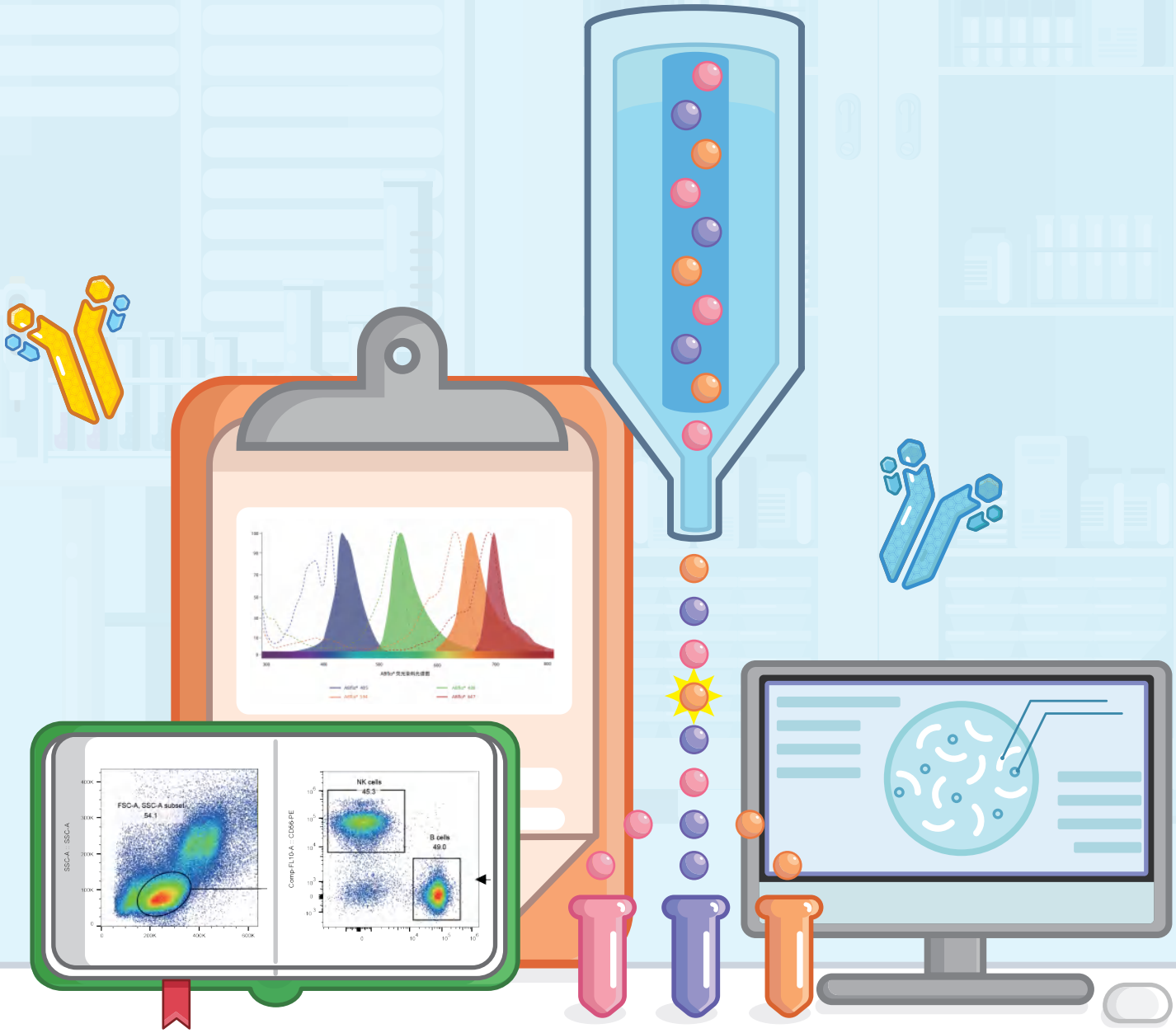
ABclonal Technology®

Flow Cytometry Technical Guide



COMPANY PROFILE

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ABclonal is an innovative growth company with the aim to provide reliable and cost-effective products and services for both basic and translational research in the cutting edges of biomedical science.

Innovation is our DNA. ABclonal has established R&D centers in the worldwide with different focuses to support technical innovation and product development for protein science and molecular biology. We always carefully listen to the opinions and feedback from talented scientists across the globe, quickly translate their needs into our product development pipeline, dedicating to develop the valuable research tools based on state-of-the-art technologies to meet the needs of innovation in a timely manner.

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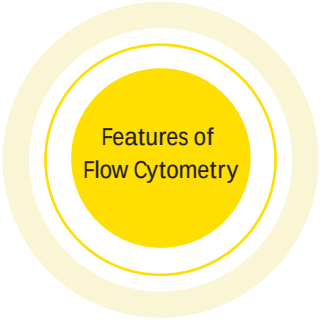
Chapter 1: Introduction to Flow Cytometry

1. Overview of Flow Cytometry

Flow cytometry (FCM) is a rapid and precise method for analyzing and sorting cells or particles based on their physicochemical propertie. This technique allows for the assessment of crucial parameters including cell size, granularity, cell surface antigen expression, DNA content. A flow cytometer with sorting capabilities can be used to sort specific subsets of cells from a large population. The sorted cells have significant potential for various applications in life science research.

2. Features of Flow Cytometry

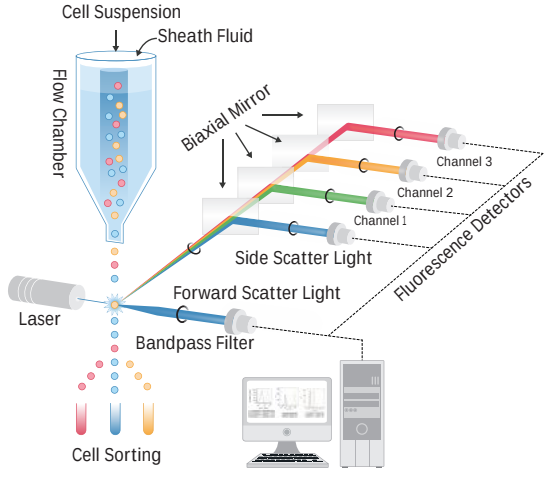


- **Targets:** Cells or Particles
- **Parameters:** Multi-parameters (size, granularity, antigen expression, DNA content, etc.)
- **Speed:** Fast, capable of processing up to tens of thousands of cells per second
- **Result:** High precision and accuracy

3. Working Principle of Flow Cytometer

3.1 Flow Cytometer

Flow cytometer is an instrument that combines the disciplines of cell and molecular biology, fluid dynamics, laser technology, optoelectronics, computer technology, cell fluorescence chemistry, and antibody techniques. It serves as a powerful tool for the comprehensive analysis and sorting of cells or particles.



Internal Structure Schematic of a Flow Cytometer

Internal Structure of a Flow Cytometer:
Optical System: Laser light source, light collection system
Fluidic System: Flow chamber, fluidic driving system
Electronic System: Photodetectors, data processing system
Cell Sorting System: Nozzle, electrostatic deflection plates, sample collector

3.2 Filters

Filters are essential components of the optical system in a flow cytometer. They allow the flow cytometer to differentiate different emitted light based on their wavelengths, enabling simultaneous analysis in multiple detection channels. Filters are mainly categorized into three types: long-pass, short-pass, and bandpass.

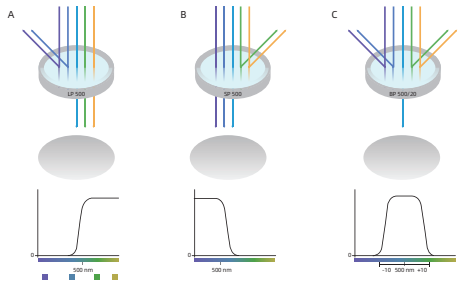


Diagram of Filters

Filter Classification:

Long-pass filter (LP): Allows only light above a specific wavelength to pass through.

Short-pass filter (SP): Allows only light below a specific wavelength to pass through.

Band-pass filter (BP): Allows only light within a certain wavelength range to pass through.

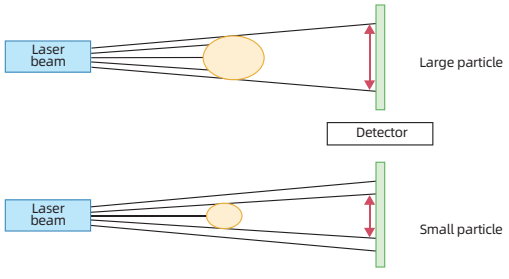
4. Types of Signals Detected in Flow Cytometry

As single cells flow into the laser illumination area of the flow chamber, they emit scatter light signals and fluorescence signals, which are collected by corresponding detectors.

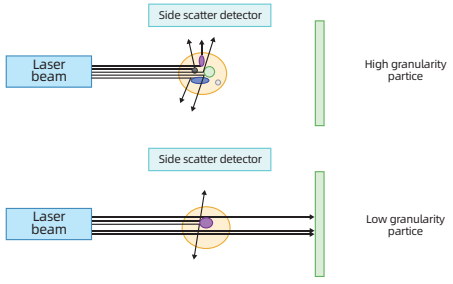
4.1 Scatter Light Signals

Forward Scatter (FSC): The signal direction of forward scatter light is parallel to the laser beam, with an angular deviation typically ranging from 1 to 6 degrees. It is also known as low-angle scatter light and primarily reflects the relative size of cells.

Side Scatter (SSC): The signal direction of side scatter light is perpendicular to the plane formed by the fluid flow. It is also known as 90-degree scatter light. The intensity of side scatter light reflects changes in cellular granularity and fine structural variations.



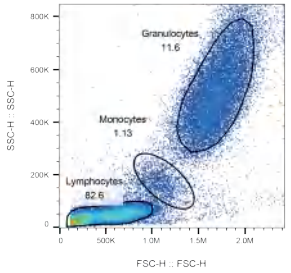
Forward Scatter Diagram



Side Scatter Diagram

Applications of Scatter Light Signals

In experiments, the FSC and SSC parameters are often utilized to differentiate different cell populations, eliminate interference from debris, dead cells, and aggregated cells.



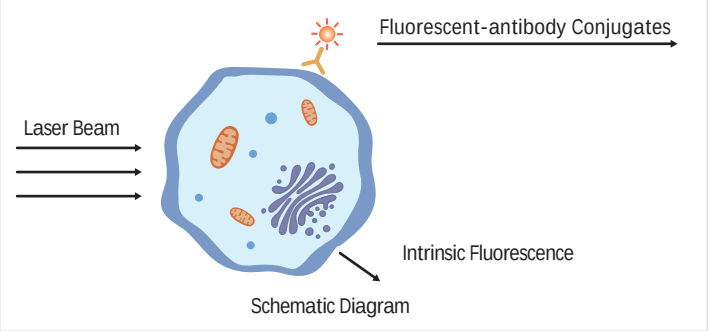
Scatter plot of human fresh peripheral blood

Using FSC to reflect cell size and SSC to reflect cell granularity, different cell populations can be distinguished.

4.2 Fluorescence Signals

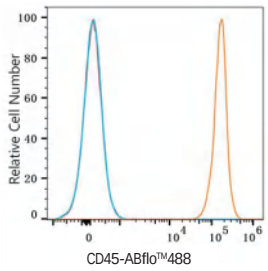
Intrinsic Fluorescence: Also called autofluorescence Naturally occurring cellular components, such as NADPH, riboflavin, and flavin coenzymes, can emit fluorescence signals that have the potential to mask antigen-specific signals.

Extrinsic Fluorescence: When cells are labeled with fluorescently conjugated antibodies or specific fluorescent dyes, the dye emits fluorescence at specific wavelengths upon excitation by corresponding lasers.



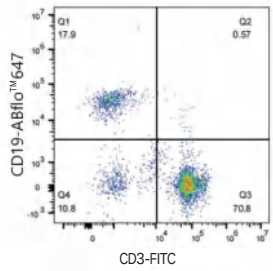
5. Graphical Representation of Flow Cytometry Data

Flow cytometry enables the analysis of large-scale cell samples, generating data points for individual cells. Various methods are employed to present flow cytometry data, including commonly used single-parameter histograms, two-parameter scatter plots, and more. As experimental parameters and complexity increase, high-dimensional data analysis techniques like tSNE and FlowSOM have been employed, enabling researchers to extract more valuable information from their data.



