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Flow cytometry

Spectral flow cytometry handbook

invitrogen

Preface

Welcome to the spectral flow cytometry handbook, an essential resource for researchers in the field of flow cytometry. This guide is designed to provide comprehensive information and practical insights into the selection and use of spectral flow cytometry, an innovative technology that enhances the capabilities of flow cytometry.

Spectral flow cytometry represents a significant advancement in the analysis of cellular populations, offering improved resolution and the ability to analyze complex samples with greater precision. This handbook covers a wide range of topics, including the fundamental differences between conventional and spectral flow cytometry, selection of appropriate fluorophores, and detailed protocols for panel design and sample preparation.

Key sections of this handbook include:

- **Conventional vs. spectral flow cytometry**—understanding the distinct workflows and applications of each method
- **Panel design**—strategies for optimizing your panels to minimize spectral overlap and maximize data quality
- **Sample preparation and staining protocols**—step-by-step instructions to help ensure accurate and reproducible results
- **Instrument setup and maintenance**—best practices for setting up and maintaining your flow cytometer to achieve optimal performance

Additionally, this handbook provides access to valuable resources, such as the Flow Cytometry Panel Design Service and the Panel Builder Tool, which can assist you in designing and troubleshooting your panels.

We hope this handbook serves as a valuable reference in your research, helping you leverage the full potential of spectral flow cytometry and achieve your scientific goals with confidence.

Learn more at **thermofisher.com/spectral** and **thermofisher.com/flow**

Contents

Conventional vs. spectral flow cytometry

Introduction

With the advent of spectral flow cytometry, it is imperative to distinguish between conventional and spectral cytometry workflows.

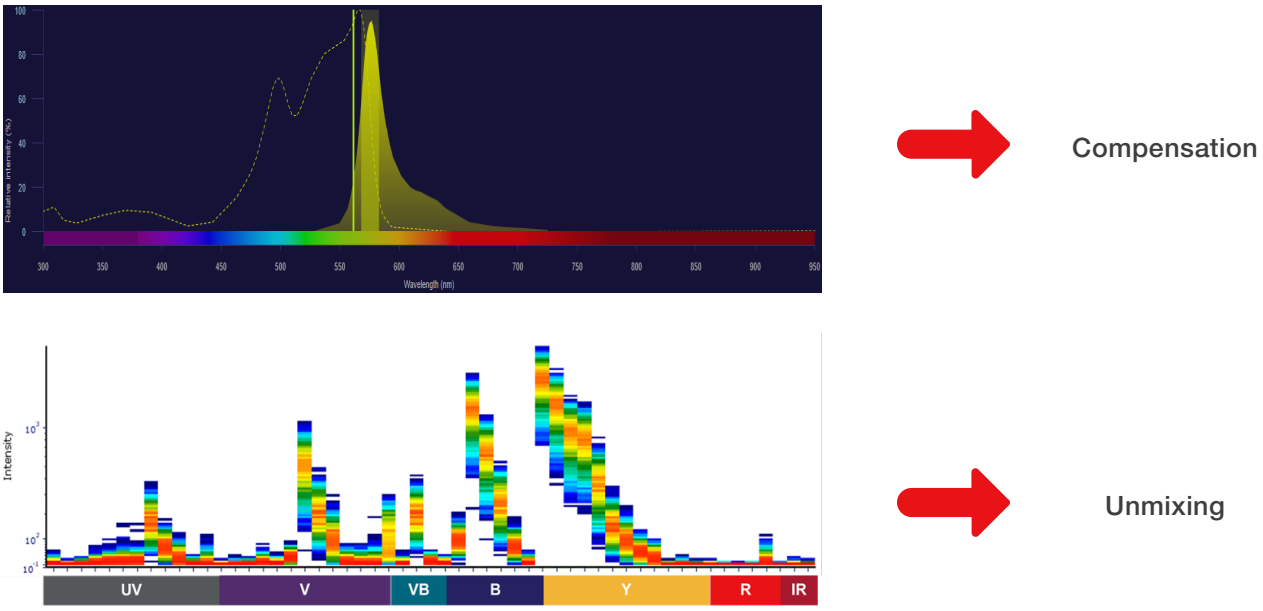


Figure 1. Schematic of the differences between conventional and spectral flow cytometry. Conventional cytometry uses one filter per fluorochrome, whereas spectral flow cytometry uses multiple detectors to generate a spectral fingerprint for each fluorochrome. Example instruments: Invitrogen™ Attune™ NxT Flow Cytometer, Invitrogen™ Bigfoot™ Spectral Cell Sorter and Invitrogen™ Attune™ Xenith™ Flow Cytometer with spectral modes.

Conventional flow cytometry

Conventional flow cytometry uses a laser and filter designed to capture peak emission of each fluorochrome to capture fluorescent light from this fluorophore. These instruments assign **one** filter/detector for **one** fluorochrome and only collect one data point for each fluorochrome. Light signals collected in off-target filters are compensated out. Example instrument: Invitrogen Attune NxT Flow Cytometer

Spectral flow cytometry

In spectral flow cytometry, all detectors are used to gather information for each fluorophore. Every fluorochrome has light emission in multiple detectors across an instrument array of filters and lasers. In spectral flow, every bit of this emission pattern is collected and assembled into a composite spectral signature. This signature will vary by the number of lasers on the instrument. Example instruments with spectral modes: Bigfoot spectral cell sorter, Attune Xenith flow cytometer (Figures 1 and 2).

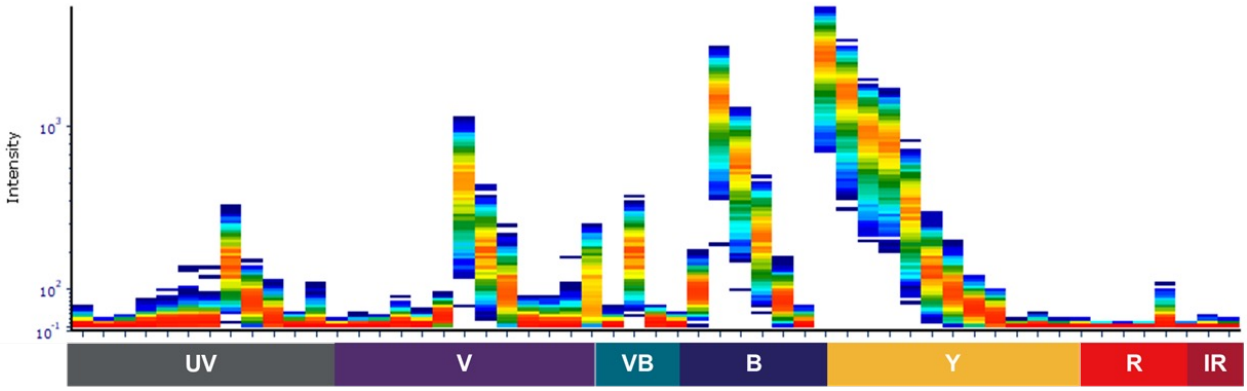
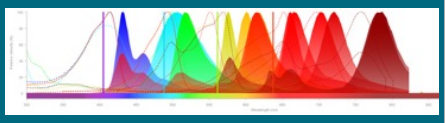
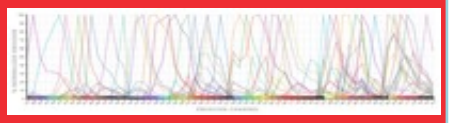


Figure 2. Representative spectral signature collected on a Bigfoot spectral cell sorter with seven lasers.

When to use conventional or spectral flow cytometry

Selecting conventional or spectral flow cytometry based on experimental conditions

Experimental conditions	 Conventional	 Spectral
Small sample size or sample is precious?	May need multiple panels and therefore high cell numbers to fully characterize or answer experimental questions.	One panel for all needs—reduces need for high numbers of cells for multiple panels.
Cells are highly auto fluorescent? (lung samples, myeloid cells)	May need to leave autofluorescence sensitive channels open to account for spread of autofluorescence. Autofluorescence can affect multiple channels in the ultraviolet, violet, blue and even yellow/green laser lines.	Autofluorescence spectral signature can be unmixed and designated as its own channel. The signal is therefore "removed" allowing easier resolution of autofluorescence sensitive channels.
Are you studying functional dyes that change their spectra over time?	Ratiometric dyes* are best done in conventional mode. Functional dyes that display changes over time acquired are simplest to capture in conventional mode.	Functional dyes can be run on spectral, but the spectra can vary in signature and can be difficult to unmix.
Controls required	Compensation can be performed without unstained cellular controls	Unmixing *requires* unstained cellular and autofluorescence controls
Panel size	<12–15 markers	20+ markers
Types of analysis	Functional, cell characterization	Deep immunophenotyping, broad and specific cell characterization

*Ratiometric dyes are functional dyes that change in their emission profiles due to the influence of binding or other cellular changes. Examples include JC-1 mitochondrial dye, some BODIPY dyes and more.

Table 1. Experimental conditions that may influence the choice of conventional vs. spectral flow cytometry include panel size, number of markers required, autofluorescence extraction, use of ratiometric or functional dyes that change spectra over time and type of analysis.

Compensation vs. unmixing

Knowing the differences between your instrument type and workflow will determine the method of data correction for overlapping fluorochromes. In conventional flow cytometry, this is termed compensation. In spectral flow cytometry, this is termed spectral unmixing.

Compensation

Simply put, this is the adjustment (removal) of the cross-excitation or spillover signal coming from non-intended fluorochromes for non-primary detectors. This is done using single-color stained controls (beads or cells) that are collected. Signal coming from non-intended fluorophores is then subtracted from the total signal detected in that channel. This is illustrated in Figure 3 with on-target signal in light green designated in the highlighted filter (red box) and off-target signal in all other detectors (dark green, yellow, light red and dark red histograms). The off-target signal is subtracted from the total signal in the selected channel to yield the signal obtained only from the light green histogram, representing the dye of interest.

Rules for compensation

— The signal from a fluorochrome must be the *brightest* in its respective channel, e.g., Invitrogen™ Alexa Fluor™ 647 dye should have a peak MFI intensity in its respective filter that is higher/stronger than any other filter on the instrument.

— Run a fully stained sample to ensure voltage/gains are set to be within the linear range of your instrument detectors. The peak of each fluorophore's histogram should also be within the linear range of its assigned detector.

— You must have a single-color control to use for compensation for every fluorochrome in your panel.

— Compensation can be done with either beads or cells.

— Signal from stained samples should not exceed the signal obtained during compensation setup.

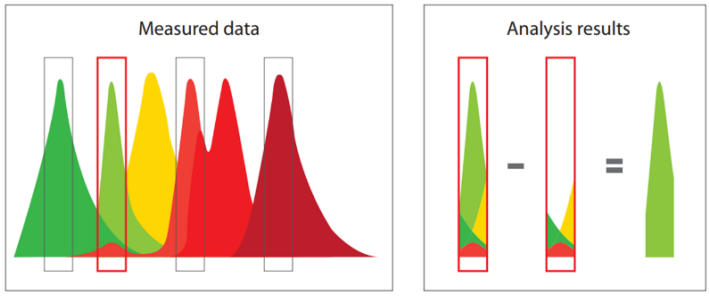


Figure 3. Schematic of compensation workflow focused on a defined primary detector channel and removal of spillover of other dyes into this channel. In this example, the light green color represents the dye of interest with a peak MFI in the channel with the red box. Note that spillover from all other dyes (dark green, yellow, light red and dark red peaks) is determined by measurement of their individual single-color controls. Removal of the off-target fluorescence from the fully stained sample should then yield the actual signal from the light green dye that peaks in this channel (assuming that compensation is appropriately applied). This is repeated mathematically for spillover of non-primary dyes into specific peak channels.

Spectral unmixing

In spectral flow cytometry, the process of adjusting the signal spillover between channels is different, as the signal for each fluorochrome is collected in every channel and the channels cover the entire spectrum.

Once unstained and single-color control signatures are collected, the first step of unmixing is normalization. Here, the signature of the stained particle is subtracted so that only the fluorescent signal of the fluorophore informs the algorithm (Figure 4). Additionally, the signal intensity of every channel is calculated relative to the peak emission channel. These normalized control signatures are then used to establish reference vectors in high dimensional space.

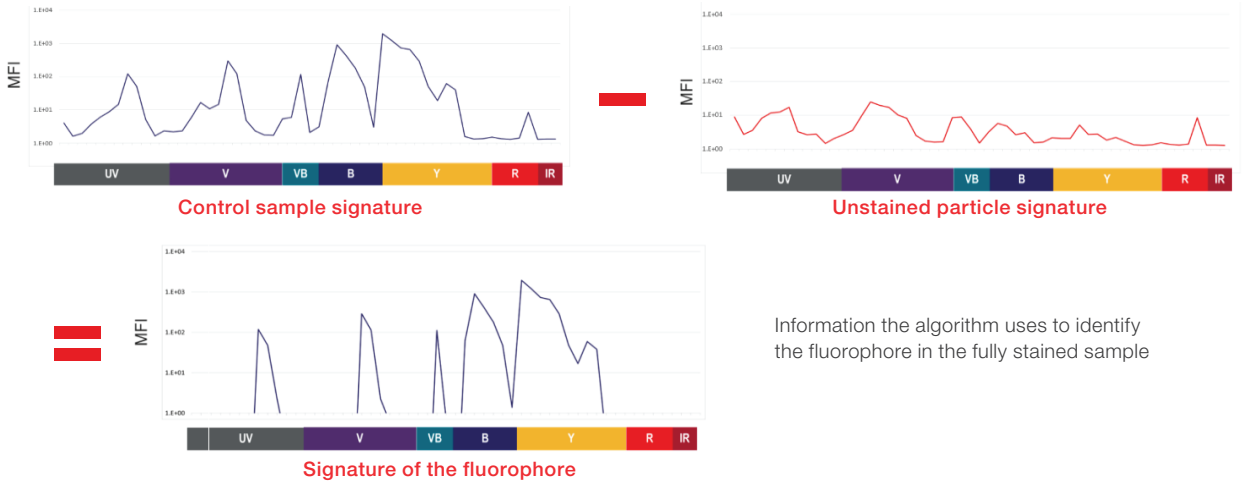


Figure 4. Schematic illustrating the use of single-color controls and unstained cells of beads to define normalized spectral signatures for use in unmixing. Spectral signatures show the peak for each channel detected in ascending spectral order for each laser line. In this case, "control sample signature" indicates the stained single-color sample for an individual dye plus either cells or beads. The "unstained particle signature" indicates unstained cells or beads (depending on the single control used). The final "signature of the fluorophore" is defined as the difference between the control sample signature and the unstained particle signature to indicate normalized fluorescence contribution from the dye alone.

Signature normalization is the first step

During the acquisition of the fully stained sample, the contribution of each reference will be measured, and the light signals acquired will be assigned to the appropriate fluorophores. In Figure 5, the reference controls for fluorescein isothiocyanate (FITC) and Invitrogen™ PE-eFluor™ 610 dyes are used to assign the light signals collected in the mixed sample to each of the fluorophores.

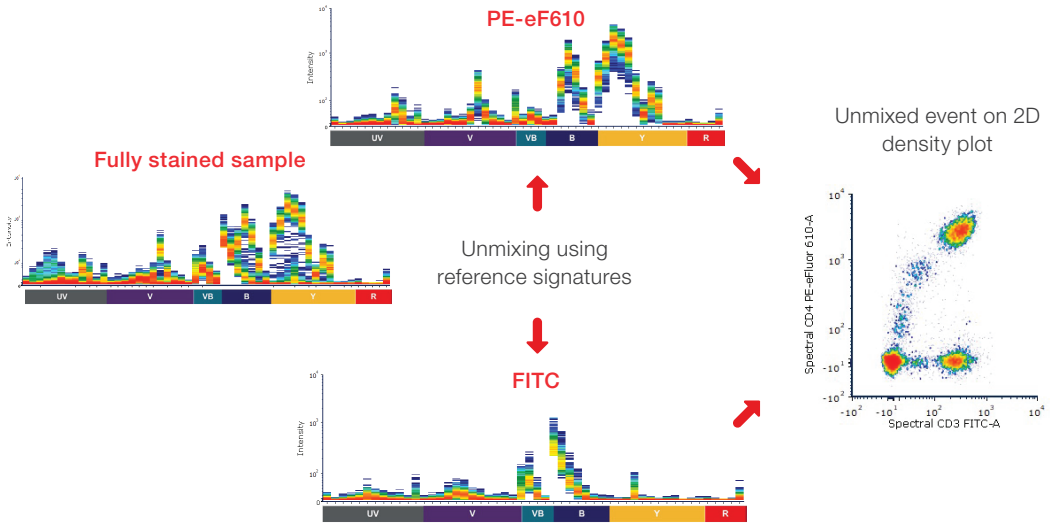


Figure 5. Unmixing the spectral signature of FITC and PE-eF610 from a fully stained sample on a 6 L Bigfoot sorter. In this example, the individual dye signatures for FITC and PE-eF610 (displayed here as a combination of cells and single-color fluorophore staining) are displayed along with the fully stained sample. The algorithm can then take the individual dye signatures and define a dye intensity that is displayed on this 2D density plot.

Rules for spectral unmixing

- Voltages/gains are often not adjusted by the user but are frequently defined using control beads so that similar settings are incorporated from experiment to experiment.
- Single-color unmixing controls can be either cells or beads.
- Unmixing controls, including beads, should generally be treated the same way as your sample, with a few notable exceptions. This includes all post-staining fixation procedures and permeabilization because those treatments can change the signature of the fluorochrome.
- Note that polymer blocking buffers, such as Invitrogen™ eBioscience™ Super Bright™ Complete Staining Buffer or Invitrogen™ Brilliant Stain Buffer, should not be used with beads since they contribute a unique dye signature.
- In order to compare the best unmixing controls, it may be useful to acquire single-color stains separately on beads or cells.
- Unstained cells should be added to inform the algorithm of the background autofluorescence pattern of the fully stained sample.
- Each sample type (tissue/cell line) may have its own unstained unmixing control to account for autofluorescence differences. The autofluorescence signature(s) can be defined either by gating on the same sample or by acquiring multiple samples that include different types of tissue or cell lines.

NovaFluor dyes engineered for narrow excitation and minimal spillover

Product description

Invitrogen™ NovaFluor™ dyes are designed with narrow excitation for minimal cross-laser excitation for spectral and conventional flow cytometry to deliver high resolution. Lower spectral spillover or overlap lessens the need for compensation, decreases spreading error and increases opportunities to add new markers. This aids in construction of flow cytometry panels with increased resolution while expanding the overall size of panels. NovaFluor conjugated antibodies represent foundational technology in the rapidly expanding world of flow cytometry (Table 2).

Note

CellBlox Plus blocking buffer should be used whenever NovaFluor dyes are used with cells.

Explore more about the advantages of using NovaFluor dyes:

- Minimal cross-laser excitation
- Minimal impact to compensation
- Decreased spillover spread
- Designed for panel expansion

Make sure to enhance NovaFluor data resolution by blocking dye-to-cell interactions by using Invitrogen™ CellBlox™ Plus Blocking Buffer.

Table 2. Available NovaFluor dyes are organized in columns by laser line and include official naming along with the wavelength of peak excitation and emission in nanometers.

