BECKMAN COULTER Life Sciences

Simplifying High Complexity Flow Cytometry Experiments: a Case Study Using an Analysis of T Cell Populations in Human Blood

Nicole Weit PhD[1], Michael Kapinsky PhD[2], Andreas Böhmler PhD[1]

[1]Beckman Coulter GmbH, Krefeld, Germany

[2]Beckman Coulter Immunotech S.A.S, Marseille, France

Corresponding Author Email Address: aboehmler@beckman.com

IN THIS PAPER YOU WILL

- Learn how adding parameters to your analysis of heterogeneous populations allows for better biological discrimination
- Learn how easy it is to use the compensation library feature enabled by CytoFLEX flow cytometer fiber array diodes
- See how to achieve even greater reproducibility in your cell population analysis using DuraClone* dry reagents
- Find a detailed gating strategy for enumeration of T cell populations including rare cell types
- Receive step-by-step instructions for CytoFLEX instrument set up for acquiring multiparameter data
- Learn how to choose fluorochromes for optimal panel performance

T Cell Biology

T cells form an integral part of the human immune system by fighting off infection and eliminating transformed cells. Over the last decades numerous T cells subsets with different functions and abilities have been identified (1). The wide spread use of monoclonal antibodies and multi-color flow cytometry allows for the routine identification of these subsets based on their immune-phenotype (2). However, the increasingly detailed understanding of different T cell subsets also results in a dramatic increase in the complexity of the antibody panels used. While two antibodies distinguishing the CD45 isoforms RA and R0 were deemed sufficient to differentiate between naïve and memory T cell subsets when these populations were first described, the recognition of effector memory and central memory, as well as CD45RA+ CD8+ effector memory cells makes the use of additional markers like CCR7 or CD62L necessary (3). In addition, some T cell subsets like Stem cell-like T cells are defined by the expression pattern of a high number of different antigens (4).

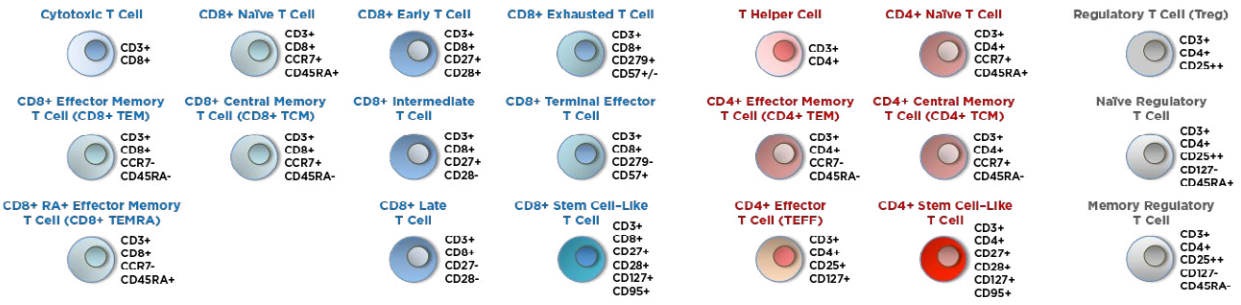

Figure 1. Depiction of various T cell populations and the surface markers used to identify them.

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Multicolor Panel Design: Some Basic Rules

The successful setup of a multicolor panel depends on some 'golden rules' that can be applied to any flow cytometer and application, because these rules are mainly based on the biological and physical properties of the antigen/fluorochrome combination, as well as the instrument configuration that is used for sample analysis. When these rules are consequently applied in panel creation, even dim populations that are usually difficult to analyze can be clearly detected.

Rule 1: Use bright fluorochromes for weak antigens. Fluorochromes differ in their quantum yield or 'brightness'. In order to resolve a weak antigen from unspecific background, it is best combined with a bright fluorochrome like PE or APC.

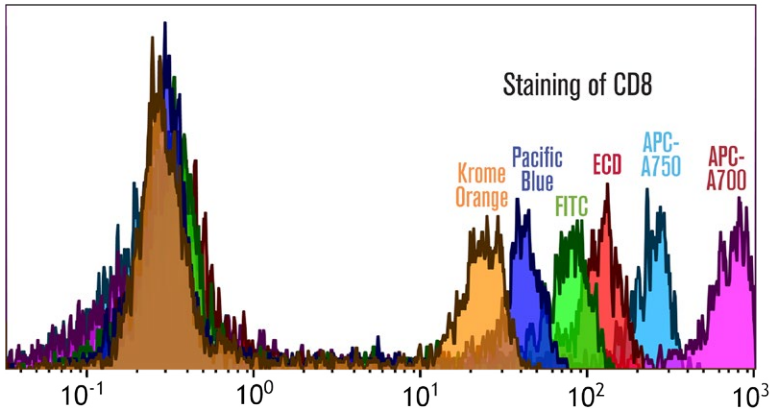

Figure 2. Fluorochrome 'brightness' as shown with CD8 combined with different fluorochromes.

Rule 2: Use dim fluorochromes for strong antigens. Based on their emission characteristics, fluorochromes are often prone to spectral overlap or 'spillover' into neighboring channels, an effect that needs to be corrected through compensation. On all digital flow cytometers, compensation may result in significant data spread, i.e. a widening of the population that ultimately leads to a loss of sensitivity within the channel where the overlap occurs. Since data spread significantly increases with combinations of strong antigens and bright fluorochromes, it makes sense to combine strongly expressed antigens with a less bright fluorochrome.

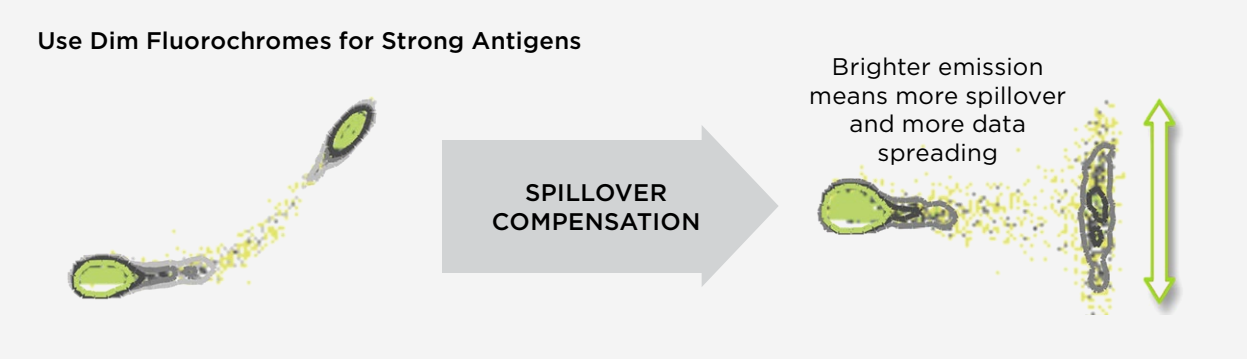

Figure 3. Effect of compensation and signal intensity on data spreading.

