Panel design by numbers: quantitative evaluation of spillover, spread, and cross excitation to build to more colors

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Abstract

Purpose: Demonstrate the process of panel design through iterative optimization using a mix of quantitative and qualitative assessments for the appropriate identification of biologically relevant cellular populations. Maximize the utilization of a flow cytometer to include all detectors through careful selection of fluorophores.

Methods: Incrementally replace and/or add fluorophores in progressive sequence. Spillover Spreading Matrices (SSM) [1], cross-excitation estimates, and compensation matrices are used to determine each fluorophore's impact on the panel's resolution.

Results: Removal of fluorophores with broad absorbance curves that are cross-excited by multiple laser sources reduces spillover-spreading and allows for the use of more detectors.

Introduction

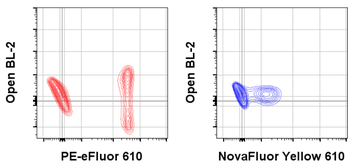
Flow cytometry panel design is often hindered by fluorophores that are cross-excited by multiple lasers and emit into more than one detector on an instrument. This is better known as fluorescence spillover. Spillover tends to increase with the size of a panel and can make it difficult to separate cellular populations. In extreme cases, it may even result in researchers avoiding certain detectors, forcing them to underutilize their instruments and lose the opportunity to otherwise collect more information about their samples.

Compensation is used to correct fluorescence spillover and is reported as a percent of a fluorophore's emission into secondary detectors. A SSM, on the other hand, characterizes changes in the standard deviation, *i.e.*, spread, of a population due to spillover, and therefore the impact that spillover has on the channel's sensitivity. Although both are related to spillover, the two provide different sets of information for evaluating panel designs.

Spillover-spreading can be evaluated by looking at single-stained samples that have been compensated against another fluorophore. In these samples, any observed signal in the secondary detector is an example of a spillover-spreading. Using the Invitrogen™ Attune™ Nxt V4 Flow Cytometer, Figure 1 looks at the relationship between Invitrogen™ PE-eFluor™ 610 (YL-2) or Invitrogen™ NovaFluor™ Yellow 610 (YL-2), and NovaFluor Blue 610-70S (BL-2) fluorophores. The SSM values in this example suggest that PE-eFluor 610 should have greater spillover-spreading into the empty BL-2 detector or NovaFluor Blue 610-70S than NovaFluor Yellow 610. Plots of compensated, single-stained PE-eFluor 610 or NovaFluor Yellow 610 graphed against the open BL-2 detector reflect this prediction. The increased spillover-spreading of PE-eFluor 610 renders BL-2 difficult to use with another marker.

Figure 1. Example SSM table and spillover-spreading in flow plots. (Left) SSM values. (Middle) PE-eFluor 610 fluorophore detected in YL-2 (red) versus BL-2. (Right) NovaFluor Yellow 610 fluorophore detected in YL-2 (blue) versus BL-2.

Fluorophore	NovaFluor Blue 610-70S
PE-eFluor 610	4.11
NovaFluor Yellow 610	0.77



In this study, we leverage evaluations like the example above to iteratively optimize the design of a panel. We start with an initial panel that has been designed with disregard to several conventional recommendations to exaggerate the potential pitfalls of poor panel design. We then provide a detailed analysis of each modification, including the contribution that cross-excitation and the resulting spread have on the inability to utilize all detectors on an instrument. We illustrate how these problems can be systematically assessed using quantitative and qualitative metrics and identify areas for additional improvement in the future.

Materials and methods

Sample Preparation

Frozen human peripheral blood mononuclear cells (PBMCs) were thawed and aliquoted at a concentration of up to $10x10^6$ cells/mL in a 96 well tissue culture plate. Briefly, up to $2x10^6$ PBMCs were washed once with phosphate buffered saline and stained for viability using a violet (405 nm) excited amine reactive viability dye for up to 30 minutes. PBMCs were washed once using flow cytometry staining buffer and stained with primary conjugated antibodies. The 12-color panel was stained against human CD3, CD4, CD8a, CD16, CD19, CD25, CD27, CD45RA, CD56, CD127, CD197 (CCR7). The 14-color panel was stained against human CD3, CD4, CD8a, CD16, CD19, CD25, CD27, CD28, CD45RA, CD56, CD127, CD185 (CXCR5), CD197 (CCR7). All samples were stained for up to 60 minutes on ice and protected from light. Samples were fixed for 30 minutes and analyzed immediately afterward.

Test Method(s)

Detector voltage settings were optimized prior to data collection. Stained PBMCs were interrogated using an Attune NxT V4 Flow Cytometer equipped with Violet (405 nm), Blue (488 nm), Yellow-Green (561 nm), and Red (637 nm) excitation lasers. All detectors were standard manufacturer recommendations. PBMCs were interrogated at a flow rate of 100 µL / min and 150,000 events were collected in the lymphocyte scatter gate.

