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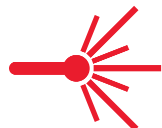

A Workflow Guide for Panel Design in Spectral Flow Cytometry Using CytoFLEX mosaic Spectral Detection Module

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Background

Spectral flow cytometry is a powerful analytical platform that expands multiparametric analysis capacity by incorporating 3–4 times more detectors than traditional cytometry. This enables the resolution of highly overlapping fluorophores through integration of their complete emission spectra, which creates unique spectral fingerprints analogous to those generated by spectrophotometric profiling. Conventional flow cytometry relies on a one-to-one match of a discrete detector with a fluorophore's peak emission wavelength.[1, 2] Spectral spillover of that fluorophore into an unintended channel is corrected by a compensation algorithm, which is a simple linear correction factor. Alternately, spectral technology uses different algorithms generally termed spectral *unmixing* to accurately differentiate fluorophores with overlapping emission profiles. These could include algorithms like Least Squares, Weighted Least Squares, or Poisson Regression models.[3, 4] The ability to use more fluorophores enables higher plex panels and greater flexibility in the design of lower plex experiments, allowing researchers to incorporate a wider range of fluorophores and markers. This capability makes spectral flow cytometry particularly advantageous for applications such as high-dimensional immune profiling, rare cell detection and complex biological investigations.

Objective

The primary objective of optimizing the assay components, sample preparation and workflow in spectral flow cytometry is to augment the overall efficiency and accuracy of the process. By refining each step in the workflow, laboratories can achieve reduced turnaround times, decrease sample processing errors, and ensure consistent, high-quality data output.

Designing an optimal spectral panel is fundamentally similar to designing a conventional panel, in that both require careful consideration of fluorophore selection and commercial availability of direct conjugates, marker expression density and patterns, instrument optics and settings, and data analysis strategies. This whitepaper aims to present guidelines and strategies for enhancing efficiency and accuracy in spectral flow cytometry. It includes recommendations for optimal panel design, iterative empirical assay testing, staining and sample considerations, and the appropriate controls necessary for accurate unmixing and gating strategies. By following these principles, researchers can maximize the potential of spectral flow cytometry for comprehensive and precise multiparametric analysis.

Panel Design

Before designing a successful panel, it's important to first determine the research question and understand the sample biology. Then identify the antigens of interest. Overall, there are certain rules that should be considered for a successful spectral flow cytometry panel. You can get more information about these rules here.[5] Before we begin building the panel, the following information is important to know:

Prerequisites

- Sample type—whether a single-cell suspension or tissue requiring dissociation—determines preparation requirements. An example with tissue dissociation can be found in an overview of the impact of collagenase and different concentrations of dispase impacting a myriad of cell surface receptors in mouse spleen. [6]
- Additionally, sample condition and cell health, whether live, fixed or cryopreserved, affect antigen density, surface receptor stability, autofluorescence and propensity for non-specific binding of reagents. Therefore, same-day analysis is often necessary to minimize changes in sample characteristics and data reliability. Limited sample volume, such as biopsies or small cell samples, necessitates careful consideration of fluorescence minus one (FMO) controls, single-color biological controls, and expression controls.
- Exogenous substances, including drugs, stimulation buffers, or other components, can contribute to unique changes in autofluorescence and must be accounted for in unmixing controls and autofluorescence extraction.
- Sample will contain multiple individual sources of autofluorescence. When gating on a single population by its scatter properties, there are many different members of that population that exist at different activation and disease states, which influences the spectral distribution of the background autofluorescence per cell. Autofluorescence extraction is often an average of these individual spectral differences, and that variance must be considered for good assay development and consistent, error-free analysis. [7, 8]
- Red blood cells are usually lysed using a commercial lysis buffer to minimize interference with fluorescence signals and reduce clogging in fluidics lines. However, treatment of stained samples with lysis and fix/perm reagents can have an impact on the integrity of a fluorophore, specifically protein-based fluorophores like PerCP, PE, APC and their tandems, and cause an inconsistent shift in the spectrum of the fluorophore. Changes to fluorophore stability need to be tested for impact on the assay performance and unmixing accuracy. An example is the difference in the integrity of the PE-Fire 700 tandem versus the generic PE-Cy5.5 under different fix/perm conditions by monitoring the percent compensation change into the donor (PE) channel. [9]
- Fixing cells prior to staining can alter epitope accessibility and fluorochrome performance, especially for tandem dyes.[10]
- Use appropriate blocking reagents (e.g., Fc receptor blockers, serum, or commercial buffers) to minimize background staining and reduce false positives. These reagents are independent of fixation and should be optimized based on cell type and antibody panel. Mouse or human serum in addition to FcR Blocking Reagent should be added to the sample prior to antibody staining to block non-specific binding of antibodies to Fc receptors on immune cells. [11]
- Monocyte blocking solutions should also be added to a cell suspension prior to cell staining. Monocyte blocking buffers prevent non-specific binding of the fluorophore to immunologically activated cells like antigen-presenting cells. This is a common artifact seen with bright PE tandem fluorophores.
- Antibody cocktails should be made in a diluent containing Brilliant Stain Buffer and/or SuperNova Staining Buffer to minimize non-specific binding of Brilliant Violet and Brilliant UV polymer dyes to themselves. When antibodies are added directly to cells, these buffers need to be added first prior to the addition of reagents.

Figure 1: Major contributors for designing a panel in spectral flow cytometry experiments.

Panel Design Principles

The fluorophore selection was guided by several key principles:

Design Principle	Explanation
Minimizing Spectral Overlap	Fluorophores must have distinct emission profiles, ideally with distinct emission peaks. The similarity of two fluorophores is calculated per channel where a maximum value of 1 or 100% would indicate that the fluorophores are identical. The complexity index is an additive score representing the total spectral spillover due to all fluorophores used in a panel or assigned to a co-expression group. The complexity index is a number that has no maximum value and increases as the total number of parameters increases. Panel optimization includes the use of fluorophore combinations with the lowest total complexity index. High spectral overlaps contribute to an unmixing artifact called spreading error which impacts population resolution. Spreading error can impact the ability to resolve two populations whether the fluorophores with high spectral overlap are co-expressed, individually expressed or present on the negative events but plotted in the frame of analysis in bivariate plots.[12]
Balancing Fluorophore Brightness	Population resolution can be impacted by the brightness of a fluorophore. A general rule is to match highly expressed markers with dim fluorophores so as not to have off-scale signals that might require an adjustment to tune down the gain on individual APDs or APD arrays. Low-expressed markers require bright fluorophores for sufficient population resolution. In addition, undesirable spreading error is also exacerbated in populations exhibiting high-intensity staining, which can especially impact the ability to gate accurately on dim versus bright populations or double positive populations on a plot.
Avoiding Crosstalk with Viability Dyes	Fluorophores assigned to live/dead dyes, though excluded from analysis, exhibit broad emission and can introduce spillover. Assigning them to less-utilized lasers, such as 808 nm, minimizes interference with immunophenotyping channels. In spectral it is even more important to titrate live/dead viability dyes to ensure that there is no residual live/dead staining of live cells and to minimize the spreading error or unmixing inaccuracies related to reagents with such bright staining. This is less of a problem when using impermeant nucleic acid stains to exclude dead cells because, when used properly, only dead and, on occasion, apoptotic cells will stain positive.[13]
Accounting for Instrument Configuration	Spectral cytometry detects the full emission spectrum of a fluorophore, therefore when matching fluorophores to an instrument, it's important to consider the impact of the different excitation sources on the reagent choice, for example the difference in excitation efficiency between the near-UV and UV excitation sources.
Ensuring Robust and Accurate Unmixing	Unmixing errors can result in inconsistent, undependable, and ultimately unusable results. Unmixing controls must be an identical spectral match to the fully stained sample to ensure accurate unmixing. They must be uncontaminated by exposure to other reagents or a poorly cleaned instrument, and the autofluorescence of the unmixing bead or cell must have a matched unstained control. Deviation from these practices will result in unmixing errors. The best practice is to create single-color biological staining controls and bead-based unmixing controls in parallel to ensure one of them will contribute to accurate unmixing of the fully stained sample.[14, 15]

Unmixing-dependent Spreading Error	Spreading error significantly impacts population resolution, data quality, and consistency. As more parameters are used in an assay, even when marker/fluorophore combinations are not co-expressed, some channels will exhibit greater spreading error. This occurs due to the application of the unmixing matrix, which can be empirically assessed only during panel optimization .[16, 17]
Enhancing Rare Population Detection	Low-frequency cell types or rare markers are often paired with bright fluorophores and marker/fluorophore combinations that minimize spreading error. This approach helps maintain sensitivity, population resolution, and reliable gating of these events.
Providing Flexibility for Future Expansion	If required, some channels may be left open to accommodate uncommitted markers. This approach allows for modifications and expansions in panel design based on future experimental needs. However, this does not mean that future marker choices will be ideal candidates for the fluorophore channels that have been left open. Panel modifications must undergo the same empirical scrutiny to maximize population resolution and minimize the impact of spreading error.

Cell Markers

In flow cytometry panel design, cell surface markers play a pivotal role in identifying and characterizing immune cell subsets, enabling precise analysis of cell populations. For instance, Figure 2 illustrates leukocyte subsets identifiable in peripheral lysed whole blood (WB) highlighting essential surface markers critical for their discrimination. It underscores the complexity of immune profiling and the necessity of thoughtful panel design and gating strategy to resolve overlapping marker combinations that define cell subsets.

Marker expression patterns across monocytes, dendritic cells, lymphocytes, NK cells, T cell subsets, and granulocytes illustrate the importance of strategic fluorophore assignments. For instance, high-expression markers like CD4 on T-helper cells can be paired with dimmer fluorophores, whereas low-expression markers on less abundantly expressed antigens require brighter dyes to achieve sufficient population resolution. By leveraging distinct expression patterns and marker combinations, researchers can design panels that accurately delineate immune cell subsets, enhancing the sensitivity and specificity of high-parameter flow cytometry experiments.

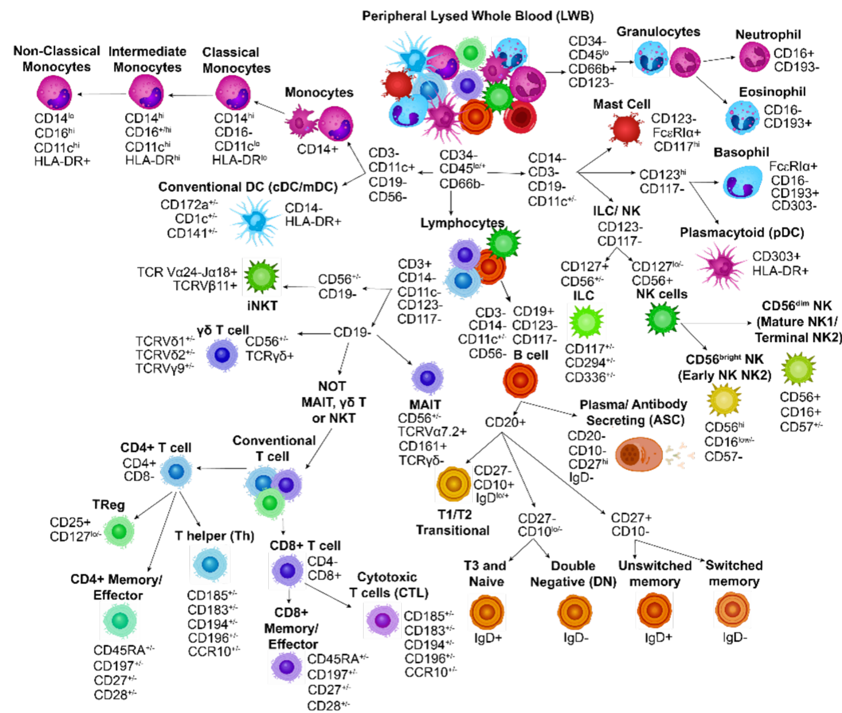


Figure 2: Comprehensive immunophenotyping map of peripheral blood leukocyte subsets (Source- FluoroFinder.com)

Based on the figure, surface markers can be grouped into 4 tiers (**Figure 3**). Tier 1 includes essential lineage markers required for broad population identification, while Tiers 2 to 4 progressively incorporate markers for detailed subset resolution, activation states, and functional profiling, respectively. This structured approach supports rational panel design by aligning marker selection with experimental priorities, gating strategy and maximizing the capabilities of high-dimensional spectral systems.