Rule 3: Put weak antigens in 'untouchable channels'. Use strong antigens with 'silent dyes'. Based on laser and filter configuration, each flow cytometer has detector channels that receive no or only very little spectral overlap from other fluorochromes. These 'untouchable channels' are best used for the detection of dimly expressed and/or sensitive antigens. Again depending on instrument setup, there are fluorochromes that show no or only minimal spill over into other channels; these 'silent' fluorochromes are ideal for strong antigens like gating markers. For each configuration of a flow cytometer, a 'spreading map' can be generated that shows the availability of untouchable channels, silent dyes and also the combinations that will result in strong spectral overlap and therefore data spreading.

Rule 4: Match co-expression patterns with spreading map. Rule 3 as described above is extremely powerful when combined with the expression patterns of the analyzed antigens. As an example, excluding antigens can easily be combined with fluorochrome combinations that will result in spectral overlap, while non-exclusive antigens are best combined with non-overlapping dyes.

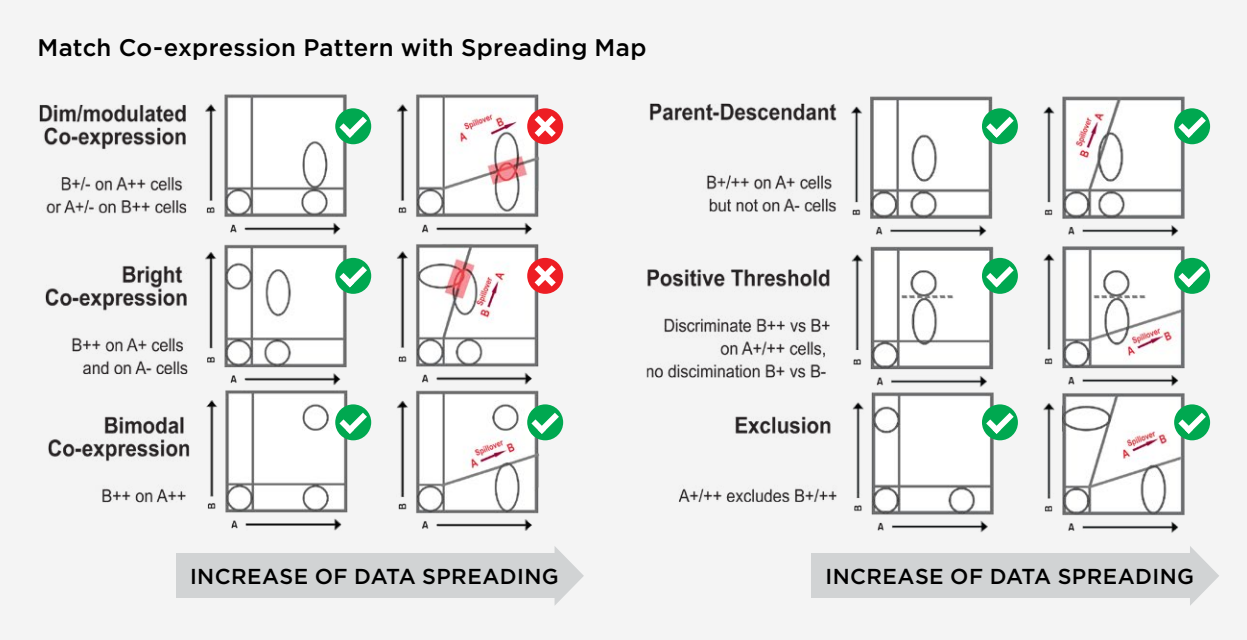

Figure 4. Antigen co-expression patterns and optimal fluorochrome combinations.

T Cell Panel Design and Gating Strategy

Here we describe the identification of different memory T cells subsets using a 13-color antibody panel based on a dry 10 color combination, DuraClone* IM T Cell Subsets tube plus 3 additional liquid drop-in markers.

488 nm					638 nm			405 nm				
FITC	PE	ECD	PC5.5	PC7	APC	A700	APC A750	Pacific Blue	Krome Orange	BV 605	BV 650	BV 785
CD 45RA	CCR7 (CD197)	CD28	PD1 (CD279)	CD27	CD4	CD8	CD3	CD57	CD45	CD95	CD25	CD127

Table 1. List of the fluorescent labels used to detect specific antigens for identification of T cell populations

Dry DuraClone* backbone

CD45 is used as a gating marker together with the SSC (side scatter) properties of the cells to identify lymphocytes. Krome Orange, a comparatively weak fluorochrome, was chosen in combination with CD45 as CD45 itself is a bright marker and Krome Orange has no negative impact on neighboring channels. CD3, CD4 and CD8 were included to identify all T cells and distinguish T-helper and cytotoxic T cells, respectively. Data spreading in the channels used for detection of APC, AF700 and APC-AF750 caused by fluorochromes excited by the 638 nm laser is generally high. The T cell lineage markers were combined with these fluorochromes to generate a “silent” backbone that does not influence the remaining 10 channels. Spillover from CD4-APC and CD8-AF700 into the channel used for detection of CD3-APC-AF750 is acceptable as CD3 is the parent marker and expressed on all CD4 and CD8 T cells. Spillover from CD8-AF700 into the channel used for the detection of CD4 is minimized by choosing AF700 over APC-AF700. Also, CD4 and CD8 are both bright markers with mutually exclusive expression patterns, so that spectral overlap can be tolerated (Figure 5).

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Several pre-cocktailed panels are available for key immune cell types. Available in convenient 25 test package. Each package includes three complete compensation kits with single color set of compensation reagents, containing lot-matched tubes for tandem dyes, specific for each panel.

