Resolving Dyes with Identical Emission Wavelengths on a 3-Laser CytoFLEX

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Introduction

Spectral flow cytometry is a relatively recent technological development, consisting of a method for unmixing different sources of light from a complex spectra of acquired light signals. Spectral flow cytometers differ from conventional flow cytometers in that they segment the emitted light into between 16 and 48 detectors that represent a variety of points across the visible spectrum. Conventional flow cytometers generally utilize only 2 to 5 detectors per laser, located at the optimal spectral ranges to collect emitted light from the most common fluorophores and tandem dyes. Beyond this, the inner workings of the instruments are similar: the light signals are digitized and converted into discrete numerical intensities by the instrument firmware.

One of the primary differences between spectral and conventional flow cytometers is how the numerical intensities are adjusted by the software. Spectral flow cytometers utilize Spectral Deconvolution to unmix the collected light intensities into the individual spectral contributions of the different fluorophores analyzed. Conventional flow cytometers utilize compensation calculations to correct for overlapping spectra from neighboring fluorophores in order to isolate the contributions of the fluorophores analyzed. Due to the advanced calculations performed by spectral deconvolution, compensation panels are said not to be required; spectral flow cytometers only require spectral (compensation) controls for each fluorophore analyzed and the software performs the calculations automatically.

Based upon our understanding of spectral deconvolution as essentially compensation, we hypothesized that we should be able to spectrally deconvolute highly overlapping fluorophores on a conventional flow cytometer using only compensation. In this poster, we demonstrate the spectral deconvolution of highly overlapping fluorophores using a 3-Laser CytoFLEX (V-B-R). We demonstrate that the CytoFLEX can easily separate FITC from VioBright-FITC and Pacific Blue from BV421 on both individual and co-labeled populations.

Materials

Item	Catalog #	Vendor
CD45-APC	IM2473U	Beckman Coulter
CD3-APC AF750	A66329	Beckman Coulter
CD3-FITC	IM1281U	Beckman Coulter
CD3-Pacific Blue	A93687	Beckman Coulter
CD8-FITC	IM0451U	Beckman Coulter
CD8-Pacific Blue	A82791	Beckman Coulter
CD4-VioBright FITC	130-113-229	Miltenyi Biotec
CD4-BV421	562425	Biolegend
VersaLyse	A09777	Beckman Coulter
3-Laser CytoFLEX V-B-R	B53000	Beckman Coulter
CytExpert Software v2.0	B49006	Beckman Coulter

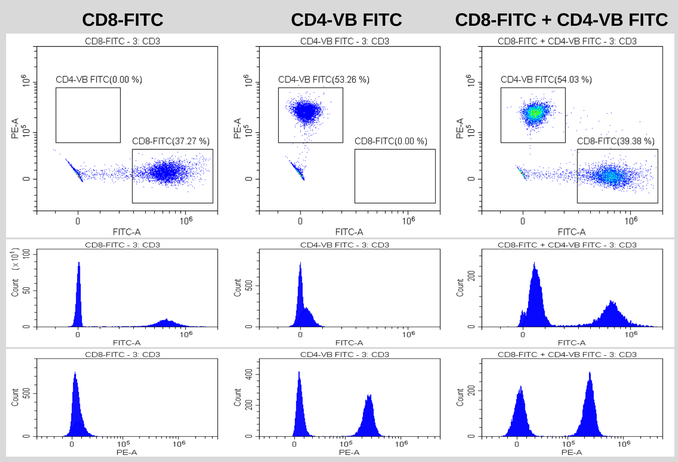
Methods

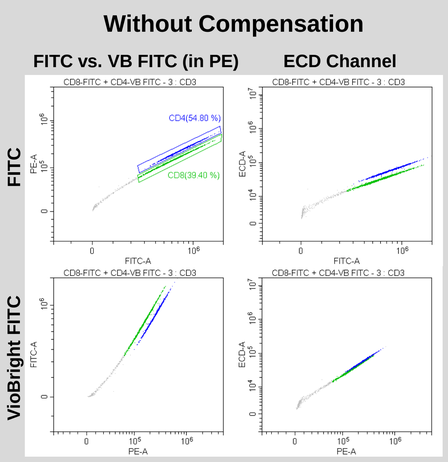
1. 100µL of fresh whole blood was stained for 20 minutes with each antibody combination.
   a) The test samples were each stained with CD45-APC + CD3-APC AF750 as the backbone for isolating T cells, and then the overlapping test fluorophore conjugates (i.e., CD4 and CD8). If CD3 was one of the overlapping test fluorophores, then only CD45-APC was used for the isolation of leukocytes.
   b) Additional single-color tubes were utilized as spectral controls.
2. After 20 minutes, the samples were lysed for 20 minutes with 1mL of VersaLyse, supplemented with 0.2% PFA (a.k.a. VersaFix), and then washed 2x with PBS.
3. The compensation (spectral deconvolution) panels were acquired, simply locating one of the overlapping fluorophores in the neighboring channel (i.e., PE or V525), without any instrument modifications.
4. Each sample was then acquired, collecting 30K events per sample.
5. Finally, the data were analyzed using CytExpert v2.0.