Ultraviolet (Ex/Em max.)	Violet (Ex/Em max.)	Blue (Ex/Em max.)	Yellow (Ex/Em max.)	Red (Ex/Em max.)
NovaFluor UV 765* (358/763 nm)	NovaFluor Violet 690* (408/691 nm)	NovaFluor Blue 510 (496/511 nm)	NovaFluor Yellow 570 (552/568 nm)	NovaFluor Red 660 (637/659 nm)
	NovaFluor Violet 745 (391/743 nm)	NovaFluor Blue 530 (509/530 nm)	NovaFluor Yellow 590 (552/590 nm)	NovaFluor Red 685 (637/685 nm)
	NovaFluor Violet 800* (408/800 nm)	NovaFluor Blue 555 (494/555 nm)	NovaFluor Yellow 610 (552/612 nm)	NovaFluor Red 700 (639/700 nm)
		NovaFluor Blue 585 (494/585 nm)	NovaFluor Yellow 660 (552/663 nm)	NovaFluor Red 710 (639/710 nm)
		NovaFluor Blue 610-30S (509/614 nm)	NovaFluor Yellow 690 (552/690 nm)	NovaFluor Red 725 (636/725 nm)
		NovaFluor Blue 610-70S (509/614 nm)	NovaFluor Yellow 700 (552/700 nm)	NovaFluor Red 755 (636/755 nm)
		NovaFluor Blue 660-40S (509/665 nm)	NovaFluor Yellow 730 (552/731 nm)	
		NovaFluor Blue 660-120S (509/665 nm)	NovaFluor Yellow 755 (552/755 nm)	
		NovaFluor Blue 690 (494/690 nm)	NovaFluor Yellow 810 (551/813 nm)	
		NovaFluor Blue 725* (492/725 nm)		
		NovaFluor Blue 760* (490/764 nm)		
		NovaFluor Blue 800 (493/806 nm)		

*This NovaFluor dye fits in a spectral space that is currently not accessible by other spectral dyes.

Learn more at **thermofisher.com/novafluor**

Panel design

What are the key principles when designing any high-parameter panel?

1. Identify the biological question that you want to answer.
2. Establish expression patterns for the markers involved in the panel.
3. Understand cytometer capabilities.
4. Match antigen density to fluorophore brightness.
5. Minimize spread and interactions between fluorochromes.
6. Consider whether markers with more similar dyes can be positioned on separate cell types.
7. Work with available, trusted and tested antibodies.

Critical note

Use our FREE flow cytometry Panel Design Service—our scientists can design panels for you as many times as you need—free of charge, with no product purchase needed.

Learn more at **thermofisher.com/paneldesign**

1. Identify the biological question.

- When building any high-parameter panel, consider the species of the sample and tissue type: blood, tissue, purified cells, cell lines, etc.
- Understand autofluorescence pattern by running unstained control—autofluorescence may be high at wavelengths below 570 nm
- Contemplate the type of assay—for example, if sample stimulation is required, remember to include biological controls since antigen expression patterns may change
- When using fluorescent proteins, take their excitation and emission patterns into account

2. Establish expression patterns for the markers involved in the panel.

Consider which markers to include in the panel to answer the biological question, and how they are expressed in the sample. Classify the marker into three main categories based on the relative expression level:

Primary: highly expressed, well characterized (surface lineage markers)

Secondary: intermediate expression, not clear negative or positive, well characterized (some memory or activation markers)

Tertiary: low or unknown expression, rare events (some transcription factors)

It is also important to identify the cellular co-expression patterns for markers included in the panel: which markers are expressed in the same cell type and which markers are mutually exclusive.

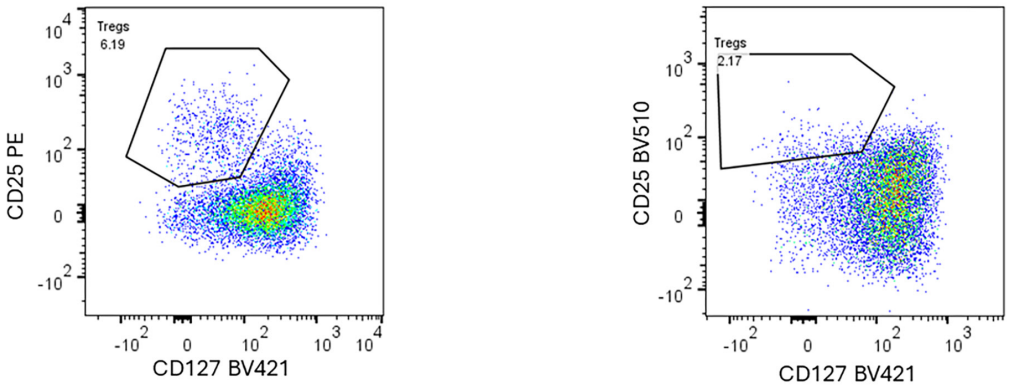
3. Understand cytometer capabilities.

The capabilities of the cytometer will determine how many antigens we can analyze in a high-parameter panel.

- Distinguish between conventional or spectral flow instruments and/or desired workflow (see the “Conventional vs. spectral flow cytometry” chapter for a comparison between conventional and spectral flow cytometry)
- Availability of lasers and filters—for example, does your cytometer have an ultraviolet (UV) laser?
- The appearance of the dye signature, relative brightness of the fluorophores and resolution of the panel are also affected by the detectors in terms of the number of detectors available and coverage of the spectra as well as whether the instrument uses avalanche photodiodes (APDs) or photomultiplier tubes (PMTs).

4. Match antigen expression levels to fluorophore brightness.

- When selecting fluorophores that we are going to use to detect our desired markers, we need to match antigen density to fluorophore brightness:
  - Use BRIGHT fluorophores for LOW-expressed antigens—LOW-expressed antigens are often tertiary markers
  - Use DIM fluorophores for HIGH-expressed antigens—HIGH-expressed antigens are likely primary markers (Figure 6)



CD25 in bright fluorophore (PE)

CD25 in dim fluorophore (BV510)

Figure 6. Example of how matching antigen density to brightness can affect data resolution. Peripheral blood mononuclear cells (PBMCs) were stained with either Cluster of Differentiation (CD) 25 in PE (bright) or Invitrogen™ Brilliant Violet™ 510 Dye (BV510) (dimmer) and co-stained with CD127 in Invitrogen™ Brilliant Violet™ 421 Dye (BV421) for Treg detection. Using a brighter fluorochrome, such as PE, allows clear identification of the CD25+ CD127- population. A dim fluorophore (BV510) does not offer full population resolution. Data and images were generated on a Bigfoot spectral cell sorter.

Comparison of the stain index or separation index allows classification of fluorophores according to their relative brightness level. The relative brightness of a fluorophore can then be matched with antigen density. Note that the specific stain index or separation index will vary based upon the detector setup of the flow cytometer.

5. Minimize spread and interactions between fluorochromes.

- Whether you are working on conventional or spectral flow, you need to select unique fluorophores with minimal overlap

  In spectral flow cytometry, in general, the more distinct the fluorophores are, the easier it will be to unmix the panel and obtain good resolution. The similarity index tells us how unique two fluorochromes are; the closer the index is to 1, the more similar the pair is, and the more difficult it will be to unmix their signals. The complexity index is a mathematical tool that helps predict how difficult it would be to unmix a panel run on the same instrument with a similar number of colors; a higher number suggesting an increase in difficulty to unmix the samples. Each instrument has its own complexity and similarity index calculations. (Figure 7).

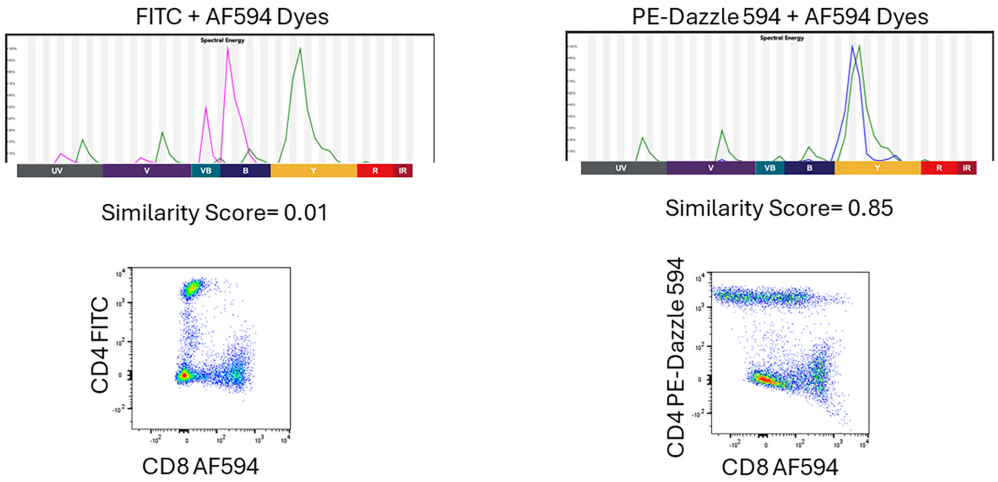


Figure 7. Comparison of dye signatures and unmixing between unique fluorophores—FITC and Invitrogen™ Alexa Fluor™ 594 (AF594) vs. using very similar fluorophores—PE-Dazzle™ 594 and AF594—to resolve CD4 and CD8 T cell populations. Using similar fluorophores leads to increased spread and more difficulty resolving populations of immune cells. Data and images were generated on a Bigfoot spectral cell sorter. Human PBMCs were gated on singlets and viable lymphocytes.

6. Consider whether markers with more similar dyes can be positioned on separate cell types.

- The final step is to understand the cellular co-expression patterns between the markers:
  - Fluorophores with high spectral overlap or high spillover should be assigned to markers that are uniquely expressed only on one cell type

The effects of spread on data resolution

For any high-parameter panel, it is fundamental to wphore when it is used in combination with another fluorophore (Figure 8).

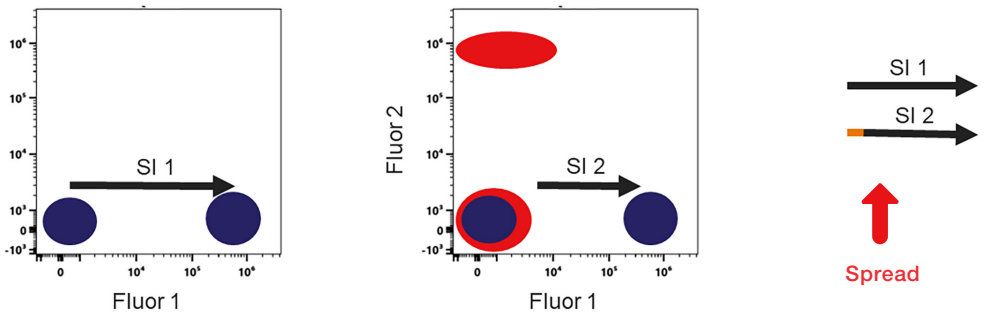


Figure 8. Illustration of the effect of spreading error. The fluorophore in red (Fluor 2) induces spreading error into the fluorophore in blue (Fluor 1), reducing the observed SI in the fully stained sample.

What can we do to overcome the effects of spread?

As a starting point, matching the relative expression of markers with fluorophore brightness will help us manage spread. The sample data in Figure 9 shows an example where mismatching required brightness affects resolution. Using increasing antigen density and a bright fluorophore (in this case PE), brightness increases in the primary detector (YL-1) and the spread into secondary detectors also increases (seen in red, pink and purple). The increased spread of the brighter conjugates results in difficulties in identifying co-stained populations. If bright PE fluor is matched to a more appropriate antigen density, such as CCR7 shown in purple, however, the separation can become more usable.

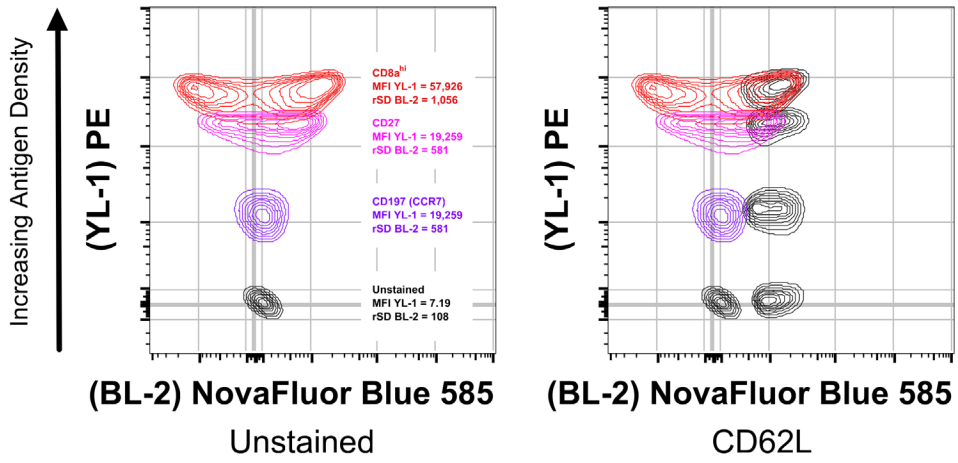


Figure 9. Demonstration of the effect of spreading error. Single-stained controls of PE (primary detector YL-1) compensated into the BL-2 detector in order of increasing antigen density: Unstained (black) Invitrogen™ eBioscience™ CD197 (CCR7) Monoclonal Antibody (3D12), PE (Cat. No. 12-1979-42); (purple) Invitrogen™ eBioscience™ CD27 Monoclonal Antibody (O323), PE (Cat. No. 12-0279-42); (pink) Invitrogen™ eBioscience™ CD8a Monoclonal Antibody (SK1), PE (Cat. No. 12-0087-42); (red) MFI (i.e., brightness) of YL-2 detector and rSD (i.e., spread) of the BL-2 detector reported next to each population (left). Single-stained controls of CD197 (CCR7) PE (purple), CD27 PE (pink), CD8 PE (red) overlayed with matched co-stained samples that include CD62L (L-Selectin) Monoclonal Antibody (DREG-56), Invitrogen™ eBioscience™ NovaFluor™ Blue 585 (Cat. No. H009T03B04) (right). For the purposes of illustrating increasing spread, CD8a was gated on the brightest expressing cells (CD8a hi). Data was collected using the stock configuration of the Attune NxT flow cytometer, blue/red/violet/yellow (V4).

In addition, spacing out fluorophores for co-expressed markers can help us mitigate the effects of spread (Figure 10).

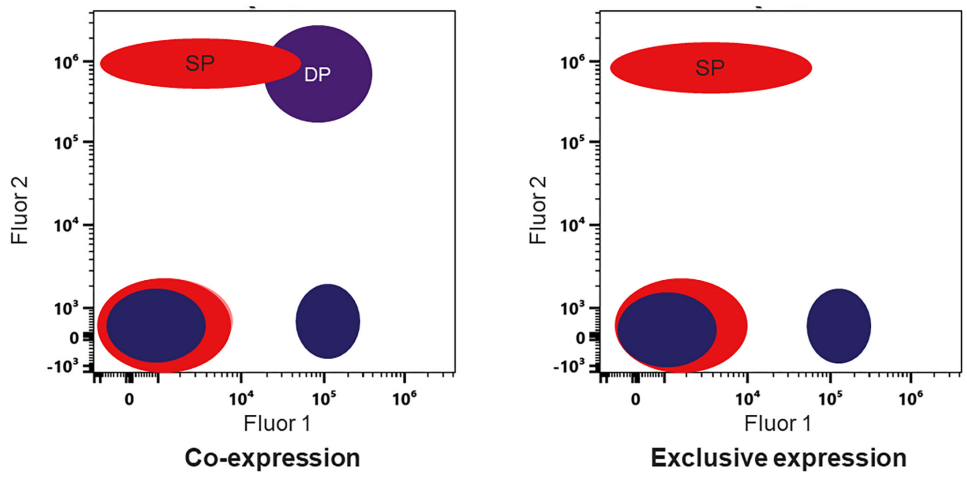


Figure 10. Illustration of how spacing out fluorophores for co-expressed markers helps mitigate the effects of spread. If Fluor 1 and Fluor 2 are assigned to markers that are co-expressed, the spread caused by Fluor 2 into Fluor 1 impairs the identification of the single positive (SP) and double positive (DP) subpopulations. Using Fluor 2 for a marker that is not co-expressed with Fluor 1 allows the identification of single positive cells for Fluor 2 despite the effect of spread. DP cells (not displayed) can be identified with a more unique fluorophore.

Using a spectral grid can be of great help in evaluating spread across lasers when building high-parameter panels, especially in spectral flow. Placing antibodies on a list makes it harder to understand the relationship between the different fluorophores. For example, in Figure 11, the spectral layout can help identify areas of potential dye cross-excitation by looking horizontally at the placement of specific markers and areas of spillover within a laser line by looking vertically.

Ultraviolet 349 nm laser bandpass filter (nm)	Fluorophore	Violet 405 nm laser bandpass filter (nm)	Fluorophore	Blue 488 nm laser bandpass filter (nm)	Fluorophore	Yellow 561 nm laser bandpass filter (nm)	Fluorophore	Red 640 nm laser bandpass filter (nm)	Fluorophore
UV1 387/11	Brilliant Ultra Violet 395								
UV2 420/10		V1 420/10	Brilliant Violet 421						
UV3 434/17		V2 434/17	Super Bright 436						
UV4 455/14		V3 455/14	eFluor 450						
UV5 473/15		V4 473/15							
UV6 507/19	Brilliant Ultra Violet 496	V5 507/19	eFluor 506	B1 507/19	FITC, Alexa Fluor 488, NovaFluor Blue 510				
UV7 549/15		V6 549/15	Pacific Orange	B2 549/15	NovaFluor Blue 555, Alexa Fluor 532				
UV8 575/15	Brilliant Ultra Violet 563	V7 575/15		B3 583/30		Y1 575/15	NovaFluor Yellow 570, PE, Alexa Fluor 561		
						Y2 589/15			
UV9 615/24		V8 615/24	Brilliant Violet 605, Super Bright 600	B4 615/24	NovaFluor Blue 610-70S, NovaFluor Blue 610-30S	Y3 605/15	NovaFluor Yellow 590, PE-eFluor 610		
						Y4 625/15	NovaFluor Yellow 610		
UV10 670/30	Brilliant Ultra Violet 661	V9 661/20	Brilliant Violet 650, Super Bright 645	B5 670/30	NovaFluor Blue 660-120S	Y5 661/20	NovaFluor Yellow 660, PE-Cy5	R1 670/30	APC, Alexa Fluor 647
						Y6 685/15			
						Y7 700/13		R2 700/13	NovaFluor Red 700
UV11 728/40		V10 710/20	Brilliant Violet 711, Super Bright 702, NovaFluor Violet 690	B6 720/60	PerCP-eFluor 710, NovaFluor Blue 725	Y8 720/24	NovaFluor Yellow 700, NovaFluor Yellow 690, PE Cyanine 5.5	R3 720/24	NovaFluor Red 710
UV12 750/LP	Brilliant Ultra Violet 737, NovaFluor UV 765, Brilliant Ultra Violet 805	V11 747/33	NovaFluor Violet 745	B7 750/LP	NovaFluor Blue 760, NovaFluor Blue 800	Y9 760/50	NovaFluor Yellow 755, PE-Cyanine 7	R4 760/50	NovaFluor Red 755, NovaFluor Red 725
		V12 770/LP	Brilliant Violet 785, Super Bright 780, NovaFluor Violet 800			Y10 800/12	NovaFluor Yellow 810	R5 770/LP	APC-eFluor 780
						Y11 832/37			
						Y12 860/LP			

Figure 11. Example of a spectral grid for a 5-laser Bigfoot spectral cell sorter. Fluorophores that may be cross-excited and affect resolution across multiple lasers can be predicted by looking across the chart horizontally, whereas fluorophores that may spill over into adjacent detectors on the same laser line can be considered by looking at the chart vertically.

