Evaluating Multicolor Panel Performance

Introduction

Designing a high quality multi-color panel can be achieved when panel design guidelines are followed. In this paper, 2 different 8-color human whole blood immunophenotyping panels are evaluated and compared in order to understand some useful panel performance evaluation tools:

- Side-by-side visual comparisons of each marker in the single stained controls to the same marker in the multicolor sample to assess sample staining adequacy and resolution;
- Cross-stain index (CSI) to quantitatively understand how each dye is impacting the resolution of the others;
- Identically gated multicolor 2-D plots with identical bi-exponential scaling to visually compare different panels that use the same markers but slightly different dye combinations.

A hierarchy highlighting the markers used in the immunophenotyping panels is shown in figure 1, and the reagents used for the two panels are shown in table 1.

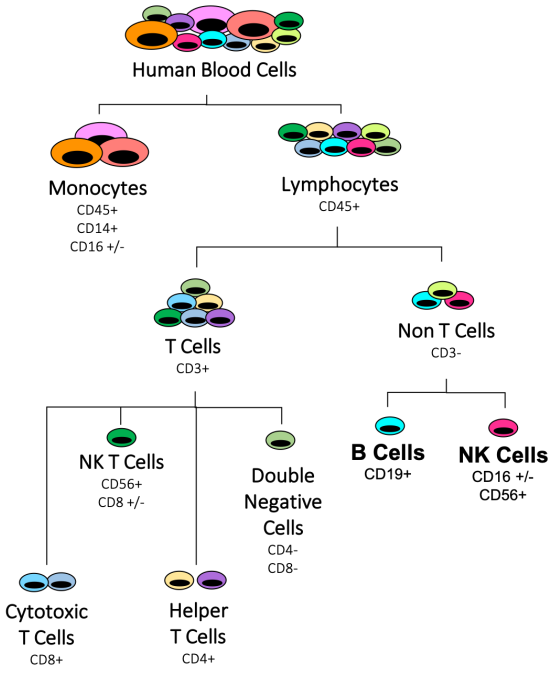

Figure 1: Hierarchy displaying cell subsets that can be identified with these 8-color blue laser excited dyes panels.

PANEL 1	Vendor	Cat. No.
CD3 FITC	BD Biosciences	349201
CD45 Alexa Fluor® 532	Thermo Fisher	58-0459-42
CD56 PE	BioLegend®	362507
CD16 PE/Dazzle™ 594	BioLegend	302054
CD4 PE/Cy™ 5	Thermo Fisher	15-0049-41
CD8 PerCP/Cy™ 5.5	Thermo Fisher	45-0088-42
CD14 PerCP-eFluor® 710	Thermo Fisher	46-0149-42
CD19 PE/Fire™ 780	BioLegend	982410
PANEL 2	**Vendor**	**Cat. No.**
CD8 FITC	BioLegend	344704
CD3 Alexa Fluor 532	Thermo Fisher	58-0038-42
CD56 PE	BioLegend	362507
CD16 PE/Dazzle™ 594	BioLegend	302054
CD45 PerCP	BD Biosciences	566392
CD4 BD Horizon™ Brilliant Blue 700	BD Biosciences	566393
CD14 PerCP-eFluor® 710	Thermo Fisher	46-0149-42
CD19 PE/Fire™ 780	BioLegend	982410

Table 1: Reagents used in each blue laser excited dyes panels.

Methods

1. Warm up the Northern Lights cytometer and run QC.
2. Prepare an unstained cell control and a single stained cell control for each reagent in the panels using a lyse/wash preparation.
3. Prepare a multi-color tube for each panel using the same lyse/wash preparation methods[1].
4. Create an experiment in SpectroFlo®. Set stopping criteria appropriately so you can clearly visualize each dye's full spectrum signature.
5. Select CytekAssaySetting user setting. Preview the unstained tube. Adjust the FSC threshold, SSC gain, and FSC gain as needed to see the lymphocytes and monocytes clearly and remove some of the debris. Create a worksheet with gating strategy for the multicolor tube. Then preview the multi-color tube to ensure signals are on scale (adjust gains if

1 - For panels with >1 Brilliant Blue reagent, add brilliant stain buffer (BDB #563794) to the multicolor sample tube prior to adding reagents to prevent polymer dye interactions.