Data Analysis

Panels were analyzed using FlowJo™ Software v10.8.1 (BD Life Sciences). Compensation was performed using single-color stained PBMCs. Fluorescence minus one controls were used to set positive gates for cell surface markers that did not show a clear separation between positive and negative populations. SSMs were calculated and compared across every panel. Graphs were generated using GraphPad Prism version 9.5.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

References

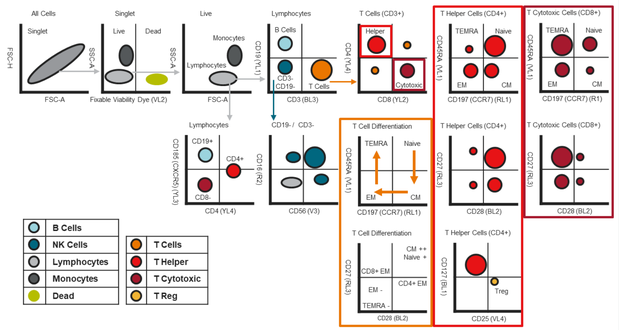
1. Nguyen R, Perfetto S, Mahnke YD, Chattopadhyay P, Roederer M. Quantifying spillover spreading for comparing instrument performance and aiding in multicolor panel design. *Cytometry A*. 2013;83(3):306-315. doi:10.1002/cyto.a.22251

Results

Panel and Gating Overview

The panel chosen for optimization focused on T cell surface antigens (CD3, CD4, CD8) and identification of subpopulations (CD25, CD127) with memory (CD45RA, CD197) and activation (CD27, CD28) markers. Also included were several antigens expressed on other lineage cell types (CD19, CD16, CD56, CD185). Regarding the gating strategy (Figure 2), we started by eliminating doublet and dead cells and gating on lymphocyte cells based on size and scatter. Lymphocytes were further divided into T and B cells. The CD3- / CD19- population was interrogated for NK cell markers. CD3+ T cells were narrowed down into T helper (CD4) and cytotoxic (CD8) subpopulations. CD4 and CD8 single positive cells were assessed for memory and activation markers. CD4 single positive cells were also evaluated for regulatory T cells (CD25+, CD127-). In Panels 5 and 6, CD28 was gated on T cells. In Panel 6, CD185 was gated on T and B cells.

Figure 2. Antigens and gating strategy.



Panel Design Progression

We started with a 12-marker panel (Panel 1) that included several fluorophores (*e.g.*, PE, PE-eFluor 610, PerCP-Cyanine5.5) that contributed significant spillover such that two detectors (BL-2 and YL-3) were left unused. Certain conjugates in this panel went against typically recommended practices, including the use of bright fluorophores with highly expressed antigens (*e.g.*, CD19 PE); however, this was done intentionally to exaggerate issues within less-than-ideal panels. We then made substitutions in order of impact. Panel 2 replaced CD19 PE to initiate opening BL-2. Panel 3 swapped PE-eFluor 610 in YL-2 with NovaFluor Yellow 610 to reduce the impact of cross-excitation into BL-2. In Panel 4, PerCP-Cyanine5.5 was swapped for NovaFluor Blue 690 to open YL-3. Panels 5 and 6 added two new conjugates, CD28 and CD185, which increased the panel from 12 to 14 markers and utilized all the cytometer's detectors.

Table 1. Building towards improved panel design. Detector names and emission filter wavelengths are indicated to the left. Cellular surface antigen markers are indicated for each detector. Grey boxes indicate detectors with significant spillover. Empty detectors are indicated as Open. Substitutions are highlighted by their laser color. Fluorophores remain unchanged unless otherwise indicated.

