set FL1 Control” as indicated with the red arrow #1 (with the PE stained press #2 and with the PC5 press #3) in Figure 5.
- 5) First click on AUTO to perform the compensation. Ensure the “Axial Compressed Log Transformation” box is checked. If necessary, the ACLT display allows the data to be manually optimized. This can be done by utilizing the scroll bars as indicated below in Figure 6 A, B and C. It can be adjusted also from the main screen after setting up compensation: just right click on the 2-parameter display and select the option “Adjust Compensation”.
- 6) Click on Save to save the settings for future use. Select Display Compensated Data to show the compensated data on the main acquisition screen and select Move Zero Channel Compensated Values into the First Decade to ensure that all the data is displayed on the plot as seen in Figure 7. Select the option to save Compensated, Uncompensated or Both data files and then press Set.



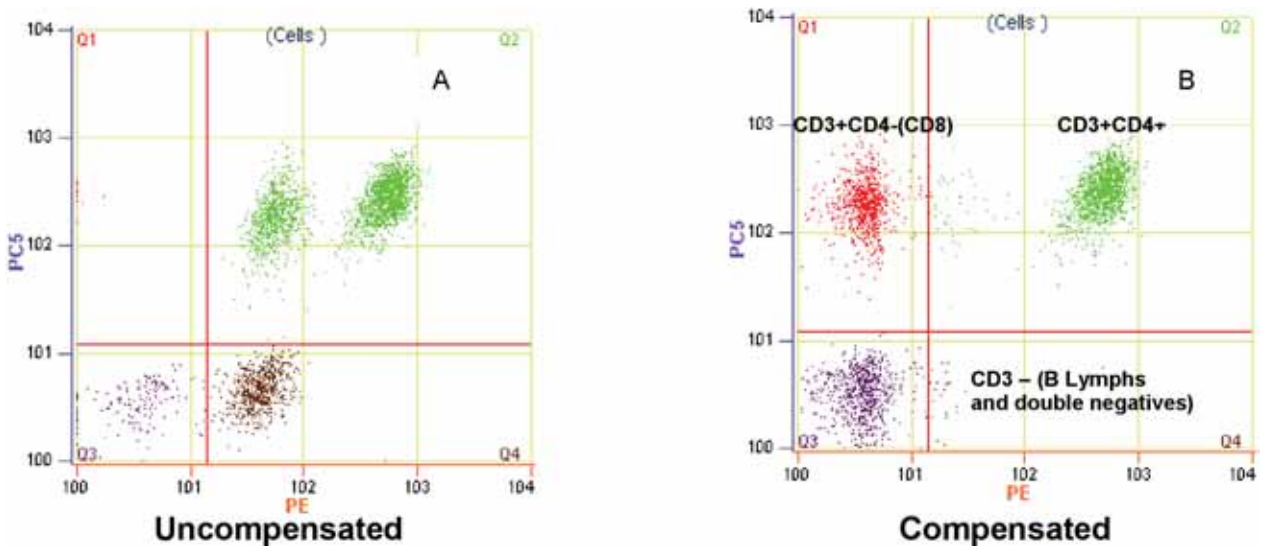
**Figure 5.**

- 7) The compensated CD45 FITC stained sample in the main screen should now look as is shown in Figure 8 A, B & C.
- 8) Perform the same procedure for PE and PC5 stained samples, see Figure 9.
- 9) Run the compensation Verification Control (tube 5) and verify the compensation settings obtained. The control should display negative and positive populations (% obtained can be verified to the CYTO-COMP™ Cell Kit package insert). Figure 10 A and B show CYTO-COMP™ Cell Kit stained with CD45-FITC, CD4-PE AND CD3-PC5; it can be seen that each population is in the correct place.





**Figure 9.** Compensation Matrix for FL1, FL2 and FL3



**Figure 10.** Run the compensation Verification Control

## Abbreviations

<i>ACLT</i>	Axial Compressed Log Transformation
<i>BP</i>	Band Pass filter
<i>CD</i>	Cluster of differentiation
<i>FITC</i>	Fluorescein Isothiocyanate
<i>HPCVs</i>	Half peak coefficient of variation
<i>LLD</i>	Lower level discriminator
<i>Log</i>	Logarithmic
<i>LP</i>	Long Pass filter
<i>MABs</i>	Monoclonal Antibodies
<i>min</i>	Minutes
<i>mL</i>	Milliliter
<i>PBMN</i>	Peripheral blood mononuclear cells
<i>PBS</i>	Phosphate Buffered Saline
<i>PC5</i>	Phycoerythrin Cyanin 5
<i>PE</i>	Phycoerythrin
<i>PMT</i>	Photomultipliers
<i>PN</i>	Part Number
<i>RT</i>	Room Temperature
<i>SC</i>	Side Scatter
<i>TM</i>	Trade Mark
<i>uL</i>	Micro liter

## Selected References

1. Shapiro HM. Practical Flow Cytometry, 4th Ed. Wiley-Liss, New Jersey 2003; 296-297.
2. Roederer M. Spectral Compensation for Flow Cytometry: Visualization Artifacts, Limitations and Caveats. Cytometry 2001; 45:194-205.
3. Diamond RA, De Maggio S. In Living Color – Protocols in Flow Cytometry and Cell Sorting. 1st Ed. Springer, Berlin 1999; 92-97.
4. Tung J, Parks D, Moore W, Herzenberg LA, Herzenberg L. New Approaches to Fluorescence Compensation and Visualization of FACS Data. Clinical Immunology 2004; 110:277-283.



*Developing innovative solutions in Systems Biology.*

**Innovate** Automate  
SIMPLIFY

Beckman Coulter, Inc. • 4300 N. Harbor Boulevard, Box 3100 • Fullerton, California 92834-3100  
Sales & Service: 1-800-742-2345 • Telex: 678413 • Fax: 1-800-643-4366 • www.beckmancoulter.com

### Worldwide Biomedical Research Division Offices:

**Australia** (61) 2 9844-6000 **Canada** (905) 819-1234 **China** (86) 10 6515 6028 **Eastern Europe, Middle East, North Africa** (41) 22 994 07 07  
**France** 01 49 90 90 00 **Germany** 49 21 513335 **Hong Kong** (852) 2814 7431/2814 0481 **Italy** 02-953921 **Japan** 03-5404-8359  
**Mexico** (55) 560-57770 **Netherlands** 0297-230630 **Singapore** (65) 6339 3633 **South Africa, Sub-Saharan Africa** (27) 11-805-2014/5 **Spain** 91 3836080  
**Sweden** 08-564 85 900 **Switzerland** 0800 850 810 **Taiwan** (886) 2 2378 3456 **Turkey** 90 216 309 1900 **U.K.** 01494 441181 **U.S.A.** 1-800-742-2345

B2006-0000

© 2006 Beckman Coulter, Inc.

Printed in U.S.A.