It is also important to consider autofluorescence when selecting fluorophores to avoid pairing dim antigens with fluorophores overlapping with autofluorescence, or, potentially, to avoid using viability dyes emitting in the autofluorescence area. Autofluorescence signals are caused by cell components, such as collagen and Nicotinamide Adenine Dinucleotide Phosphate (NADPH), that emit light and are intrinsic to the cell of study—they are often highest in the detector arrays of the UV and violet lasers.

7. Work with available, trusted and tested antibodies.

Implementing a high-parameter panel can be a time-consuming process. Using validated antibodies will simplify the panel implementation and increase the possibilities of success. At Thermo Fisher Scientific, we provide high-quality, validated antibodies that are covered by our Antibody Performance Guarantee.

Learn more at **thermofisher.com/antibodyperformanceguarantee**

Resources for panel design

Free flow cytometry Panel Design Service with scientists and Panel Builder Tool

Work with our scientists using our free **Panel Design Service** to build your flow cytometry panels or use our **Panel Builder Tool** to create the panels you need yourself. We provide options for all experience levels, and you can use these resources as many times as you need, free of charge and with no product purchase required.

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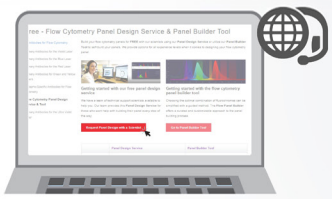
Just follow these 4 steps

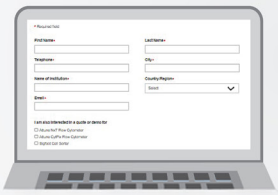
Step 1

Go to thermofisher.com/paneldesign



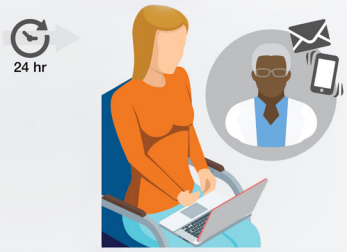
Click red button on left-hand side of screen to request a panel design

Step 2



Complete the panel design request form

Step 3



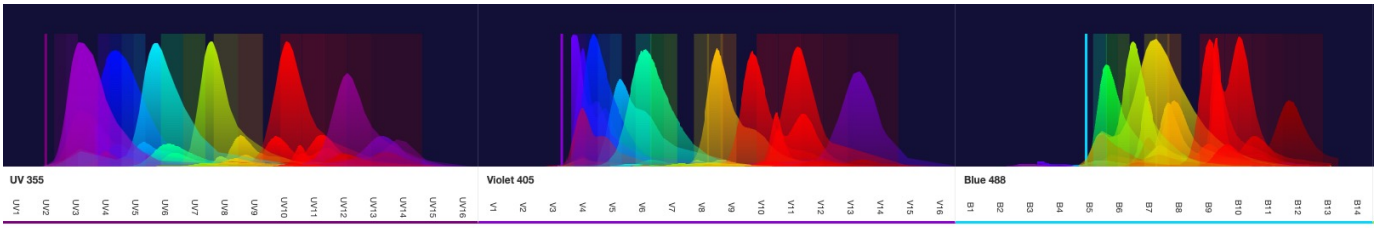
A scientist will contact you within 24 hours to discuss your needs

Step 4

A minimum of two scientists create, review, and optimize your panel

Self-design your panels with our Panel Builder Tool

A guided method can simplify choosing the optimal combination of fluorochromes. The Panel Builder Tool offers a curated and customizable approach.



Target	Fluorochrome	Complexity
CCR4 Human	PE	1.0
NKp46 (CD335) Human	PerCP-eFluor 710	1.1
CXCR3 Human	PE-eFluor 610	1.9
CCR6 Human	Super Bright 436 (SB436)	1.9
Human IgM Human	Brilliant Violet 480 (BV480)	1.9
FAS (CD95) Human	Super Bright 600 (SB600)	2.0
CD1c Human	Super Bright 645 (SB645)	2.0
Fixable **Viability**	LIVE/DEAD Fixable Blue Dead Cell Stain	2.1
Human IgD Human	FITC	2.1
Placeholder **CD39** Human	Brilliant Ultraviolet 563 (BUV563)	2.1
Placeholder **CD11c** Human	Brilliant Ultraviolet 661 (BUV661)	2.2
CCR7 Human	Brilliant Violet 786 (BV786)	2.3

Get started at **thermofisher.com/paneldesign**

Comparison of Panel Design Service and Panel Builder Tool

Choose the right option for your flow cytometry needs

	Panel Design Service	Panel Builder Tool
What	Take advantage of expert panel design when: • Designing a new panel • Troubleshooting an existing panel • Revising and optimizing an existing panel with new markers	Intuitive interface that: • Organizes fluorochrome marker selections • Reports predicted spillover values • Shows fluorescence spectra according to your cytometer configuration • Offers a pre-configured panel match
Target users	All flow cytometry users (all users—from naïve to experienced—are welcome to use)	All flow cytometry users confident in their ability to design/optimize panels on their own
Time frame	Initial response within 24 hours	Instant
Who	Live scientists with panel design expertise	Online tool
Product purchase	Not needed	Not needed
Cost	Free	Free
What to expect	Panel design, panel optimization, complete, end-to-end service with final panel design and review with a minimum of two experts	Design panels in five easy steps • Preconfigured panels—quick and easy access to panels designed by our experts, including many with supporting datasets • Streamlined input interface gets you to your panel quickly
Mode of communication	First, complete the panel **design service request form**. Our experts will then contact you by email or phone as needed.	Saved panels can be used to communicate with experts using our free Panel Design Service.

Table 3. Choose your path. Select the Panel Design Service to work with a live scientist or build your own panel using our Panel Builder Tool.

Get started at **thermofisher.com/paneldesign**

Single-color control beads for compensation and spectral unmixing

Introduction

Invitrogen™ UltraComp eBeads™ Plus Compensation Beads react with antibodies of human, rabbit, mouse, rat and hamster origin and are immunoglobulin light chain independent. Each drop of beads contains two populations: a positive population that will capture any mouse, rat, or hamster antibody and a negative population that will not react with antibodies. When a fluorochrome-conjugated antibody is added to the beads, both positive and negative populations result. This bimodal distribution can be used for single-color compensation or spectral unmixing controls in multicolor flow cytometry experiments.

UltraComp eBeads Plus compensation beads are broadly in use with many fluorophores and animal species, including all fluorochromes excited by ultraviolet (355 nm), violet (405 nm), blue (488 nm), green (532 nm), yellow-green (561 nm) and red (633–640 nm) lasers. UltraComp eBeads Plus compensation beads are optimized to perform better with the >650 nm part of the spectrum and more optimally replicate the dye signature of dyes such as Invitrogen™ Brilliant Violet™ 711, Invitrogen™ Brilliant Violet™ 786, Invitrogen™ Super Bright™ 702 and Invitrogen™ Super Bright™ 780, as compared to first generation Invitrogen™ UltraComp eBeads™ Compensation Beads.

Accurate single-color controls are essential for accurate multicolor flow cytometry data. As outlined in the section on compensation and unmixing in the "Panel design" chapter, knowing which instrument type you work with will determine the method of data correction for overlapping fluorochromes. In conventional flow cytometry, this is termed compensation. In spectral flow cytometry this is termed spectral unmixing.

Single-color controls can often be created by staining the cell type of interest. However, compensation beads can improve consistency and convenience and are essential if the target of interest is poorly expressed on the cells.

Critical note

Polymer blocking buffers are NOT compatible with UltraComp eBeads compensation beads and UltraComp eBeads Plus compensation beads. If either eBioscience Super Bright complete staining buffer or Brilliant stain buffer are used with the beads, then a dye signature from the blocking buffer will appear and it will not properly compensate or unmix the sample.

Note

UltraComp eBeads Plus compensation beads are compatible with standard staining buffers that contain Phosphate-Buffered Saline (PBS) or Hank's Balanced Salt Solution (HBSS), proteins, such as bovine serum albumin (BSA) or fetal bovine serum (FBS) and sodium azide. No other additives should be used.

For more information, please contact Technical Support.

Note

When first testing a new panel, it may be useful to run both cells and beads independently as single-color controls and then decide which is most helpful in unmixing an experiment.

Note

Goat and sheep host species should use single-color cell and Fluorescence Minus One (FMO) controls, not beads.

Protocol

Materials

- UltraComp eBeads Plus Compensation Beads (**Cat. No. 01-3333-41**) or Invitrogen™ UltraComp™ Spectral eBeads (**Cat. No. U20250**), contact **flowplex.support@thermofisher.com** for **Cat. No. U20250**
- Unstained cells
- Primary antibodies (directly conjugated)
- Invitrogen™ eBioscience™ Flow Cytometry Staining Buffer (**Cat. No. 00-4222-26**)
- 12 x 75 mm round-bottom test tubes
- Reagents for fixation and permeabilization (optional)

Learn more at **thermofisher.com/compbeads**

Experimental procedure

Step I: Preparation of single-color compensation or unmixing controls with beads

1. Label a tube for each fluorochrome that will be used in the experiment.
2. Mix beads by vigorously inverting at least 10 times or pulse-vortexing.
3. Add 1 drop of UltraComp eBeads Plus compensation beads to each tube.
4. Add 1 test or less of antibody conjugate to each tube.
5. Mix well by flicking, inverting vigorously or pulse-vortexing.
6. Incubate at 2–8°C for 15–30 minutes in the dark.
7. Add 2 mL of eBioscience Flow Cytometry staining buffer to each tube and centrifuge at 400–600 x *g* for 3–5 minutes.
8. Decant or aspirate supernatant and add 0.2-0.4 mL of eBioscience Flow Cytometry staining buffer to each tube.
9. Mix briefly by flicking or pulse vortexing before analysis.

Critical note

The spectral signature between cells and compensation beads may differ, leading to compensation and spectral unmixing errors. It is strongly recommended that cells be used as single-color controls if unmixing or compensation errors are identified when using UltraComp eBeads Plus compensation beads.

Step II: General compensation and spectral unmixing setup principles

1. Before acquiring beads, run unstained and fully stained cells on a cytometer to determine appropriate voltage or gain settings for fluorescence detectors in a compensation-based workflow. Note that in a spectral-based workflow, there may already be recommended gains or voltages. Do not change these settings while acquiring compensation or spectral unmixing setup, with the exception of Forward Scatter/Side Scatter (FSC/SSC) parameters.
2. Run a sample of beads to adjust FSC/SSC, if necessary, to visualize beads (this can even be a single-colored stained bead). It is acceptable to adjust the FSC/SSC to get the beads in view.
3. Run each single-stained bead sample to help ensure the positive peaks are on scale. Voltages or gains should be decreased (as minimally as possible) for any positive bead peak that is off-scale. Do not record any data until all single-color stained beads have been reviewed. Make sure that the resulting settings are still appropriate for the cell sample (there should be minimal change).
4. Run each single-stained bead sample to perform setup for compensation or spectral unmixing and record files for each tube with beads.
5. Run the compensation wizard or manually set compensation or use spectral unmixing tool.
6. Readjust FSC/SSC settings for cell samples, if necessary, and acquire experimental samples.
7. Collect and record the unstained cell sample and all experimental samples.

Blocking buffers

Introduction to blocking buffers

Often, when immunologists think of blocking buffers, they think specifically about blocking cells from binding the Fc portion of antibodies and giving a false positive as compared to target binding by the Fab antigen-specific portion of the antibody.

However, there are multiple types of interactions that can lead to false data, including cell-antibody, dye-dye and cell-dye interactions (Figure 12).

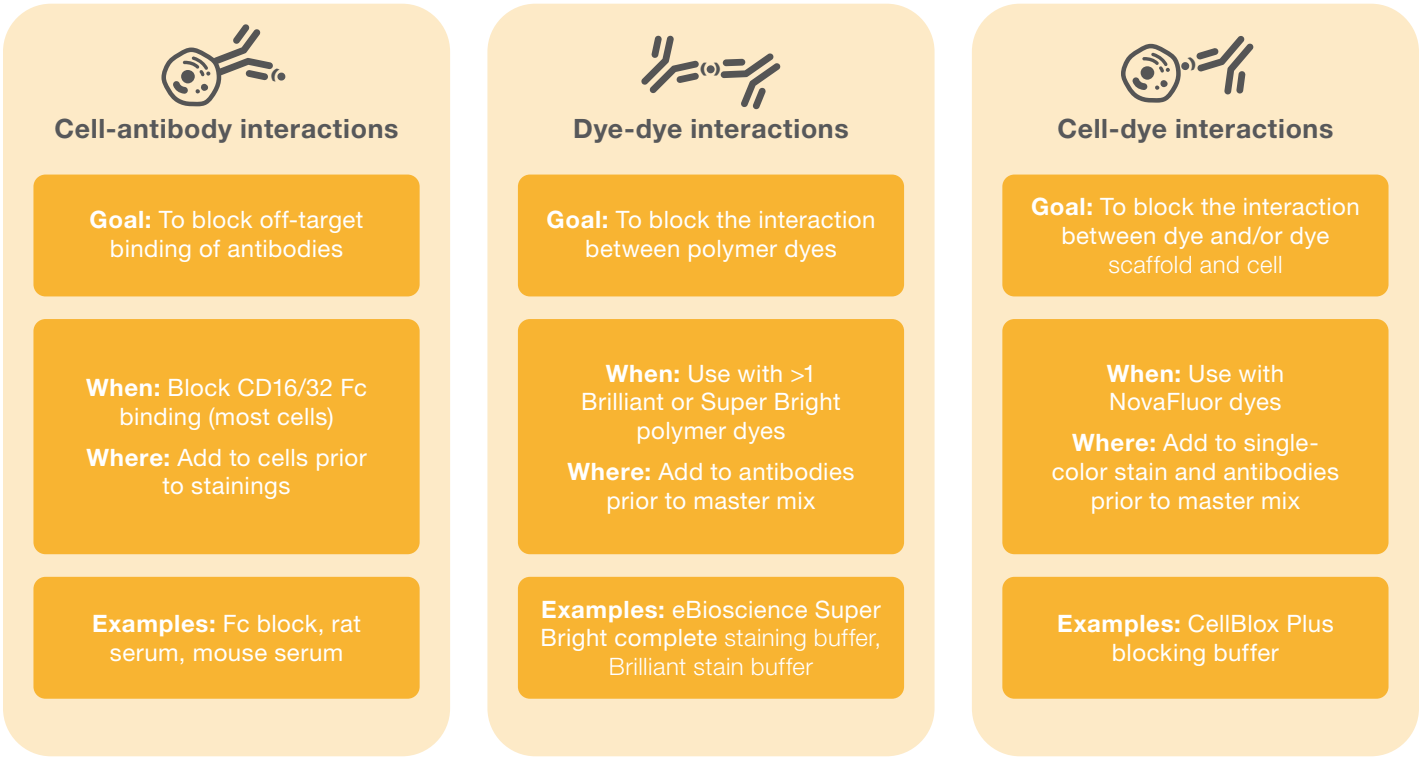


Figure 12. Diagram of types of interactions that can alter flow cytometry data paired with specific blocking solution.

Blocking cell-antibody interactions (Fc block, serum)

Immune cell types, such as neutrophils, monocytes, macrophages, B-cells, natural killer cells and some T cells, can express low or high-affinity Fc receptors. These cellular Fc receptors can then bind antibodies nonspecifically and provide confusion in data interpretation since they are not binding through the Fab antigen-specific portion. These interactions can be blocked with anti-CD16/CD32 antibodies, Fc block, as well as species-specific sera. Cells are typically pre-blocked for 10–20 minutes with an Fc block or serum prior to addition of antibodies or antibody cocktail to the cells.

Blocking dye-dye interactions (polymer dyes)

Polymer-based dyes, such as Invitrogen™ Brilliant Ultra Violet™, Invitrogen™ Brilliant Violet™ and Invitrogen™ Super Bright™ dyes, can interact and lead to dye-dye interactions that can affect the dye signatures. When more than one polymer dye-conjugated antibody is included in a panel, e.g., Invitrogen™ Brilliant and/or Super Bright dyes, either Invitrogen eBioscience Super Bright Complete Staining Buffer (**Cat. No. SB-4401-75**) or Invitrogen Brilliant Stain Buffer (**Cat. No. 00-4409-75**) should be included in the antibody cocktail prior to polymer-conjugated antibody addition. These polymer-blocking buffers prevent nonspecific polymer-to-polymer interactions that can result

in data appearing under- or over-compensated. eBioscience Super Bright complete staining buffer is provided in a 5 µL/test format, while Brilliant stain buffer is provided in a 50 µL/test format. Either blocking buffer must be added to the cocktail before polymer dyes are added to prevent polymer-to-polymer interactions.

These buffers can be used interchangeably. Though either eBioscience Super Bright complete staining buffer or Brilliant stain buffer can be used, the spectral signature of a polymer dye may change, so the single-color cellular controls may also include the matching blocking buffer. Both buffers are compatible with traditional fluorochromes, Brilliant Violet, Brilliant Ultra Violet, Super Bright dyes and standard flow cytometry protocols.

However, neither polymer blocking buffer is compatible with UltraComp eBeads and Invitrogen UltraComp eBeads Plus compensation beads. If either eBioscience Super Bright complete staining buffer or Brilliant stain buffer is used with the beads, then a dye signature from the blocking buffer will appear and it will not properly compensate or unmix the sample

Blocking cell-dye interactions (NovaFluor dyes)

When using any Invitrogen™ NovaFluor™-conjugated antibody for cell surface staining, Invitrogen CellBlox Plus Blocking Buffer (**Cat. No. C001T06F01**) is required to block nonspecific, cell-NovaFluor dye interactions. In the absence of CellBlox Plus blocking buffer, the background is significantly increased, which makes it appear that the separation of positives and negatives is much worse. This is a non-antibody blocking solution formulated to reduce background labeling and must be used whenever a NovaFluor-conjugated antibody is used for labeling any cell type, including single-color controls. CellBlox Plus blocking buffer has enhanced blocking capabilities compared to the original Invitrogen™ CellBlox™ Blocking Buffer, specifically in terms of observed B cell background. In addition, CellBlox Plus blocking buffer has the added benefit of blocking nonspecific binding of monocytes, macrophages and other cells to cyanine-based or tandem dyes and thus an additional monocyte blocker is not required.

CellBlox Plus blocking buffer is compatible with all fluorophores and LIVE/DEAD fixable dead cell stains and can be used with other blocking reagents. CellBlox Plus blocking buffer can be added to an antibody cocktail mixture prior to staining cells. CellBlox Plus blocking buffer is provided in a 5 µL/test format. Add 5 µL per sample (regardless of the number of NovaFluor dyes in your panel) to the antibody cocktail.

CellBlox Plus blocking buffer is not required for use with compensation beads when staining with NovaFluor-conjugated antibodies (since there are no cells present to bind the NovaFluor dyes). However, in contrast to the eBioscience Super Bright complete staining buffer or Brilliant stain buffer, CellBlox Plus blocking buffer is fully compatible with UltraComp eBeads and UltraComp eBeads Plus compensation beads, and no measurable signature is observed if CellBlox Plus blocking buffer is included during bead staining.

Blocking a fully stained sample (all three blocking buffers)

When staining a sample that includes at least one NovaFluor dye and >1 polymer dye (Brilliant and/or Super Bright), all three of the described blocking buffers should be used. Cell-antibody interactions should be blocked with Fc block and/or serum (as needed) by pre-blocking cells for 10–20 minutes prior to staining with antibodies. Polymer-blocking buffers (eBioscience Super Bright complete staining buffer or Brilliant stain buffers) should be added to the antibody cocktail PRIOR to adding any polymer-conjugated antibodies. We recommend adding CellBlox Plus blocking buffer at the same time (prior to antibody addition) to simplify the workflow and prevent cell-dye interactions. The cells (blocked with Fc block and/or serum) can then be mixed with the final antibody cocktail containing the polymer blocking buffer and CellBlox Plus blocking buffer.