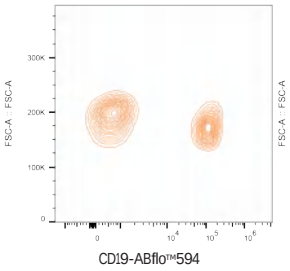
Histogram:
The number of cells relative to fluorescence intensity

- Proportions of negative and positive cell populations
- Mean or median fluorescence intensity (MFI)



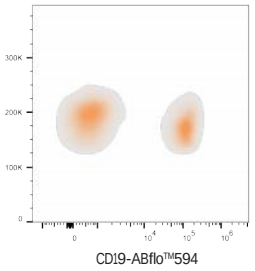
Scatter Plot:
Representation of information from two channels

- Provides a more intuitive representation of cell information
- Enables analysis of specific cell populations or subpopulations



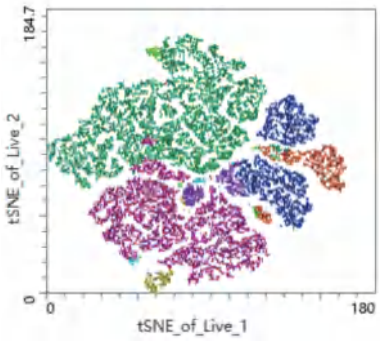
Contour Plot:
Representation of the density of cells

- Contour lines represent areas with the same cell density
- Provides a visual depiction of the concentration of cell populations



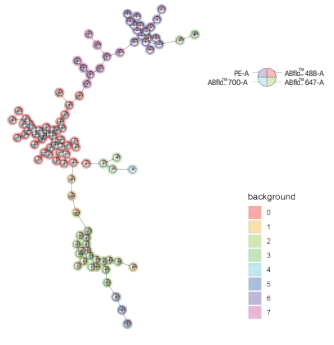
Density Plot:
Three-dimensional representation of data

- Displays the relative quantity of events in a given area
- Reflects the overall density distribution of cell populations



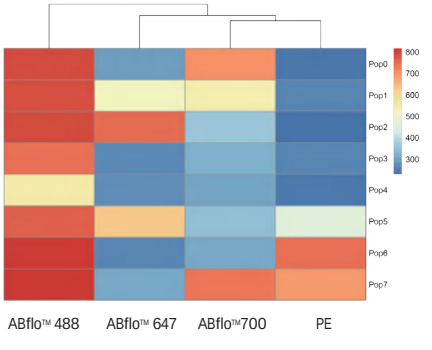
tSNE Dimensionality Reduction Analysis Plot:
Non-linear dimensionality reduction method

- Visualizes high-dimensional data in a lower-dimensional space
- Measures the similarity between samples in the high-dimensional space



FlowSOM Clustering Analysis Plot:
High-dimensional data clustering algorithm

- Partially restores the high-dimensional structure in the data
- Increases the possibility of discovering rare cell populations



Heatmap: The most common visualization method

- Displays expression intensity using color gradients from cool to warm
- Rich color variations and vivid representation of information

6. Applications of Flow Cytometry

Flow cytometry exhibits key characteristics such as multi-parameter analysis, high speed, excellent accuracy, and high precision. This powerful technique allows for the sorting of target cells, making it a valuable tool for analyzing complex populations. Flow cytometry has found extensive applications in scientific research, clinical settings, and the biotechnology industry, significantly advancing the progress of basic scientific research, disease diagnosis and treatment, and drug discovery.

6.1 Applications of Flow Cytometry in Scientific Research

6.1.1 Immunophenotyping

One of the most common applications of flow cytometry is to analyze multiple parameters of immune cell populations using immunological cell markers. For example, analyzing the characteristics of different T cell subsets (CD4+ T cells, CD8+ T cells, antigen-specific T cells) can provide valuable information for immunological research and treatment of autoimmune diseases, cancer, and other diseases.

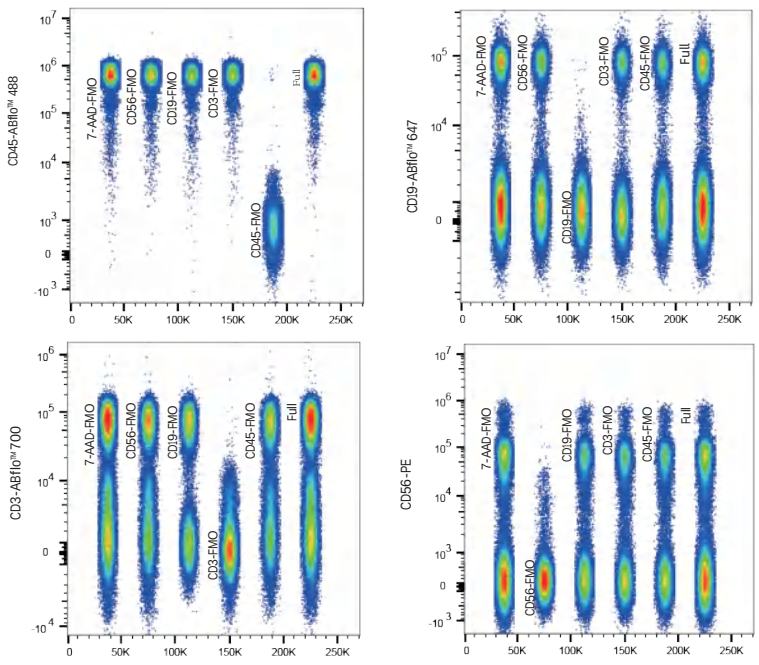
>> Case study: Common Immunophenotyping of Immune Cells (T, B, NK cells)

(1) Panel Design

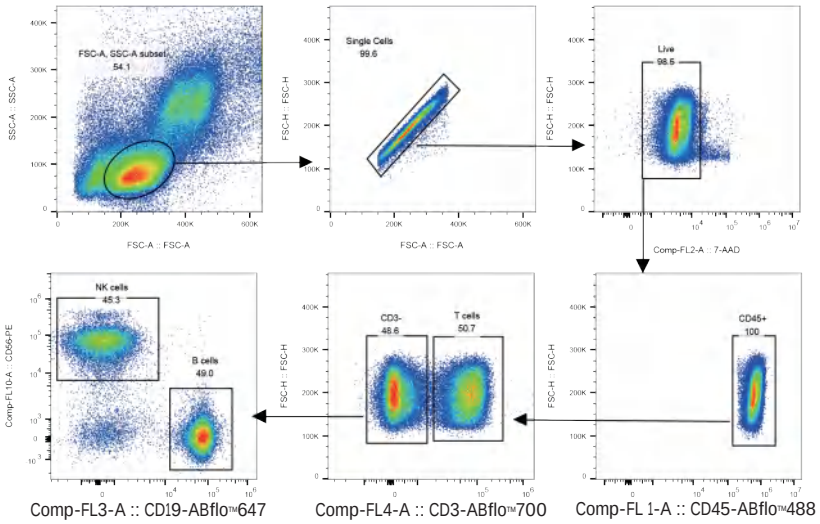
Dye	7-AAD-FMO	PE-FMO	ABflo™ 647-FMO	ABflo™ 700-FMO	ABflo™ 488-FMO	Full Panel	Species	Cat.No.	Sample
ABflo™ 488	CD45-ABflo™ 488	CD45-ABflo™ 488	CD45-ABflo™ 488	CD45-ABflo™ 488	-	CD45-ABflo™ 488	Human	A22494	
ABflo™ 647	CD19-ABflo™ 647	CD19-ABflo™ 647	-	CD19-ABflo™ 647	CD19-ABflo™ 647	CD19-ABflo™ 647	Human	A23009	
ABflo™ 700	CD3-ABflo™ 700	CD3-ABflo™ 700	CD3-ABflo™ 700	-	CD3-ABflo™ 700	CD3-ABflo™ 700	Human	A24943	Human PBMC
PE	CD56-PE	-	CD56-PE	CD56-PE	CD56-PE	CD56-PE	Human	A22826	
7-AAD	-	7-AAD	7-AAD	7-AAD	7-AAD	7-AAD	Human	-	

(2) Multicolor Manual Gating Analysis

FMO Controls



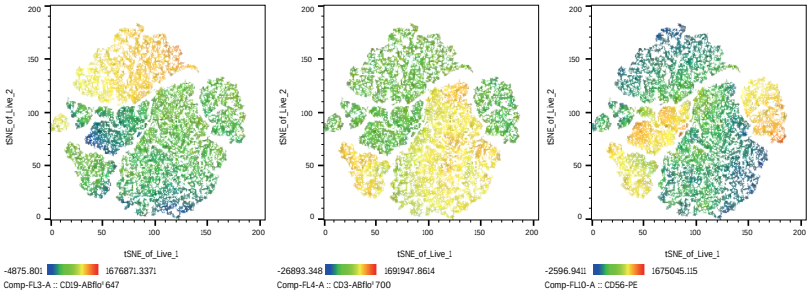
Result: FMO controls for 7-AAD, CD56-PE, CD19-AB-flo™ 647, CD3-ABflo™ 700, and CD45-ABflo™ 488 show clear separation between negative and positive populations, allowing accurate distinction of positive and negative cell populations. Other fluorescent dyes in the experimental panel have minimal impact on the observed signals in the channels used for detecting the target fluorescent dyes.



Full Stained Sample

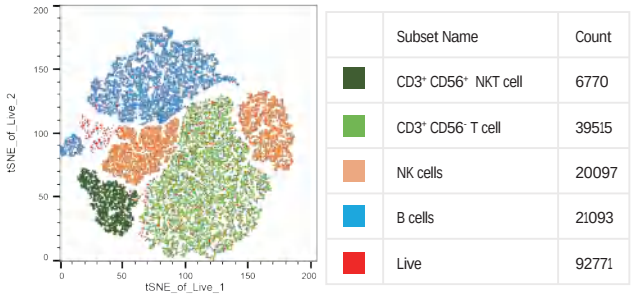
Result : Multicolor experiment with CD45, CD19, CD3, CD56, and 7-AAD. Clear separation of CD3$^+$ T cells, CD3$^-$ CD19+ B cells, and CD3$^-$ CD19$^-$ CD56$^+$ NK cells.

(3) tSNE Dimensionality Reduction Analysis of Signal Distribution for Each Marker



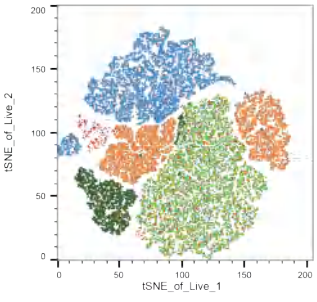
Result: In this plot, the color gradient from red to blue represents the expression of the corresponding markers. The signal distribution plots of CD3, CD19, and CD56 are consistent with the clustering of T cells, B cells, and NK cells, respectively.

(4) Comparison of Manual Gating and Automatic tSNE Analysis



	Subset Name	Count
	CD3$^+$ CD56$^+$ NKT cell	6770
	CD3$^+$ CD56$^-$ T cell	39515
	NK cells	20097
	B cells	21093
	Live	92771

Manual Gating

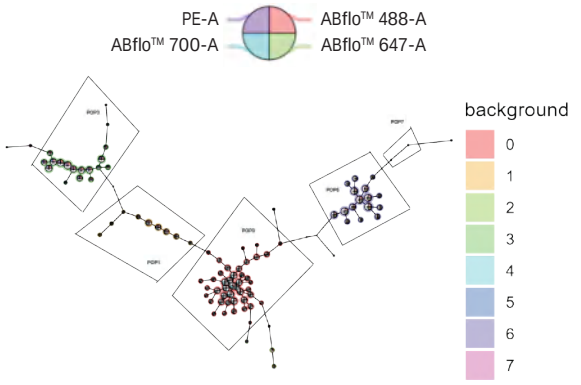


	Subset Name	Count
	FlowSOM.Live.Pop1.NKT cell	7647
	FlowSOM.Live.Pop0.T cell	39203
	FlowSOM.Live.Pop3.NK cell	20069
	FlowSOM.Live.Pop6.B cell	21452
	Live	92771

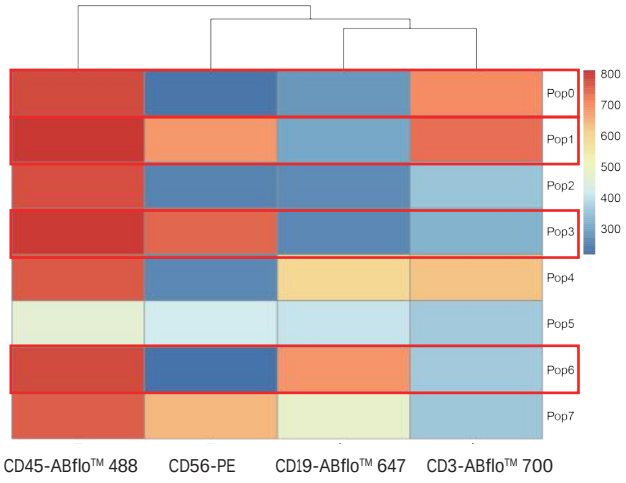
Automatic tSNE Analysis

Result: In this plot, the red color represents the live cell population gated from lymphocytes. The blue color represents the CD19$^+$ B cell population (CD45$^+$ CD3$^-$ CD19$^+$) gated from the live cell population. The orange color represents the NK cell population (CD45$^+$ CD3$^-$ CD56$^+$) gated from the live cell population. The green color represents the CD3$^+$ CD56$^-$ T cell population (CD3$^+$ CD56$^-$) gated from the live cell population. The dark green color represents the CD3$^+$ CD56$^+$ NKT cell population gated from the live cell population. The manual gating and FlowSOM automatic analysis show consistent clustering and cell counts.