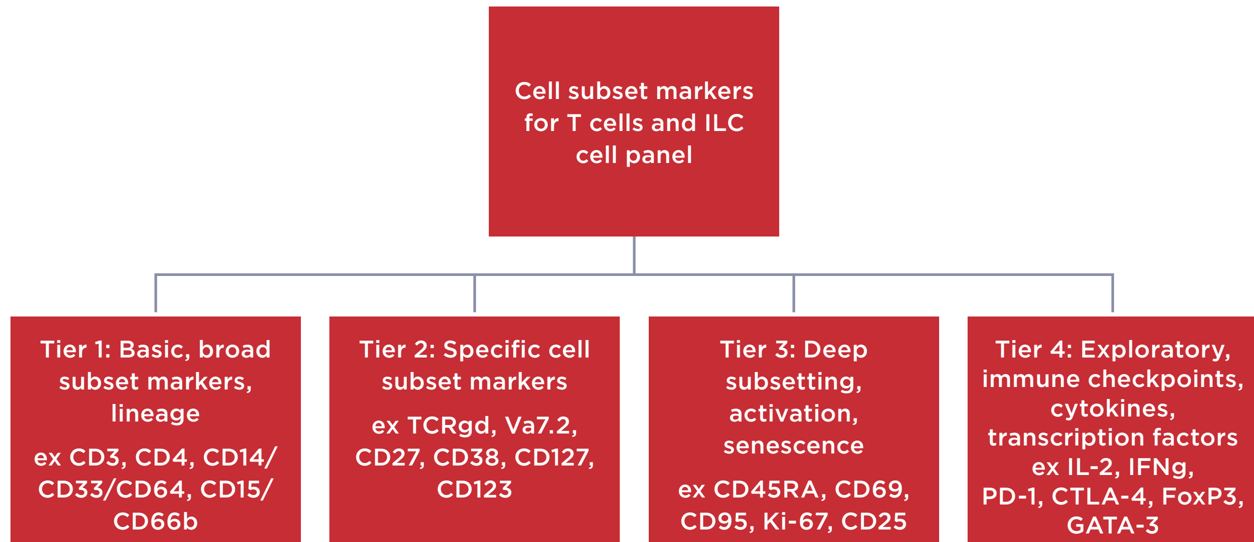


Figure 3: Tiered classification of cell subset markers for T cells and ILC panel for spectral flow cytometry panel design

Fluorophores: In spectral flow cytometry panel design, understanding the interplay between stain index and median fluorescence intensity (MFI) is important to understand population resolution. The MFI is an instrument-specific value derived from the inherent brightness of an individual fluorophore generally, determined by its quantum yield (QY) and extinction coefficient (EC), the degree of labeling (DOL), which is how many fluorophores are conjugated to a single antibody, and the biologically relevant cellular antigen (Ag) density. MFI is also dependent upon instrument configuration, laser wattages and APD gain values. In this context the assumption is that the instrument factors are internally consistent.

Brightness (MFI+) = (ECxQY of Individual Fluorophore) x DOL of Antibody x Cellular Antigen Density

However, brightness is only one factor that influences the ability to resolve a population. Staining index further integrates the loss of resolution due to autofluorescence or other non-specific sources of staining intensity impacting the negative population (MFI-) divided by the standard deviation (SD^{neg}) or the widening distribution of the negative population that might impinge on the ability to distinguish positive from negative, an artifact of spreading error that often arises in high parameter spectral applications.

Staining Index (SI)= (MFI+-MFI-)/ (2x SD^{neg})

Strategically assigning fluorophores based on their inherent brightness ensures that the stain index aligns with antigen expression levels, maximizing sensitivity and specificity in high-parameter experiments. **Figure 4** shows the stain index of single-color stained CD4 conjugated fluorophores specific to the CytoFLEX mosaic Spectral Detection Module (mosaic 88 attached to the CytoFLEX LX instrument with the 355 nm and 808 nm laser options) configuration providing a critical visual representation of their relative brightness and impact on population resolution, which is essential for multicolor panel design.

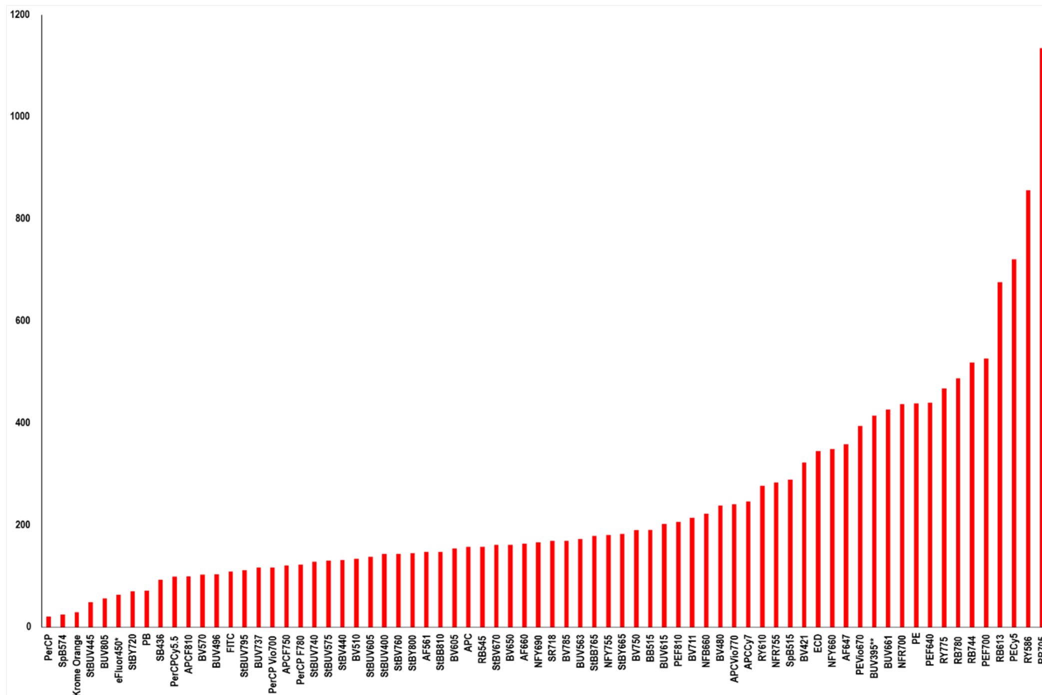


Figure 4: Stain index of commonly used fluorophores plotted by using CD4 conjugated with different fluorophores and plotted using the same Log-Linear coefficient range for every fluorophore. (Note: The experiment was conducted as a single run on the same biological sample with Fc receptor and monocytes blockers used.)

Additionally, in spectral flow cytometry, the selection of fluorophores with minimal spectral overlap is critical to reduce spreading error in multicolor panels. These days various tools are available to help with panel design for instance, **Figure 5** shows a theoretical similarity matrix for the selected panel. Here, we present a similarity matrix to evaluate the spectral relationships between 37 fluorophores generated with the CytExpert for spectral software with real single colors. The matrix quantifies pairwise similarity on a scale from 0 to 1, where values closer to 1 indicate significant spectral overlap, and values near 0 suggest distinct emission profiles.