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necessary). Record the control tubes at medium flow rate. Live unmix the experiment, then record the multicolor tubes.

Evaluating Panel Performance

Once a dataset exists for the panel that includes an unstained control, single stained cell controls, and a multicolor sample, there are several steps to complete to understand if the panel is working well, or if it could use further optimization:

1. Compare staining resolution of the single stained cell controls to the multicolor sample
2. Assess if spread is well managed in the panel
3. Determine if autofluorescence extraction is needed

Comparing Controls & Multicolor Staining Resolution

The first step in assessing panel performance is to compare the staining resolution of the marker for the cells in the single stained controls with the same marker when the cells are in the multicolor sample. The ideal situation is that the staining pattern and resolution seen in the single stained controls is identical to that in the multi-color tube. Often, the multi-color resolution can change due to increased spread or issues with the reagents when combined together. These changes must be assessed marker by marker. If the resolution change is minor and not impacting the readout of the assay, then the panel is working. If the changes are impacting the readout of the assay, then the panel design needs further optimization by experimenting with different antibody titers and/or assigning markers to different dyes. In figure 2, histograms for each marker are shown for the single stained cell control tubes compared to the multicolor tube using identical bi-exponential scaling. For both panels 1 and 2, the separation between the positive and negative cells in each control is maintained and is quite good. The width of the negatives in some of the histograms broaden slightly in the multi-color setting; for