(5) FlowSOM Clustering Analysis



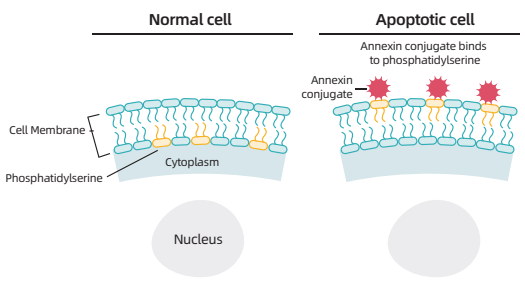
Result : This plot shows the clustering of FlowSOM using a minimum spanning tree. Cells with the same background color belong to the same cluster, and the size of the bubbles indicates the size of each cluster. The fan-shaped plots represent the expression of corresponding marker colors, with larger fan areas indicating higher expression and smaller areas indicating lower expression.



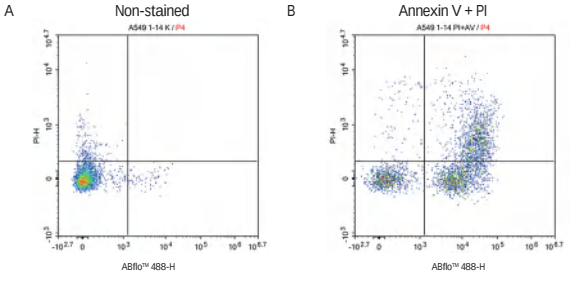
Result : This plot shows the FlowSOM heatmap, where the color gradient from red to blue represents the expression of the corresponding markers. Pop0 represents the $CD3^+$ $CD56^-$ T cell subset ($CD45^+$ $CD3^+$ $CD19^-$ $CD56^-$), Pop1 represents the $CD3^+$ $CD56^+$ NKT cell subset ($CD45^+$ $CD3^+$ $CD19^-$ $CD56^+$), Pop3 represents the NK cell subset ($CD45^+$ $CD3^-$ $CD19^-$ $CD56^+$), and Pop6 represents the CD19+ B cell subset ($CD45^+$ $CD3^-$ $CD19^+$ $CD56^-$).

6.1.2 Apoptosis Analysis

Apoptosis is a form of programmed cell death and is frequently examined in immunology and other research fields. Flow cytometry offers various methods for detecting apoptosis, such as Annexin V staining to detect phosphatidylserine externalization on the cell membrane, TUNEL assay to detect DNA fragmentation, or detection of activated Caspase enzymes.



The principle of apoptosis detection based on Annexin V staining.

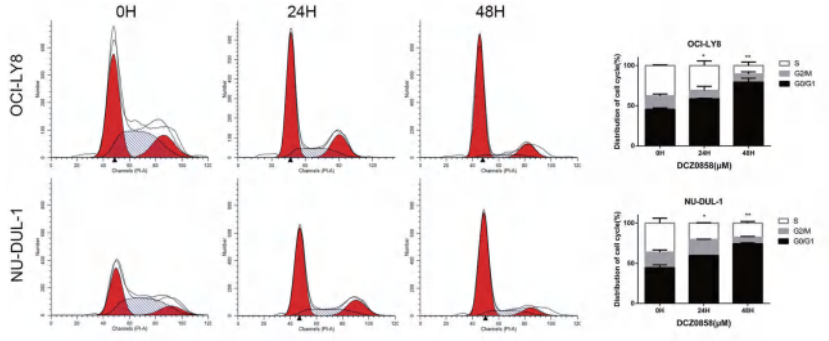


ABflo™ 488 Annexin V/PI Apoptosis Detection Kit (RK05875)

A549 cells (human non-small cell lung cancer cells) were treated with an apoptosis inducer for 5 hours, and then left unstained (A) or double-stained using ABflo™ 488 Annexin V and PI from the kit (B).

6.1.3 Cell Cycle Analysis

The commonly used method for cell cycle analysis is to measure DNA content. This is achieved by using fluorescent dyes that bind to DNA, such as PI, and analyzing the fluorescence intensity based on the different DNA content in each phase of the cell cycle. This allows for the determination of the proportion of cells in each phase. Cell cycle analysis is of great value in tumor research, diagnosis, and prognosis.



Source: doi: 10.1038/s41392-020-0123-0.

Under treatment with 10 μM DCZ0858, the proportion of cells in the G0/G1 phase significantly increased with prolonged treatment time.

6.1.4 Cell Sorting

Cell sorting is an immensely valuable function of flow cytometry. Flow cytometers equipped with cell sorting capabilities can effectively isolate specific cell types from a heterogeneous population. This enables the purification of transfected cells, stem cells, tumor cells, lymphocyte subsets, and tumor-infiltrating lymphocytes, facilitating subsequent in-depth analysis.

6.2 Clinical Applications of Flow Cytometry

6.2.1 Analysis of Lymphocyte Subsets

The analysis of lymphocyte subsets offers valuable insights into the functional status of the immune system under various conditions, encompassing both cellular and humoral immune functions. In clinical practice, it serves as an important adjunct diagnostic tool for diseases that have a significant impact on the immune system. It aids in the analysis of disease pathogenesis, monitoring of disease progression, assessment of treatment efficacy, and prognosis evaluation. The primary parameters measured include lymphocyte subsets (TBNK), T lymphocyte phenotyping, and detection of regulatory T cells (Tregs).

6.2.2 Immunophenotyping of Leukemia

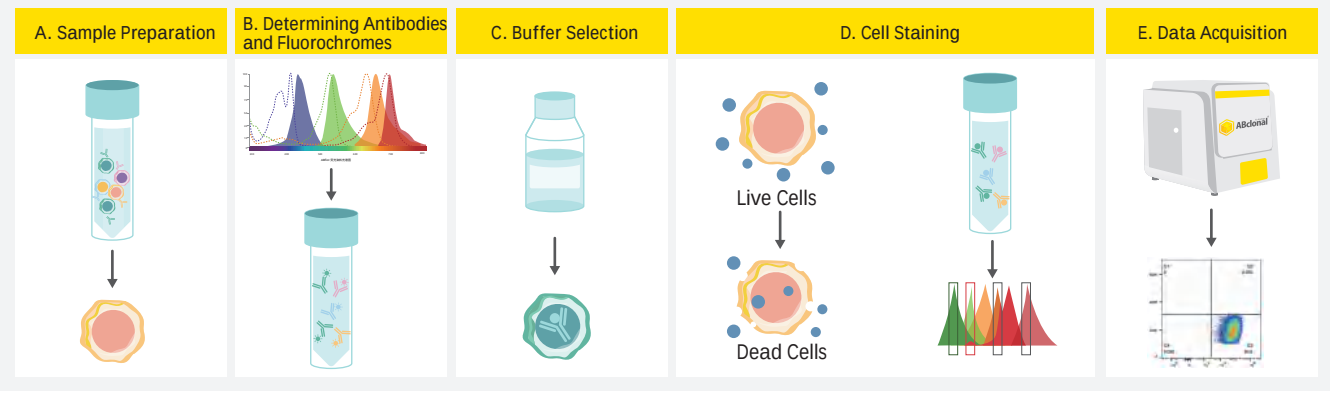
Immunophenotyping of leukemia plays a crucial role in the diagnosis of hematological malignancies, and flow cytometry is the internationally recognized method for this purpose. Flow cytometry analysis of leukemia immunophenotype enables differentiation of cell origins and classification of their differentiation stages. It holds immense value in diagnosing and classifying leukemia, guiding treatment selection, assessing prognosis, and conducting research on the disease's pathogenesis. The primary parameters measured include acute leukemia, chronic lymphoproliferative disorders (CLD)/lymphoma, and other relevant detections.

6.2.3 HLA-B27 Testing

The association between HLA-B27 and ankylosing spondylitis is widely recognized and represents one of the strongest and most characteristic HLA-disease associations. HLA-B27 serves as a crucial marker for ankylosing spondylitis, making the detection of HLA-B27 of significant diagnostic value for the disease. Flow cytometry is currently regarded as the preferred method for HLA-B27 testing and is internationally recommended for this purpose.

Chapter 2: Experimental Procedure and Precautions for Flow Cytometry

1. Experimental Procedure



1.1 Sample Preparation

To perform flow cytometry, it is important to have the sample in the form of a single-cell suspension. However, for adherent cells or solid tissues, it becomes necessary to prepare a single-cell suspension prior to flow cytometry staining. Common methods for achieving this include enzymatic digestion or mechanical tissue dissociation. The following are commonly used sample processing methods:

1.1.1 Suspension Cells

1. Gently pipette up and down to mix the suspension cells.
2. Collect the cells in a 15 mL or 50 mL centrifuge tube (depending on the cell quantity).

1.1.2 Adherent Cells

1. Discard the culture medium and wash the cells with 10 mL of 1×PBS, pH 7.4. Then, add 3 mL of 0.05% Trypsin-EDTA solution at room temperature and incubate for 3 minutes. Observe under a microscope for the detachment of a large number of cells.
2. Terminate the reaction with 15 mL of culture medium and gently pipette up and down to wash the adherent cells. Collect the cells in a 15 mL or 50 mL centrifuge tube (depending on the cell quantity).

1.1.3 Lymphoid Tissue

1.1.3.1 Spleen Tissue

1. Remove the spleen and place it in a 10 cm dish containing 20 mL of RPMI1640 medium supplemented with 1×P/S.

2. Place the spleen in a 75 μm cell strainer and use a pestle to grind the spleen against the strainer, ensuring movement of the strainer while grinding. Rinse the cells off the strainer with medium to disperse them in the medium.
3. Transfer the medium into a 50 mL centrifuge tube. Rinse the dish with fresh medium and transfer it to the centrifuge tube as well, keeping the total volume of medium not exceeding 30 mL.
4. Red Blood Cell (RBC) Lysis: Centrifuge at room temperature at 400 g for 5 minutes, discard the supernatant, and keep the cells. Add 13 mL of room temperature RBC lysis solution to lyse the red blood cells. Gently pipette to disperse cell clumps and incubate for 1 minute. Add 37 mL of basic culture medium and mix well to stop the RBC lysis.
5. Centrifuge at room temperature at 400 g for 5 minutes, discard the supernatant, and keep the cells. Add 40 mL of basic culture medium at room temperature to resuspend the cells and gently pipette to disperse cell clumps, completing the first wash.
6. Centrifuge at room temperature at 400 g for 5 minutes, discard the supernatant, and keep the cells. Add 20 mL of 20% FBS culture medium at room temperature to resuspend the cells and gently pipette to disperse cell clumps. Pass the resuspended cells through a cell strainer to remove clumped cells.
7. Centrifuge at room temperature at 400 g for 5 minutes and discard the supernatant.
8. Add 20 mL of complete culture medium (RPMI1640 + 10% FBS + 1×P/S) to resuspend the cells and prepare for cell counting.

1.1.3.2 Thymus Tissue

1. Remove the thymus and immerse it in a 10 cm culture dish containing 10 mL of serum-free medium with 1% Penicillin-Streptomycin Solution.
2. Place the thymus in a 200-mesh sieve and gently grind it with a tissue grinder until no obvious clumps are present.
3. Rinse the sieve with 15 mL of PBS, collect the rinse solution in a 15 mL centrifuge tube, centrifuge at 300 g for 5 minutes, and discard the supernatant.
4. Resuspend the thymus cells in complete culture medium, pass the suspension through a 200-mesh sieve, centrifuge at 300 g for 5 minutes, discard the supernatant, and resuspend the thymus cells in complete culture medium, adjusting the cell concentration to 1×10^7/mL.