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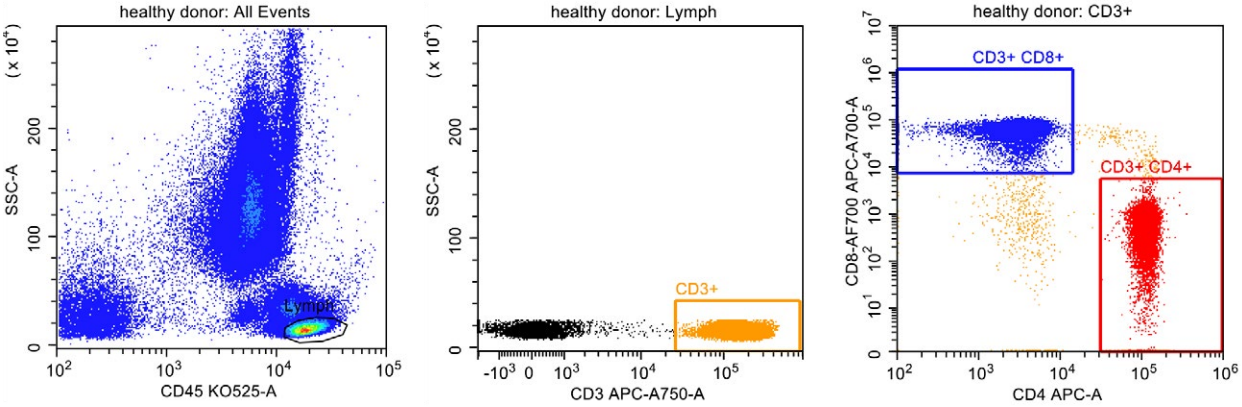

Figure 5. Sequential gating to distinguish CD3+ CD4+ helper T cells from (blue) CD3+ CD8+ cytotoxic T cells (red).

CD45RA and CCR7 were included into the panel in order to distinguish different memory T cell subsets.

The chemokine receptor CCR7 shows a weak and modulated expression and was therefore combined with the bright fluorochrome PE. CD45RA expression is sufficiently bright to be combined with FITC, which is detected in an "untouchable" channel not suffering from spillover from other fluorochromes.

CD45RA+ CCR7+ co-expression characterizes naïve T cells, which have not yet encountered their specific antigen. Lack of both CD45RA and CCR7 expression defines effector memory T cells (TEM), that are fast to produce cytokines in response to re-stimulation and have largely lost their ability to home to peripheral lymphoid organs (5, 6). Central memory T cells (TCM) are distinguished by their CCR7 expression and the absence of the CD45RA isoform. These cells have a high proliferative potential in response to re-stimulation but require a prolonged period of differentiation before they can produce cytokines (7). These subsets can be found both in the CD4+ helper T cell and in the CD8+ cytotoxic T cell compartments. Terminally differentiated effector T cells show a CD45RA+ CCR7- phenotype and are recognized in the CD8+ compartment as TEMRA (7) (Figure 6).

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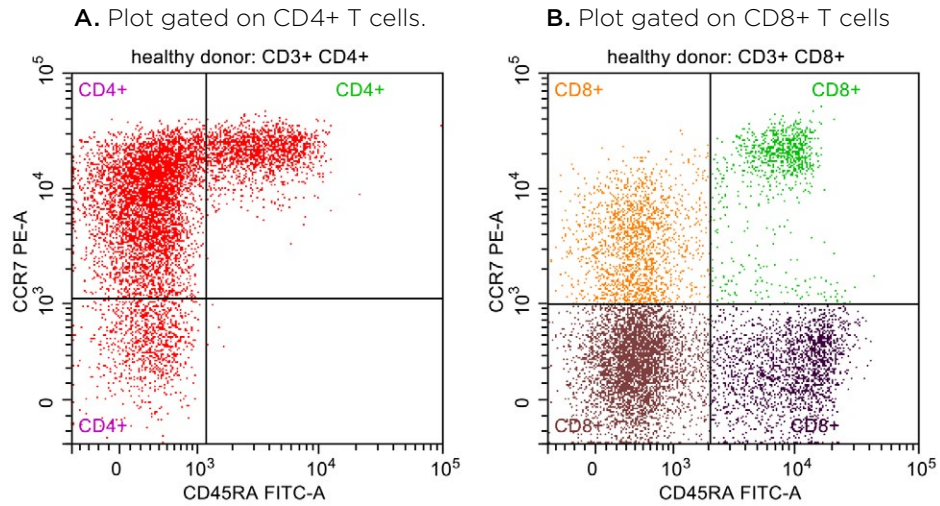

Figure 6. Identification of memory T cell subsets using CCR7 and CD45RA. A) Plot gated on CD4+ T cells. B) Plot gated on CD8+ T cells. Green: naïve CD8+ T cells, orange: CD8+ TCM, brown: CD8+ TEM, black: CD8+ TEMRA

The co-stimulatory molecules CD27 and CD28 can be used to further define CD8+ T cells. For CD4+ T cells this model has not been well defined yet. Both markers show bimodal expression patterns and can therefore be placed into channels that might suffer from some spillover from neighboring channels.

When gating on CD8+ T cells, naïve T cells and TCM express both CD27 and 28. Effector memory T cells can be divided into early (CD27+ CD28+), intermediate (CD27+ CD28-) and late (CD27- CD28-) TEM (8) (Figure 7). These subsets represent different stages of differentiation during which cytotoxic T cells acquire cytolytic properties and the ability to produce cytokines (9).

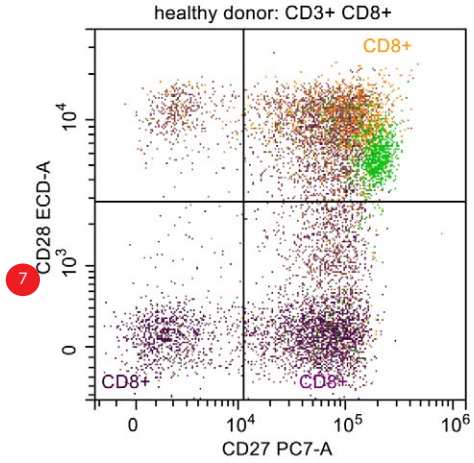

Figure 7. CD27 and CD28 expression patterns differentiate between different stages of CD8+ T cell development. Green: naïve CD8+ T cells, orange: CD8+ TCM, brown: CD8+ TEM, black: CD8+ TEMRA

CD57 expression on CD8+ T cells was shown to correlate with a senescent state with decreased proliferative potential (10). However, this state of late differentiation of CD57+ CD8+ T cells can be reversed under some stimulation conditions. CD279 (PD-1) is a mediator of inhibitory signals in T cells promoting immunologic tolerance. It is overexpressed on exhausted CD8+ T cells in chronic infectious states (11). These cells cannot be induced to proliferate any longer (12). The combination of CD57 and PD-1 in one tube allows for the distinction between exhausted CD8+ T cells (CD279/PD-1+ CD57+/-) and terminal effector CD8+ T cells (CD279/PD-1- CD57+) (Figure 8).