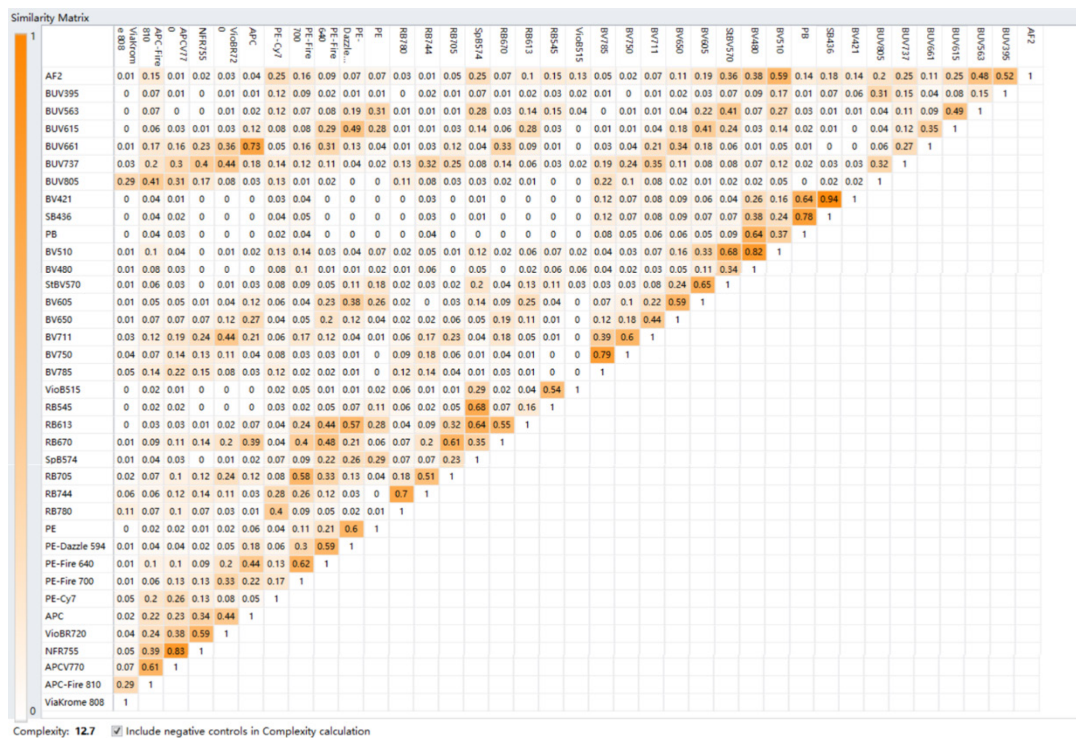
For instance, SuperBright 436 (SB436) and Brilliant Violet 421 (BV421) exhibit a high similarity score of 0.94, indicating substantial overlap, while Brilliant UltraViolet 615 (BUV615) and SuperBright 436 show a low score of 0.01, suggesting suitability for combined use. This analysis aids in the rational design of fluorophore panels for spectral flow cytometry by identifying combinations that minimize crosstalk and thus spillover spreading error in multicolor experiments. Similarly, **Figure 6** illustrates the emission spectra of all fluorophores used in the panel, across various emission channels. Each spectral ribbon represents the normalized emitted intensity of a fluorophore, highlighting regions of spectral overlap that are critical for designing multicolor flow cytometry panels with minimal spillover.

However, the similarity matrix and graphs of spectral overlap are only panel building tools and do not provide information about the directional nature of spectral spillover that exacerbates the severity of spreading error specifically . Since the “trumpet” distribution or the effect of a population that becomes wider with an increasing MFI, the impact of spreading error on population resolution must be empirically assessed individually for each panel and cannot be generalized. A similarity matrix assigns the same similarity score to both fluorophores it compares. A spillover spreading matrix assigns a value based on the directional spill of one of those fluorophores into another, a non-intended channel and vice versa.

For example, in the normalized view of the spectral spillover between Alexa Fluor 647 (peak emission channel R2) and PE-Cy5 (peak emission channel Y4), you can tell that PE-Cy5 spills over quite a bit into R2 but Alexa Fluor 647 does not spill over equally into Y4 (**Figure 7**). These spectral plots are normalized, which means the amplitude of the peaks they display are plotted on a relative intensity scale where peak emissions are plotted to the same 100% in order to provide a good view of their spectral distributions. The amplitudes do not accurately represent the actual brightness of either of these fluors nor the true empirical impact they will finally have on spreading error. Each of these tools is one of many to help the end user make informed decisions about the compatibility of fluorophore combinations in a panel.

Example panel:

For this study, we used a 36-color panel that enables the in-depth immunophenotyping of unconventional and conventional CD3+ T cell subsets (CD4, CD8), natural killer (NK) cell subsets (CD16, CD57, CD94, CD56) , γδ T cell subsets (TCRαβ, TCR Vδ1, TCR Vδ2, TCR Vγ9), innate lymphoid cell subsets (ILC; CD127, CD117,CD294, CD56), NKT cells (TCRVa24-Ja18, TCRVb11) and mucosal-associated invariant T cells (MAIT; CD161, TCR Vα7.2). We included markers to assess T helper subsets (CD183, CD185, CD194, CD196, CD161, CD294), Treg (CD25, CD127) and activation markers (CD45RA, CD27, CD197). In addition, this panel includes myeloid markers for cDC (CD11c, HLA-DR), pDC (CD123, HLA-DR, CD303), monocytes (CD14, CD16), and basophils (CD193, FceR1a, CD123). This unique combination of markers enables a thorough phenotypic characterization of these cell subsets.