Blocking and staining protocol

Cell suspensions

Materials

- Fisherbrand™ Premium Microcentrifuge Tubes, 1.5 mL (**Fisher Scientific Cat. No. 05-408-136**)
- Globe Scientific Inc.™ Culture Tubes, 12 x 75 mm (**Fisher Scientific Cat. No. 22-171-606**) or Falcon™ 96-Well, Non-Treated, U-Shaped-Bottom Microplates (**Fisher Scientific Cat. No. 08-772-54**)
- **Primary antibodies** (directly conjugated or purified)
    - Secondary detection reagents, if primary antibodies are not directly conjugated
- Invitrogen eBioscience Flow Cytometry Staining Buffer (**Cat. No. 00-4222-26**)
- Required fluorophore buffers:
    - Invitrogen eBioscience Super Bright Complete Staining Buffer (**Cat. No. SB 4401-75**) or Invitrogen Brilliant Stain Buffer (**Cat. No. 00-4409-42**)
- Optional: For blocking nonspecific Fc-mediated interactions
    - Anti-mouse CD16/32 antibody (e.g., Invitrogen™ eBioscience™ CD16/CD32 Monoclonal Antibody [93]) (**Cat. No. 14-0161-81**)
    - Anti-human Fc receptor binding inhibitor antibody (e.g., Invitrogen™ eBioscience™ Fc Receptor Binding Inhibitor Polyclonal Antibody) (**Cat. No. 14-9161-71**)
    - Invitrogen™ Normal Mouse Serum (**Cat. No. 10410**)
    - Invitrogen™ Normal Rat Serum (**Cat. No. 10710C**)
- Viability staining
    - Fixable stains: Invitrogen™ LIVE/DEAD™ Fixable Dead Cell Stain Sampler Kit (**Cat. No. L34960**)
    - Non-fixable stains:
        > Invitrogen™ eBioscience™ 7-AAD Viability Staining Solution (**Cat. No. 00-6993-50**)
        > Invitrogen™ eBioscience™ Propidium Iodide Staining Solution (**Cat. No. 00-6990-50**)
        > Invitrogen™ SYTOX™ Dead Cell Stain Sampler Kit (**Cat. No. S34862**)

Experimental procedure

1. **Prepare single-cell suspension** as described in BestProtocols: Cell Preparation for Flow Cytometry Protocols at **thermofisher.com/cellpreparation**.

2. **Optional: Block nonspecific Fc-mediated interactions.** This is necessary when working with neutrophils, monocytes, macrophages, B-cells, natural killer cells and some T cell subsets. Positive staining with anti-CD16/32 on cells will show cells that express Fc-gamma receptor.

   - For mouse cells: Preincubate the cells with 0.5–1 µg of anti-mouse CD16/CD32 antibody per 100 µL for 10–20 minutes at 2–25°C before staining. Resuspend in 50 µL of eBioscience Flow Cytometry staining buffer
   - For human cells: Pre-incubate the cells with 20 µL of anti-human Fc receptor binding inhibitor antibody per 100 µL for 10–20 minutes at 2–25°C before staining. Resuspend in 50 µL of eBioscience Flow Cytometry staining buffer
   - Preincubate the cells with 2 µL of 2% mouse serum to human cells and 2 µL of 2% rat serum to mouse cells per 100 µL of cells. Incubate for 15 minutes at 2–25°C. Do not wash prior to staining cells with antibodies.

3. **Aliquot 50 µL of cell suspension** (from 10^5–10^8) to each tube or microwell plate.

4. **Block cells with the buffers associated with eBioscience Super Bright,** Brilliant Violet and Brilliant Ultra Violet dyes and/or NovaFluor dyes. Antibody mixtures should be made and used fresh (Table 4).

Note

Antibody-binding kinetics are temperature-dependent. Staining on ice may require longer incubation times. Furthermore, some antibodies may require non-standard incubation conditions that will be noted on the technical data sheet provided with the antibody.

Note

Mouse and rat serum are both required when using NovaFluor dyes with mouse cells.

Protocol tip

To determine the size of the sample (number of cells) that will provide a given precision, the equation r=(100/CV) x2 is used, where *r* is the number of cells and *CV* is the coefficient of variation of a known positive control.

Table 4. Type of blocking buffer, function and volume to use in antibody cocktail. Note that polymer blocking buffers should be added prior to polymer-conjugated antibody addition.

Buffer	Use	Amount
eBioscience Super Bright complete staining buffer	Reduces background when using antibodies labeled with Super Bright dyes, Brilliant Violet dyes or Brilliant Ultra Violet dyes	5 µL per sample to the antibody cocktail
Brilliant stain buffer (alternative to eBioscience Super Bright complete staining buffer)	Reduces background when using antibodies labeled with Super Bright dyes, Brilliant Violet dyes or Brilliant Ultra Violet dyes	50 µL per sample to the antibody cocktail
BD Horizon Brilliant™ Stain Buffer Plus	Reduces background when using antibodies labeled with Super Bright dyes, Brilliant Violet dyes or Brilliant Ultra Violet dyes	10 µL per sample to the antibody cocktail
CellBlox Plus blocking buffer	Reduces background when antibodies labeled with NovaFluor dyes and cyanine-based dyes. CellBlox Plus blocking buffer is recommended for best NovaFluor dye performance.	5 µL per sample to the antibody cocktail

When using antibodies labeled with polymer dyes (e.g., Super Bright dyes, Brilliant Violet dye, Brilliant Ultra Violet dye), block nonspecific dye interactions by adding volume of eBioscience Super Bright complete staining buffer or Invitrogen™ Brilliant Stain Buffer directly to antibody cocktails/master mixes **PRIOR** to adding antibodies to cocktail or master mix; cells can then be stained by combining antibody cocktail/master mix with cells.

When using antibodies labeled with NovaFluor dyes or cyanine-based dyes, we recommend adding CellBlox Plus blocking buffer to antibody cocktails/master mixes prior to combining with cells. Add 5 µL per sample (regardless of the number of NovaFluor dyes in your panel) to use the antibody cocktail as intended. CellBlox Plus blocking buffer is required every time a NovaFluor dye is used with cells.

1. Combine the recommended quantity of each primary or fluorophore-labeled antibody in an appropriate volume of eBioscience Flow Cytometry staining buffer so that the final staining volume is 100 µL (i.e., 50 µL of cell sample + 50 µL of antibody mix) and add to cells. Pulse-vortex gently to mix.
2. Incubate for 30 minutes at 2–8°C. Protect from light.
3. Wash the cells by adding eBioscience Flow Cytometry staining buffer. Use 2 mL per tube or 200 µL per microplate well. Centrifuge at 400–600 x *g* for 5 minutes at room temperature. Discard supernatant.
4. Repeat Step 7.
5. Dilute the appropriate fluorophore-labeled secondary detection reagent in 100 µL of eBioscience Flow Cytometry staining buffer and add to cells. Incubate for at least 30 minutes at 2–8°C or on ice. Protect from light.
6. Wash the cells by adding eBioscience Flow Cytometry staining buffer. Use 2 mL per tube or 200 µL per microplate well. Centrifuge at 400–600 x *g* for 5 minutes at room temperature. Discard supernatant.
7. Repeat Step 7.
8. Optional: Stain samples with a viability dye according to LIVE/DEAD Fixable Dead Cell Stains Protocol or BestProtocols: Cell Preparation for Flow Cytometry Protocols at **thermofisher.com/cellpreparation.**
9. Optional: For storage of samples before analysis, resuspend cells in 100 µL of eBioscience Flow Cytometry staining buffer and add 100 µL of Invitrogen™ eBioscience™ IC Fixation Buffer or 2 mL of Invitrogen™ eBioscience™ 1-step Fix/Lyse Solution.
10. Analyze samples by flow cytometry analyzer or isolate cells with a flow cytometry cell sorter.

Critical note

eBioscience Super Bright complete staining buffer is only necessary when using more than one polymer dye-conjugated antibody in the same sample to minimize nonspecific polymer dye interactions, which can result in data appearing under-compensated. eBioscience Super Bright complete staining buffer is compatible with traditional fluorophores.

Critical note

Invitrogen Brilliant stain buffer or BD Horizon Brilliant Stain Buffer Plus can be used as an alternative to eBioscience Super Bright complete staining buffer. It is not necessary to add both.

Note

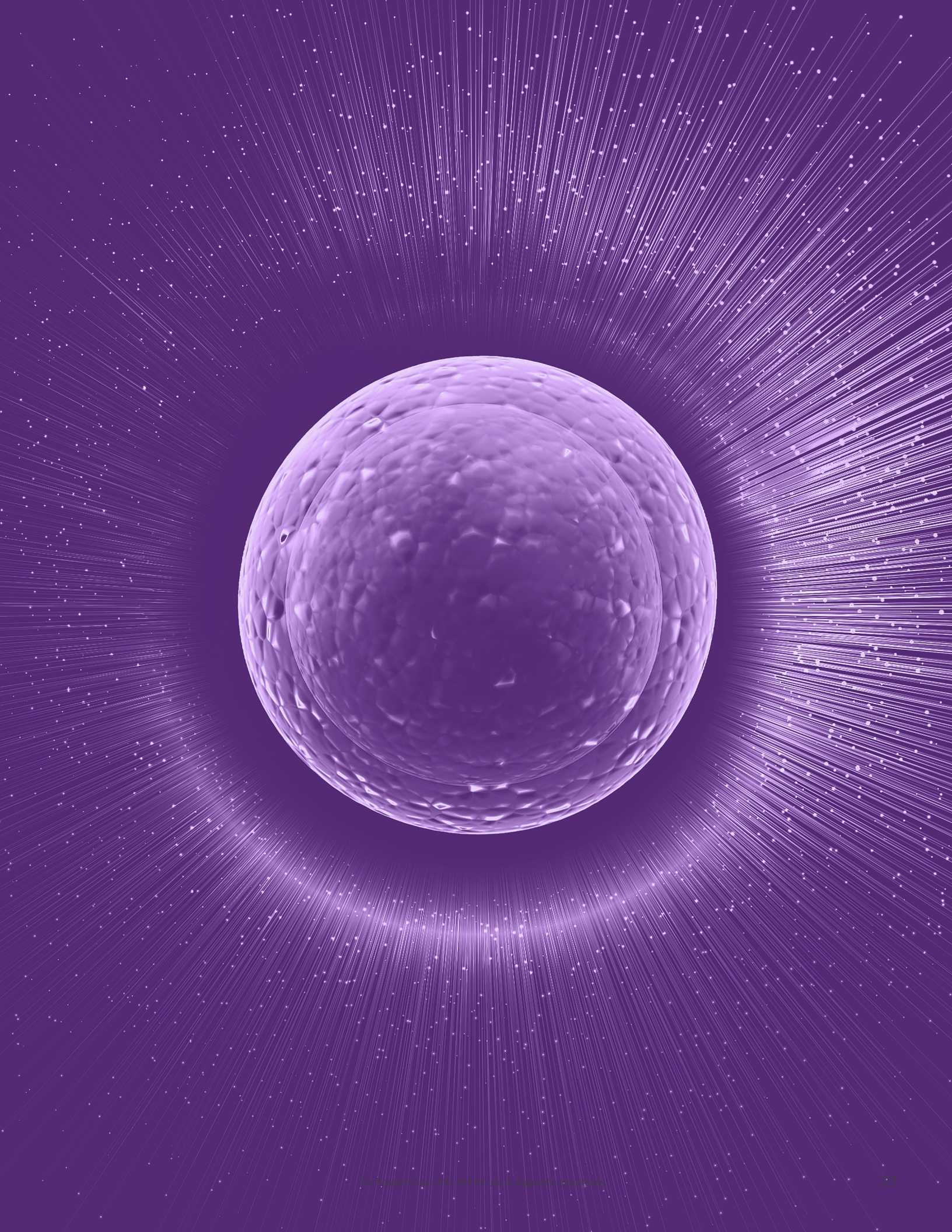
When using purified or biotinylated antibodies, incubate for one hour at 2–8°C.

Note

Use a LIVE/DEAD fixable dead cell stain with NovaFluor dyes. Impermeant DNA-binding dyes, such as 4',6-diamidino-2-phenylindole (DAPI), propidium iodide and SYTOX stains are not compatible with NovaFluor dyes.

Note

SYTOX reagents, eBioscience 7-AAD viability staining solution and eBioscience propidium iodide are not compatible with cells that have been fixed. Resuspend cells in an appropriate volume of eBioscience Flow Cytometry staining buffer (i.e., 10^5 cells in microtiter plate will be resuspended in less than 500 µL of buffer.)



Blocking nonspecific binding with CellBlox Plus blocking buffer

Product description

CellBlox Plus blocking buffer is formulated to block nonspecific binding of Invitrogen™ NovaFluor™ labels with cells. These nonspecific interactions can result in higher background labeling. CellBlox Plus blocking buffer should be used every time a NovaFluor dye is used for labeling any cell type to minimize background labeling.

CellBlox Plus blocking buffer is also recommended for use with cyanine-based dyes or cyanine-based tandem dyes to block nonspecific interactions with monocytes, macrophages and other cell types to minimize background labeling.

Use of CellBlox Plus blocking buffer requires no change to flow cytometry staining protocols. Add 5 µL of CellBlox Plus blocking buffer directly to a cell suspension containing 10^3–10^8 cells during a blocking step prior to the addition of an antibody. Or, add 5 µL of CellBlox Plus blocking buffer per sample to an antibody cocktail prior to labeling cells.

Note

CellBlox Plus blocking buffer should be used whenever NovaFluor dyes are used with cells.

Tips for success

- Always use CellBlox Plus blocking buffer with NovaFluor dyes when labeling cells for excellent background reduction
- CellBlox Plus blocking buffer is compatible with all fluorophores and with Invitrogen™ LIVE/DEAD™ Fixable Dead Cell Stains
- CellBlox Plus blocking buffer can be used with any fluorophore-antibody conjugate as a high-performance monocyte and macrophage-blocking solution
- CellBlox Plus blocking buffer is compatible with other blocking reagents, such as Fc block, blocking proteins, Brilliant stain buffer and eBioscience Super Bright complete staining buffer
- CellBlox Plus blocking buffer is not required when labeling antibody-capture beads

Experimental protocol

Materials

- Invitrogen CellBlox Plus Blocking Buffer (Cat. Nos. C001T02F01, C001T03F01, C001T06F01)
- Invitrogen eBioscience Flow Cytometry Staining Buffer (Cat. No. 00-4222-26)
- Primary conjugated antibodies
- 12 x 75 mm round-bottom polystyrene test tubes or U- or V-bottom polystyrene microplates

Protocol when adding CellBlox Plus blocking buffer to antibody cocktail

1. Prepare single-cell suspension as described in BestProtocols: Cell Preparation for Flow Cytometry Protocols at thermofisher.com/cellpreparation.
2. Aliquot cell suspension containing 10^3–10^8 cells to each sample tube or well.
3. Add 5 µL of CellBlox Plus blocking buffer for every sample to be labeled directly into the antibody mixture for a final staining volume of 100 µL per sample. For example, if preparing enough antibody mixture for use with 10 samples, add 50 µL of CellBlox Plus blocking buffer to the antibody mixture.
4. Prepare an antibody mixture of conjugated antibodies at predetermined optimal concentrations of each antibody conjugate. Mix well after addition of each antibody.
5. Add volume of antibody mixture containing CellBlox Plus blocking buffer to aliquoted cell samples, with 100 µL as a final staining volume per sample.
6. Incubate samples for 30 minutes at 2–8°C, protected from light.
7. Wash the cells by adding 2 mL of eBioscience Flow Cytometry staining buffer per sample. Centrifuge at 400–600 x *g* for 5 minutes. Discard supernatant.
8. Repeat step 7.
9. Resuspend cells in an appropriate volume of eBioscience Flow Cytometry staining buffer.
10. Analyze samples by flow cytometry, or, if staining for intracellular targets, proceed with BestProtocols: Staining Intracellular Antigens for Flow Cytometry at thermofisher.com/intracellularstaining.

Protocol when adding CellBlox Plus blocking buffer to bulk cell samples

1. Prepare single-cell suspension as described in BestProtocols: Cell Preparation for Flow Cytometry Protocols at thermofisher.com/cellpreparation.
2. Prepare bulk cell suspension containing 10^3–10^8 cells for every sample to be labeled.
3. Add 5 µL of CellBlox Plus blocking buffer to the bulk cell suspension for every sample to be labeled. For example, if preparing enough bulk cells for use with 10 samples, add 50 µL of CellBlox Plus blocking buffer to the bulk cell suspension.
4. Aliquot cell suspension containing CellBlox Plus blocking buffer to each sample tube or well.
5. Add appropriate amount of each antibody conjugate to cell suspension aliquot containing CellBlox Plus blocking buffer, with 100 µL as a final staining volume per sample.
6. Incubate samples for 30 minutes at 2–8°C, protected from light.
7. Wash the cells by adding 2 mL of eBioscience Flow Cytometry staining buffer per sample. Centrifuge at 400–600 x *g* for 5 minutes. Discard supernatant.
8. Repeat step 7.
9. Resuspend cells in an appropriate volume of eBioscience Flow Cytometry staining buffer.
10. Analyze samples by flow cytometry, or, if staining for intracellular targets, proceed with BestProtocols: Staining Intracellular Antigens for Flow Cytometry at thermofisher.com/intracellularstaining.

Protocol when adding CellBlox Plus blocking buffer directly to cell samples

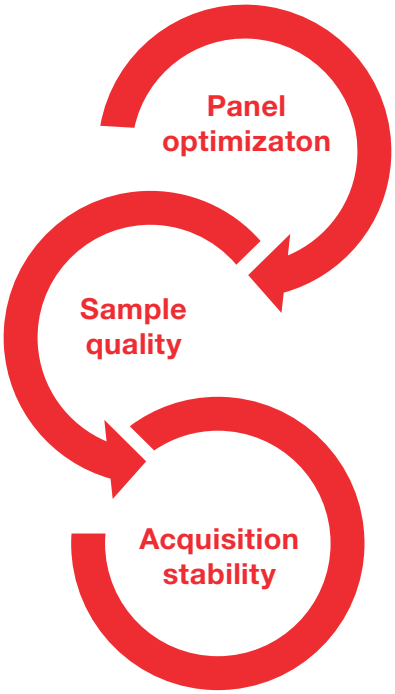
1. Prepare single-cell suspension as described in BestProtocols: Cell Preparation for Flow Cytometry Protocols at thermofisher.com/cellpreparation.
2. Aliquot cell suspension containing 10^3–10^8 cells to each sample tube or well.
3. Add 5 µL of CellBlox Plus blocking buffer directly to each sample prior to staining cells. Note: Other blocking reagents can be added at this step and incubation can occur if required for other reagents.
4. Add appropriate amount of each antibody conjugate to cell suspension containing CellBlox Plus blocking buffer for a final staining volume of 100 µL per sample.
5. Incubate samples for 30 minutes at 2–8°C, protected from light.
6. Wash the cells by adding 2 mL of eBioscience Flow Cytometry staining buffer per sample. Centrifuge at 400–600 x *g* for 5 minutes. Discard supernatant.
7. Repeat step 6.
8. Resuspend cells in an appropriate volume of eBioscience Flow Cytometry staining buffer.
9. Analyze samples by flow cytometry, or, if staining for intracellular targets, proceed with BestProtocols: Staining Intracellular Antigens for Flow Cytometry at thermofisher.com/intracellularstaining.