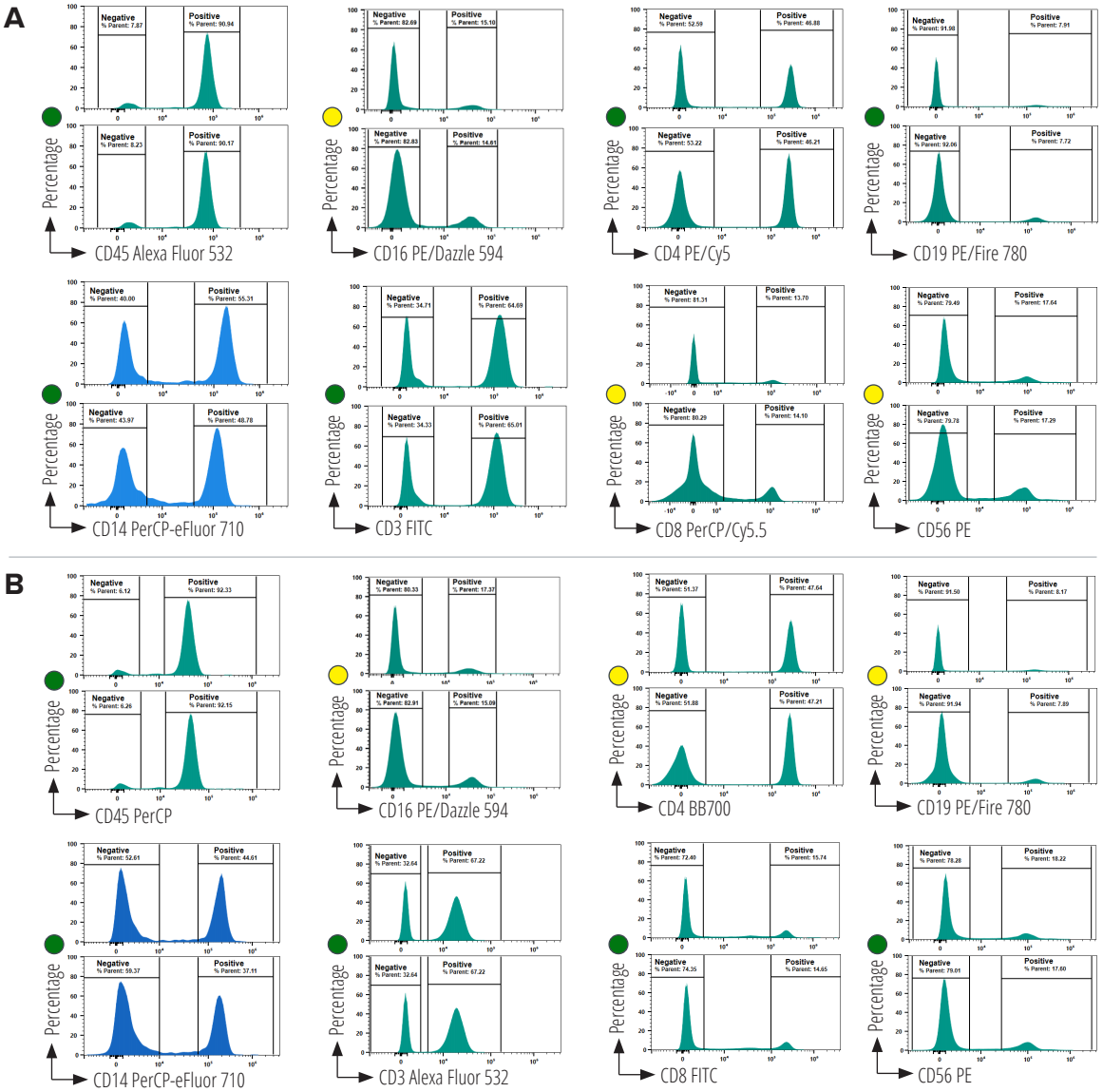

Figure 2: Comparison of staining pattern of single stained cell control tube (top plot of each plot pair) and multi-color cell tube for each reagent used in panel 1 (A) and panel 2 (B). Histogram data shown in blue is derived from monocyte scatter gate, and data shown in teal is derived from lymphocyte scatter gate.

Plot pairs with a green circle demonstrate comparable staining between the single stained cells control and the multicolor sample.

Plot pairs with a yellow circle demonstrate some resolution loss in the multicolor sample compared to the single stained control and need review to determine if the resolution is acceptable or not.

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example, in CD16, CD8 and CD56 in figure 2A and in CD16, CD4 and CD19 in figure 2B. However, the dim populations are still distinguishable so this slight decrease in resolution is acceptable.

Assessing Spread in a Multicolor Panel

In the last section, spread in both panels was easily observed by the broadening of some of the negative populations in the multicolor samples. This spread can be quantified with a metric called the cross-stain index (CSI). The formula is similar to stain index (SI), but instead of looking at the robust standard deviation (rSD) of the negatives for the dye of interest, denoted with a Y, CSI uses the rSD of the positive events of a dye X to understand how it spreads into and impacts dye Y:

$$SI_Y = \frac{Pos\ MFI_Y - Neg\ MFI_Y}{2\ (Neg\ rSD_Y)}$$

$$CSI_{YX} = \frac{Pos\ MFI_Y - Neg\ MFI_Y}{2\ (Pos\ rSD_X)}$$

To visually understand CSI, take a look at figure 3 showing two different fluorochromes and how they impact each other. Fluorochrome Y is shown in green and does not have any spread into fluorochrome X. Fluorochrome X shown in blue does spread into fluorochrome Y, so the stain index of fluorochrome Y will decrease from arrow 1 to arrow 2 when in the presence of fluorochrome X. The resolution of fluorochrome Y in the presence of fluorochrome X is captured mathematically using the CSI_{YX} formula above. To visualize fluorochrome Y's stain index reduction in the presence of fluorochrome X for every fluorochrome in a multi-color panel, the CSI and SI results can be presented as a color coded matrix where each cell in the matrix represents the following:

$$1 - \frac{CSI_{YX}}{SI_Y}$$

A reference CSI matrix for all Cytek instrument configurations is available in a document called the Fluorochrome Guideline, downloadable from the Resources section of the Cytek website (www.cytekbio.com) and from the help section inside SpectroFlo® software. The CSI matrix in the fluorochrome guideline was generated using single stained human CD4 cells. Since CD4 expression from donor to donor is fairly consistent, and since CD4 is expressed at a medium frequency in helper T cells from

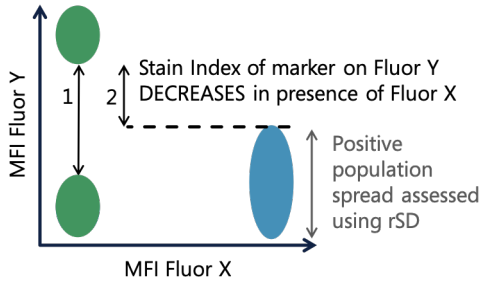

Figure 3: Example of how one fluorochrome (X shown in blue) can introduce spread and impact the resolution of another fluorochrome (Y shown in green). This behavior can be quantified using CSI.