Similarity Matrix

	ViaKrome 808	APC-Fire 810	APCV770	NFR755	VioBR720	APC	PE-Cy7	PE-Fire 700	PE-Fire 640	PE-Dazzle...	PE	RB780	RB744	RB705	SpB574	RB670	RB613	RB545	VioB515	BV785	BV750	BV711	BV650	BV605	StBV570	BV480	BV510	PB	SB436	BV421	BUV805	BUV737	BUV661	BUV615	BUV563	BUV395	AF2
AF2	0.01	0.15	0.01	0.02	0.03	0.04	0.25	0.16	0.09	0.07	0.07	0.03	0.01	0.05	0.25	0.07	0.1	0.15	0.13	0.05	0.02	0.07	0.11	0.19	0.36	0.38	0.59	0.14	0.18	0.14	0.2	0.25	0.11	0.25	0.48	0.52	1
BUV395	0	0.07	0.01	0	0.01	0.01	0.12	0.09	0.02	0.01	0.01	0	0.02	0.01	0.07	0.01	0.02	0.03	0.02	0.01	0	0.01	0.02	0.03	0.07	0.09	0.17	0.01	0.07	0.06	0.31	0.15	0.04	0.08	0.15	1	
BUV563	0	0.07	0	0	0.01	0.02	0.12	0.07	0.08	0.19	0.31	0.01	0.01	0.01	0.28	0.03	0.14	0.15	0.04	0	0.01	0.01	0.04	0.22	0.41	0.07	0.27	0.03	0.01	0.01	0.04	0.11	0.09	0.49	1		
BUV615	0	0.06	0.03	0.01	0.03	0.12	0.08	0.08	0.29	0.49	0.28	0.01	0.01	0.03	0.14	0.06	0.28	0.03	0	0.01	0.01	0.04	0.18	0.41	0.24	0.03	0.14	0.02	0.01	0	0.04	0.12	0.35	1			
BUV661	0.01	0.17	0.16	0.23	0.36	0.73	0.05	0.16	0.31	0.13	0.04	0.01	0.03	0.12	0.04	0.33	0.09	0.01	0	0.03	0.04	0.21	0.34	0.18	0.06	0.01	0.05	0.01	0	0	0.06	0.27	1				
BUV737	0.03	0.2	0.3	0.4	0.44	0.18	0.14	0.12	0.11	0.04	0.02	0.13	0.32	0.25	0.08	0.14	0.06	0.03	0.02	0.19	0.24	0.35	0.11	0.08	0.08	0.07	0.12	0.02	0.03	0.03	0.32	1					
BUV805	0.29	0.41	0.31	0.17	0.08	0.03	0.13	0.01	0.02	0	0	0.11	0.08	0.03	0.03	0.02	0.01	0	0	0.22	0.1	0.08	0.02	0.01	0.02	0.02	0.05	0	0.02	0.02	1						
BV421	0	0.04	0.01	0	0	0	0.03	0.04	0	0	0	0	0.03	0	0.01	0	0	0	0	0.12	0.07	0.08	0.09	0.06	0.04	0.26	0.16	0.64	0.94	1							
SB436	0	0.04	0.02	0	0	0	0.04	0.05	0	0	0	0	0.03	0	0.01	0	0	0	0	0.12	0.07	0.08	0.09	0.07	0.07	0.38	0.24	0.78	1								
PB	0	0.04	0.03	0	0	0	0.02	0.04	0	0	0	0	0.04	0	0	0	0	0	0	0.08	0.05	0.06	0.06	0.05	0.09	0.64	0.37	1									
BV510	0.01	0.1	0.04	0	0.01	0.02	0.13	0.14	0.03	0.04	0.07	0.02	0.05	0.01	0.12	0.02	0.06	0.07	0.02	0.04	0.03	0.07	0.16	0.33	0.68	0.82	1										
BV480	0.01	0.08	0.03	0	0	0	0.08	0.1	0.01	0.01	0.02	0.01	0.06	0	0.05	0	0.02	0.06	0.06	0.04	0.02	0.03	0.05	0.11	0.34	1											
StBV570	0.01	0.06	0.03	0	0.01	0.03	0.08	0.09	0.05	0.11	0.18	0.02	0.03	0.02	0.2	0.04	0.13	0.11	0.03	0.03	0.03	0.08	0.24	0.65	1												
BV605	0.01	0.05	0.05	0.01	0.04	0.12	0.06	0.04	0.23	0.38	0.26	0.02	0	0.03	0.14	0.09	0.25	0.04	0	0.07	0.1	0.22	0.59	1													
BV650	0.01	0.07	0.07	0.07	0.12	0.27	0.04	0.05	0.2	0.12	0.04	0.02	0.02	0.06	0.05	0.19	0.11	0.01	0	0.12	0.18	0.44	1														
BV711	0.03	0.12	0.19	0.24	0.44	0.21	0.06	0.17	0.12	0.04	0.01	0.06	0.17	0.23	0.04	0.18	0.05	0.01	0	0.39	0.6	1															
BV750	0.04	0.07	0.14	0.13	0.11	0.04	0.08	0.03	0.03	0.01	0	0.09	0.18	0.06	0.01	0.04	0.01	0	0	0.79	1																
BV785	0.05	0.14	0.22	0.15	0.08	0.03	0.12	0.02	0.02	0.01	0	0.12	0.14	0.04	0.01	0.03	0.01	0	0	1																	
VioB515	0	0.02	0.01	0	0	0	0.02	0.05	0.01	0.01	0.02	0.06	0.01	0.01	0.29	0.02	0.04	0.54	1																		
RB545	0	0.02	0.02	0	0	0	0.03	0.02	0.05	0.07	0.11	0.06	0.02	0.05	0.68	0.07	0.16	1																			
RB613	0	0.03	0.03	0.01	0.02	0.07	0.04	0.24	0.44	0.57	0.28	0.04	0.09	0.32	0.64	0.55	1																				
RB670	0.01	0.09	0.11	0.14	0.2	0.39	0.04	0.4	0.48	0.21	0.06	0.07	0.2	0.61	0.35	1																					
SpB574	0.01	0.04	0.03	0	0.01	0.02	0.07	0.09	0.22	0.26	0.29	0.07	0.07	0.23	1																						
RB705	0.02	0.07	0.1	0.12	0.24	0.12	0.08	0.58	0.33	0.13	0.04	0.18	0.51	1																							
RB744	0.06	0.06	0.12	0.14	0.11	0.03	0.28	0.26	0.12	0.03	0	0.7	1																								
RB780	0.11	0.07	0.1	0.07	0.03	0.01	0.4	0.09	0.05	0.02	0.01	1																									
PE	0	0.02	0.02	0.01	0.02	0.06	0.04	0.11	0.21	0.6	1																										
PE-Dazzle 594	0.01	0.04	0.04	0.02	0.05	0.18	0.06	0.3	0.59	1																											
PE-Fire 640	0.01	0.1	0.1	0.09	0.2	0.44	0.13	0.62	1																												
PE-Fire 700	0.01	0.06	0.13	0.13	0.33	0.22	0.17	1																													
PE-Cy7	0.05	0.2	0.26	0.13	0.08	0.05	1																														
APC	0.02	0.22	0.23	0.34	0.44	1																															
VioBR720	0.04	0.24	0.38	0.59	1																																
NFR755	0.05	0.39	0.83	1																																	
APCV770	0.07	0.61	1																																		
APC-Fire 810	0.29	1																																			
ViaKrome 808	1																																				

Complexity: **12.7** ☑ Include negative controls in Complexity calculation

Figure 5: Spectral overlap analysis of fluorophores in the select panel using a Similarity Matrix (source: CytExpert for Spectral software).