Maximizing accuracy of immunophenotyping using spectral unmixing with the Attune Xenith Flow Cytometer: essential strategies for optimizing sample quality and acquisition stability

Introduction

In the field of immunophenotyping, the accurate analysis of complex panels relies on the seamless coordination of a flow cytometer's optical, electronic and fluidic components. However, instrument errors and poor sample quality can significantly impact the reliability of results.

This technical note explores the importance of sample quality and acquisition stability in achieving in-depth immunophenotyping, and highlights the capabilities of the Attune Xenith flow cytometer in overcoming these challenges.



Instrument platforms utilized in this work:

- Invitrogen Attune Xenith Flow Cytometer (Cat. No. A59358)
- Invitrogen™ Countess™ 3 FL Automated Cell Counter (Cat. No. A49893)

Additional resources:

- Thermo Fisher Scientific Technical Reference Library
- Thermo Fisher Scientific Flow Cytometry Learning Center

Helping ensure sample quality with appropriate preparation steps

To help minimize aggregates and inappropriate expression profiles, it is crucial to handle samples properly from collection to data acquisition. Dead cells, free-floating debris and damage-associated molecular patterns (DAMPs) negatively affect downstream immunophenotyping.

Where appropriate, modified sample digestion and enzymatic treatment may improve sample quality. Researchers should consider dissociation, lysis, enzymatic treatments and cleanup steps (such as filtration and extra rinse steps) in the sample preparation pipeline (Figure 13). Additional preparation steps to remove debris, such as gradient centrifugation, must be balanced with sample handling time, cost and effects on cell health and population dynamics. These steps should be modified to account for tissue type, populations and molecular targets of interest.

Once preparation steps are determined, antibody titrations should be performed on samples prepared following these steps exactly, as each decision point may alter antigen presentation or population abundance. Single-stained and unstained cell controls should also be prepared in the same manner, to help ensure that these controls are appropriate for and reflective of the data expected from fully stained samples.

Large spectral panels require extensive optimization efforts over several months. However, even with well-designed panels, the basics of sample preparation and instrument stability cannot be overlooked. Proper sample handling and ensuring acquisition stability are essential factors that can significantly impact the success of large spectral panel optimization.

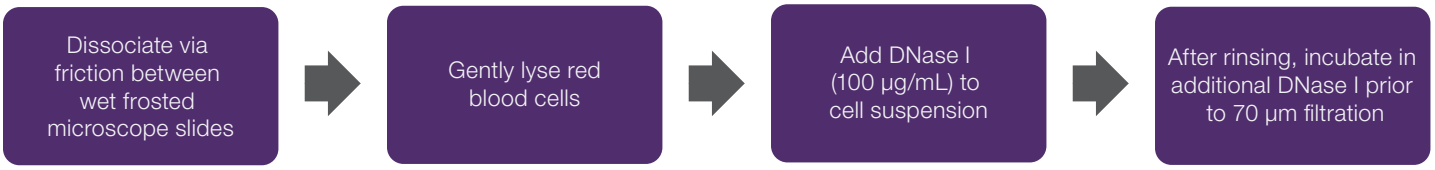


Figure 13. Recommended steps to help reduce aggregation and DAMPs generation prior to immunophenotyping staining. For tissue digests, reducing shear forces in manual digestion or overexposure to chemical treatments is critical. This workflow shows several steps that help ensure that manually digested spleen samples are of high quality, including DNase treatment and filtration.

Additional sample quality checks

To monitor sample quality, we recommend incorporating live/dead differentials during both the preparation and acquisition stages. Small volumes (≤10 µL) of homogenized sample may be used for this evaluation with the Countess 3 FL automated cell counter in under 2 minutes. Adding viability dyes to your flow cytometry panel allows for dead cell removal in gating steps and is a critical quality step, especially for tissue and tumor digests.

Even with thorough preparation steps, certain factors, such as sample collection methods, can introduce quality concerns. In flow cytometry acquisitions, low-quality samples may exhibit large debris fields with low FSC/SSC, indicating compromised data integrity. While scatter and live/dead gating can help eliminate many of these events, the effects on the cells around them during incubation, staining and analysis must be considered when evaluating overall sample quality during acquisition and analysis (Figure 14).

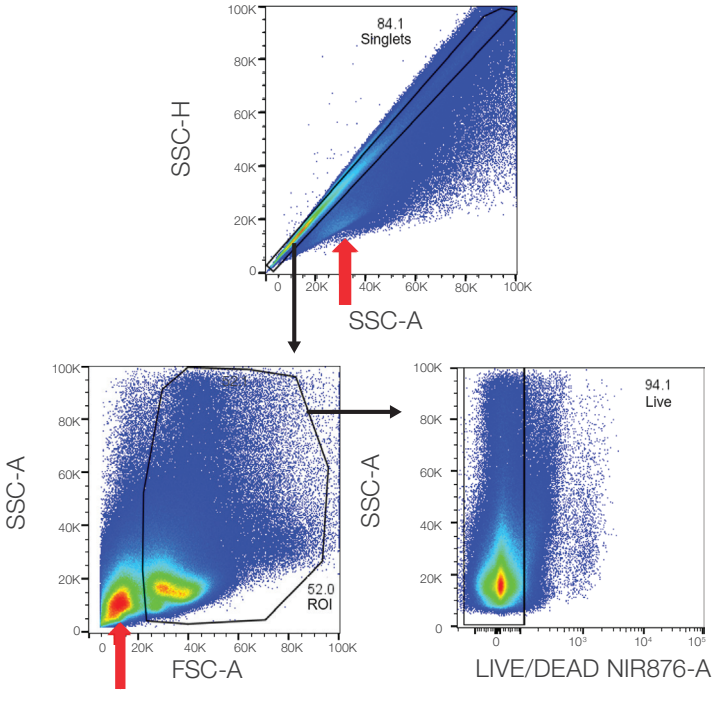


Figure 14. Poor sample quality in mouse splenocytes leads to inaccurate flow cytometry results. Doublets/aggregates and large debris fields (red arrows) observed during acquisition are indicative of residual sample debris. These events may indicate an overall sample quality concern that will present inappropriate antigen expression. Additionally, poor-quality events may escape cleanup gates (here, singlets, region of interest (ROI) and a live cell gate), causing additional downstream errors. LIVE/DEAD NIR876 = Invitrogen™ LIVE/DEAD™ Fixable NIR 876 viability dye.

Importance of sample acquisition stability

Assessing sample stability through time plots enables users to help ensure the preservation of data integrity. These plots can be generated during either the acquisition or data analysis steps. Figure 15 demonstrates highly stable data even for the poor-quality sample shown in Figure 14. Despite this suboptimal sample quality, the Attune Xenith flow cytometer demonstrates remarkable acquisition stability, contributing to reliable and reproducible results.

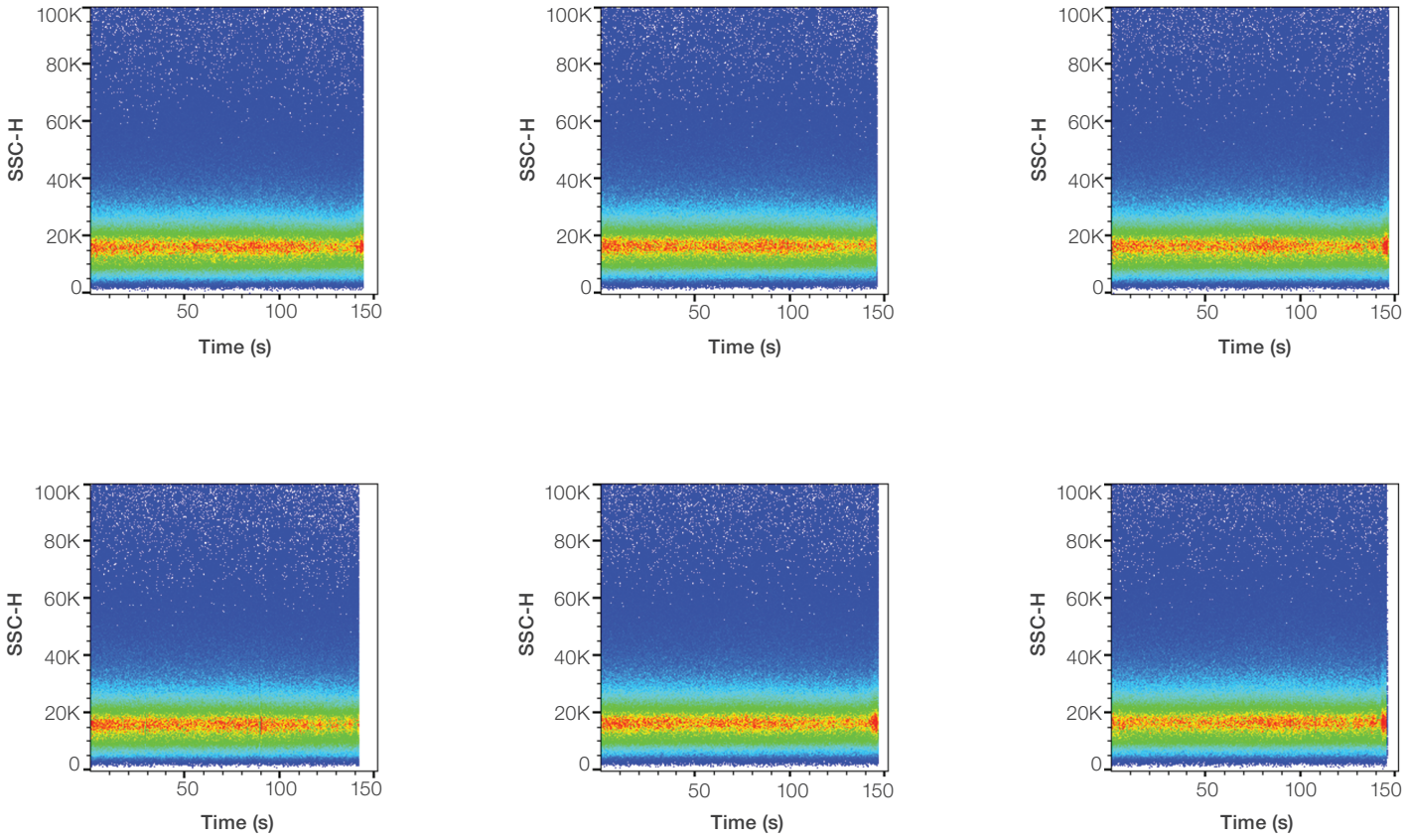


Figure 15. Assessing data acquisition quality through time plots. Where lower-quality samples must be used, the quality control of acquisition is even more critical. Here, time plots against a scatter channel are used to detect any abnormalities that may result from especially clog-prone samples, such as the low-viability tissue digest used here. Downstream data processing tools and time-assisted gating may be necessary to mitigate suboptimal acquisitions. These samples show consistent acquisition rates, and data were not time-gated prior to further analysis. These plots can be generated during either the acquisition or data analysis steps.

Impact of sample quality on spectral unmixing resolution

The Attune Xenith flow cytometer's acoustic focusing technology can accommodate samples prone to clogging. However, it is important to note that data resolution is still heavily influenced by sample quality (Figure 16).

Proper sample collection and handling techniques can help minimize the impact of tissue death on immunophenotyping results. Evaluation of sample health with a live/dead differential on the Countess 3 FL automated cell counter during sample preparation is a preferred way to gain early insights into sample quality before proceeding with the time-consuming and costly efforts of in-depth immunophenotyping. Where sample quality concerns cannot be avoided, such as with unhealthy tissues, additional debris removal prior to staining may be appropriate and should be investigated for its effects on population abundance and cell recovery.

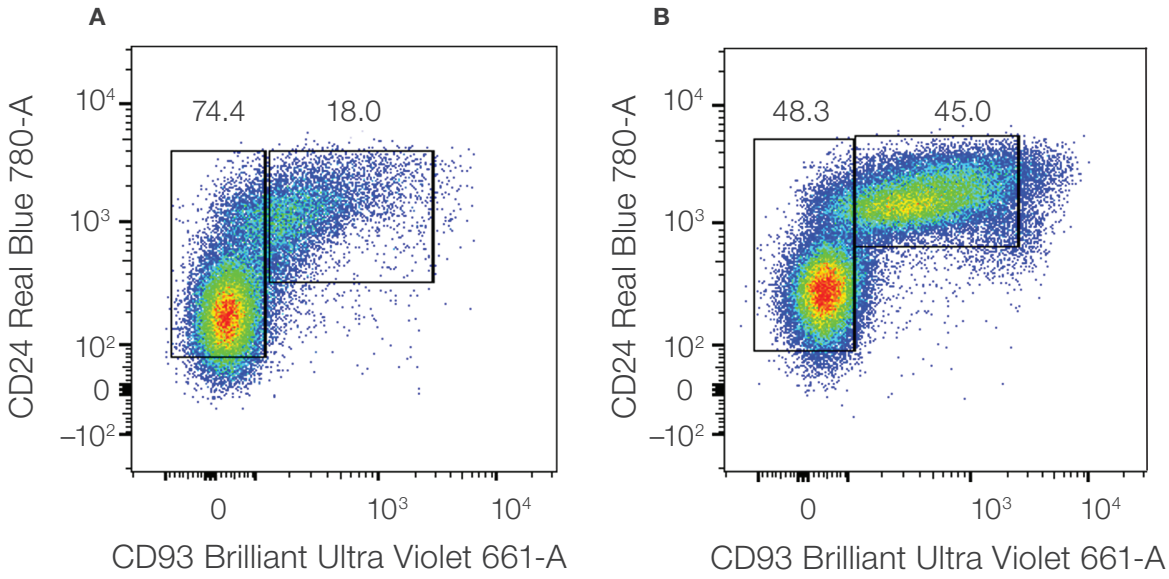


Figure 16. Impact of sample quality on expression profiles. Developing B cells from mouse bone marrow are gated on $CD19^+$ $B220^+$ cells and are being compared for different levels of viability. (A) This sample showed a high percentage of dead cells, as evaluated with cell counts and trypan blue exclusion prior to surface marker staining. In the final analysis, this sample exhibits inappropriate expression profiles. (B) This sample confirms the analysis of the sample in panel A, as it demonstrates high viability during the same quality checks and displays appropriate expression patterns. Even with upstream gating for debris and dead cell removal, the sample plots vividly illustrate the significance of sample quality in high-parameter spectral data sets. Real Blue 780 = Real Blue™ 780 fluorophore. Brilliant Ultra Violet 661 = Brilliant Ultra Violet™ 661 fluorophore.

Conclusion

Achieving in-depth immunophenotyping requires meticulous attention to sample quality and acquisition stability. The Attune Xenith flow cytometer, with its advanced optical detection system and fluidics design, offers excellent data quality even for challenging samples. Evaluating sample quality with rapid cell counts and viability assays using the Countess 3 FL automated cell counter is recommended during the initial stages of preparation before proceeding with further preparation, as a high level of debris or dead cells may be detrimental to downstream staining and analysis.

By implementing data quality checkpoints and optimizing sample handling protocols, researchers can optimize the performance of flow cytometers and obtain reliable immunophenotyping results.

Example sample preparation steps for this application

Table 5 lists the supplies necessary to perform the assay used to generate the results we have shown. Table 6 provides information for acquiring the necessary fluorophores and antibodies.

Table 5. Immunophenotyping preparation supplies.

Product	Supplier	Cat. No.
Cell strainers (70 µm, nylon)	Corning	352350, or equivalent
Fisherbrand™ Premium Frosted Microscope Slides, Superfrost™ marking area	Fisher Scientific	12-544-2
Gibco™ Trypan Blue, 0.4% w/v	Thermo Fisher Scientific	15250061
Invitrogen™ Countess™ Cell Counting Chamber Slides and Holder, disposable	Thermo Fisher Scientific	T10282
Gibco™ Fetal Bovine Serum (FBS), Premium, heat-inactivated	Thermo Fisher Scientific	A5670502
Gibco™ RPMI 1640 Medium	Thermo Fisher Scientific	11875093
Gibco™ PBS, pH 7.4	Thermo Fisher Scientific	10010023
Invitrogen™ eBioscience™ 10X RBC Lysis Buffer (Multi-species)	Thermo Fisher Scientific	00-4300-54
Invitrogen eBioscience Flow Cytometry Staining Buffer	Thermo Fisher Scientific	00-4222-26
Invitrogen eBioscience CD16/CD32 Monoclonal Antibody (93), functional grade	Thermo Fisher Scientific	16-0161-82
Invitrogen eBioscience IC Fixation Buffer	Thermo Fisher Scientific	00-8222-49
Invitrogen UltraComp eBeads Plus Compensation Beads	Thermo Fisher Scientific	01-3333-42
DNase I, from bovine pancreas	Sigma	11284932001
BALB/c or C57BL/6 spleens, collected same day or shipped overnight at 4°C		

Table 6. Antibody and viability kits.

Fluorophore	Antigen	Clone	Supplier	Cat. No.
Brilliant Ultra Violet 661	CD93	AA4.1	BD Biosciences	741574
Real Blue 780	CD24	M1/69	BD Biosciences	755892
Invitrogen LIVE/DEAD Fixable NIR 876	Dead cells	–	Thermo Fisher Scientific	L34982
Invitrogen™ Brilliant Ultra Violet™ 496	CD19	eBio1D3	Thermo Fisher Scientific	364-0193-82
Invitrogen™ Brilliant Ultra Violet™ 805	CD45R/B220	RA3-6B2	Thermo Fisher Scientific	368-0452-82

Reagent preparation

Note: All reagents should be used at 4ºC unless otherwise noted or directed by the manufacturer.

1. **Lysis buffer:** Prepare working solution of lysis buffer by adding 1 part eBioscience 10X RBC Lysis Buffer to 9 parts ultrapure water. Store at 4ºC until use.

2. **Harvest medium:** Prepare by adding 1 part heat-inactivated Fetal Bovine Serum, Premium, to 9 parts RPMI 1640 Medium. Invert several times to homogenize and use at 4ºC.

3. **DNase medium:**
   a. Prepare stock solution by dissolving lyophilized DNase I in ultrapure water to a concentration of 5 mg/mL. Store aliquots at –20ºC as directed by he manufacturer.
   b. Prepare final **DNase medium** as 100 µg/mL DNase I by adding 1 part of 5 mg/mL DNase I to 49 parts of **harvest medium**. Use at 4ºC.

4. **LIVE/DEAD working solution:**
   a. Prepare LIVE/DEAD Fixable NIR 876 viability dye by adding 50 µL of DMSO to the vial as per instructions for this dye.
   b. LIVE/DEAD Fixable NIR 876 dye should be titrated for best live/dead separation with minimal background. We recommend starting at a 1:1,000 dilution in 1X PBS.

Sample digestion and staining

Note: This assay is scaled for 3 spleens. Scale reagent volumes up or down in initial tissue dissociation steps as appropriate.