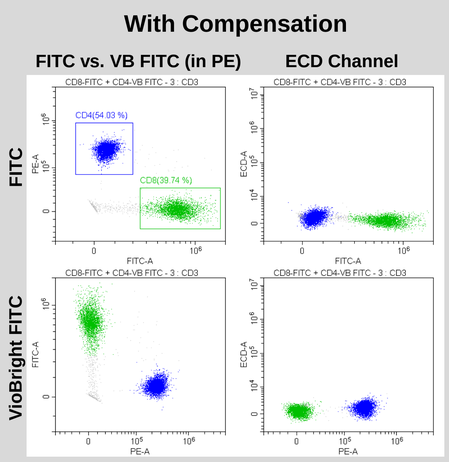
Results

Identical Emissions on Different Cells

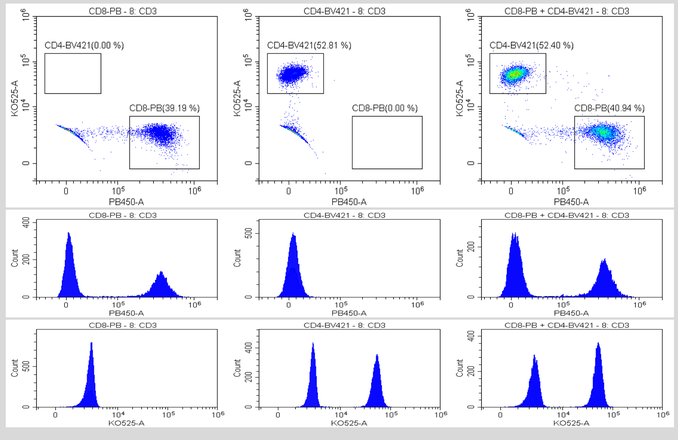
CD8-FITC vs. CD4-VioBright FITC

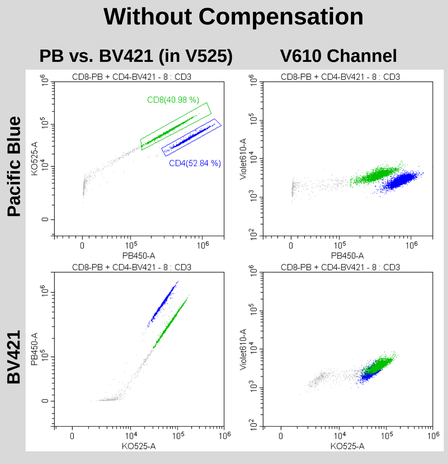


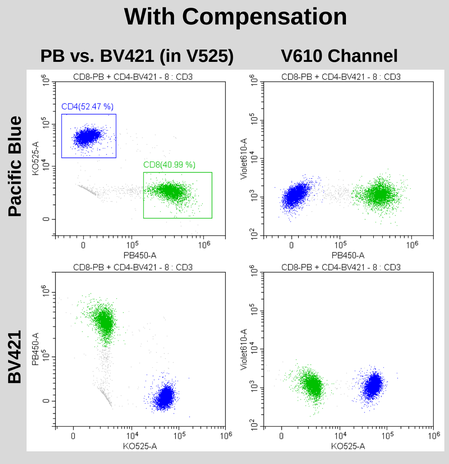




CD8-PB vs. CD4-BV421

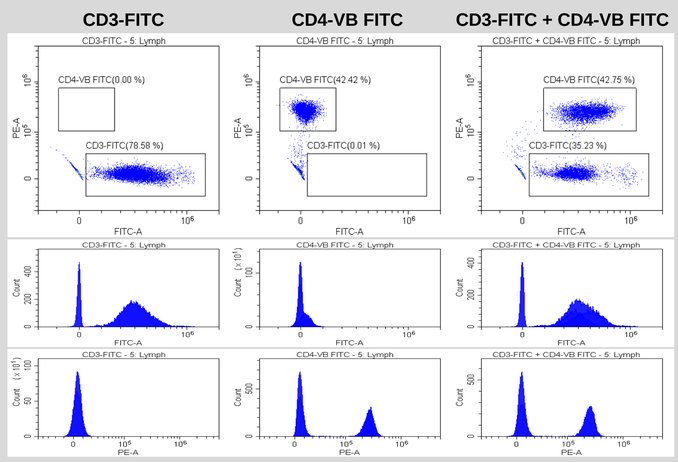




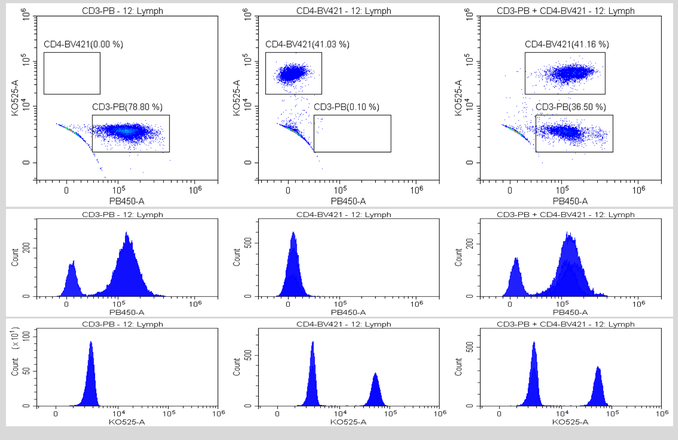


Identical Emissions on the Same Cell

CD3-FITC vs. CD4-VioBright FITC



CD3-PB vs. CD4-BV421



Discussion

In this poster, we have demonstrated that the CytoFLEX flow cytometer can deconvolute highly overlapping fluorophores on either different cells or the same cell using only compensation. This was performed by simply assigning one of the overlapping fluorophores to a neighboring channel in the compensation panel, and required no special instrument modifications. Ultimately, we conclude that spectral deconvolution is just compensation by a different name, but without the ability to make adjustments to the compensation matrix, otherwise known as black-box compensation. Rather than conferring ease-of-use, the inability to adjust compensation matrices may actually be detrimental to the ability to properly analyze flow-cytometry experiments.

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