1.2 Cell Surface Staining

1. Cell Counting: Collect the cells in a 50 mL centrifuge tube and count them. Centrifuge at 500 g for 4 minutes, discard the supernatant.
2. Preparation of Single Cell Suspension: Resuspend the collected cells in 1 mL of 0.5% BSA/PBS solution. Centrifuge at 400 g for 3 minutes, discard the supernatant, and repeat this step twice. Resuspend the cells in 0.5% BSA/PBS solution and distribute them into a 96-well plate, adding 50 µL of cell suspension per well.
3. Blocking (optional): Depending on the staining protocol and cell species, incubate with 10% goat serum/10% mouse serum/commercial blocking reagent at room temperature for 30-60 minutes to block non-specific binding mediated by Fc receptors.
4. Incubation with Primary Antibody: Add 50 µL of primary antibody dilution to each well. Shake gently and incubate at room temperature for 20 minutes.
5. Wash: Centrifuge at 400 g for 5 minutes, discard the supernatant.
6. Incubation with Secondary Antibody: If using a fluorescent dye-conjugated secondary antibody, add 100 μL of fluorescent secondary antibody to each well. Resuspend gently in the dark and incubate at room temperature for 20 minutes. If using directly-labeled antibodies, skip to step 9.
7. Centrifuge at 400 g for 5 minutes, discard the supernatant.
8. Fixation (optional): If not immediately analyzing the stained cells, resuspend the cells in 100 μL of 4% paraformaldehyde, incubate at room temperature for 10 minutes, and wash twice with 0.5% BSA/PBS solution.
9. Wash: Add 200 µL of 0.5% BSA/PBS solution, centrifuge at 400 g for 5 minutes, discard the supernatant, and repeat this step twice.
10. Resuspend the cells in 200 µL of 0.5% BSA/PBS solution and analyze them using the appropriate instrument.

1.3 Intracellular Staining

1.3.1 Formaldehyde/Saponin Method (Cytoplasmic Proteins)

1. Sample Preparation: Collect the cells to be tested, count and assess viability. Then, resuspend the cells in 1 mL of 0.5% BSA/PBS solution, wash twice, centrifuge at 400 g for 3 minutes, and discard the supernatant.
2. Viability Staining: Use a dilution of L/D dye, stain at room temperature in the dark for 15 minutes, centrifuge and discard the supernatant, then wash twice with 1 mL of 0.5% BSA/PBS solution.
3. Cell Fixation: Resuspend the cells in 4% paraformaldehyde fixation solution, rotate and incubate in the dark for 15 minutes, followed by one wash with 0.5% BSA/PBS solution.
4. Cell Permeabilization: Prepare a permeabilization solution by adding 0.1% saponin to 0.5% BSA/PBS solution. Resuspend the cells in this permeabilization solution, incubate in the dark for 1 hour, then centrifuge and discard the supernatant. (All buffers used after this step should contain 0.1% saponin since saponin permeabilization is reversible.)
5. Incubation with Primary Antibody: Resuspend the cells in 0.5% BSA/PBS solution containing 0.1% saponin, distribute them into a 96-well plate (50 µL per well, with a cell count of ≤1E6 per well), and add the primary antibody dilution (diluted in 0.5% BSA/PBS solution containing 0.1% saponin), 50 µL per well. Mix well. Place the 96-well plate on a shaker, incubate at room temperature in the dark for 30 minutes.
6. Wash to Remove Unbound Primary Antibody: After incubation, centrifuge at 400 g for 5 minutes and discard the supernatant. Wash each well with 200 µL of 0.5% BSA/PBS solution containing 0.1% saponin, centrifuge at 400 g for 5 minutes, and discard the supernatant.
7. Incubation with Secondary Antibody: If the primary antibody is not directly labeled with a fluorescent dye, add the appropriate fluorescent secondary antibody dilution (diluted in 0.5% BSA/PBS solution containing 0.1% saponin) for the corresponding species, 100 µL per well. Incubate in the dark at room temperature on a shaker for 30 minutes. If the primary antibody is already labeled with a fluorescent dye, proceed to step 9 after repeating step 6.
8. Wash to Remove Unbound Secondary Antibody: After incubation, centrifuge at 400 g for 5 minutes and discard the supernatant. Wash each well twice with 200 µL of 0.5% BSA/PBS solution containing 0.1% saponin, centrifuge at 400 g for 5 minutes, and discard the supernatant.
9. Resuspend for Analysis: Resuspend the cells in 100-200 µL of 0.5% BSA/PBS solution containing 0.1% saponin and analyze using the appropriate instrument.

1.3.2 Formaldehyde/Triton Method (Nuclear Proteins)

1. Sample Preparation: Collect the cells to be tested, count and assess viability. Then, resuspend the cells in 1 mL of 0.5% BSA/PBS solution, wash twice, centrifuge at 400 g for 3 minutes, and discard the supernatant.
2. Viability Staining: Use a dilution of L/D dye, stain at room temperature in the dark for 15 minutes, centrifuge and discard the supernatant, then wash twice with 1 mL of 0.5% BSA/PBS solution.
3. Cell Fixation: Resuspend the cells in 4% paraformaldehyde fixation solution, rotate and incubate in the dark for 15 minutes, followed by one wash with 0.5% BSA/PBS solution.
4. Cell Permeabilization and Blocking: Resuspend the cells in Triton X-100 permeabilization solution, rotate and incubate in the dark for 15 minutes, centrifuge and discard the supernatant, then resuspend in Fc receptor blocking solution, incubate for 1 hour, and centrifuge and discard the supernatant.
5. Incubation with Primary Antibody: Resuspend the cells in 0.5% BSA/PBS solution, distribute them into a 96-well plate (50 µL per well, with a cell count of ≤1E6 per well), and add the primary antibody dilution (diluted in 0.5% BSA/PBS solution), 50 µL per well. Mix well. Place the 96-well plate on a shaker, incubate at room temperature in the dark for 30 minutes.
6. Wash to Remove Unbound Primary Antibody: After incubation, centrifuge at 400 g for 5 minutes and discard the supernatant. Wash each well with 200 µL of 0.5% BSA/PBS solution, centrifuge at 400 g for 5 minutes, and discard the supernatant.

7 Incubation with Secondary Antibody: If the primary antibody is not directly labeled with a fluorescent dye, add the appropriate fluorescent secondary antibody dilution for the corresponding species, 100 µL per well. Incubate in the dark at room temperature on a shaker for 30 minutes. If the primary antibody is already labeled with a fluorescent dye, proceed to step 9 after repeating step 6.

8 Wash to Remove Unbound Secondary Antibody: After incubation, centrifuge at 400 g for 5 minutes and discard the supernatant. Wash each well twice with 200 µL of 0.5% BSA/PBS solution, centrifuge at 400 g for 5 minutes, and discard the supernatant.

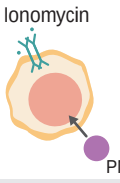
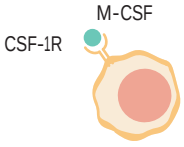
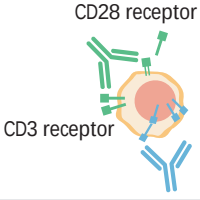
9 Resuspend for Analysis: Resuspend the cells in 100-200 µL of 0.5% BSA/PBS solution and analyze using the appropriate instrument.

2. Precautions

2.1 Sample Preparation

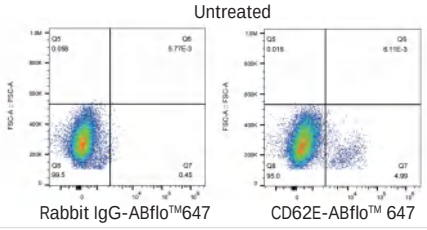
2.1.1 Cell Handling

The selection of effective methods and conditions for handling samples is crucial for the success of the experiment and ensuring the expression of the target in the samples. For instance, immune cells often require activation to rapidly proliferate or differentiate into mature cells. Activated cells often exhibit high expression of specific transcription factors, cytokines, and other targets, which can be detected using flow cytometry. Prior to commencing the experiment, it is advisable to design the experiment and handle the samples based on the specific cell type, target expression level, and experimental conditions.

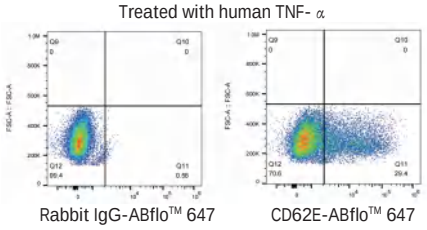
A	B	C	D	E
				
PMA and Ionomycin stimulate T cells to produce cytokines	Lipopolysaccharide (LPS) activates monocytes to secrete cytokines	M-CSF factor is used to regulate monocyte proliferation and differentiation	Phytohemagglutinin A (PHA) induces T cell activation and expansion	CD3/CD28 functional antibodies are used for T cell activation and expansion

>> Case study 1: Detection of CD62E Expression in HUVEC Endothelial Cells

CD62E is not expressed in normal endothelial cells. However, when endothelial cells are stimulated by inflammatory factors such as TNF-α, the expression of CD62E increases.



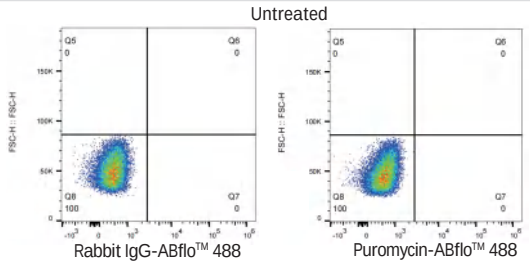
Sample: HUVEC cells (untreated)
Antibodies: ABflo™ 647 Rabbit anti-Human CD62E/E-Selectin mAb (A24309)
Isotype control: ABflo™ 647 Rabbit IgG isotype control (A22070)



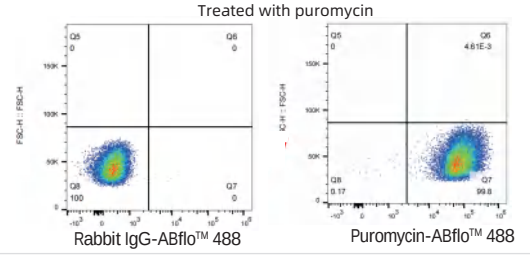
Sample: HUVEC cells
(treated with human TNF-α,Cat. No. RP00993,10 ng/mL,16 h)
Antibodies:ABflo™ 647 Rabbit anti-Human CD62E/E-Selectin mAb (A24309)
Isotype control: ABflo™ 647 Rabbit IgG isotype control (A22070)

>> Case study 2: Detection of Protein Synthesis Levels in C6 Cells Using Puromycin Incorporation

Puromycin is a protein synthesis inhibitor that can be incorporated into the process of protein translation, leading to premature termination of protein synthesis. Peptides containing puromycin are released from the ribosome. By using puromycin antibodies, techniques such as Western blotting (WB), immunofluorescence (IF), and flow cytometry (FC) can be employed to detect and analyze protein synthesis.



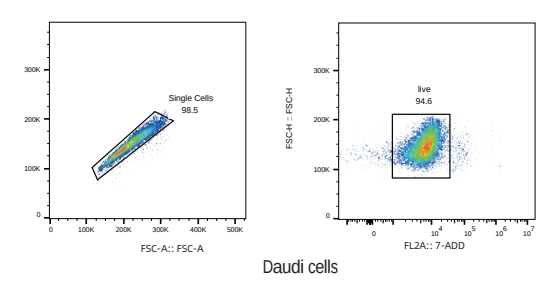
Sample: C6 cells (untreated)
Antibodies: ABflo™ 488 Rabbit anti-puromycin mAb (A23130)
Isotype control: ABflo™ 488 Rabbit IgG isotype control (A22069)



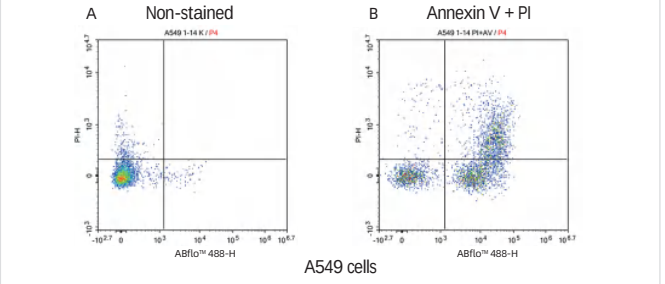
Sample: C6 cells (treated with puromycin,20 μg/mL, 4 h)
Antibodies: ABflo™ 488 Rabbit anti-puromycin mAb (A23130)
Isotype control: ABflo™ 488 Rabbit IgG isotype control (A22069)

2.1.2 Cell Viability Assay

Cell viability assays are used in flow cytometry to exclude dead cell populations, enabling clearer cell subpopulation identification and precise recognition of target cell populations. Therefore, distinguishing dead cells to exclude false-positive cells is a crucial step in ensuring the accuracy of flow cytometry. In flow cytometry, sensitive dyes and reagents can be used for labeling, and cell viability analysis can be performed through flow cytometry. The principle is mainly based on the specific fluorescence labeling of cells due to changes in membrane permeability after cell death. Common methods include staining cells with DNA dyes such as PI, 7-AAD, and DAPI, or using protein conjugates such as fluorescently labeled Annexin V to bind to phosphatidylserine on apoptotic cell surfaces. Additionally, cell viability can also be assessed by measuring changes in cell size and physicochemical properties.