1. **Process pooled tissue from 3 mice over petri dish with fluid from collection. This is typically ~10 mL RPMI medium.**
   a. Physically dissociate tissue between two wet frosted microscope slides. **Note:** It is important to keep the slides wet with medium to allow the cell suspension to flow towards the petri dish. Use the frosted section of one slide to gently dissociate tissue against the second slide.
2. Filter the suspension through a 70 µm cell strainer into a fresh 50 mL conical tube.
3. Bring final volume to 30 mL with **harvest medium**, rinsing slides and petri dish into the same conical tube through the cell strainer.
4. Centrifuge at 400 x *g* for 5 minutes at 4°C, and discard supernatant by gently decanting.
5. Resuspend cell pellet in 1.0 mL **harvest medium**, pipetting gently to homogenize.
6. Add 3 mL RBC **lysis buffer** working solution. Incubate for 3 minutes at room temperature with occasional agitation.
7. Add 20 mL **DNase medium** to halt lysis and prevent cell aggregation by degrading free DNA.
8. Gently invert cell suspension several times to homogenize, and filter through 70 µm cell strainer into fresh 50 mL conical tube.
9. Centrifuge at 400 x *g* for 5 minutes at 4°C, and discard supernatant by gently decanting.
10. Resuspend cell pellet in 10 mL **DNase medium**, pipetting gently to homogenize, and incubate for 15 minutes at 4ºC.
11. Count cells to determine appropriate resuspension volume (see later steps).
12. Add 20 mL **harvest medium** and invert to homogenize.
13. Centrifuge at 400 x *g* for 5 minutes at 4°C, and discard supernatant by gently decanting.
14. Resuspend cells in 10 mL **harvest medium**, pipetting gently to homogenize.
15. Add an additional 20 mL **harvest medium** and invert to homogenize.
16. Centrifuge at 400 x *g* for 5 minutes at 4°C, and discard supernatant by gently decanting.
17. Resuspend cells to 3 x 10^7 live cells/mL in eBioscience Flow Cytometry staining buffer, based on counts above.
18. Add mouse CD16/32 Monoclonal Antibody (1 µL per 100 µL of cell suspension) and incubate at 4°C for 5 min.
19. Prepare mixed live/dead cell suspension:
    a. Heat-shock 100 µL cell suspension for 5 minutes at 65°C
    b. Allow heat-shocked cells to cool to 4°C, and add 50 µL fresh cell suspension to prepare a mixed sample for live/dead controls.
20. Add appropriate volume of each antibody or master mix to the respective pre-labeled tubes.
21. Aliquot 100 µL cell suspension (3 x 10^6 cells) to each sample and control tube. Pipette gently to homogenize. **Note:** Use mixed live/dead cells for the live/dead control.
22. Incubate for 30 minutes at 4°C, protected from light.
23. Add 3 mL 1X PBS to each cell suspension.
24. Centrifuge at 400 x *g* for 5 minutes at 4°C, and discard supernatant by gently decanting.
25. Resuspend each cell pellet in 3 mL 1X PBS, pipetting gently to homogenize.
26. Centrifuge at 400 x *g* for 5 minutes at 4°C, and discard supernatant by gently decanting.
27. Resuspend each cell pellet in the residual fluid volume (~100 µL), pipetting gently to homogenize.
28. Add 10 µL of **L/D working solution** to fully stained sample, appropriate FMO controls and live/dead control.
29. Incubate for 20 minutes at 4°C, protected from light.
30. Add 3 mL eBioscience Flow Cytometry staining buffer to each cell suspension.

31. Centrifuge at 400 x *g* for 5 minutes at 4°C, and discard supernatant by gently decanting.
32. Resuspend each cell pellet in 3.0 mL eBioscience Flow Cytometry Staining Buffer, pipetting gently to homogenize.
33. Centrifuge at 400 x *g* for 5 minutes at 4°C, and discard supernatant by gently decanting.
34. Resuspend each cell pellet in the residual fluid volume (~100 µL), pipetting gently to homogenize.
35. Proceed to immediate analysis or to cell fixation with eBioscience IC fixation buffer.

Note: LIVE/DEAD Fixable viability dyes may be used on fresh samples and are stable with a wide range of fixatives. If an alternative viability dye is used, ensure that dead cell staining is consistent before and after fixation by testing this during assay development.

Bead control preparation

1. Prepare UltraComp eBeads Plus compensation beads per manufacturer directions.
2. Proceed to bead fixation with eBioscience IC fixation buffer or to immediate analysis.

Note: Bead controls should be treated in a manner similar to cell controls following staining. Thus, if fixation and/or permeabilization is performed on cell controls and samples, the same procedures should be completed on bead controls.

Panel design and analysis of natural killer cell populations using spectral unmixing on the Attune Xenith Flow Cytometer

Introduction

This application note presents a panel specifically designed to study natural killer (NK) cells. NK cells are a group of immune cells responsible for identifying and eliminating infected or cancerous cells through the recognition of specific markers or their absence (Figure 17). This study aims to investigate various markers related to NK cell activation, inhibition and maturation.

The spectral panel consists of 25 markers and is optimized for use with the Attune Xenith flow cytometer, which offers rapid analysis of a large number of cells. We provide an overview of the panel design, the markers included and their significance in NK cell research.

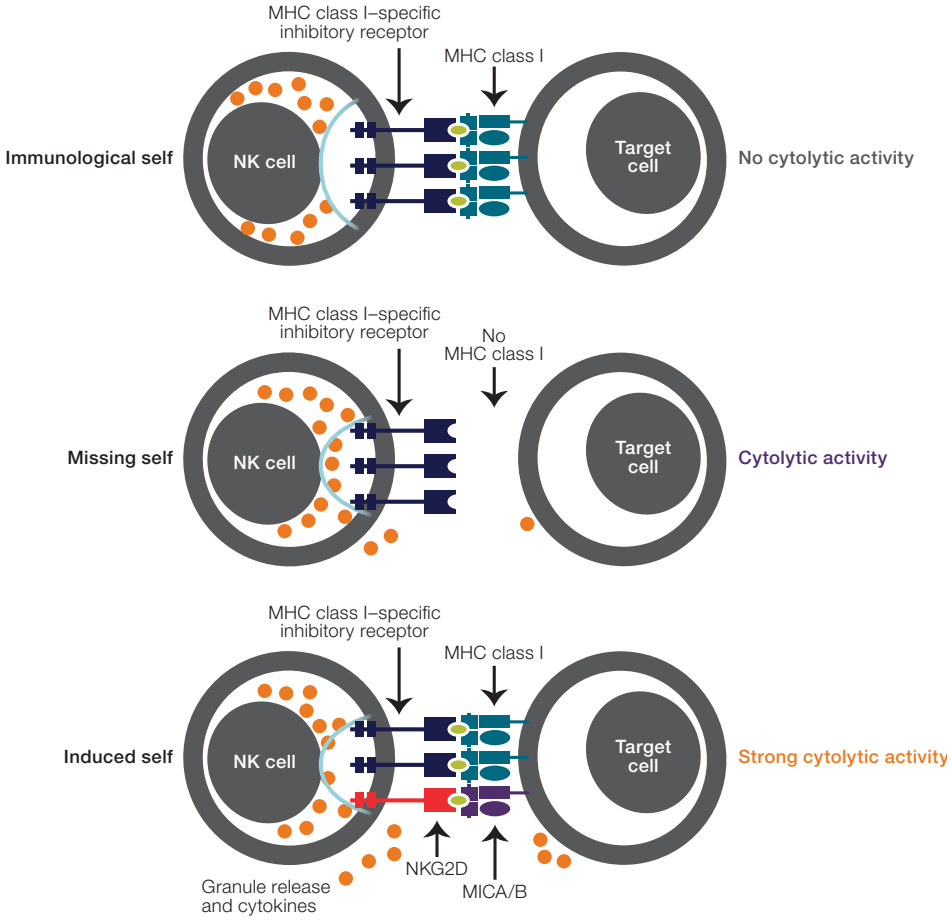


Figure 17. NK cells recognize the presence or absence of specific cell markers and react accordingly.

Flow cytometry analysis

The lymphocyte population is gated for singlets. Dead cells are excluded using LIVE/DEAD Fixable Near IR (876) viability dye, which utilizes infrared (IR) detection on the Attune Xenith flow cytometer. Further exclusion of unwanted cell types, such as T cells, B cells, HLA-DR–positive cells and CD127-positive cells, allows for focused analysis of the NK population. Within the NK population, the maturation stages, including immature, mature and terminal NK cells, are identified by CD56 and CD16 markers.

Panel design

With the tools provided in Invitrogen™ Sasquatch software, users can identify panel expansion opportunities. These tools include the spectral energy plot (Figure 18), similarity index and complexity score values. Additionally, users may visualize the effects of unmixing on single-color controls to identify fluorophore pairs where overlap may influence data resolution.

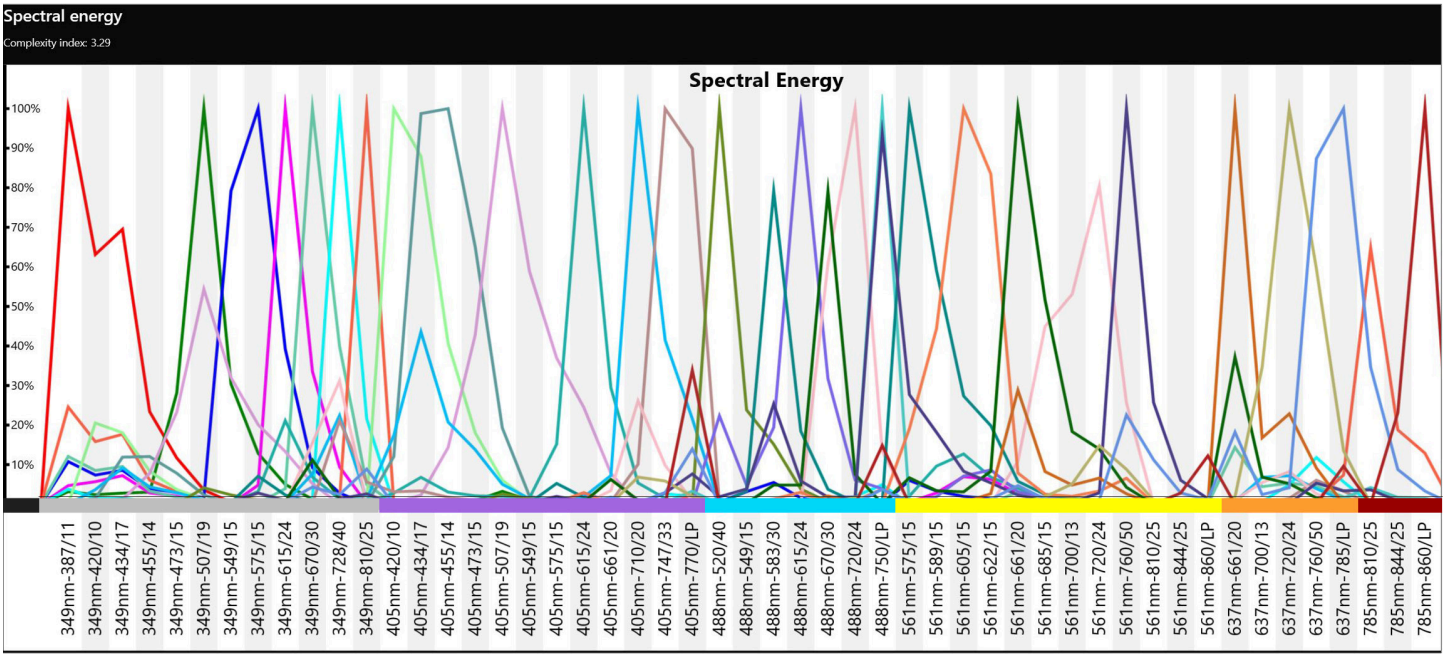


Figure 18. Sasquatch software tools for the Attune Xenith flow cytometer facilitate advanced panel design. In the spectral energy plot, the panel fluorophores are graphed against all detectors to identify areas with minimal overlap where the panel can be further expanded. A specific emission curve can be shaded by highlighting it in the Selected Fluorophores list in the Sasquatch software.

Panel design and analysis of natural killer cell populations using spectral unmixing on the Attune Xenith flow cytometer

Table 7 presents the natural killer cell panel design grid, which shows selected fluorophores for each antigen and for differentiation of dead cells. For this assay, bead controls were used for all fluorophores and antigens with the exception of CD127 and HLA-DR. These fluorophores are mapped across detectors in the spectral energy plot in Figure 18.

Antigen expression analysis

The panel includes several pan-NK markers for which varying expression levels are expected across the NK populations evaluated. The expression of these markers is assessed using histograms representing the various maturation stages of NK cells. The panel includes additional pan markers such as CD11b, expressed on all circulating NK cells, and CD161, expressed to varying degrees in different NK cells.

Activation and inhibition marker analysis

Activation markers play a crucial role in elucidating the readiness and activity of NK cells. The panel includes markers such as NKG2D, NKG2C, CD2 and CD38, which exhibit different expression patterns across the maturation stages of NK cells. NKG2C, for example, is primarily expressed in mature NK cells, while NKG2D is expressed in almost all NK cell populations. In contrast, inhibitory markers, including NKG2A, KIR markers and KLRG1, are analyzed using forward scatter of mature NK cells. Histograms demonstrate the expression of these markers in different maturation stages, highlighting their importance in NK cell populations.

To assess the functionality and responsiveness of the panel, cells were stimulated with a cytokine cocktail, as certain markers are not expressed on naive NK cells; these are stimulated overnight with a cytokine cocktail (IL-2, IL-15, and IL-21). CD69 and TIGIT are among these markers, which show significant shifts in expression upon stimulation, particularly in mature NK cells. NKP46, a pan-NK marker expressed to some degree in almost all NK cells, also exhibits notable shifts in expression following activation.

Table 7. Natural killer cell panel.

Laser and channel	Filter	Fluorophore	Antigen	Clone	Fluorescence-minus-one (FMO) controls
349 UV-1	387/11	Brilliant Ultra Violet™ 395	NKG2D	1D11	Yes
349 UV-6	507/19	Brilliant Ultra Violet 496	CD11b	ICRF44	Yes
349 UV-8	575/15	Brilliant Ultra Violet™ 563	CD56	NCAM16.2	
349 UV-9	615/24	Brilliant Ultra Violet™ 615	PD-1	EH12.1	Yes
349 UV-10	670/30	Brilliant Ultra Violet 661	KLRG1	Z7-205.rMab	Yes
349 UV-11	728/40	Brilliant Ultra Violet™ 737	NKp30	p30-15	Yes
349 UV-12	750/LP	Brilliant Ultra Violet 805	CD2	RPA-2.0	Yes
405 V-1	420/10	Brilliant Violet 421	NKG2C	134591	Yes
405 V-3	455/14	eFluor™ 450	CD57	TB01	Yes
405 V-5	507/19	Brilliant Violet 510	CD3	OKT3	
405 V-8	615/24	Super Bright™ 600	CD244	eBioC1.7	Yes
405 V-10	710/20	Super Bright 702	CD127	eBioRDR5	Yes
405 V-11	747/33	Brilliant Violet™ 750	CD69	FN50	Yes
488 B-1	520/40	FITC	KIRDL2/3	DX27	Yes
488 B-2	549/15	NovaFluor™ Blue 610-70S	HLA-DR	LN3	
488 B-6	720/24	PerCP-eFluor™ 710	TIGIT	MBSA43	Yes
488 B-7	750/LP	Real Blue 780	CD19	SJ25C1	
561 Y-1	575/15	PE	KIRDL1	124	Yes
561-Y-3	605/15	PE-Dazzle 594	NKG2A	S19004C	Yes
561 Y-5	661/20	PE-Cy®5	NKp46	9E2	Yes
561 Y-9	760/50	PE-Cy®7	CD161	HP-3G10	Yes
637 R-1	661/20	APC	CD226	11AB7.4	Yes
637 R-3	720/24	Alexa Fluor™ 700	CD38	HIT2	Yes
637 R-5	785/LP	APC-eFluor™ 780	CD16	eBioCB16	Yes
781 IR-3	860/LP	LIVE/DEAD Fixable Near IR (876)	Dead cells	N/A	Yes

Results

Utilizing the panel described above with the Attune Xenith flow cytometer's high acquisition rates (200 µL/min), the activation and maturation of NK cells was characterized. Immature, mature and terminal NK populations were identified from human peripheral blood mononuclear cells (PBMCs), and surface marker expression of each of these subpopulations was compared (Figure 19). For instance, the expression profiles of NK cell subsets NKp30, NKp46, CD226 and CD244 were further investigated (Figure 20), as were the shifts in expression levels of pan-NK markers that occurred when cells were stimulated with human IL-2, IL-15, and IL-21 (Figure 20).

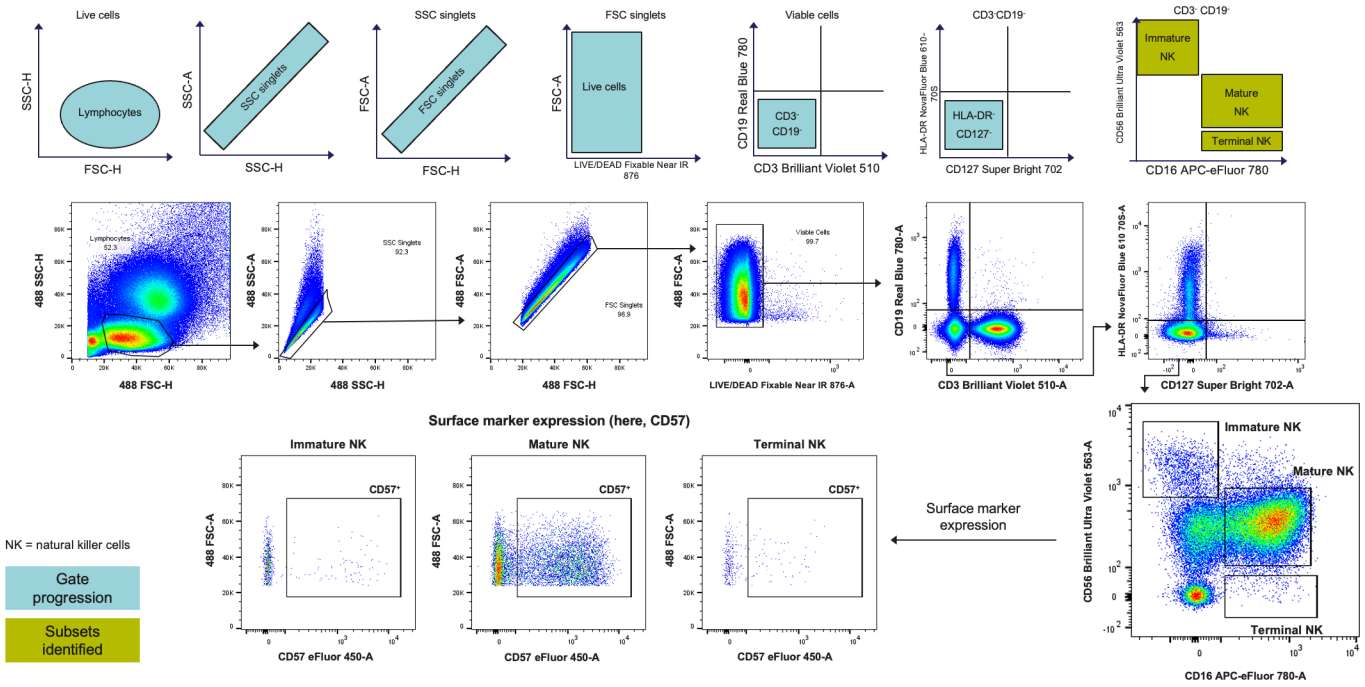


Figure 19. Gating scheme for 25-color natural killer cell panel. A 25-color flow cytometry panel was employed in the analysis of NK cells. Starting with the identification of viable lymphocytes, sequential gating steps were applied to exclude doublets, dead cells and non-NK cell populations. The final gated population represents the NK cells, characterized by specific surface markers. Each color corresponds to a different fluorophore-conjugated antibody used in the panel, enabling the precise identification and analysis of NK cell subsets.