CD57 was combined with Pacific Blue, a “silent” fluorochrome to avoid spillover artifacts due to the high expression level.

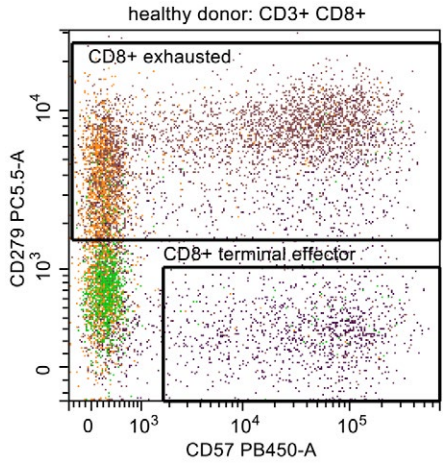

Figure 8. CD8+ T cells can be divided into exhausted cells unable to proliferate and terminal effector cells based on CD279/PD-1 and CD57 expression. Green: naïve CD8+ T cells, orange: CD8+ TCM, brown: CD8+ TEM, black: CD8+ TEMRA

Regulatory T cells (Treg) play a crucial role in down-modulating immune responses and thereby promoting tolerance and preventing autoimmunity. They are CD4+ and are characterized by an increased expression of the IL-2 receptor alpha chain CD25 and low/neg expression for the IL-7 receptor alpha chain CD127. Treg cells can be further subdivided in CD45RA+ naïve Treg and CD45RA- memory Treg (13). Figure 9 shows how CD4dim CD25++ Treg cells were gated (black). In the 2nd step, looking at all CD4+ CD25+ cells that include activated effector T cells (Teff) in addition to Treg, Treg show a lower CD127 expression than effector T cells (red). Additionally, the Treg subpopulation is subdivided in naïve CD127low CD45RA+ and memory CD127low CD45RA- cells.

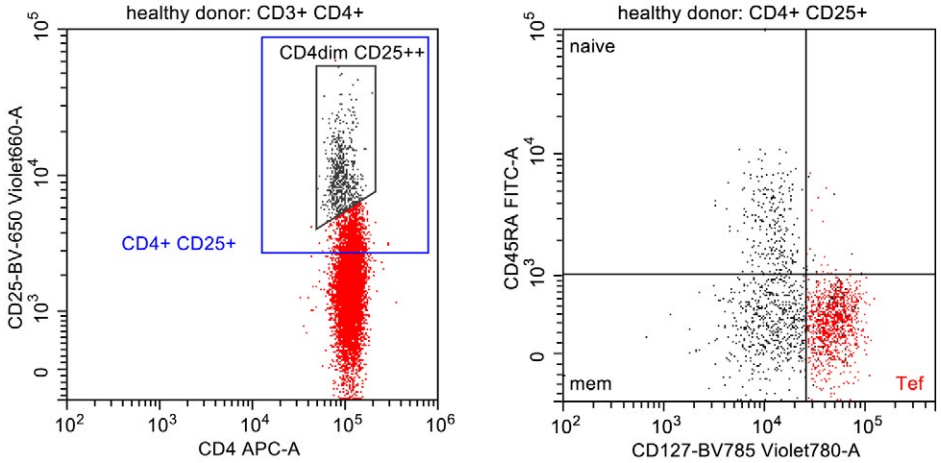

Figure 9. Detection of naïve and memory Treg populations after surface staining of CD4, CD25, CD127 and CD45RA.

Only recently a T cell memory subpopulation displaying stem-cell like properties like self-renewal and multipotency could be identified in humans and in mice (14). This cell type is currently moving more into the focus of clinical research as the persistence of the cells in humans could be shown up to 12 years after transplantation. This cell type is characterized by the expression of CD45RA, CCR7, CD27, CD28, CD95 and CD127. An example of the gating strategy can be found in Figure 10.

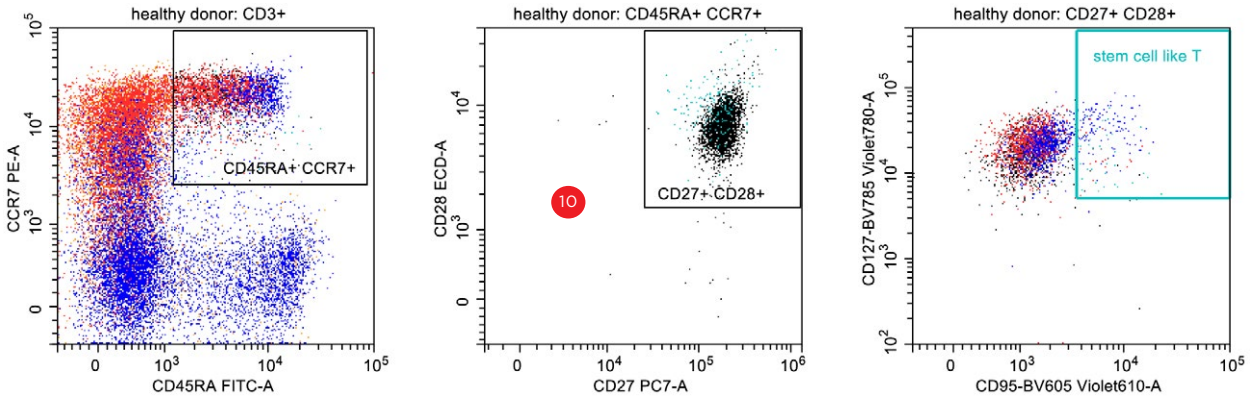

Figure 10. Sequential gating strategy for the identification of stem cell like memory T cells (stem cell like T).

Protocol

PREPARATION OF CONTROLS FOR COMPENSATION SET-UP

Each DuraClone* IM kit includes tubes of reagents in dry format for the multicolor panel as well as the single color conjugates in order to adjust detector and compensation settings.

Reagent	Supplier	Part Number
DuraClone* IM T Cell Subsets Kit	Beckman Coulter	B53328
VersaComp Antibody Capture Bead Kit	Beckman Coulter	B22804

1. Single color staining for compensation setup using DuraClone* Compensation Kit and VersaComp beads:
    a. Add 1 drop of well mixed VersaComp Antibody Capture Negative Beads and 1 drop of well mixed VersaComp Antibody Capture Positive Beads to the bottom of each tube of the lot-specific Compensation Kit included in the DuraClone* IM Kit. Vortex thoroughly.
    b. Incubate in the dark at room temperature (20 – 30 °C) for 15 min.
    c. Add 1mL of buffer (see VersaComp IFU) to each tube containing VersaComp beads.
    d. Vortex.
    e. Centrifuge at 300 x g for 6 min. Decant the supernatant.
    f. Resuspend the beads in 600 µL of buffer. Store bead solution in the dark until acquisition.