Cell Viability Assay using 7-AAD dye

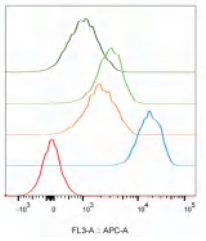


Cell Viability Assay using ABflo™ 488-Annexin V+PI

2.1.3 Fc Blocking

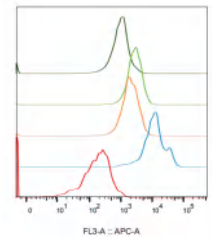
The antibodies used in flow cytometry experiments typically contain an Fc region. Some cell types, such as monocytes, macrophages, and B cells, express Fc receptors on their surfaces. During the antibody staining process, the Fc region of the antibody binds to the Fc receptors on the cell surface, resulting in non-specific staining. Therefore, the choice of blocking agent can be made based on the sample conditions to achieve better staining results and signal-to-noise ratio in flow cytometry.

>> Case study: Comparison of Detection Results between Cells Expressing Fc Receptors with and without Blocking



- Commercial Blocking Agent
- Mouse IgG antibody blocking
- 10% mouse serum blocking
- Unblocked
- Blank control

Sample: U-937 cells



- Commercial Blocking Agent
- Mouse IgG antibody blocking
- 10% mouse serum blocking
- Unblocked
- Blank control

Sample: HL-60 cells

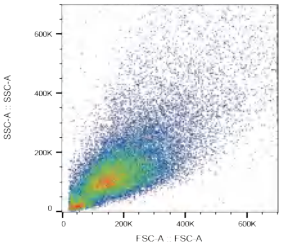
The results show that unblocked cells exhibit strong non-specific signals after staining with rabbit non-immune serum. However, after blocking, the non-specific signals significantly decrease.

2.1.4 Intracellular and Nuclear Staining

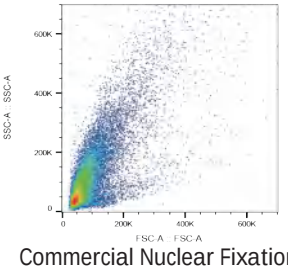
The cell staining method depends on the expression location of the target protein in the cell. For detecting cytoplasmic or nuclear proteins, appropriate cell fixation and membrane permeabilization should be performed before staining. The treated cells should still maintain their morphology and scattering characteristics. Staining and detection of intracellular molecules help in identifying different cell subpopulations and determining cell states.

>> Case study 1: Influence of Different Fixation and Permeabilization Methods on Cell Subpopulation

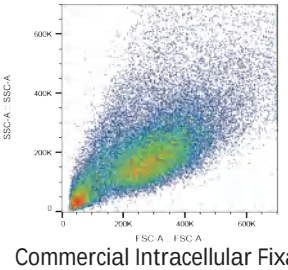
K562 cells were treated using nine different fixation and permeabilization methods. The results showed significant differences in the FSC-SSC characteristics of the cells following the various treatments. For cytoplasmic protein staining, it is recommended to use the 4% PFA + 0.5% saponin method, while for nuclear protein staining, the 4% PFA + 0.3% Triton X-100 method is recommended.



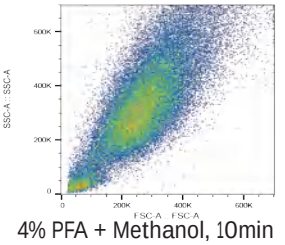
4% PFA + 0.3% Triton X-100



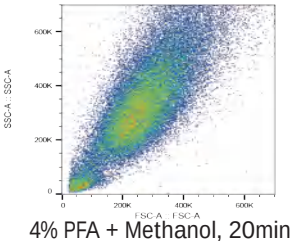
Commercial Nuclear Fixation and Permeabilization Solution



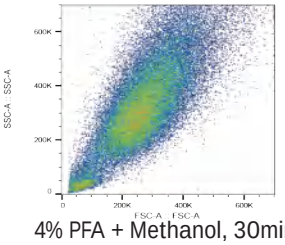
Commercial Intracellular Fixation and Permeabilization Solution



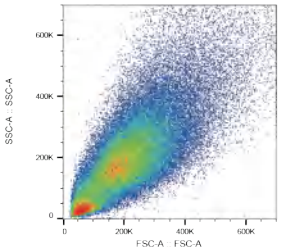
4% PFA + Methanol, 10min



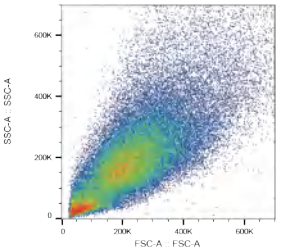
4% PFA + Methanol, 20min



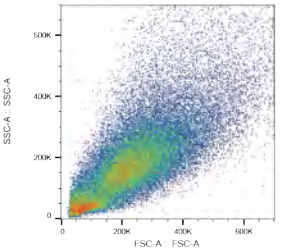
4% PFA + Methanol, 30min



4% PFA + 0.1% saponin

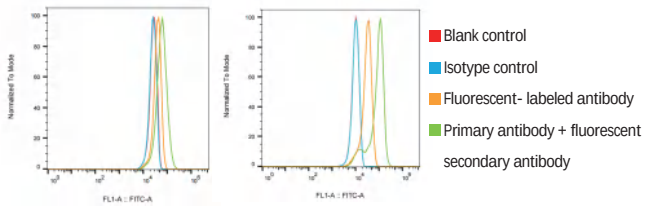


4% PFA + 0.3% saponin



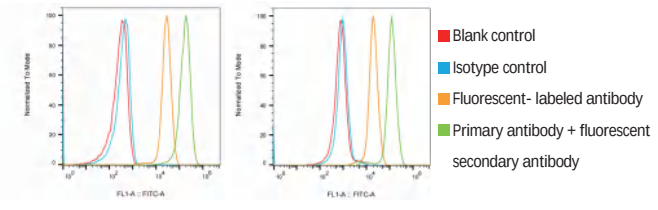
4% PFA + 0.5% saponin

>> Case study 2: Non-specific Staining Due to Improper Fixation and Permeabilization Solution Selection (Cytoplasmic Protein using Nuclear Staining Method)



HeLa cells (negative control) Jurkat cells

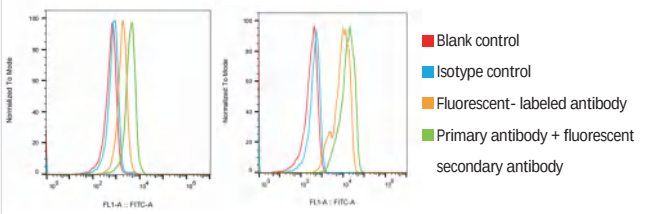
Target: Human ZAP70
Localization: Cytoplasm
Correct Fixation and Permeabilization Solution: 4% PFA + 0.5% saponin



HeLa cells (negative control) Jurkat cells

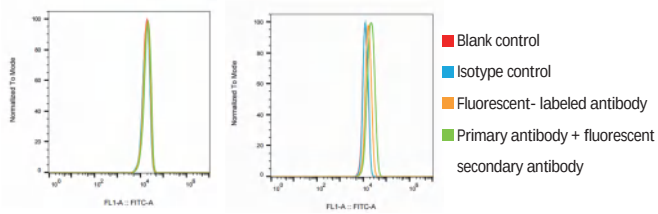
Target: Human ZAP70
Localization: Cytoplasm
Incorrect Fixation and Permeabilization Solution: 4% PFA + 0.3% Triton X-100

>> Case study 3: No Signal Due to Improper Fixation and Permeabilization Solution Selection (Nuclear Protein using Cytoplasmic Protein Staining Method)



K562 cells (negative control) MOLT4 cells

Target: ERG
Localization: Mainly nucleus, secondary cytoplasm
Correct Fixation and Permeabilization Solution: 4% PFA + 0.3% Triton X-100



K562 cells (negative control) MOLT4 cells

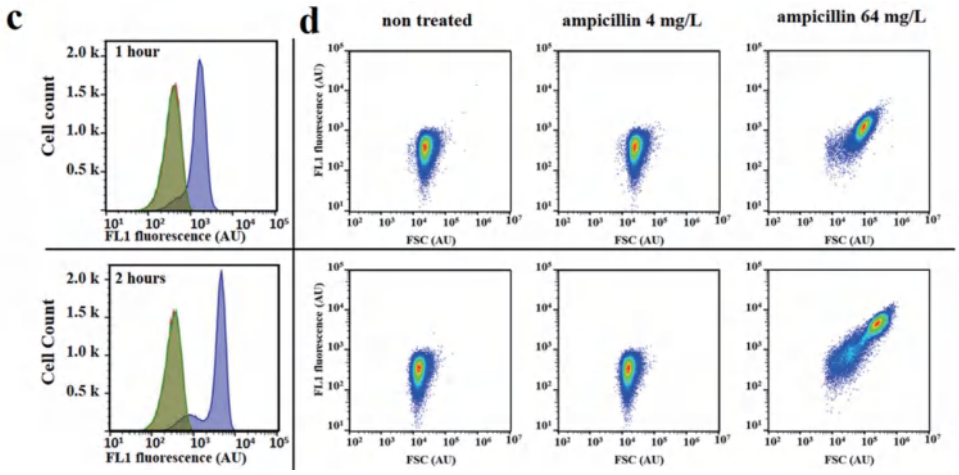
Target: ERG
Localization: Mainly nucleus, secondary cytoplasm
Incorrect Fixation and Permeabilization Solution: 4% PFA + 0.5% Saponin

2.2 Experimental Controls

The proper use of controls is essential in flow cytometry experiments to avoid false-positive or false-negative results, ensuring more objective and accurate analysis of the data. Commonly used controls in flow cytometry include blank controls, single-stain controls, isotype controls, and fluorescence minus one (FMO) controls. These controls help in establishing baseline fluorescence levels, distinguishing specific staining from background noise, and setting appropriate gating strategies for data interpretation.

2.2.1 Blank control

A blank control refers to a control tube where no additional fluorescent staining is added. The blank control is set up to distinguish the background autofluorescence of cells and to determine whether external treatments such as drugs introduce additional autofluorescence in cells. It can be used to adjust the voltage settings for each channel.



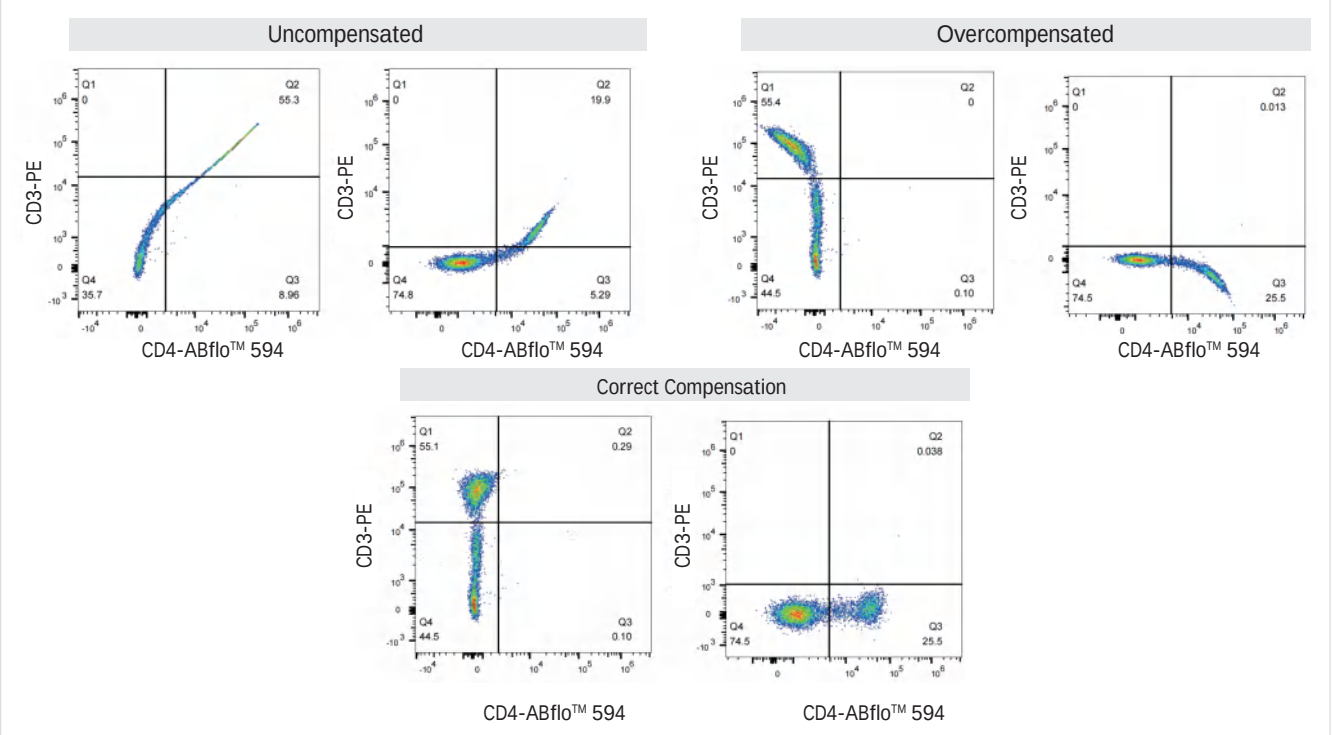
Source: DOI: 10.1038/s41598-018-30623-2

Enhanced autofluorescence of cells induced by Ampicillin.