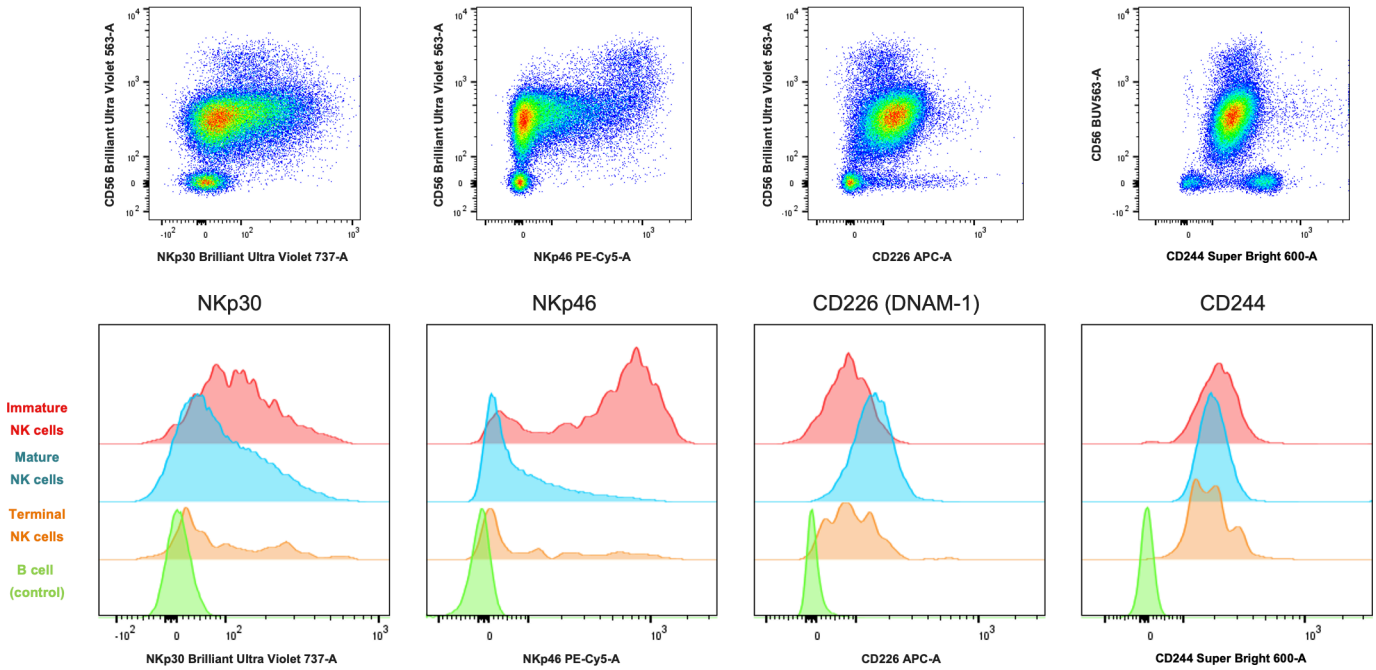


Figure 20. Pan-NK markers. Natural killer cell expression profiles of NKp30, NKp46, CD226 and CD244 can be further investigated between cell subsets.

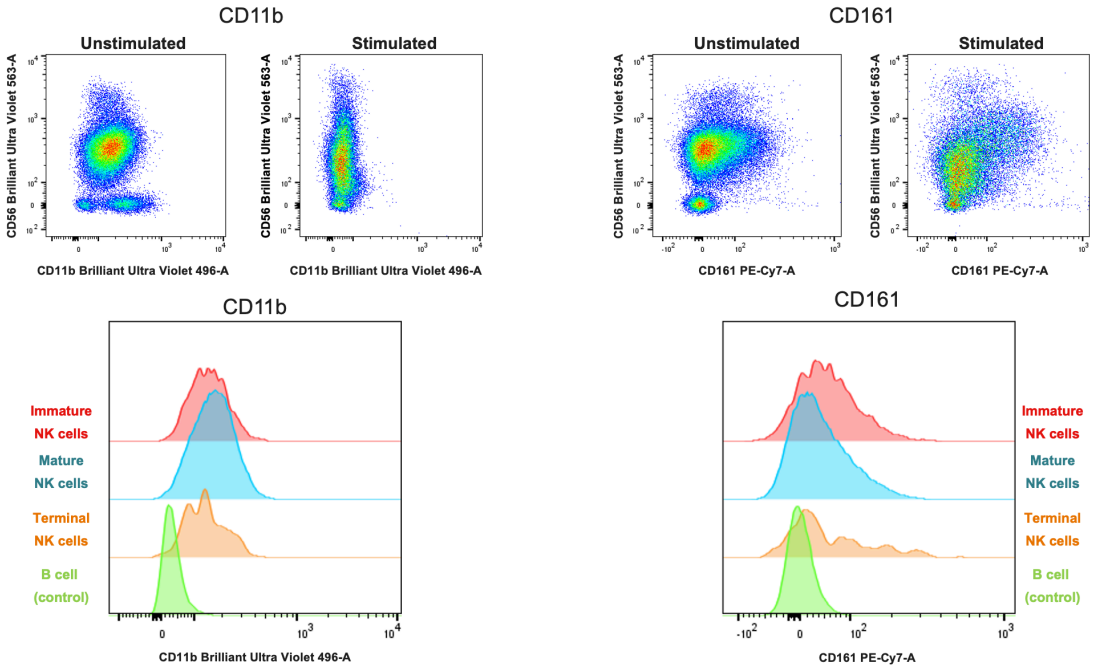


Figure 21. Pan-NK markers shift after cell stimulation. Expression level of CD11b or CD161 changes when NK cells are stimulated overnight with IL-2 at 100 IU/mL, IL-15 at 10 ng/mL and IL-21 at 25 ng/mL.

Several markers of cellular activation were also investigated on immature, mature and terminal populations. These markers are important for NK effector functions, and many increase in expression as the NK cells mature (Figures 22 and 23). In conjunction with activation markers, we also explored inhibitory antigen expression on the various NK cell populations, which will also change as cells mature and respond to cytokines or other biological stimuli.

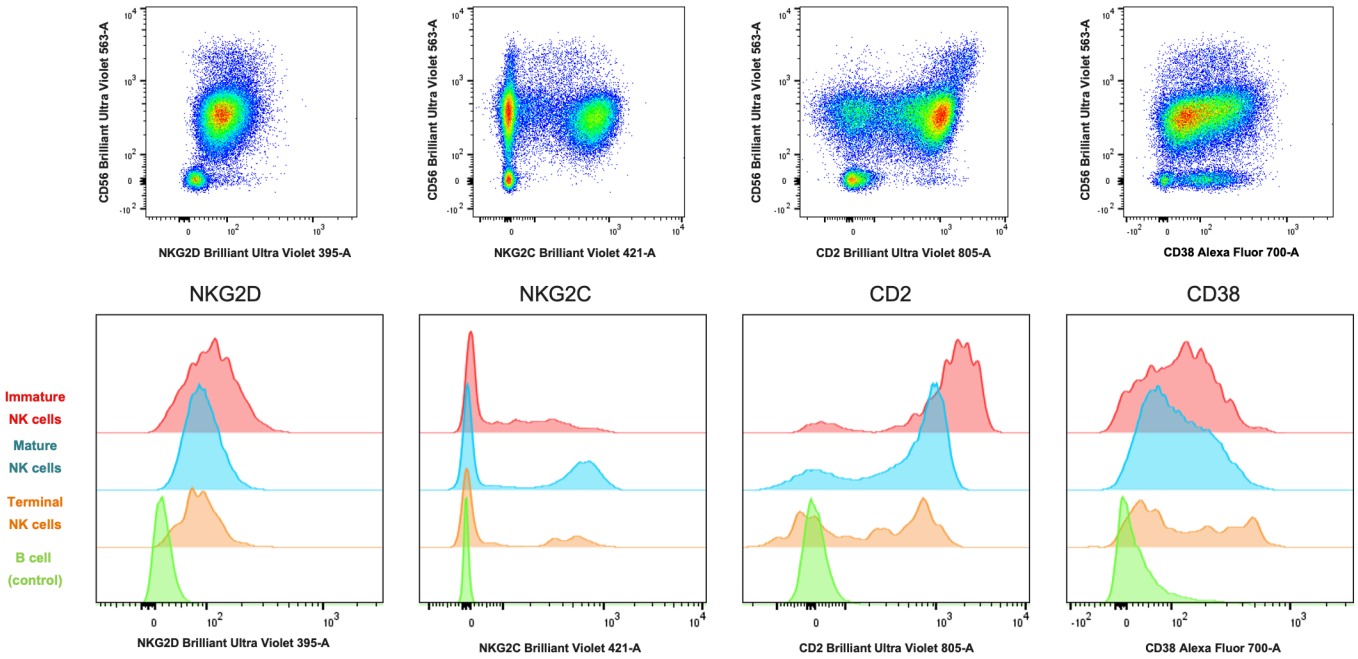


Figure 22. Activation markers in NK populations. Varied levels of maturation antigens are expressed in mature NK cell populations. The expression of markers such as NKG2C, CD2 and CD38 may be tracked during viral infections or activation by tumor cells.

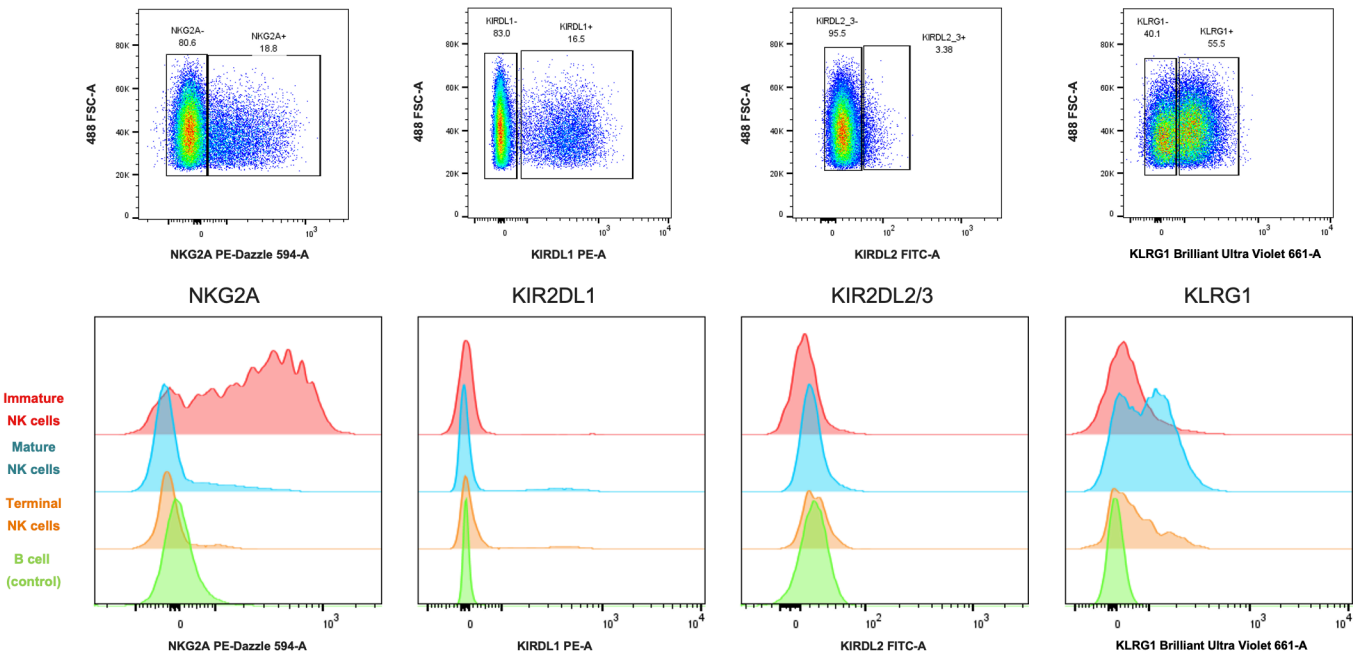


Figure 23. Inhibitory antigens in NK cell populations. Expression levels of NKG2A, KIR2DL1, KIR2DL2/3 and KLRG1, which regulate NK cell activation and function in response to tumors and viral infections, are shown.

Panel design and analysis of natural killer cell populations using spectral unmixing on the Attune Xenith flow cytometer

Figure 24 displays the reaction of NK cell activation markers to stimulation of PBMCs. In NK cells, the level of expression can vary between populations at different levels of maturation, as demonstrated by 1D analysis of different markers upon stimulation.

The sensitivity of the Attune Xenith flow cytometer and the NK panel also allowed for the detection of rare events without the need for concentrating samples (Figure 25). High sample throughput reduced time-to-results without compromising population resolution. The sensitivity of detection helped ensure robust data resolution, enabling the differentiation of even rare populations in high-complexity panels. In Figure 25, antigen expression was evaluated in human NK cells that were incubated with or without an IL-2, IL-15 and IL-21 cytokine cocktail for a duration of 48 hours. The Attune Xenith flow cytometer demonstrated strong sensitivity in detecting changes across different stimulation procedures, helping to ensure reliable and accurate characterization of these rare-cell subsets. These results highlight the potential of the panel for conducting in-depth analysis and gaining a deeper understanding of NK cell subsets.

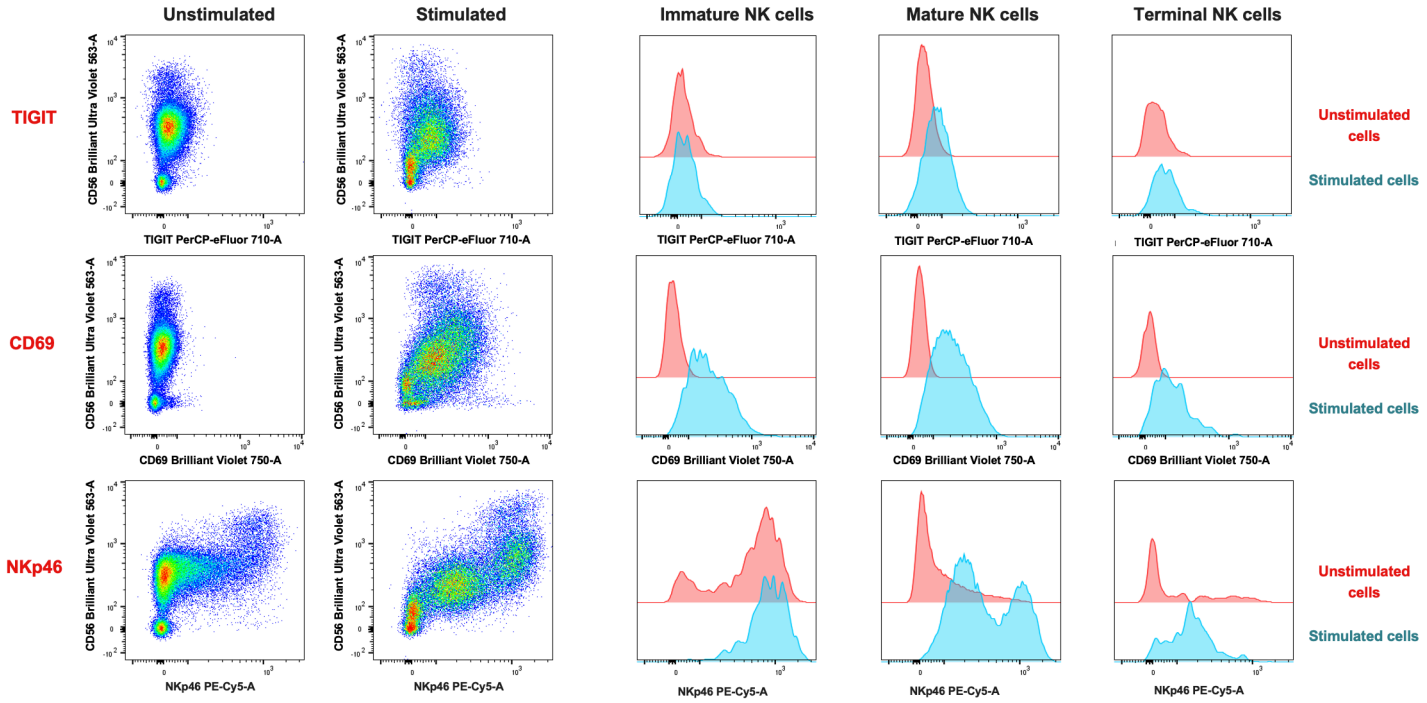


Figure 24. Expression of activation antigens in NK cells. When PBMCs are stimulated, NK cells display diverse expression of activation markers such as TIGIT, CD69 and NKp46. The levels of expression may vary between immature, mature and terminal NK cells.

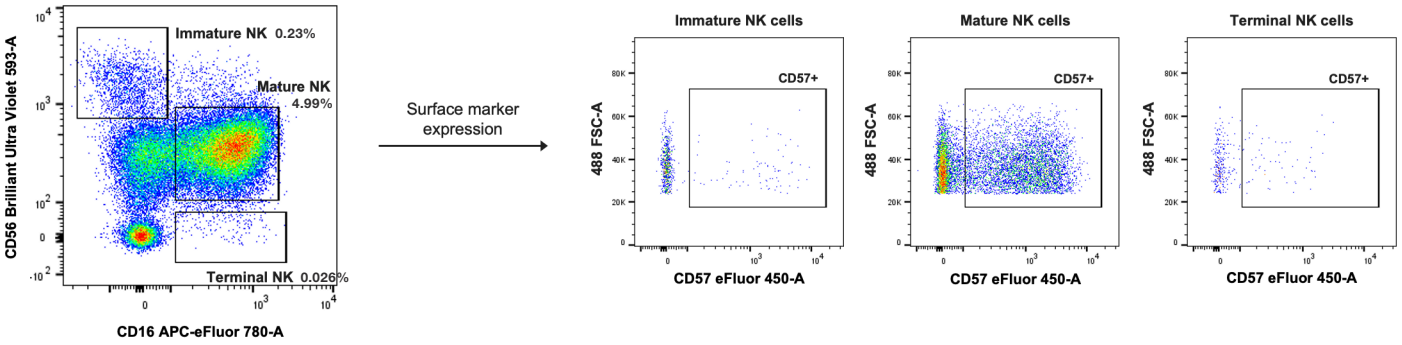


Figure 25. Precision in rare-event detection using a 25-color NK cell panel. Over a million events were rapidly collected (200 µL/min) to analyze NK cells from immature, mature and terminal NK populations. Percentages of these populations as a portion of total cell count are shown in the first panel.

Discussion

Spectral flow cytometry has allowed for the expansion of panel size for a deeper investigation of populations of interest. Here we used 25 colors to perform an in-depth analysis of various NK populations from human PBMCs. Expression of key markers in the immature, mature and terminal NK populations was analyzed to elucidate characteristics of NK activation and inhibition states. Various markers were used to label human PBMCs, both with and without cytokine stimulation, revealing significant changes in NK populations post-stimulation. Changes were observed in the expression of several important markers, including CD69, NKp46 and others, which align with general expectations of NK cell behavior under these conditions. This confirms the Attune Xenith flow cytometer's capability, along with the applied panel, to accurately investigate this important lineage in immuno-oncology research.

The Sasquatch software tools and unmixing results suggest that the panel has not yet fully maximized the detection capabilities of the system. As the panel evolves, further research should explore additional characterization of non-NKs within the PBMC samples and additional activation/inhibition markers within the NK subsets outlined here. This ongoing development will enhance the panel's utility for more comprehensive study of immune cell populations.

Methods – Supply information

Table 8 presents purchasing information for supplies necessary to perform the comprehensive NK cell panel analyses.

Table 8. Supplies for comprehensive NK cell panel analysis.