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SAMPLE PREPARATION

Reagent	Supplier	Part Number
DuraClone* IM T Cell Subsets Kit	Beckman Coulter	B53328
VersaLyse Lysing Solution	Beckman Coulter	A09777 or* IM3648
IOTest 3 Fixative Solution	Beckman Coulter	A07800 or* IM3515
Brilliant Violet 605 anti-human CD95	BioLegend	305628
Brilliant Violet 650 anti-human CD25	BioLegend	302634
Brilliant Violet 768 anti-human CD127	BioLegend	351330

* Depending on geography.

1. Pipet 100 µL of whole blood onto the bottom of the DuraClone* tube. Add 5 µL each of Brilliant Violet 605™ anti-human CD95, Brilliant Violet 650™ anti-human CD25, and Brilliant Violet 768™ anti-human CD127 antibodies (BioLegend)
2. Vortex the DuraClone* tube for 6-8 seconds.
3. Incubate in the dark at room temperature (20 – 30 °C) for 15 min.
4. Add 2 mL of VersaLyse Lysing Solution to each DuraClone* tube and vortex at high speed immediately for 1-3 seconds.
5. Incubate at room temperature for 15 minutes, protected from light.
7. After erythrocyte lysis, centrifuge the tube at 200 x g for 5 minutes and discard the supernatant by aspiration.
8. Re-suspend the pellet by addition of 3 mL of PBS to each tube.
9. Centrifuge the tube at 200 x g for 5 minutes and discard the supernatant by aspiration.
10. Re-suspend the pellet by addition of 0.5 mL of PBS supplemented with 0.1% of IOTest3 fixative solution.
11. The sample is now ready for acquisition.

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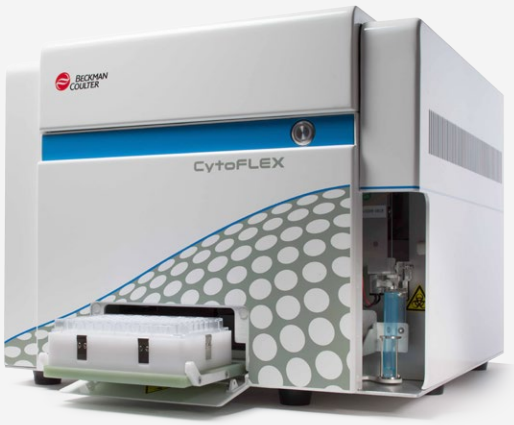

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CytoFLEX INSTRUMENT PREPARATION

Reagent	Supplier	Part Number
CytoFLEX Daily Quality Control Fluorospheres	Beckman Coulter	B53230
CytoFLEX Sheath Fluid	Beckman Coulter	B51503
Contrad 70 Cleaning Solution	Beckman Coulter	81911
FlowClean Cleaning Agent	Beckman Coulter	A64669

1. Run the CytoFLEX System Startup Program.
2. Run quality control (QC) procedure according to the CytoFLEX user manual.
3. Verify the detector configuration.

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer with the following laser and filter configuration:

- 405 nm, 80 mW solid-state diode laser
- 488 nm, 50 mW solid-state diode laser
- 638 nm, 50 mW solid-state diode laser

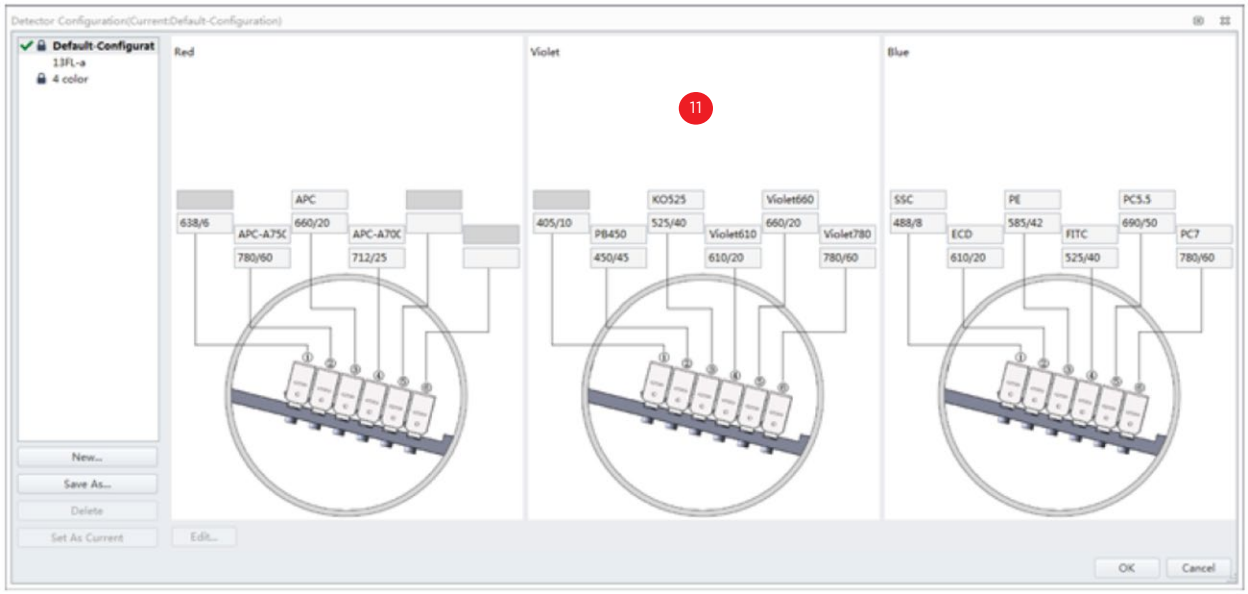

Figure 11. Standard filter configuration of a 3 laser / 13 color CytoFLEX flow cytometer.

As differences exist in the performance between analyzers, the authors cannot guarantee a similar appearance with the use of other Flow Cytometers.

ADD SINGLE STAINS TO THE COMPENSATION LIBRARY

NOTE: Perform the steps outlined in this section for every new lot of detection antibody.

1. Select “New Compensation” from the File menu for the acquisition of the single stains (Figure 12).

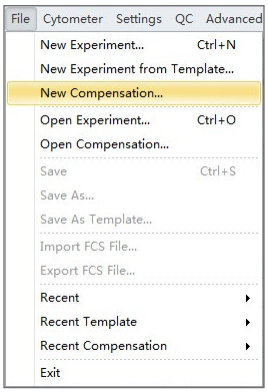

Figure 12. CytExpert File sub menu options.