peripheral human blood, the fluorochrome guideline CSI matrix is a good middle ground for examining and starting to understand where spread is likely to occur in a multicolor panel. Resolution loss due to spread can be predicted using the CD4-based CSI matrices if the level of expression of each marker relative to CD4 is known. To quantify loss of resolution due to spread in a specific panel with a variety of markers expressed at different levels, the CSI matrix can be calculated using single stained cell controls specific to that panel. To create the CSI matrix, request an Excel template from a Cytek technical application specialist, or follow this video tutorial to calculate it using FCS Express: https://youtu.be/O_PJ3w_e0hw.

There are several flavors of panel spread tools like the CSI matrix that have slight differences in their underlying math[2], but the general result and purpose are the same - to assess the impact of spread from one dye into another in a multicolor panel. In the CSI matrix, the impact one dye has on another is represented with a white and red gradient. Areas of the matrix that are dark pink to red have a lot of impact on each other, and areas of the matrix that are light pink to white have little or no impact on each other. For example, take a look at the two blue arrows in figure 5 where we've plotted the same CD3 negative lymphocyte subset from each panel to look at CD8 versus CD56 expression with identically matched bi-exponential scaling for each marker. When looking at the $CD8^-CD56^-$ cells on each plot, notice the width of this population. It is clear that this population in panel 2 is narrower, or has less spread, compared to panel 1. The spread is great enough in panel 1 that the $CD8^+CD56^+$ NK cells are more

2 - Another way to calculate spread: Nguyen, R. et al. Quantifying Spillover Spreading for Comparing Instrument Performance and Aiding in Multicolor Panel Design, Cytometry A. 2013 Mar; 83(3): 306-315.

Panel 1	FITC	Alexa Fluor 532	PE	PE/Dazzle 594	PE/Cy5	PerCP/Cy5.5	PerCP-eFluor 710	PE/Fire 780
FITC		36%	43%	31%	0%	23%	0%	0%
Alexa Fluor 532	0%		42%	47%	25%	41%	0%	4%
PE	0%	0%		53%	35%	51%	0%	10%
PE/Dazzle 594	0%	0%	0%		49%	60%	11%	25%
PE/Cy5	0%	0%	0%	20%		92%	80%	82%
PerCP/Cy5.5	0%	0%	0%	0%	58%		69%	80%
PerCP-eFluor 710	0%	0%	0%	1%	60%	88%		87%
PE/Fire 780	0%	0%	0%	0%	1%	49%	0%	

Panel 2	FITC	Alexa Fluor 532	PE	PE/Dazzle 594	PerCP	BB700	PerCP-eFluor 710	PE/Fire 780
FITC		84%	57%	45%	0%	20%	0%	0%
Alexa Fluor 532	0%		0%	17%	0%	13%	0%	2%
PE	0%	11%		57%	0%	43%	0%	17%
PE/Dazzle 594	0%	0%	0%		0%	55%	8%	29%
PerCP	0%	0%	0%	0%		65%	22%	41%
BB700	0%	16%	0%	41%	64%		82%	87%
PerCP-eFluor 710	0%	16%	0%	15%	35%	88%		86%
PE/Fire 780	0%	0%	0%	0%	0%	45%	0%	

Figure 4: Cross-stain index matrices for panels 1 and 2. Fluorochrome in the row impacts fluorochrome in the column. For example: in panel 1, PE/Cy5 has a lot of impact on PerCP/Cy5.5 shown by the dark red color, whereas PE has very little impact on FITC shown by the white color.