Product	Supplier	Cat. No.
Cell strainers (70 µm, nylon)	Corning	352350 or equivalent
Invitrogen Countess Cell Counting Chamber Slides and Holder, disposable	Thermo Fisher Scientific	T10282
Gibco Trypan Blue, 0.4% w/v	Thermo Fisher Scientific	15250061
Gibco Fetal Bovine Serum (FBS), Premium, heat-inactivated	Thermo Fisher Scientific	A5670502
Gibco™ AIM V™ Medium, liquid (research grade)	Thermo Fisher Scientific	12055091
Gibco™ CTS™ NK-Xpander™ Medium	Thermo Fisher Scientific	A5019001
Gibco™ Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	15140148
Gibco PBS, pH 7.4	Thermo Fisher Scientific	10010023
Gibco™ Human IL-2 Recombinant Protein, PeproTech™	Thermo Fisher Scientific	20002
Gibco™ Human IL-15 Recombinant Protein, PeproTech™	Thermo Fisher Scientific	200-15-50UG
Gibco™ Human IL-21 Recombinant Protein, PeproTech™	Thermo Fisher Scientific	200-21-50UG
Invitrogen eBioscience Flow Cytometry Staining Buffer	Thermo Fisher Scientific	00-4222-26
Invitrogen eBioscience IC Fixation Buffer	Thermo Fisher Scientific	00-8222-49
Invitrogen UltraComp eBeads Plus Compensation Beads	Thermo Fisher Scientific	01-3333-42
Invitrogen™ Countess™ 3 Automated Cell Counter	Thermo Fisher Scientific	A49862
Human BD Fc Block™	BD Biosciences	564219
Invitrogen Brilliant Stain Buffer	Thermo Fisher Scientific	00-4409-42
Invitrogen CellBlox Plus Blocking Buffer	Thermo Fisher Scientific	C001T02F01
Human PBMCs, stored in vapor nitrogen phase	N/A	N/A

Conclusion

This application note has provided an overview of a comprehensive panel designed for the study of NK cells acquired on the Attune Xenith flow cytometer. The panel encompasses markers related to activation, inhibition and maturation, allowing for a detailed analysis of NK cell populations.

This work highlights the potential of the panel and the Attune Xenith flow cytometer for conducting in-depth analysis and gaining a deeper understanding of NK cell subsets. Future plans include expanding the panel and addressing any queries related to its design, stimulation methods, marker selection and fluorophore/antigen choices.

Table 9 displays detailed information on the necessary fluorophores.

Table 9. Fluorophore information.

Fluorophore	Antigen	Clone	Supplier	Cat. No.
Brilliant Ultra Violet 395	CD5	53-7.3	BD Biosciences	740206
Brilliant Ultra Violet 496	CD19	eBio1D3	Thermo Fisher Scientific	364-0193-82
Brilliant Ultra Violet 563	CD44	IM7	Thermo Fisher Scientific	365-0441-82
Brilliant Ultra Violet 615	CD11c	N418	Thermo Fisher Scientific	366-0114-82
Brilliant Ultra Violet 661	CD93	AA4.1	BD Biosciences	741574
Brilliant Ultra Violet 737	TCRgd	GL3	BD Biosciences	748991
Brilliant Ultra Violet 805	B220	RA3-6B2	Thermo Fisher Scientific	368-0452-82
Brilliant Violet 421	Siglec H	551	BD Biosciences	567815
Super Bright 436	F4/80	BM8	Thermo Fisher Scientific	62-4801-82
eFluor 450	CD21	eBio4E3	Thermo Fisher Scientific	48-0212-82
Brilliant Violet 480	CD62L	MEL-14	Thermo Fisher Scientific	414-0621-82
Brilliant Violet 510	CCR2	475301	BD Biosciences	747970
Brilliant Violet 570	Ly6G	1A8	BioLegend	127629
Super Bright 600	I-A/I-E	M5/114.15.2	Thermo Fisher Scientific	63-5321-82
Super Bright 645	CD25	PC61.5	Thermo Fisher Scientific	64-0251-82
Super Bright 702	IgD	11-26c	Thermo Fisher Scientific	67-5993-82
Brilliant Violet 750	CD4	GK1.5	BioLegend	100467
Super Bright 780	CD11b	M1/70	Thermo Fisher Scientific	78-0112-82
FITC	CD38	90	Thermo Fisher Scientific	11-0381-82
RB545	TCRb	H57-597	BD Biosciences	756204
NovaFluor Blue 610-70S	CD8	53-6.7	Thermo Fisher Scientific	M003T02B06-A
NovaFluor Blue 660-120S	CD205	205yekta	Thermo Fisher Scientific	M051T03B08
PerCP-eFluor 710	CD1d	1B1	Thermo Fisher Scientific	46-0011-82
Real Blue 780	CD24	M1/69	BD Biosciences	755892
PE	CD127	A7R34	Thermo Fisher Scientific	12-1271-82
RY586	CD27	LG.3A10	BD Biosciences	753482
PE-eFluor 610	IgM	II/41	Thermo Fisher Scientific	61-5790-82
PE-Cy5	CD3	17A2	Thermo Fisher Scientific	15-0032-82
PE-Cy5.5	CD49b	DX5	Thermo Fisher Scientific	35-5971-82
PE-Cy7	CD23	B3B4	Thermo Fisher Scientific	25-0232-82
PE/Fire 810	CX3CR1	SA011F11	BioLegend	149057
APC	CD335	29A1.4	Thermo Fisher Scientific	17-3351-82
NovaFluor Red 700	CD274	MIH5	Thermo Fisher Scientific	M036T03R03-A
Alexa Fluor 700	CD45	30-F11	Thermo Fisher Scientific	56-0451-82
APC-eFluor 780	Ly6C	HK1.4	Thermo Fisher Scientific	47-5932-82
APC-Fire 810	TER-119	TER-119	BioLegend	116264
LIVE/DEAD Fixable Near IR (876)	Dead cells	–	Thermo Fisher Scientific	L34982

Reagent preparation

Note: All reagents should be used at 4ºC unless otherwise noted or directed by the manufacturer.

1. **LIVE/DEAD working solution:**
   a. Prepare LIVE/DEAD Fixable Near IR 876 dye per manufacturer directions.
   b. Prepare working solution by adding 1 µL of LIVE/DEAD Fixable Near IR 876 suspension to 49 µL of 1X PBS, and pipette gently up and down to homogenize.
2. **Prepare cytokine aliquots as directed by the manufacturer, storing at –80°C until use.**
   a. Final concentrations added to the medium of choice will be 100 U/mL IL-2, 10 ng/mL IL-15, and 25 ng/mL IL-21.
3. **Prewarm AIM-V Medium in a 37°C water bath.**
4. **If cell stimulation is to be utilized:**
   a. Prepare complete NK-Xpander Medium with 5% heat-inactivated FBS and 100 U/mL penicillin-streptomycin.
   b. Prewarm in 37°C water bath.

PBMC thawing

1. **Add tightly capped cryopreserved PBMC vials to a 37°C water bath for ~90 sec,** then gently invert 3–4 times every 15–20 sec until vial is ~75% liquid.
2. **Remove from water bath and wipe outside of vial to remove any remaining water.**
3. **For each vial of cryopreserved PBMCs (~1 mL volume, ~5 x 107 cells):**
   a. Gently pour PBMCs into a 50 mL conical tube, and add 10 mL prewarmed AIM-V Medium
   b. Slowly add 2 mL AIM-V Medium to each PBMC vial and pour into the same 50 mL conical tube used in step 3a. Repeat.
   c. Centrifuge at 400 x *g* for 5 minutes. Discard supernatant by gently pouring off.
   d. Resuspend PBMCs in 1X PBS by gently pipetting up and down (cells can be resuspended in any volume between 1 and 10 mL of PBS, depending on the desired concentration)
   e. Gently invert cell suspension several times to homogenize, and record concentration and viability with the Countess 3 automated cell counter.

Note: If viability is <90%, preparing another sample of cells from a fresh vial is recommended.

Cell stimulation

Cells are stimulated for 48 hours in an IL-2, IL-15, IL-21 cocktail. After 48-hour stimulation, cells are collected and stained as described in this and the next subsection. We recommend collecting an unstained sample of the stimulated cells, as they will have a slightly different autofluorescence (AF) profile than unstimulated cells and can be used as a separate negative control and AF parameter in unmixing experiments.

1. **Divide cell suspension for cell stimulation.**
   a. Aliquot appropriate volumes of cell suspension to separate conical tubes. A minimum of 9 x 106 live cells is recommended for stimulation treatment.
   b. Centrifuge at 400 x *g* for 5 minutes. Discard supernatant by gently pouring off.
   c. Resuspend the cells in each stimulation tube in NK-Xpander Medium to 3 x 106 live cells/mL.
   d. Resuspend cells in the negative control (no stimulation) tube in 1X PBS to 1 x 106 live cells/mL, and move directly to cell staining procedures.
2. **To the cells to be stimulated, add IL-2 to 100 U/mL**, IL-15 to 10 ng/mL and IL-21 to 25 ng/mL final concentrations.
3. **Aliquot to 6-well tissue culture plates** with a minimum volume of 1 mL per well.
4. **Incubate at 37°C for 48 hours**
   a. After 48 hours, transfer cell suspensions from culture plates to conical tubes, pipetting several times to homogenize.
   b. Gently invert cell suspension tube several times to homogenize, and record concentration and viability with the Countess 3 automated cell counter.

Cell staining

PBMCs should be in 1X PBS at a concentration of 1 x 106 live cells/mL.

1. **PBMCs should be in 1X PBS at a concentration of 1 x 106 live cells/mL.**
2. **Gently invert the cell suspension tube several times to homogenize,** and filter through a 70 µm cell filter into a fresh 50 mL conical tube.
3. **Prepare heat-shocked cells for viability dye single-stain control:**
   a. Add 1 mL (1 x 106 cells) to a microcentrifuge tube and place on heat block to incubate suspension for 5 minutes at 65°C. Remove from heat block and place on ice for 2–3 minutes.
   b. Add 500 µL of fresh PBMC suspension to cooled microcentrifuge tube from heat block incubation.
4. **Separate sufficient PBMCs for unstained** and single-color controls (no viability dye added).
5. **To remaining suspension,** add 1 µL L/D working solution per 1 mL of PBMCs, pipetting gently several times to homogenize.
6. **Incubate all suspensions 30 minutes at 4°C, protected from light.**
7. **Centrifuge at 400 x *g* for 5 minutes at 4°C and discard supernatant,** drawing off residual fluid with brief contact against an absorbent wipe.
8. **Resuspend each cell pellet to 1 x 106 cells/mL** in eBioscience Flow Cytometry staining buffer, pipetting gently to homogenize.
9. **Block each cell suspension by adding 20 µL Human BD Fc Block™,** 10 µL Brilliant stain buffer, and 5 µL CellBlox Plus blocking buffer per every 105 cells (i.e., 100 µL of resuspended pellet) in suspension. Gently vortex to homogenize.
10. **Incubate for 15 minutes at 4°C, protected from light.**
11. **Transfer 100 µL of blocked cells to individual tubes.**
    a. Samples will be the bulk PBMCs stained with LIVE/DEAD Fixable Near IR 876 dye.
    b. The viability single-color control will use the stained suspension containing heat-shocked cells.
    c. Unstained and all remaining single-color cell controls will use the unstained suspension.
12. **Add an appropriate volume of each antibody** to the relevant samples and controls. Gently vortex to homogenize.

Cell staining (continued)

13. **Incubate for 30 minutes at 4°C, protected from light.**
14. **Wash samples by adding 1 mL of eBioscience Flow Cytometry staining buffer.**
15. **Centrifuge at 400 x *g* for 5 minutes at 4°C and discard supernatant,** drawing off residual fluid with brief contact against an absorbent lab wipe.
16. **Resuspend cell pellet in 1 mL eBioscience Flow Cytometry staining buffer,** pipetting gently to homogenize.
17. **Centrifuge at 400 x *g* for 5 minutes at 4°C and discard supernatant,** drawing off residual fluid with brief contact against an absorbent lab wipe.
18. **Resuspend cell pellet in 200 µL eBioscience Flow Cytometry staining buffer,** pipetting gently to homogenize.
19. **Proceed to immediate analysis or to cell fixation with eBioscience IC fixation buffer.**

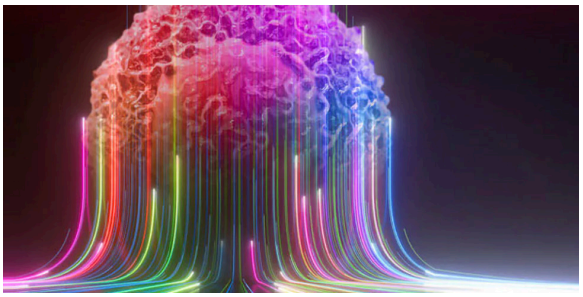
Note: LIVE/DEAD Fixable viability dyes may be used on fresh samples and are stable with a wide range of fixatives. If an alternative viability dye is used, ensure that dead cell staining is consistent before and after fixation by testing this during assay development.

Bead control preparation

1. **Prepare UltraComp eBeads Plus compensation beads per manufacturer directions.**
2. **Proceed to bead fixation with eBioscience IC fixation buffer, or to immediate analysis.**

Note: Bead controls should be treated in a manner similar to cell controls following staining. Thus, if fixation and/or permeabilization is performed on cell controls and samples, the same procedures should be completed on bead controls.

Recommended resources



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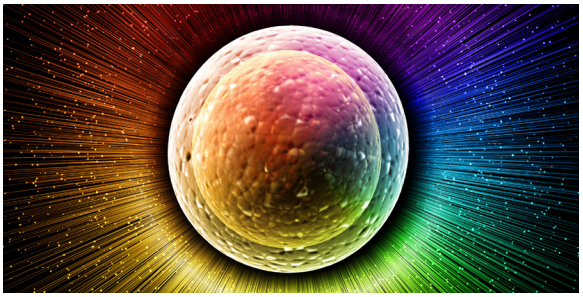

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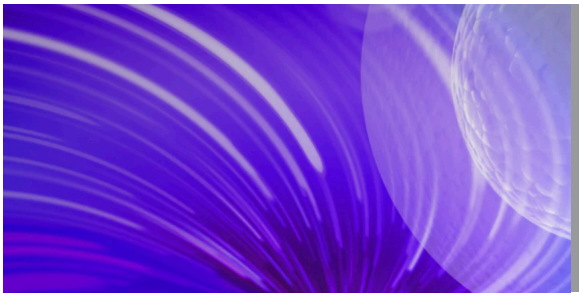

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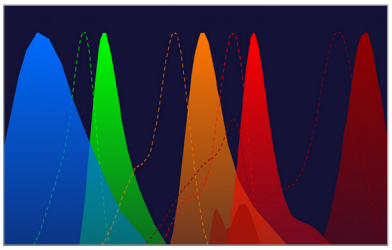

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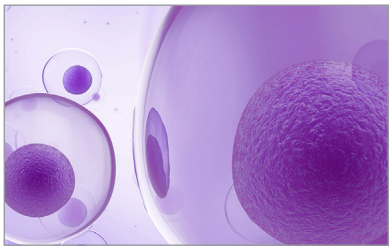

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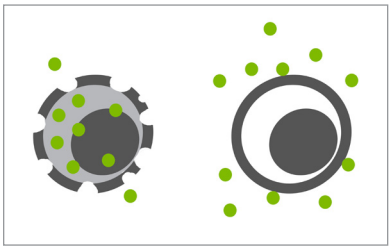

LIVE/DEAD fixable viability dyes

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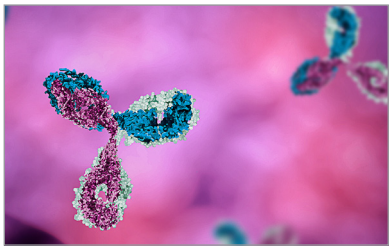

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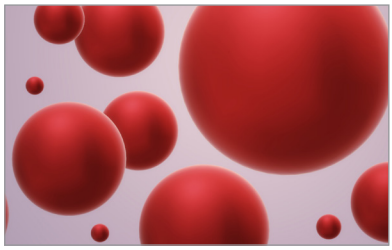

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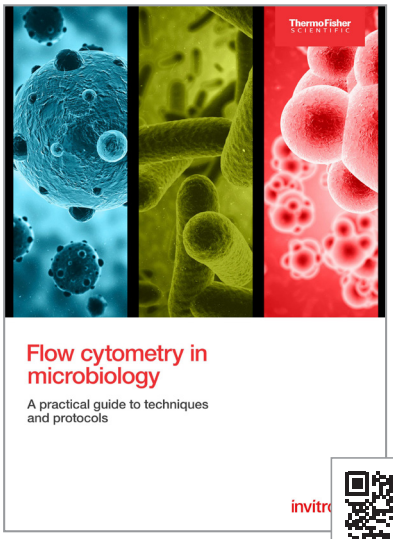
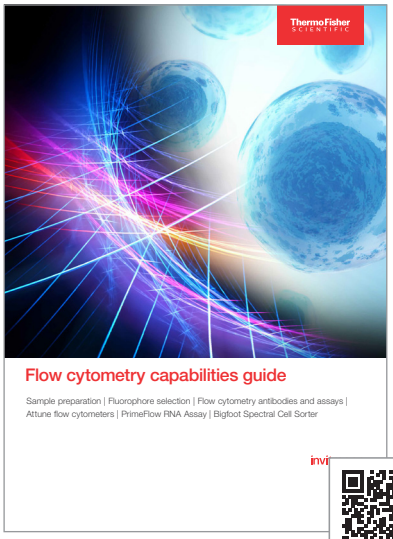
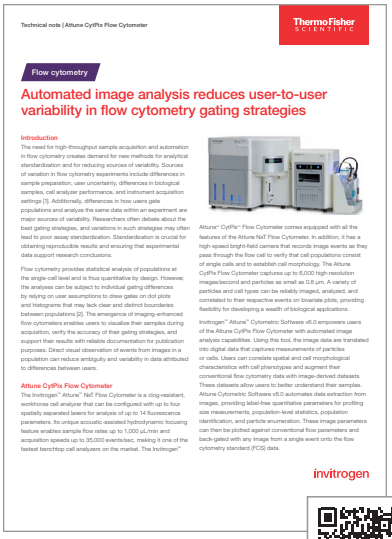


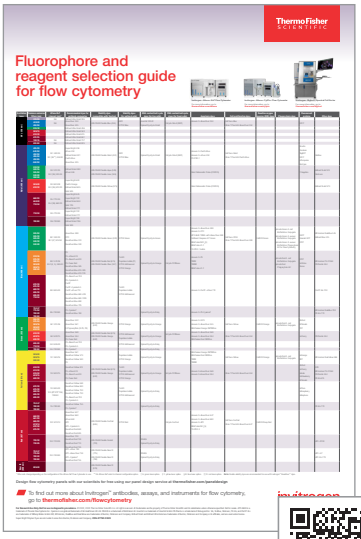
Flow cytometry in microbiology handbook



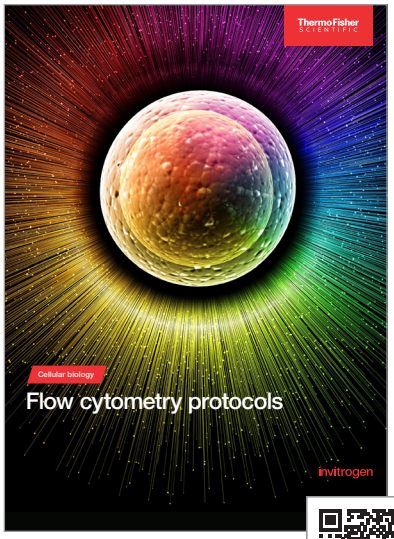
Flow cytometry capabilities guide



Automated image analysis technical note



Fluorophore and reagent selection guide for flow cytometry



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