2. Select a file location and save the new compensation as “DuraClone* xxx Lot yyy”, replacing xxx by the name of the panel, e.g. “IM T Cell” and yyy with the Lot number of the DuraClone* IM kit (Figure 13).

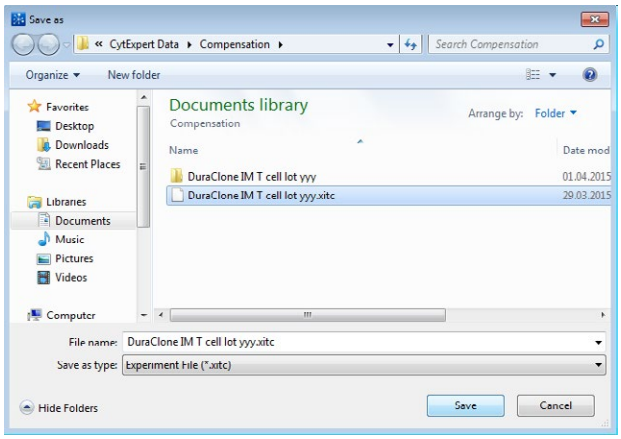

Figure 13. Recommended file naming conventions for CytExpert compensation library data.

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3. In the ‘Compensation Setup’ window (Figure 14), select tubes for every fluorochrome used, include the antibody used for staining as ‘Label’ and include the Lot no. for easy identification. Choose ‘Bead’ as sample type.

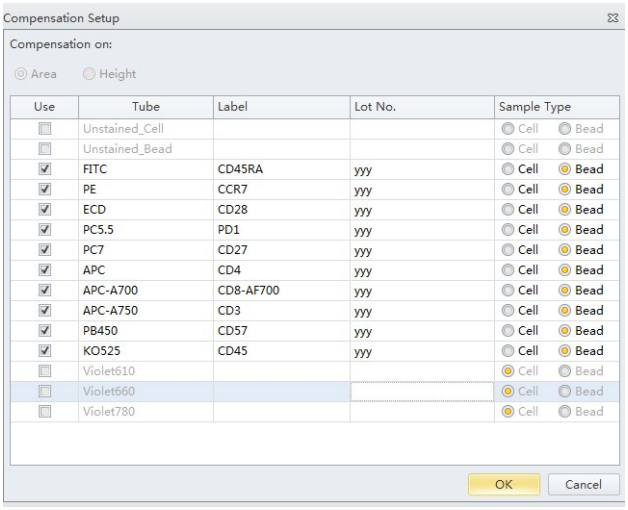


Figure 14. CytExpert Compensation Set-up window.

4. Import gain settings:
   a. Select “Acq. Setting…” on the left side of the screen. The Acq. Setting window appears (Figure 15).
   b. Select the “Gain” tab in the Acq. Setting window.
   c. Select “Recommended” to work with the instrument’s QC settings.

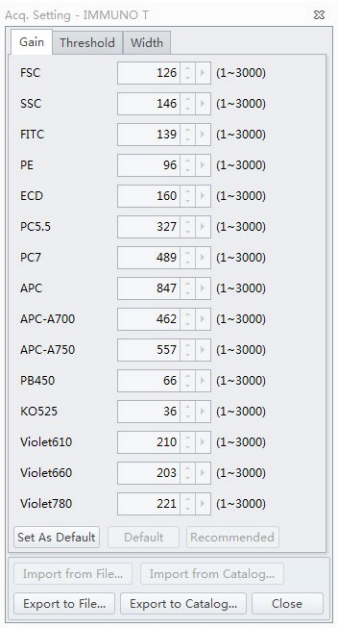


Figure 15. CytExpert window for setting Gain values for each fluorochrome channel.

5. Run the single positive control samples:

   a. Place the single positive tube in sample loading position.

   b. Select the appropriate, corresponding tube in the CytExpert software.

   c. Move the gate in the FSC/SSC plot so that it encloses the desired population. If necessary, move the positive gate so that it encloses the positive population (Figure 16).

   NOTE: Adjust the threshold in the first tube by using the threshold tool. Use the "Auto" function in the Plot Properties Menu to facilitate the display of the VersaComp beads.

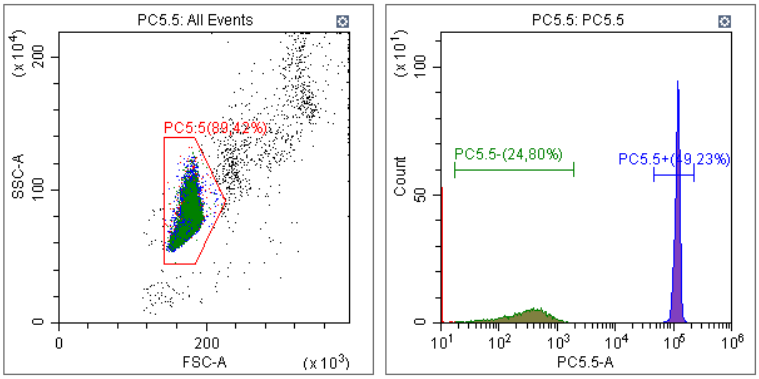


Figure 16. Establish the gates for the desired population.

6. Check the data from all acquired sample tubes and confirm that the gating is appropriate.

7. Select "Compensation Calculation" in the Compensation menu (Figure 17) to calculate the compensation values. The Compensation Matrix window (Figure 18) appears, displaying the calculated compensation values.

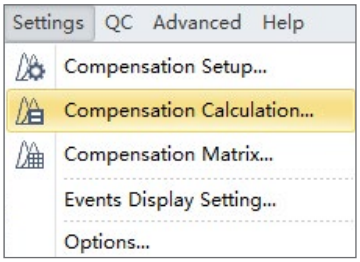
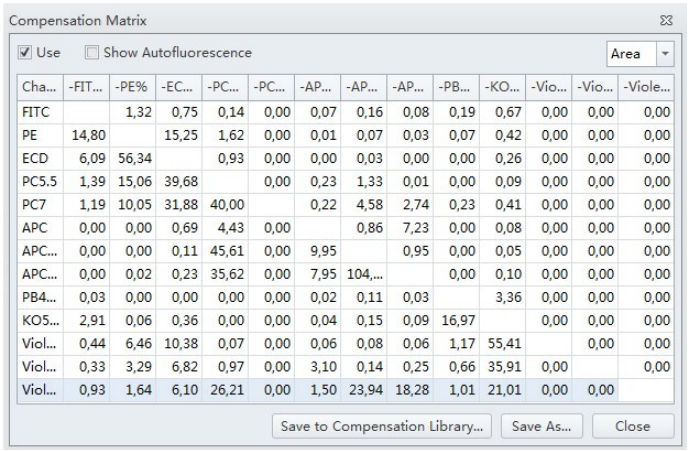


Figure 17. CytExpert settings submenu showing the command for setting the Compensation Calculations.