2.2.2 Single-stain control

A single-stain control, also known as a single-positive control, is primarily used for compensation adjustment between fluorescence channels. It can also assist in adjusting the voltages to prevent positive signals from exceeding the measurement range of the instrument. Compensation adjustment between flow cytometry fluorescence channels is necessary because in multicolor flow cytometry experiments, different fluorescence dyes in different channels may exhibit spectral overlap after being excited by corresponding lasers, leading to fluorescence spillover between different channels. Once the fluorescence compensation is properly set, the cell population should be located in the single-positive region, where the cell populations in the upper-left and lower-right positions should be perpendicular or parallel to the x and y axes.

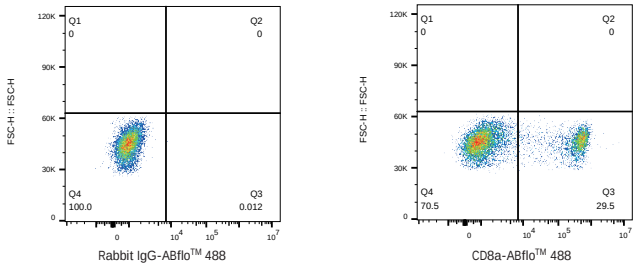
>> Case study : Compensation Adjustment for ABflo™ 594 and PE Double Staining



2.2.3 Isotype control

An isotype control is an antibody derived from the same species as the primary antibody, with the same immunoglobulin subtype and labeled with the same fluorescent dye. It should be used at the same final concentration as the primary antibody in the experiment. Its purpose is to eliminate background staining caused by non-specific binding of the primary antibody. Examples of isotype control selection are as follows:

Type	Name	Host	Subtype	Fluorochrome
Primary Antibody	ABflo™ 488 Rabbit anti-Human/Monkey CD8a mAb（A23345）	Rabbit	Rabbit IgG	ABflo™ 488
Isotype Control	ABflo™ 488 Rabbit IgG isotype control (A22069)	Rabbit	Rabbit IgG	ABflo™ 488

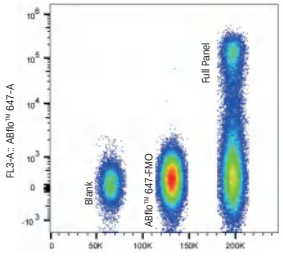


Human PBMC

Example image of staining results with isotype control and primary antibody.

2.2.4 Fluorescence Minus One (FMO) Control

Fluorescence Minus One (FMO) control refers to cell samples in which one specific fluorescence is subtracted from the experimental design. It allows the assessment of fluorescence dye interferences and compensation backgrounds, aiding in the proper gating of multicolor experiments.

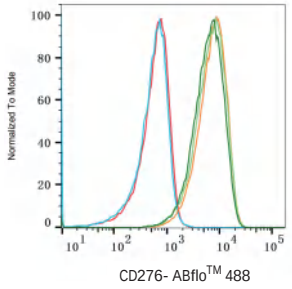


Dye	Blank Control	ABflo™ 647-FMO	Full Panel
ABflo™ 647	-	-	CD16-ABflo™ 647
ABflo™ 700	-	CD3-ABflo™ 700	CD3-ABflo™ 700
PE	-	CD56-PE	CD56-PE

2.3 Antibody Concentration

Determine the optimal concentration of the antibody for the experiment through antibody titration.

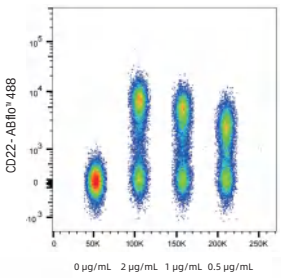
Human/Mouse CD276-Cat.No.A22156



Sample: PC-3 cells
Recommended antibody concentration: 0.5 μg/mL

- 0.5 μg/mL
- 1 μg/mL
- 2 μg/mL

Mouse CD22-Cat.No.A24627



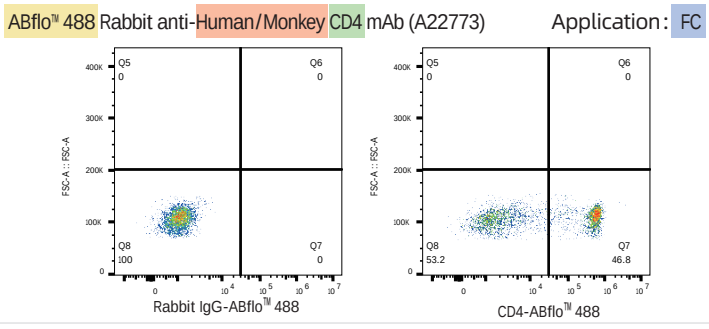
Sample: C57BL/6 mouse Splenocytes
Recommended antibody concentration: 2 μg/mL

Chapter 3: Flow Cytometry Antibody Selection and Panel Design

1. Key Points for Flow Cytometry Antibody Selection

Choosing the right flow cytometry antibody is one of the key factors to ensure the success of flow experiment. When selecting flow cytometry antibodies, the following key points should be considered:

- Determine the target to be detected, such as the expression location of the target antigen in the target cells (membrane expression, intracellular expression), and pay attention to the antigen selection section provided by the manufacturer.
- Determine the species of the experimental samples.
- Check if the antibody datasheet indicates the species to be detected and its suitability for flow cytometry applications.
- Whenever possible, choose commercially available validated antibodies for titration to reduce experimental steps and procedures.

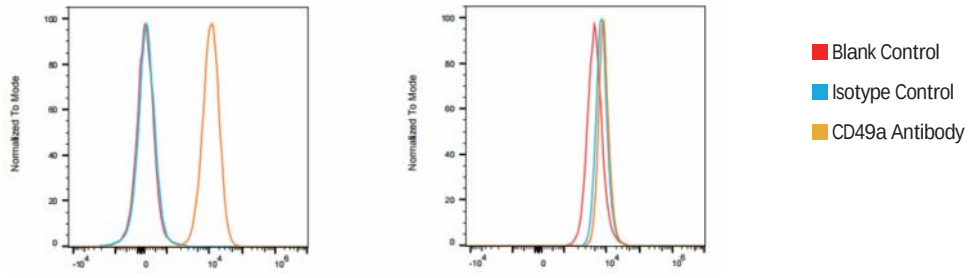


2. Flow Cytometry Panel Design

With the continuous advancement of flow cytometers and the increasing variety of fluorescent dyes, multicolor flow cytometry offers more possibilities. Its advantages include the ability to simultaneously detect multiple markers on single cells, gather comprehensive cellular experimental data, and achieve more precise identification of cell populations. Furthermore, the demand for smaller sample volumes and higher sample throughput has significantly enhanced the efficiency of flow cytometry experiments. However, the detection of a larger number of antigens and fluorescent dyes adds complexity to the design of multicolor flow cytometry panels. When designing a flow cytometry panel, careful consideration should be given to the following factors:

- Instrument configuration: Prior to panel design, it is essential to understand the configuration of your instrument (lasers, filters).
- Fluorescence intensity: Different dyes have varying fluorescence intensity, which can be compared using stain index. Higher stain index indicates brighter fluorescence.
- Antigen expression and dye pairing: low expressed antigens can be paired with brighter fluorescent dyes, while high expressed antigens can be paired with weaker fluorescent dyes.
- Minimize spectral overlap: When combining different fluorescent dyes in different channels, choose dyes with minimal overlap in emission spectra.
- Fluorescent interference: Fluorescent dyes that interfere with each other can be used to label different cells, and then each population can be gated and analyzed separately.
- Cell viability detection dyes: Flow cytometry panels should include cell viability detection dyes to exclude dead cells and cell debris.

>> Case study : Detection of Low-Expressed Antigen using Strong or Weak Fluorescent Dyes



Target: Human CD49a Positive Sample: SH-SY5Y Cells Expression Level: 1.6 nTPM

The results show that for a low-expressed antigen, the strong fluorescent dye ABflo™ 647 can effectively distinguish between negative and positive cell populations, while the weak fluorescent dye PerCP cannot differentiate them. Therefore, when designing a multicolor flow cytometry panel, in addition to considering fluorescence spillover, the expression level of the target protein in the sample should also be taken into account. Proper dye pairing is essential to effectively detect the expression of the target protein.

3. Features of ABflo™ Fluorescent Dyes

High fluorescence intensity

The ABflo™ series dyes exhibit excellent fluorescence intensity, allowing for better detection and discrimination of weakly expressing cell populations.

Wide pH tolerance range

The dyes exhibit stable fluorescence across a broad pH range, maintaining stability within a wide pH range.

ABflo™ fluorescent dyes

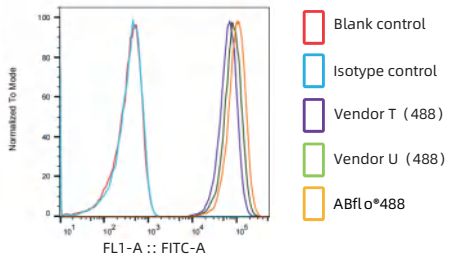
High stability

These dyes have minimal decay in luminescence stability, enabling long-term acquisition and analysis of high-quality fluorescence data.

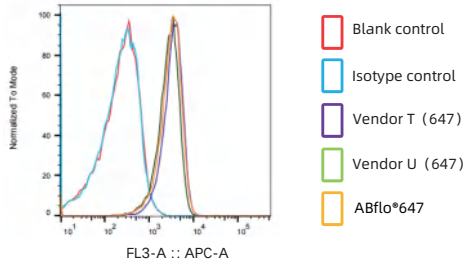
Broad instrument compatibility

ABflo™ dyes have good spectral compatibility with common flow cytometry analysis and sorting devices, ensuring reliable data.

Flow cytometry experiments were conducted under same conditions using ABclonal's recombinant rabbit monoclonal antibodies targeting the same antigen, sourced from the same batch. A comparative analysis was performed between ABflo™ dyes and other dyes with similar spectra for labeling. The results showed that the ABflo™ series fluorescent dyes exhibited superior or comparable brightness levels when compared to widely used fluorescent dyes.