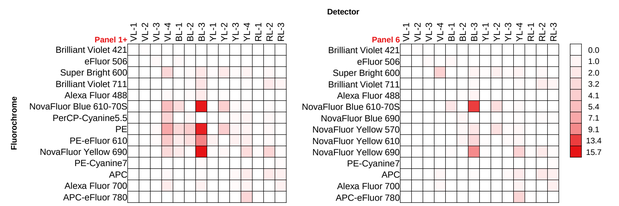
Attune Detectors		Surface Marker	Panel 1	Panel 2	Panel 3	Panel 4	Panel 5	Panel 6
VL-1	440/50	CD45RA	Brilliant Violet™ 421					Brilliant Violet 421
VL-2	512/25	Viability	Fixable Viability Dye eFluor 506					Fixable Viability Dye eFluor 506
VL-3	603/48	CD56 (NCAM)	Super Bright™ 600					Super Bright 600
VL-4	710/50	CD25	Brilliant Violet 711					Brilliant Violet 711
BL-1	530/30	CD127	Alexa Fluor™ 488					Alexa Fluor 488
BL-2	620/15	CD28	Open	Open	Open	Open	NovaFluor Blue 610-70S	
BL-3	695/40	CD3	PerCP-Cyanine5.5			NovaFluor Blue 690		
YL-1	585/16	CD19	PE	NovaFluor Yellow 570				
YL-2	620/15	CD8a	PE-eFluor 610		NovaFluor Yellow 610			
YL-3	695/40	CD185 (CXCR5)	Open	Open	Open	Open	Open	NovaFluor Yellow 690
YL-4	780/60	CD4	PE-Cyanine7					PE-Cyanine7
RL-1	670/14	CD197 (CCR7)	APC					APC
RL-2	720/30	CD16	Alexa Fluor 700					Alexa Fluor 700
RL-3	780/60	CD27	APC -eFluor 780					APC -eFluor 780

Results

Theoretical SSM

We determined the expected impact of our panel optimizations using theoretical SSMs for Panels 1 and 6 (Figure 3). These SSMs were created using human PBMCs stained with CD4 single-color controls each acquired with the same instrument. Cells were gated in FlowJo on the lymphocyte population and the AutoSpill / AutoSpread function was used to calculate the compensation and SSMs for each panel. The SSM for Panel 1 included the addition of NovaFluor Blue 610-70S and NovaFluor Yellow 690 (labeled as Panel 1+) for a direct comparison to Panel 6. The SSMs suggested that trying to occupy all 14 detectors with Panel 1 would have greater spillover spreading issues prior to the replacement of PE, PE-eFluor 610, and PerCP-Cyanine5.5.

Figure 3. Theoretical SSM heat maps using CD4 conjugates to compare fluorophore performance. SSMs of expanded Panel 1+ (left) and Panel 6 (right).



Opening Detectors and Reducing Spread

In Panel 1, high cross-excitation of the fluorescent proteins and tandems PE, PE-eFluor 610 and PerCP-Cyanine5.5, prohibited use of the BL-2 and YL-2 detectors. As illustrated by Figure 4, these problems were assessed using the absorbance spectrum of each fluorophore and visualization of the effects in the open BL-2 and YL-3 detectors. Swapping PE for NovaFluor Yellow 570 and PE-eFluor 610 for NovaFluor Yellow 610 reduced the spillover-spreading into BL-2 due to lower 488 nm cross-excitation (4 A, B). Similarly, the replacement of PerCP-Cyanine5.5 for NovaFluor Blue 690 reduced the spillover into YL-3 and the spread into VL-4 (4 C).

Figure 4. Comparing panel replacements. (Left) Estimated percent cross-excitation of off-target lasers with Median Fluorescent Intensity (MFI) and Standard Deviation (SD) of secondary detectors. (Middle) Absorbance spectra of original and replaced fluorophores overlaid with 405 nm (violet), 488 nm (blue), and/or 561 nm (yellow) excitation sources. (Right) Flow plots of Panel 1 (red) and Panels 2, 3, or 4 (blue) of primary against secondary detectors. (A) PE and NovaFluor Yellow 570 from Panels 1 and 2. (B) PE-eFluor 610 and NovaFluor Yellow 610 from Panels 1 and 3. (C) PerCP-Cyanine5.5 and NovaFluor Blue 690 from Panels 1 and 4.

A.

	Panel 1	Panel 2
488 nm Excitation	53.3 %	17.3 %
MFI (BL-2)	12,320	421
SD (BL-2)	5,283	303

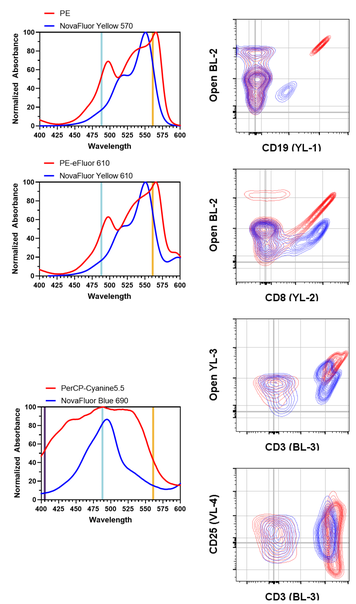
B.

	Panel 1	Panel 3
488 nm Excitation	48.1 %	16.6 %
MFI (BL-2)	5,065	747
SD (BL-2)	3,023	393

C.