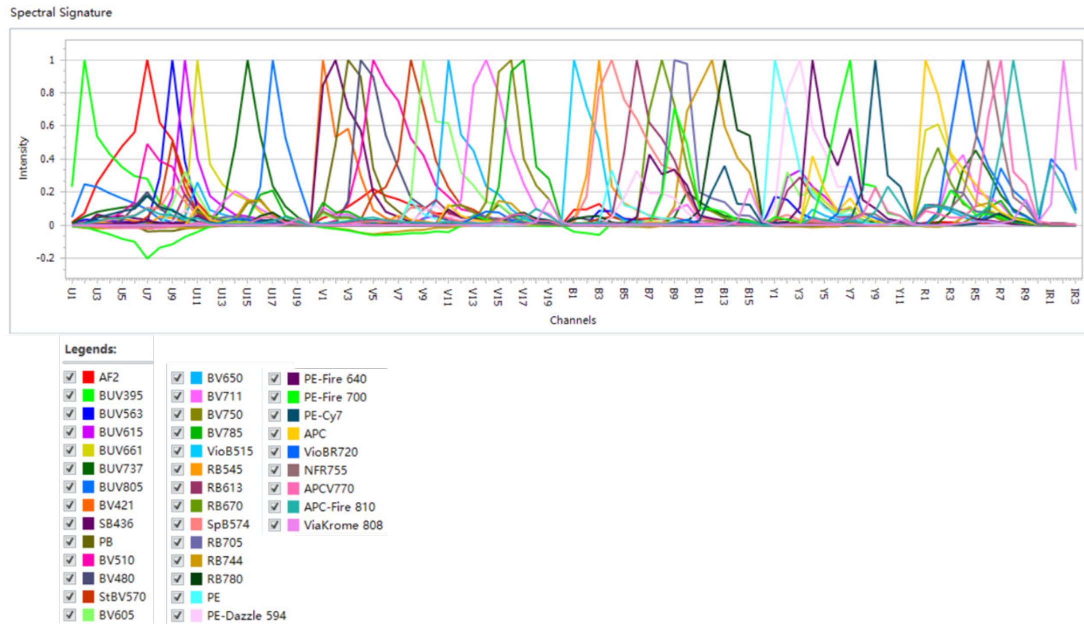


Figure 6: Spectral emission profiles of fluorophores (source: CytExpert for Spectral software).

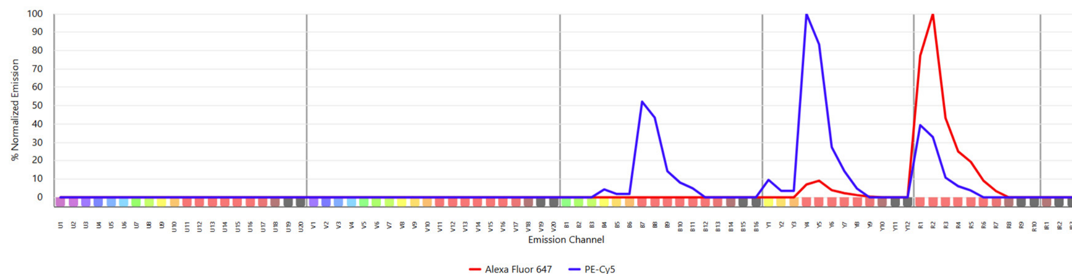


Figure 7: Spectral emission profiles of Alexa Fluor 647 and PE-Cy5 Across different detection channels (source: FluoroFinder).

Instrument configurations

Instrument configurations play a pivotal role in multicolor flow cytometry panel design, as the number and type of detectors, laser wavelengths, and optical filters directly influence the ability to resolve fluorophore signals. For spectral flow cytometry, instruments equipped with full-spectrum detectors, such as those covering 350–850 nm, enable the use of fluorophores with overlapping emission profiles, by unmixing their signals through computational algorithms. We are using a CytoFLEX mosaic 88 spectral detection module compatible with the CytoFLEX LX (UV) instrument with 6 lasers and 88 detectors for this study, and FluoroFinder, a web-based tool that helps with panel design based on our instrument settings. We begin by understanding the impact on panel design by the instrument configuration to be used:

- **355 nm UV or 375 nm NUV Laser Fluorophores:** Fluorophores useful on this laser would be the Brilliant UV (BUV) and StarBright UV fluorescent polymer families. The UV and NUV lasers do not excite these fluorophores equally, and the difference in efficiency would be reflected in the stain index calculation. For example, BUV805 is already a dim fluorophore and the inefficient excitation by the 375 nm NUV laser requires that it be used on a highly abundant antigen. Fluorophores off this laser are often used for lineage and key immune markers. In this panel they are dedicated to Tier 1 basic immunophenotypic markers such as HLA-DR, CD14, FceR1a, TCRαβ, CD11c and CD303 to minimize overlap with visible spectrum fluorophores.