- Sample: U266 Cells
- Unconjugated Antibody: Rabbit anti-Human CD48 mAb
- Results: ABflo™ 488 > Vendor U (488) > Vendor T (488)

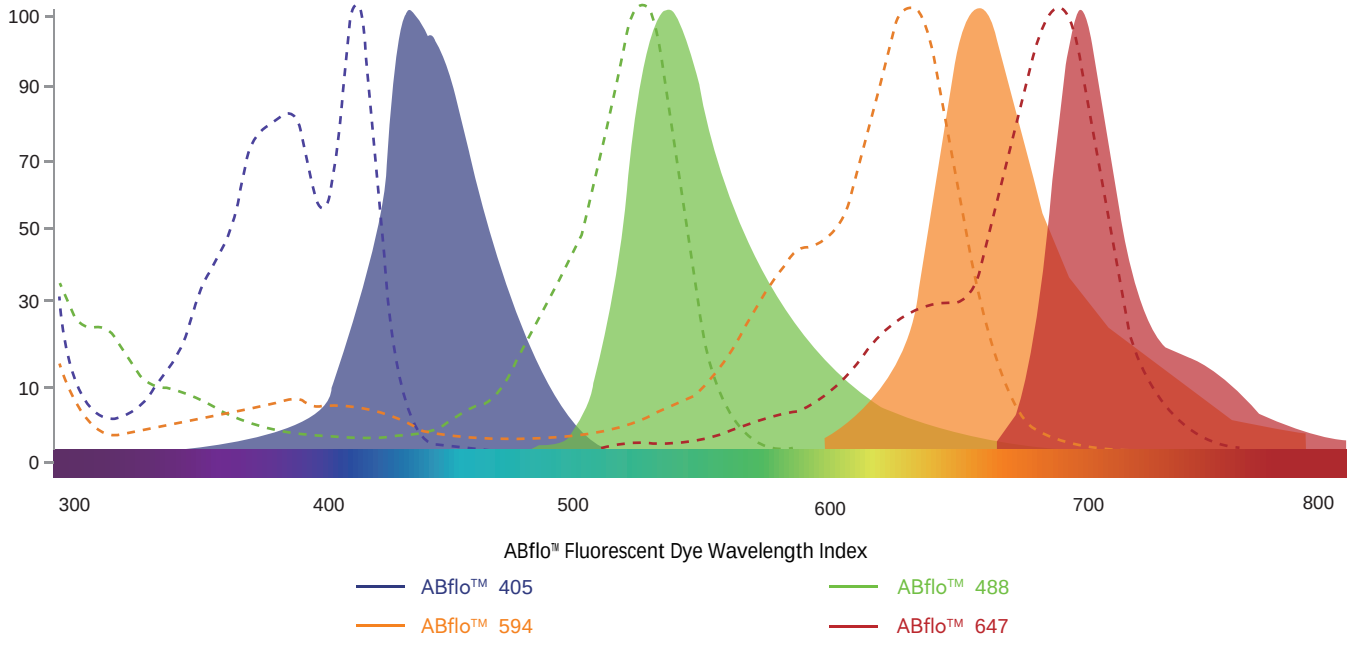


- Sample: Raji Cells
- Unconjugated Antibody: Rabbit anti-Human CD40 mAb
- Results: ABflo™ 647 ≈ Vendor T (647) > Vendor U (647)

4. Common Fluorescent Dyes

Dye Name	Maximum Excitation Wavelength (nm)	Maximum Emission Wavelength (nm)	Fluorescent Dyes in the Same Channel (Partial Listing)
ABflo™ 405	403	427	Pacific Blue, Alexa Fluor™ 405, eFluor450
ABflo™ 488	491	516	FITC, BB515, Alexa Fluor™ 488
FITC	491	516	ABflo™ 488, Alexa Fluor™ 488, BB515
ABflo™ 594	588	604	Alexa Fluor™ 594, ECD
PE	565	574	-
PerCP	482	678	PerCP-Cy5.5, BB700
PerCP-Cy5.5	482	695	PerCP, BB700
ABflo™ 647	648	664	APC, Alexa Fluor™ 647, eFluor660
APC	650	660	ABflo™ 647, Alexa Fluor™ 647, eFluor660
ABflo™ 700	690	713	Alexa Fluor™ 700, APC-Alexa Fluor™ 700
PE-Cy5	565	666	PE-Alexa Fluor™647
PE-Cy7	565	778	PE-Vio™770
APC-Cy7	651	779	APC-H7

Intensity of Common Fluorescent Dyes: PE-Cy5>PE>PE-Cy7>ABflo™ 647>APC>APC-Cy7>ABflo™ 594>ABflo™ 700>ABflo™ 488> PerCP-Cy5.5>ABflo™ 405>FITC>PerCP



Chapter 4: Common Flow Cytometry Markers for Major Cell Types

1. Common Cell Markers

Cell Type	Species (Human)
Leukocyte Common Antigen	CD45
T Cells	CD3
Helper T Cells	CD3, CD4
Cytotoxic T Cells	CD3, CD8
Regulatory T Cells	CD4, CD25, Foxp3, CD127(low/-)
Th1, Th2, Th17 Cells	CD4,IFN- γ ,IL-4,IL-17A
Th9, Th22 Cells	CD4,IL-9,IL-22
Tfh Cells	CD4,CD278,CD185 (CXCR5),CD279 (PD-1)
B Cells	CD19, CD20
Dendritic Cells	CD11c, MHC class II, CD141, CD209
NK Cells	CD3(-), CD16, CD56

Cell Type	Species (Human)
NKT Cells	CD3,CD56
Monocytes	CD14, CD16(±)
Macrophages	CD11b, CD68
Hematopoietic Stem Cells	Lin (-), CD34, CD38 (-), CD90, CD117
Megakaryocytes / Platelets	CD41, CD42a, CD42b, CD61, CD62P
Red Blood Cells	CD235a
Neutrophils	CD11b, CD15, CD16
Eosinophils	CD11b, CD193, Siglec-8, EMR1
Basophils	FcεRIα, CD123, CD203c, CD117 (-)
Mesenchymal Stem Cells	CD73,CD105,CD90 (Thy1),CD29,CD44, HLA-DR,CD34,CD45
Tumor Stem Cells	CD24,CD44,CD45

Cell Type	Species (Mouse)
Leukocyte Common Antigen	CD45
T Cells	CD3
Helper T Cells	CD3, CD4
Th1, Th2, Th17 Cells	CD4,IFN- γ ,IL-4,IL-17A
Cytotoxic T Cells	CD3, CD8
Regulatory T Cells	CD4, CD25, Foxp3
B Cells	CD19, B220(CD45R)
Dendritic Cells	CD11c, MHC class II
NK Cells	CD3(-), NK1.1(Limited Mouse Strains), CD49b(DX5)

Cell Type	Species (Mouse)
Monocytes	CD11b, CD115, Ly-6G(Gr-1)
Macrophages	F4/80, CD68
Hematopoietic Stem Cells	Lin(-/low), Sca-1, CD117, CD150
Megakaryocytes / Platelets	CD41, CD61, CD62P
Red Blood Cells	TER-119
Neutrophils	CD11b, Ly-6G(Gr-1)
Eosinophils	CD11b, CD193, Siglec-F, F4/80
Basophils	CD49b, Fc ε RI α , CD200R3
Mesenchymal Stem Cells	CD29,CD44,CD106,CD34,CD45

2. Selected Common Flow Cytometry Panels

Immune Cell Phenotyping Panel	Species	Markers
T/B/NK Analysis	Human	CD45
	Human	CD3E
	Human	CD4
	Human	CD8a
	Human	CD19
	Human	CD16
	Human	CD56
Th1/Th2/Th17 Cells	Human	CD3E
	Human	CD4
	Human	IL-17A
	Human	IFN-γ
	Human	IL-4
T Cell Activation Status Analysis	Human	CD3E
	Human	CD4
	Human	CD8a
	Human	CD25
	Human	CD69
Naive/Memory T Cells	Human	CD3E
	Human	CD4

Immune Cell Phenotyping Panel	Species	Markers
Naive/Memory T Cells	Human	CD45RA
	Human	CD197 (CCR7)
B Cell Subpopulation Analysis	Human	CD45
	Human	CD19
	Human	CD20
	Human	CD27
	Human	CD38
	Human	IgD
	Human	IgM
M1 Macrophages	Human	CD3E
	Human	CD20
	Human	NCAM1/CD56
	Human	CD86
	Human	HLA-DR
M2 Macrophages	Human	CD3E
	Human	CD20
	Human	NCAM1/CD56
	Human	CD163
	Human	MMR/CD206

Panel Name	Species	Markers
T Lymphocyte Subpopulations	Mouse	CD3e
	Mouse	CD4
	Mouse	CD8a
Th1/Th2/Th17 Cells	Mouse	CD3e
	Mouse	CD4
	Mouse	IFN-γ
	Mouse	IL-4
	Mouse	IL-17A
Naive/Memory T Cells	Mouse	CD3e
	Mouse	CD4
	Mouse	CD44

Panel Name	Species	Markers
Naive/Memory T Cells	Mouse	CD62L
T Lymphocyte Activation	Mouse	CD3e
	Mouse	CD4
	Mouse	CD8
	Mouse	CD25
	Mouse	CD69
Macrophages	Mouse	CD45
	Mouse	CD11b
	Mouse	F4/80
	Mouse	CD86
	Mouse	CD206

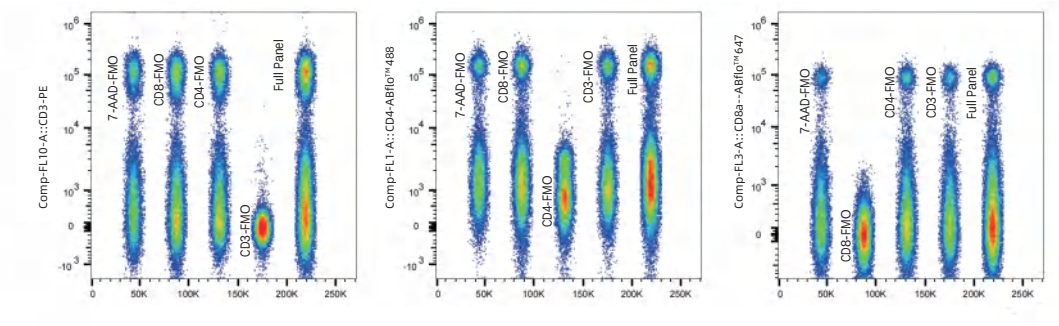
3. Common Flow Cytometry Panel Case Studies

3.1 T Cell Phenotyping

(1) Panel Design

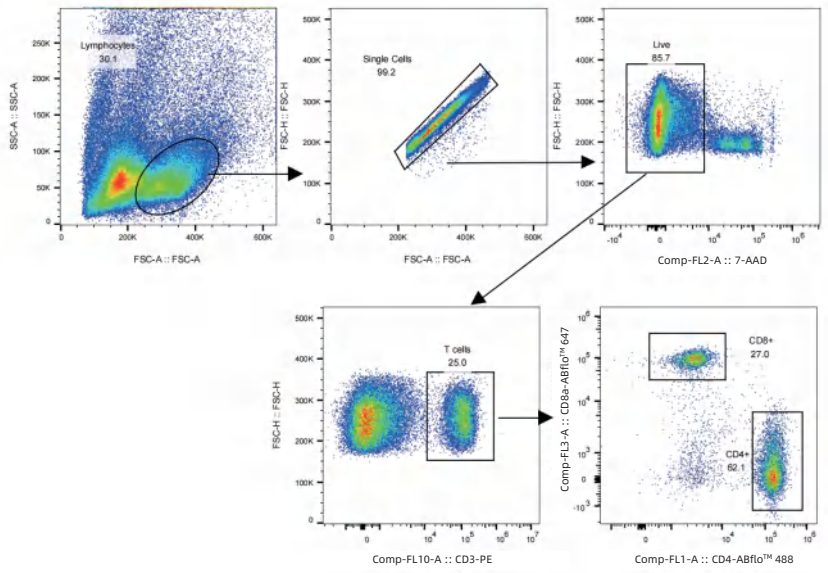
Dye	7-AAD-FMO	ABflo™ 647-FMO	ABflo™ 488-FMO	PE-FMO	Full Panel	Species	Cat.No.	Sample
ABflo™ 488	CD4-ABflo™ 488	CD4-ABflo™ 488	-	CD4-ABflo™ 488	CD4-ABflo™ 488	Mouse	A24907	Mouse Spleen
ABflo™ 647	CD8a-ABflo™ 647	-	CD8a-ABflo™ 647	CD8a-ABflo™ 647	CD8a-ABflo™ 647	Mouse	A24942	
PE	CD3-PE	CD3-PE	CD3-PE	-	CD3-PE	Mouse	A23321	
7-AAD	-	7-AAD	7-AAD	7-AAD	7-AAD	Mouse	-	

(2) FMO Controls



Result : Staining of FMO control groups with 7-AAD-FMO, CD8a-ABflo™ 647-FMO, CD4-ABflo™ 488-FMO, CD3-PE-FMO showed clear separation of positive and negative cell populations.

(3) Full Stained Sample



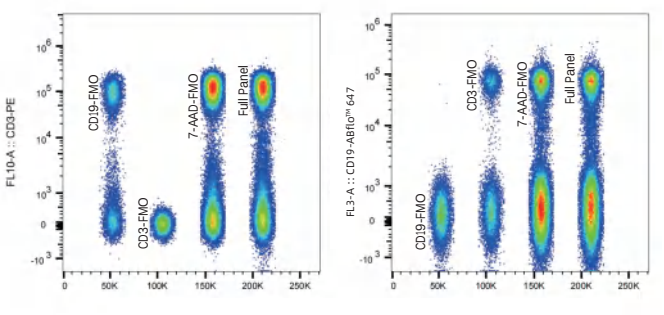
Result : Multicolor staining with CD3, CD4, CD8, and 7-AAD. Clear separation of $CD3^+$ T cells, $CD4^+$ T cells, and $CD8^+$ T cells was observed.