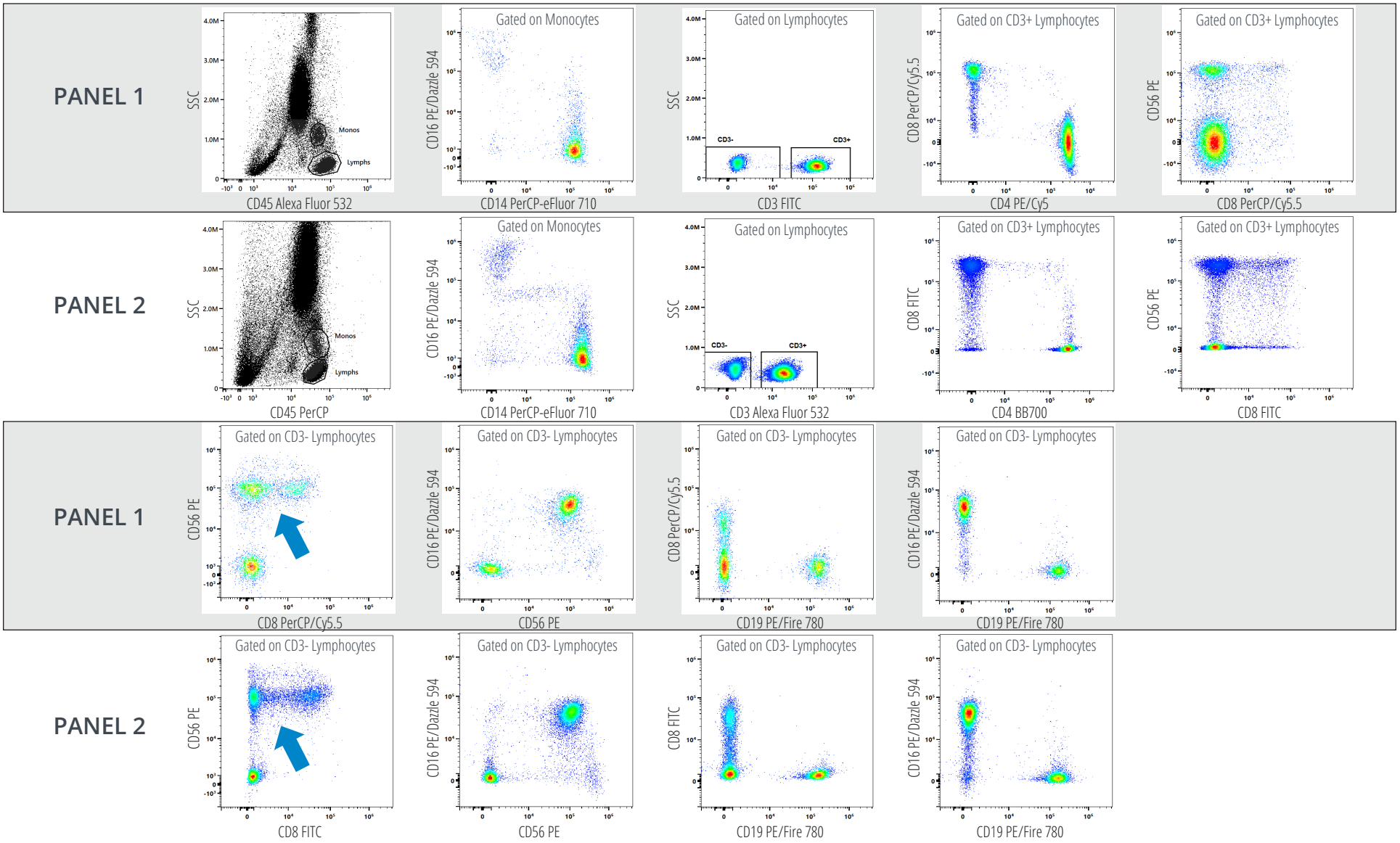

Figure 5: Side-by-side comparison of the cell subsets visualized from the panel 1 multi-color tube and from the panel 2 multi-color tube. Bi-exponential scaling is identical between the panels for each marker.

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challenging to discern from the CD8$^-$CD56$^+$ cells, whereas in panel 2 the separation of these two groups is much better. Next, take a look at the CSI matrices for each panel in figure 4. The impact of CD56 PE into CD8 PerCP/Cy5.5 in panel 1 has a medium impact indicated by the pink color, whereas the impact of CD56 PE into CD8 FITC has little impact indicated by the white color. Hence the calculated color coding of the CSI matrices aligns with the earlier visual observations.

Further visual comparisons of the spread in the multicolor panels can be made by continuing to compare each panel 1 plot with the corresponding panel 2 plot in figure 5, always using identical bi-exponential scaling in the 2 plots being compared. Panel 2 clearly does a better job of minimizing the amount of spread in the data, providing superior resolution over panel 1 with all cell subsets easily identified.