Cha...	-FIT...	-PE%	-EC...	-PC...	-PC...	-AP...	-AP...	-AP...	-PB...	-KO...	-Vio...	-Vio...	-Viole...
FITC		1,32	0,75	0,14	0,00	0,07	0,16	0,08	0,19	0,67	0,00	0,00	0,00
PE	14,80		15,25	1,62	0,00	0,01	0,07	0,03	0,07	0,42	0,00	0,00	0,00
ECD	6,09	56,34		0,93	0,00	0,00	0,03	0,00	0,00	0,26	0,00	0,00	0,00
PC5.5	1,39	15,06	39,68		0,00	0,23	1,33	0,01	0,00	0,09	0,00	0,00	0,00
PC7	1,19	10,05	31,88	40,00		0,22	4,58	2,74	0,23	0,41	0,00	0,00	0,00
APC	0,00	0,00	0,69	4,43	0,00		0,86	7,23	0,00	0,08	0,00	0,00	0,00
APC...	0,00	0,00	0,11	45,61	0,00	9,95		0,95	0,00	0,05	0,00	0,00	0,00
APC...	0,00	0,02	0,23	35,62	0,00	7,95	104,...		0,00	0,10	0,00	0,00	0,00
PB4...	0,03	0,00	0,00	0,00	0,00	0,02	0,11	0,03		3,36	0,00	0,00	0,00
KO5...	2,91	0,06	0,36	0,00	0,00	0,04	0,15	0,09	16,97		0,00	0,00	0,00
Viol...	0,44	6,46	10,38	0,07	0,00	0,06	0,08	0,06	1,17	55,41		0,00	0,00
Viol...	0,33	3,29	6,82	0,97	0,00	3,10	0,14	0,25	0,66	35,91	0,00		0,00
Viol...	0,93	1,64	6,10	26,21	0,00	1,50	23,94	18,28	1,01	21,01	0,00	0,00	

Figure 18. Example of the compensation matrix window obtained in the CytExpert software.

8. Select “Save as…” and choose a location and a name to save the generated compensation matrix, e.g. “DuraClone* xxx Lot yyy”.
9. Select “Save to Compensation Library…” and assign a key word, e.g. “DuraClone* xxx Lot yyy” to save the single color compensation values to the compensation library.

SETTING UP A NEW EXPERIMENT

1. Select “File - “New Experiment”.
2. Select location and a name, e.g. “DuraClone* xxx”, replacing xxx by the name of the panel, e.g. “IM T Cell”.
3. Select “Set Label” (Figure 19) from the Settings menu, and enter the antibodies used for staining as label description.

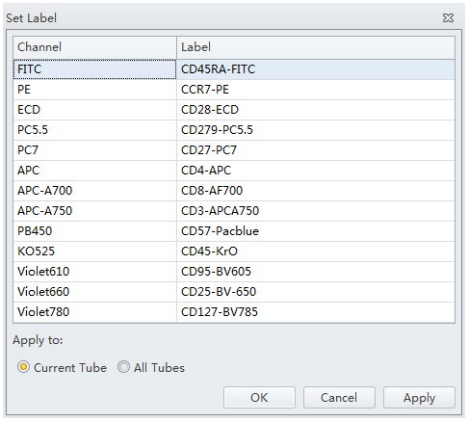


Figure 19. Dialogue box for selecting the labels used in the protocol.

4. Import the gains and compensation matrix by selecting “Compensation Matrix” from the “settings” menu (Figure 20) and selecting the “Import” function (Figure 21) and selecting the lot specific DuraClone* compensation matrix. Choose to import the compensation matrix and gain as shown in Figure 22.

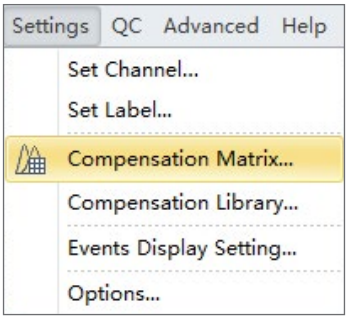


Figure 20. Settings submenu showing the Compensation Matrix command.

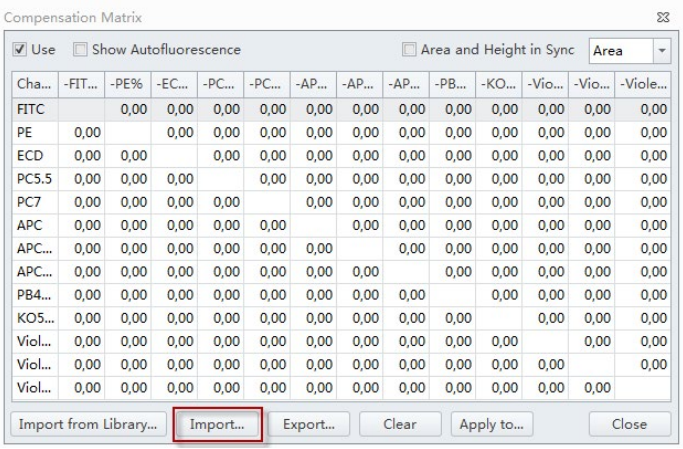


Figure 21. Compensation Matrix window with the Import button highlighted.

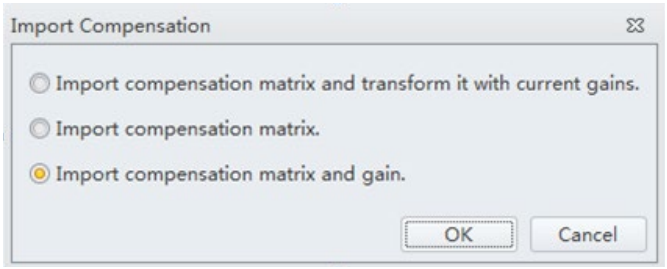


Figure 22. Import Compensation dialogue box with the correct option selected.