- StarBright UV445 and BUV496 are fluorophores that fall at the same emission peak as the most common autofluorescence source, NADH, and this should be considered when using these fluorophores in highly metabolically active and autofluorescent samples. However, generally, there is less cross-beam excitation of other fluorophores that would lead to unwanted spectral spillover into the channels off the UV/ NUV laser. UV fluorophores are also typically much dimmer than fluorophores excited by other lasers, making them more useful for abundant markers that are more ubiquitously expressed, such as lineage markers. The exception to this would be StarBright UV 665 and Brilliant Violet 661, which both have significant cross-beam excitation by the 633 nm red laser that can cause unwelcome spectral spillover and spreading error with Alexa Fluor 647 and APC.
- **405 nm Violet Laser Fluorophores:** There are bright polymer fluorophores, such as SuperNova (SNv), Brilliant Violet (BV), SuperBright (SB) and StarBright (StB) brands for the violet laser, and dimmer, small organic fluorophores like eFluor 450, Pacific Blue, V500 and Krome Orange. In lower parameter number panels, BV421, SNv 428 and StBv440 channels should be reserved for important markers and low-abundant antigens in panels. Although it is possible to use different combinations of SB436, BV421, SNv428, StBv440 and eFluor 450/ Pacific Blue, they are highly overlapping combinations that can compromise population resolution and limit the benefits for use on the important, low abundance markers.
- Ensure that, when used in the same assay, events stained with combinations of these fluorophores will not be present on the same data plot. The same is true, but to a lesser extent, when using BV480 with BV510 and BV750 with BV711 and BV785. In this panel, SB436 was assigned to a very low-abundant marker, CD117, which phenotypes both progenitors, mast cells and the ILC immune subset, all of which are rare in cryopreserved PBMC and generally in peripheral blood. BV421 was assigned to CD193/CCR3, which is expressed predominantly on basophils and eosinophils and a small subset of T cells. Pacific Blue, a dim fluorophore, was assigned to CD57, which is expressed only on NK and NKT cells and is expressed abundantly. Of these three highly overlapping fluorophores, Pacific Blue emits at the longest wavelength and thus, due to the directional nature of the spectral spillover, it can be used on the more abundant fluorophore with less impact on the other two fluorophores. Additionally, none of these three fluorophores will be present on the same analysis plot, either co-expressed on the same cell or present on an analysis plot in the negative population once tier 1 subsetting is complete.
- Other violet laser fluorophores were assigned to lineage, subsetting and dump channel markers such as CD25, CD16, CD8, CD127, TCRVα7.2 and CD3 to balance brightness and prevent crosstalk. CD3 will define all T cells. CD25 and CD127 will define regulatory T cells. CD16 is a subsetting marker for NK cells and monocytes. TCRVα7.2 defines a subset of conventional T cells called mucosal-associated invariant T cells (MAIT). And finally, CD8, which is a basic phenotypic marker for T cells. Besides the BV711/BV750/BV785 combination, the fluorophores on these markers have very little spectral overlap into neighboring channels. This is why BV750 is being used as a dump channel for CD19/CD34 in this assay.
- **488 nm Blue and 561 nm Yellow-Green Laser Fluorophores:** On any flow cytometer, the FITC channel is the least spilled-into or “cleanest” channel. However, FITC itself is not a very bright fluorophore. For that reason, it is the perfect home for a marker that is abundantly expressed (high MFI), has a high variability of expression (wide CV of the MFI) and is commonly expressed on many cell types within the panel. In this panel, CD45RA fits that description . However, for some added intensity, we used VioBright B515 instead of FITC.
- Older-generation fluorophores, like PerCP and PE tandem fluorophores PerCP-Cy5.5, PE-Cy5 and PE-Cy5.5, which exhibit promiscuous excitation by the 355, 405, 488 and 561nm lasers to varying degrees, are being replaced in panels by the RealBlue and RealYellow fluorophores, which are very bright but, more importantly, exhibit much more discrete excitation profiles leading to less problematic spreading error. These new fluorophore chemistries make >30-color panels much more accessible without compromising data integrity and quality. As they are not yet commercially available in every marker we require, the markers chosen replaced second

tier antigen markers important for deeper immune subsetting like TCRVg9, TCRVd2, CD161, and CD27. The use of RealBlue conjugates for CD56 and CD94, basic immunophenotypic markers, also limits the impact of spectral spillover on these markers that are more ubiquitously expressed on NK cells. The few fluorophores that the RealBlue and RealYellow are unlikely to displace are PE itself and bright, stable fluorophores like PE-CF594 and PE-Dazzle 594, and in this panel PE is dedicated to a difficult-to-resolve iNKT population that is very low in abundance in peripheral blood defined by TCRvα24-Jα18.

- In this panel, clone and vendor restrictions for CD197, CD185, and CD194 required the use of PE tandems. Also, the fluorophore PE-Fire 810 has been demonstrated to be highly susceptible to photobleaching in the OMIP-069 update. In this panel PE-Fire 810 was tested conjugated to CD294, which will be used to phenotype a small ILC subset. It was removed from this panel because the instability of the tandem proved it to be unreliable in unmixing accuracy.
- **633 nm Red Laser Fluorophores:** Although the 633 nm laser has limited options, it can be reserved for critical subset markers such as CD294 for Th2 subsets and CD336 to subset ILC, both of which are low to medium expression levels, to leverage high brightness of APC and AF647. However the channels off the red laser will also experience the greatest degree of additive spectral spillover from reagents emitting off the other lasers. This is called cross-beam excitation/emission.
- Although APC and AF647 can be used together, it is not recommended to use any high-spillover reagent combinations unless necessary. Therefore, in this panel, we decided to remove CD336 AF647 with the option to keep AF647 open for that marker in the future. If used together, ensure the markers are both on antigens with middle to low abundance when possible, and dedicated to markers that will not be co-expressed on the same cell or present on events displayed on the same analysis plot . For example, avoid using BUV661, BV650, PE-Cy5 or PE-Fire 640, RB670 and APC-Fire 810 on markers defining the same population, as those also have markers conjugated to APC and AF647. The fact that the resolution of these channels can be impacted by these reagents is one of the reasons that live/dead fixable probes like Zombie NIR and Zombie R718 are popular when dead cells are excluded from analysis.
- **808 nm IR Laser Fluorophores:** 808 nm is very low-energy excitation, and any fluorophore excited by this laser, unless it is packaged into a polymer, also has very low efficiency in an aqueous environment. Together, these are both strengths for using this laser for live/dead detection where dim fluorophores excel. However, for immunophenotyping, it limits what can provide sufficient population resolution, even for moderately expressed antigens. A recommendation in panel development would be to reserve IR820 for CD45 or CD8.

No.	Excitation Range	Fluorophore	Fluorophore relative brightness (theoretical)	Marker	Antigen Density	Justification
1	UV	BUV395	Medium	HLA-DR	High	HLA-DR is highly expressed on antigen-presenting cells such as macrophages, B-cells, monocytes and dendritic cells.
2	UV	BUV 496	Low to medium	CD14	High	CD14 is a surface antigen primarily expressed on monocytes and macrophages. Any marker used in this channel must exceed the intensity of the innate NADH emission at this peak.
3	UV	BUV563	Low to medium	FcεRIα	High	FcεRIα (Fc epsilon RI alpha) marks specific subsets such as mast cells and basophils, crucial for allergic responses.
4	UV	BUV615	Medium	TCRαβ	High	TCRαβ is used in this panel to differentiate alpha/beta from gamma/delta T cell subsets. The detection of TCRγδ is clonal and conformationally dependent. TCRαβ is preferred so as not to compete with the binding of TCRVδ1, TCRVδ2 and TCRVɣ9.