The CSI matrix is useful for comparing panel designs. When optimizing a panel, the goal is to minimize spread in parts of the gating strategy where dim cells of interest may become indiscernible, and manage spread in areas where the panel can handle it. Understanding the gating strategy for the panel, the brightness of the dyes, and the expression level for each marker is necessary to make good guided fluorochrome choices with the help of the CSI matrix.

Determining if Auto-Fluorescence Extraction is Needed

When unmixing data in SpectroFlo, there is an option to extract auto-fluorescence as a parameter. This option can be useful if the unstained cells have high auto-fluorescence, where "high" is median fluorescence intensity (MFI) greater than 10^4 or 10^5. For samples that have little to no auto-fluorescence, choosing to extract auto-fluorescence is not helpful and can make the data look worse. This is because adding the auto-fluorescence parameter to the equation results in over-fitting the best-fit model that the least squares unmixing algorithm determines. To demonstrate this, take a look at the unstained sample full spectrum signature (figure 6) and 2-D plots of lymphocyte CD3 expression when unmixing Panel 1 with and without auto-fluorescence extracted (figure 7). In the unstained sample, the highest signal is in channel B3 at approximately 10^3, which is not very high. When panel 1 is unmixed with this very low intensity auto-fluorescence signal extracted, the spread of the negatives increased in the non-T cells, which decreased the separation of the T cells from the non-T cells.

For most cell types, auto-fluorescence signal emits in shorter wavelengths when excited by UV or Violet lasers, and exhibit little to no emission when excited by the Blue laser. The decision to extract auto-fluorescence should be made on an assay by assay basis with close attention paid to the signature of the unstained sample.

Summary

Using the 1-laser blue Northern Lights cytometer, a high quality 8-color human immunophenotyping panel is achievable. Panel optimization may be needed and can be determined using these tools: comparison of single cell control and multicolor sample staining resolutions; assessing spread with the CSI matrix and with identically scaled gated multicolor data; and determining if auto-fluorescence extraction is needed. For additional Northern Lights application examples, please visit the Resources page at cytekbio.com.

ORDERING INFORMATION
NL-1000 B14 Cytometer: N7-00006
AMS Plate Loader Option: N7-00020-0A
Violet Laser Upgrade: N7-00034
Red Laser Upgrade: N7-00035

Find out more at www.cytekbio.com/pages/northern-lights

Please contact sales@cytekbio.com with any questions

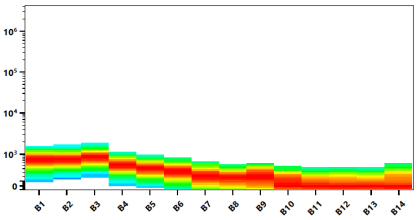

Figure 6: Unstained cells full spectrum signature. Detectors B1 through B14 are shown on the x-axis, and MFI is displayed along the y-axis. MFI in channels B1, B2, and B3 are close to 10^3.

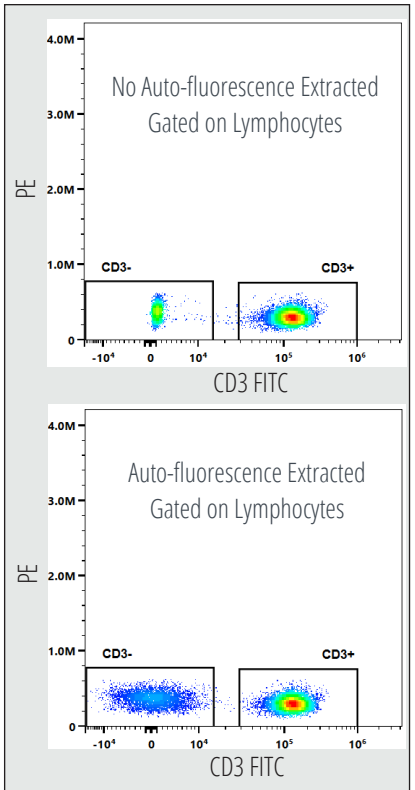

Figure 7: A side-by-side comparison of panel 1 multi-color data when unmixed with and without extracting auto-fluorescence. Bi-exponential scaling for each marker is identical to enable visual comparison of the staining patterns. Extracting auto-fluorescence (AF) as a parameter increases the amount of spread observed in the non-T cells.

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