5. Generate the desired plots and gating strategy. Please make sure to include the plots of interest for the intended application and include suitable stopping conditions.
6. Run a tube stained with the full antibody panel and check for plausibility of patterns and populations.
7. If necessary, adjust the gain setting of each channel under the Gain tab in the Acq. Setting window. Raising the gain increases the signal. Lowering the gain reduces the signal.
8. Alternatively, use the Gain Control button on the toolbar in the graphic control area to adjust the gain values for cell population data to their desired levels, directly on the plots where the data appears during data collection. To save the adjusted gain settings as default go to “Cytometer” → “Acq. Settings...” → “Gain” → “Set as default”
9. Select “File” → “Save as template...” to generate an Experiment template for Routine use, e.g. “DuraClone* xxx”, replacing xxx by the name of the panel, e.g. “IM T Cell” (Figure 23).

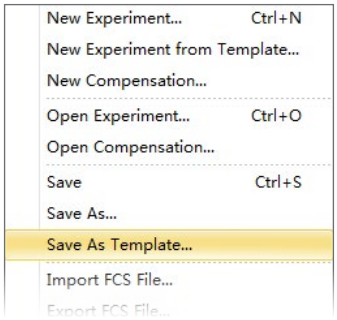


Figure 23. File submenu showing the save as template command.

CREATING AN EXPERIMENT FROM TEMPLATE

1. Open the “New Experiment from Template” dialogue box in the File menu (Figure 24).

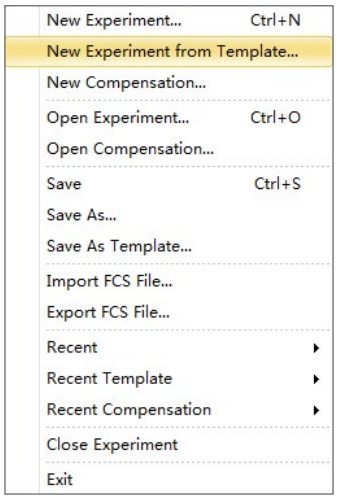


Figure 24. File submenu showing the New Experiment from Template command.

2. Select the previously saved template (e.g. “DuraClone* xxx”) and define a name and file location for the new experiment, e.g. “DuraClone* xxx date”
3. Open the “Acq. Settings” window and select “Gain”.
   a. Select “Recommended” to work with the QC settings.
   b. Select “Default” or select settings from the library to work with previously defined settings.
   4. Import Compensation from Library.
   c. Open the Compensation Matrix Window (Figure 25).
   d. Choose “Import from Library...” (Figure 26).

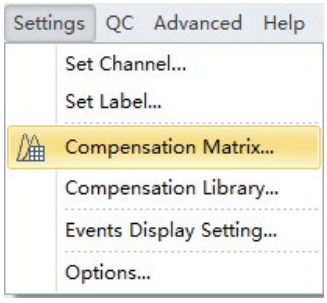


Figure 25. Setting submenu showing the Compensation Matrix command.

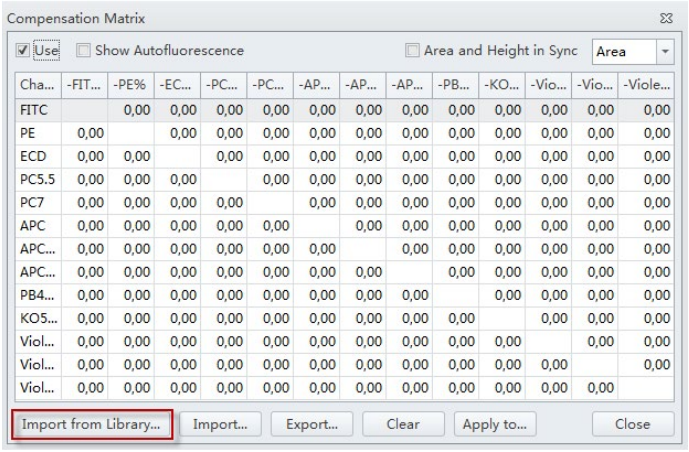


Figure 26. Compensation Matrix window highlighting the Import from Library button.

5. Choose the appropriate key word ("DuraClone* xxx Lot yyy"), load the single stains and click "OK" (Figure 27).

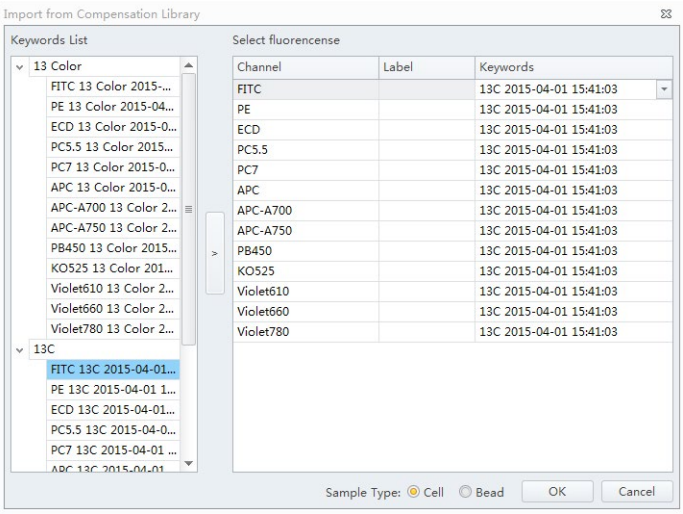


Figure 27. Demonstration of using the keyword finder to locate the appropriate compensation library.

6. Select "Import compensation matrix and transform with current gain" and click "OK" to import the compensation values (Figure 28).

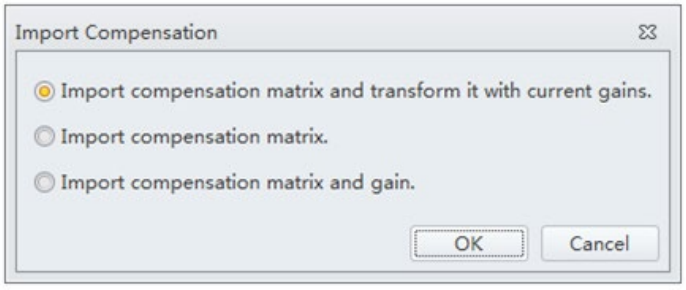


Figure 28. Import Compensation dialogue box showing the correct option selected.

7. Add tubes to the experiment and label as desired.
8. Start sample acquisition.

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15. CytoFLEX Flow Cytometer Instructions for Use PN B49006AB.

NOTE: The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer with 488 nm / 638 nm / 405 nm laser configuration. As differences exist in the performance between analyzers, the authors cannot guarantee a similar appearance with the use of other Flow Cytometers.

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