5	UV	BUV661	Medium-to-high	CD303	High	CD303 is selectively expressed on plasmacytoid dendritic cells (pDCs). Although not required to subset pDC, it is recommended to differentiate pDC from basophils.
6	UV	BUV737	Low	CD11c	Low	CD11c is a basic phenotypic marker for dendritic cells, especially cDC. However, it also marks specific subsets of activated B, NK and monocytes.
7	UV	BUV805	Low	CD123	High	CD123 is highly expressed on plasmacytoid dendritic cells (pDC) and, to a lesser extent, on basophils.
8	Violet	BV421	Medium –to high	CD193	Medium	In this panel, CD193/CCR3 is a marker for eosinophils, basophils and Th2-like lymphocytes. It can also be expressed on mast cells, CD34+ progenitors and platelets.
9	Violet	SB436	Medium	CD117	Low	CD117 is a low-abundance protein expressed on hematopoietic stem cells, mast cells, ILC and germ cells.
10	Violet	Pacific Blue	Low	CD57	High	CD57 is a highly abundant protein on NK cells that serves as a marker for terminal differentiation on certain immune cells, including CD8+ T cells and a subset of natural killer (NK) cells.
11	Violet	BV480	Medium	CD25	Low	CD25 is expressed on activated T cells, regulatory T cells (Tregs), and some B cells.
12	Violet	BV510	Low	CD16	High	CD16 is a marker for certain immune cells, such as natural killer (NK), neutrophil, and monocyte subsets.
13	Violet	SBV570	High	CD8a	High	CD8a is a glycoprotein found on the surface of cytotoxic T cells.
14	Violet	BV605	Low to medium	CD196 (CCR6)	Medium	CD196 is a marker for certain immune cells, including subsets of T cells including Th9, Th17 and Th22, B lymphocytes, and immature dendritic cells.
15	Violet	BV650	Low to medium	TCR Vα7.2	Medium	TCR Vα7.2 is a marker for mucosal-associated invariant T (MAIT) cells but can also be expressed on non-MAIT T cells. It requires the co-expression of CD161 to define MAIT cells.
16	Violet	BV711	Medium	CD127	Low	CD127 is also known as the interleukin-7 receptor alpha chain (IL-7Rα). It's expressed on various immune cells, including thymocytes, most peripheral T lymphocytes and a subset of monocytes. CD127 expression is downregulated on Treg cells, making it a useful marker for distinguishing Tregs from other T cell subsets.
17	Violet	BV750	Medium	Dump channel (Exclusion Markers- CD19, CD34)	Medium to high	CD19 is expressed on B cells (irrelevant for this panel); CD34 is expressed on hematopoietic progenitors – also not of interest here.
18	Violet	BV785	Medium	CD3	High	CD3 is a marker for both conventional and unconventional mature T cells.
19	Blue	VioBright B515	Medium	CD45RA	Medium	CD45RA is a marker for naïve T cells and certain subsets of B cells.
20	Blue	RealBlue 545	Medium	CD94	Medium	CD94 is expressed by NK cells, a subset of γδ T cells, and NKT cells.
21	Blue	Spark Blue 574	Low	CD4	High	CD4 is typically a Tier 1 marker—critical for lineage identification of T helper cells.
22	Blue	RealBlue 613	High	CD56	Low	CD56 is a marker for NK cells and is expressed on some T cells, often called NKT-like cells.
23	Blue	RealBlue 670	Medium to High	TCR Vg9	Medium	TCR Vγ9 is a marker for a subset of γδ T cells, often paired with Vδ2.
24	Blue	RealBlue 705	High	CD161	Low to Medium	CD161 is expressed by NK cells, T helper 17 (Th17) cells and MAIT cells.
25	Blue	RealBlue 744	High	CD27	Medium	CD27 is a differentiation marker for conventional and γδT cells (memory and effector cells), and NK cells.

26	Blue	RealBlue 780	High	TCR Vδ2	Medium	TCR Vδ2 is a marker for a subset of γδ T cells.
27	Yellow Green	PE	High	TCR Vα24-Jα18	Low	TCR Vα24-Jα18 is a marker for invariant natural killer T (iNKT) cells. It can also be expressed on conventional T cells and thus requires the co-expression of TCRVβ11 to confirm iNKT subsets.
28	Yellow Green	PE-Dazzle 594	Medium -to high	CD197 (CCR7)	Low	CD197 (CCR7) is a marker for central memory conventional and γδT cells and is expressed on some dendritic cells.
29	Yellow Green	PE-Fire 640	High	CD14	High	CD14 is a pattern-recognition receptor expressed primarily on monocytes and macrophages.
30	Yellow Green	PE-Fire 700	High	CD185 (CXCR5)	Low	CD185 (CXCR5) is a marker for follicular helper T cells (Tfh). It is a homing protein for both T and B cells for the B cell follicle.
31	Yellow Green	PE-Cy7	High	CD194 (CCR4)	Low	CD194 (CCR4) is a marker for Th2, Th17 and Th22 cells and some regulatory T cells.
32	Red	APC	Medium	CD294	Low	CD294 is expressed on the surface of Th2 CD4+ and ILC subsets.
33	Red	VioBright R720	Medium	TCR Vδ1	Low	TCR Vδ1 is a marker for a subset of γδ T cells.
34	Red	NovaFluor Red 755	Medium	CD45	High	CD45 is a pan-leukocyte marker expressed on all immune cells.
35	Red	APC-Vio 770	Medium	TCR Vβ11	Medium	TCR Vβ11 is a marker for iNKT cells.
36	Red	APC-Fire 810	Low	CD183 (CXCR3)	Low to Medium	CD183 (CXCR3) is a marker for Th1 and Th9 cells and some NK cells.
37	IR	ViaKrome 808	N/A	Viability marker	N/A	Live/dead fixable viability dyes are amine-reactive probes that differentially label intracellular proteins accessible when the cell membrane is permeant.

Table 1: Details of the final panel iteration

Note: Relative brightness values shown here are theoretical approximations based on quantum yield, extinction coefficient, and typical antibody conjugation. Actual performance may vary depending on instrument configuration (laser power, detector gain), antigen density, and sample preparation.

Conclusion

Spectral flow cytometry helps in high-dimensional immune profiling by leveraging full-spectrum detection and advanced unmixing algorithms. Careful panel design—guided by principles such as minimizing spectral overlap, balancing fluorophore brightness, and ensuring robust unmixing—is essential to achieve accurate and reproducible results. Incorporating appropriate controls, optimizing sample preparation, and aligning fluorophore choices with instrument configuration further enhance data quality. By following these strategies, researchers can maximize resolution, reduce spreading error, and enable comprehensive characterization of complex cellular systems in both basic and translational research.

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