	Panel 1	Panel 4
561 nm Excitation	42.9 %	15.3 %
MFI (YL-3)	2,462	798
SD (YL-3)	1,174	688

	Panel 1	Panel 4
405 nm Excitation	53.2 %	7.2 %
MFI (VL-4)	409	186
SD (VL-4)	1,859	1,521



Comparing Compensation

Compensation matrices supplemented the SSM and cross-excitation plots. Comparisons between panels indicated panel improvement based on fluorophore choice (Table 2). The replacements of PE and PE-eFluor 610 resulted in significantly less spillover in BL-2 with very minor effects in YL-3. The replacement of PerCP-Cyanine5.5 with NovaFluor Blue 690, however, did not significantly impact the compensation matrix.

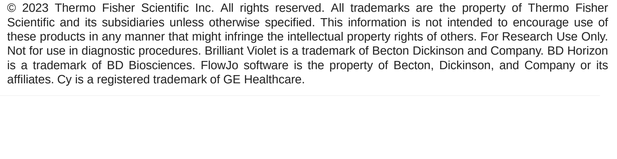
Table 2. Compensation differences after fluorophore replacement. Percent difference between Panels 1 and 4 in BL-2 and YL-3.

Panel 1	Panel 4	BL-2	YL-3
PE	NovaFluor Yellow 570	-66.15%	9.66%
PE-eFluor 610	NovaFluor Yellow 610	-39.97%	4.58%
PerCP-Cyanine5.5	NovaFluor Blue 690	3.60%	-5.02%

Utilizing All Detectors

As we moved from Panel 1 to 6, some benefits and drawbacks were observed. The switch from PerCP-Cyanine5.5 to NovaFluor Blue 690 reduced the spread in VL-4 (Figure 5, left and middle - left) and to a lesser degree in YL-4 (middle - right). There was a slight increase in spread in YL-2 due to the addition of NovaFluor Blue 610-70S (middle - right). The addition of new conjugates into the previously open detectors resulted in spread in BL-3 (right). However, these small changes in spread in YL-2 and BL-3 were offset by the addition of two previously unused detectors.

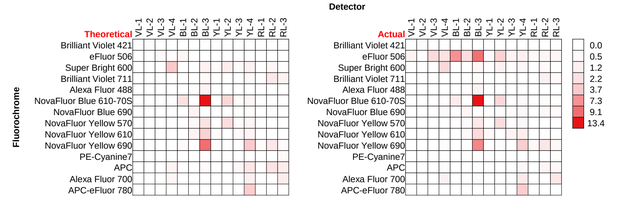
Figure 5. Changes in spread when comparing Panels 1 (red) and 6 (blue). (Left and middle - left) Flow plots of CD127 Alexa Fluor 488 (BL-1) versus CD25 Brilliant Violet 711 (VL-4) gated on CD3+, CD4+ T cells. (Middle - right) CD4 PE-Cyanine7 (YL-4) versus CD8 (YL-2) gated on CD3+ T cells. (Right) CD3 (BL-3) and CD19 (YL-1) gated on all lymphocytes.



Summary

We compared the theoretical SSM of Panel 6 against the actual SSM (Figure 6) as a measurement of our success. Visual comparison confirmed many of the predicted results, although, there were some unexpected results with eFluor 506 to be addressed in a follow-up experiment.

Figure 6. Panel 6 theoretical and actual SSM heat maps. Theoretical SSM (using CD4 conjugates, left) versus the actual SSM (incorporating both antigen density and dye performance, right).



For each panel, we also compared the actual SSM full matrix sum to the theoretical matrix sum using CD4 conjugates (Table 3). With every fluorophore replacement, we observed a decrease in the matrix sum value for both the theoretical and actual SSMs. As expected, the matrix sum increased with the number of detectors utilized; this is counter-balanced with the gain of deeper immunological assessment. Accordingly, a direct SSM comparison between different sized panels is not recommended.

Table 3. Evaluation of SSM full matrix sums for Panels 1 through 6. Comparison between theoretical and actual matrix sums for each panel.

Panel #	1	2	3	4	5	6
# of Detectors Utilized	12	12	12	12	13	14
Theoretical SSM Matrix Sum	54.97	55.29	46.14	40.61	66.39	87.17
Actual SSM Matrix Sum	66.3	66.4	61	56.1	90.7	110.8

Conclusions

Fluorophores with high spillover and/or spread may obscure co-expressed conjugates causing difficulties in population resolution or a reduction in total cellular markers within a desired panel. By only swapping fluorophores, not all the spillover-spreading issues may be resolved. Antigen selection is important, too. SSMs can provide a framework to assess changes in panel design and is most useful between similar sized panels. By using a combination of SSM, antigen density, and fluorophore characteristics to plan an experiment, a researcher can fully utilize their instrument while maintaining acceptable separation for cell identification.

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