3.2 B Cell Phenotyping

(1) Panel Design

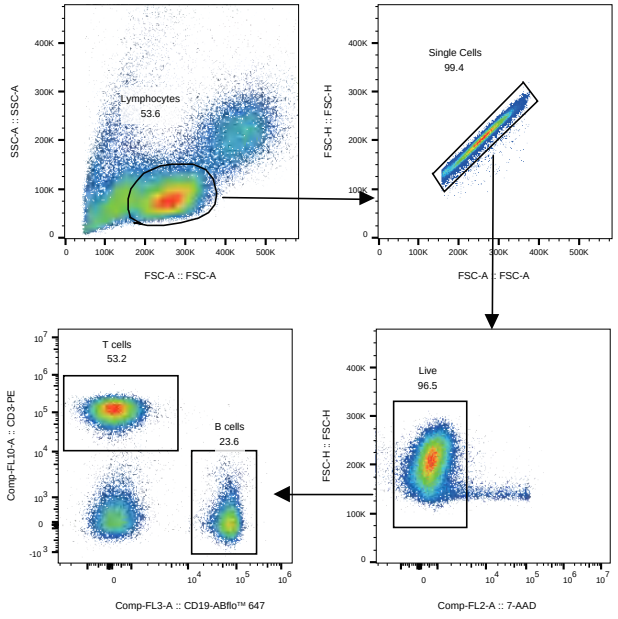
Dye	7-AAD-FMO	ABflo™ 647-FMO	PE-FMO	Full Panel	Species	Cat.No.	Sample
ABflo™ 647	CD19-ABflo™ 647	-	CD19-ABflo™ 647	CD19-ABflo™ 647	Human	A23009	Human PBMC
PE	CD3-PE	CD3-PE	-	CD3-PE	Human	A22795	
7-AAD	-	7-AAD	7-AAD	7-AAD	Human	-	

(2) FMO Controls



Result : Staining of FMO control groups with CD19-ABflo™ 647-FMO, CD3-PE-FMO, 7-AAD-FMO showed clear separation of positive and negative cell populations.

(3) Full Stained Sample



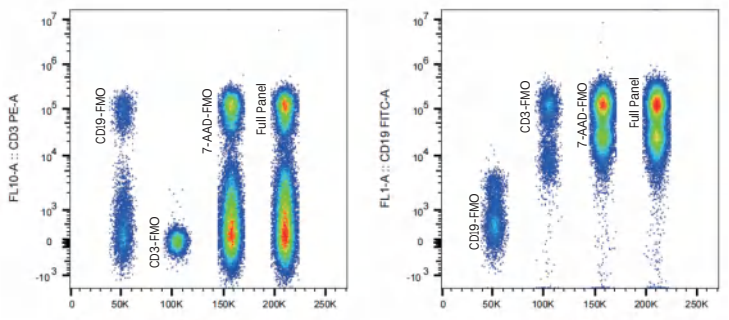
Result : Multicolor staining with CD3, CD19, and 7-AAD. Clear separation of $CD3^+$ T cells and $CD3^-CD19^+$ B cells was observed.

3.3 B Cell Phenotyping

(1) Panel Design

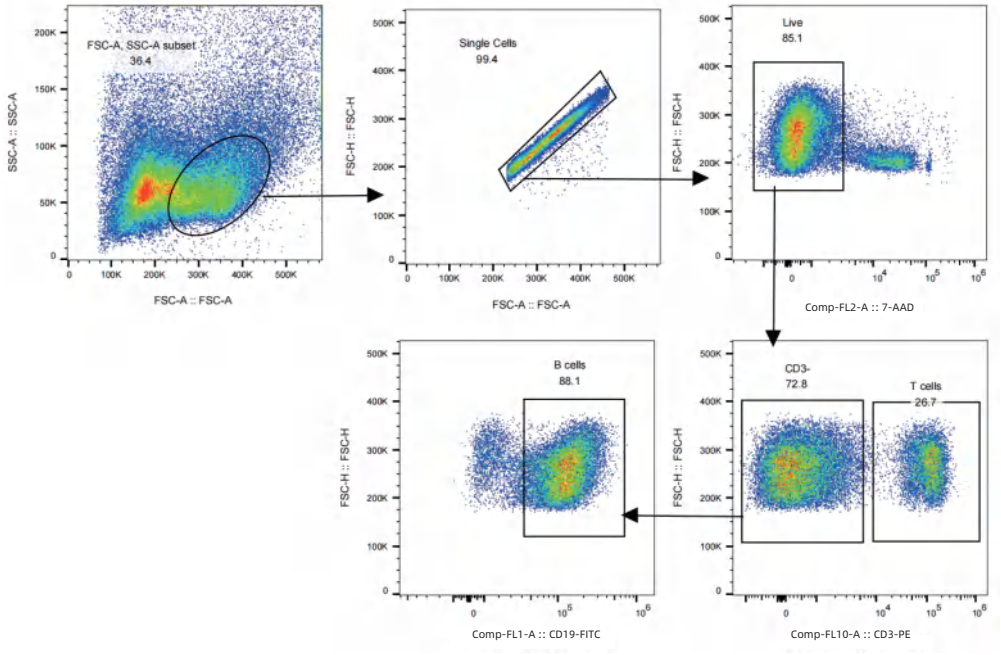
Dye	7-AAD-FMO	FITC-FMO	PE-FMO	Full Panel	Species	Cat.No.	Sample
FITC	CD19-FITC	-	CD19-FITC	CD19-FITC	Mouse	A23330	Mouse Spleen
PE	CD3-PE	CD3-PE	-	CD3-PE	Mouse	A23321	
7-AAD	-	7-AAD	7-AAD	7-AAD	Mouse	-	

(2) FMO Controls



Result : Staining of FMO control groups with CD19-FITC-FMO, CD3-PE-FMO, 7-AAD-FMO showed clear separation of positive and negative cell populations.

(3) Full Stained Sample



Result : Multicolor staining with CD3, CD19, and 7-AAD. Clear separation of positive and negative cells was observed.

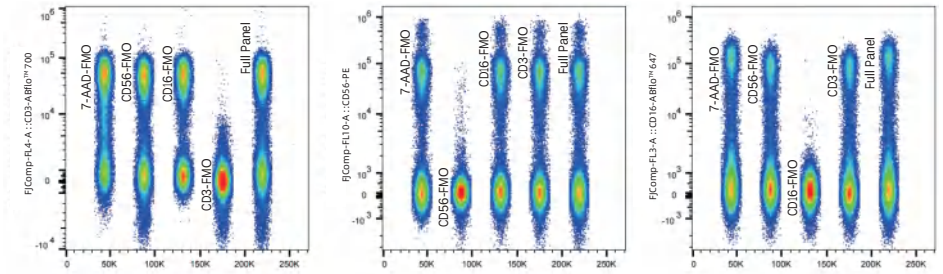
3.4 NK Cell Phenotyping

(1) Panel Design

Dye	7-AAD-FMO	PE-FMO	ABflo™ 647-FMO	ABflo™ 700-FMO	Full Panel	Species	Cat.No.	Sample
ABflo™ 647	CD16-ABflo™ 647	CD16-ABflo™ 647	-	CD16-ABflo™ 647	CD16-ABflo™ 647	Human	A23400	Human PBMC
ABflo™ 700	CD3-ABflo™ 700	CD3-ABflo™ 700	CD3-ABflo™700	-	CD3-ABflo™ 700	Human	A24943	
PE	CD56-PE	-	CD56-PE	CD56-PE	CD56-PE	Human	A22826	
7-AAD	-	7-AAD	7-AAD	7-AAD	7-AAD	Human	-	

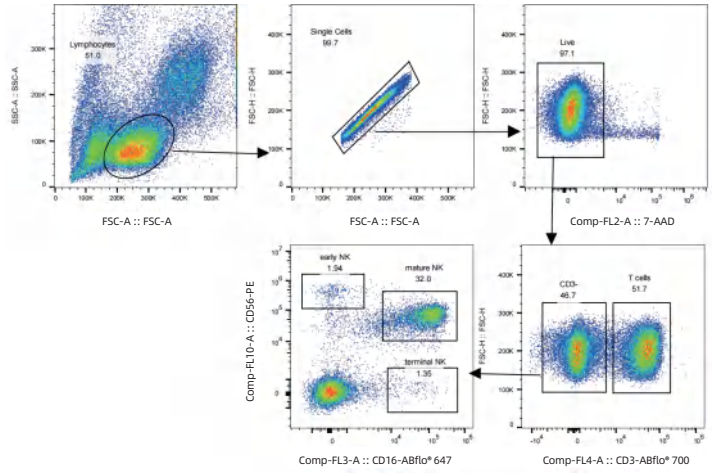
(2) Multicolor Manual Gating Analysis

FMO Controls

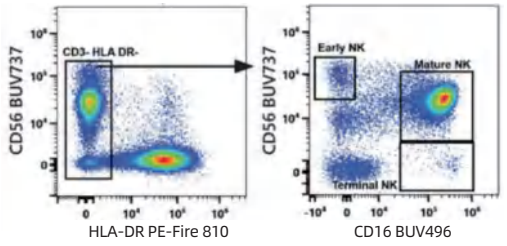


Result : Staining of FMO control groups with 7-AAD-FMO, CD56-PE-FMO, CD16-ABflo™ 647, CD3-ABflo™ 700 showed clear separation of positive and negative cell populations.

Full Stained Sample



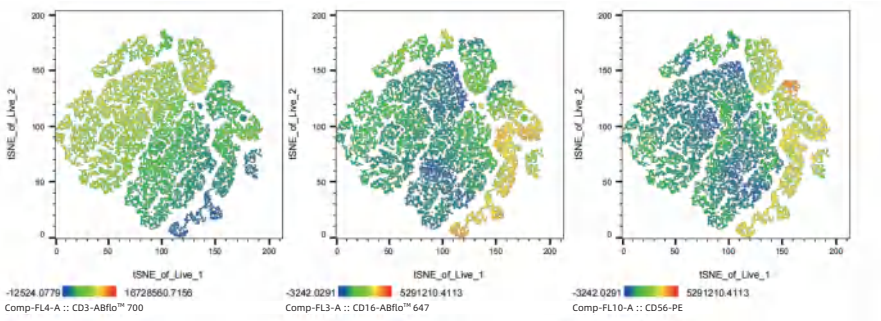
NK Cell Subtyping According to Reference



Source: DOI: 10.1002/cyto.a.24213

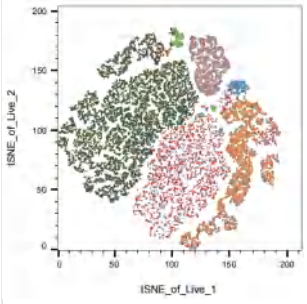
Result : Multicolor staining with CD3, CD16, CD56, and 7-AAD. Clear separation of $CD16^+CD56^+$ cells, $CD16^+CD56^-$ cells, and $CD16^-CD56^+$ cells was observed, consistent with the subtyping described in the reference.

(3) tSNE Dimensionality Reduction Analysis of Signal Distribution for Each Marker



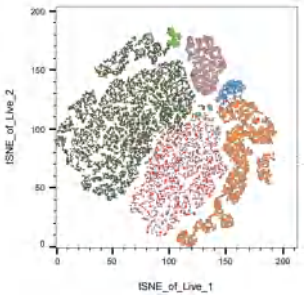
Result : In this plot, color ranging from red to blue represents the expression level of the corresponding marker. The signal distribution maps of CD3, CD16, and CD56 were consistent with the subtyping of T cells and NK cells.

(4) Comparison of Manual Gating and Automatic tSNE Analysis



	Subset Name	Count
	$CD3^+$ $CD56^+$ NKT cell	3508
	$CD3^+$ $CD56^-$ T cell	23760
	terminal NK	516
	mature NK	7012
	early NK	520
	Live	53833

Manual Gating

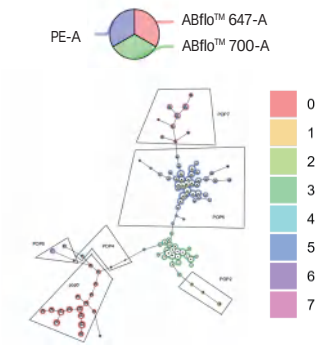


	Subset Name	Count
	FlowSOM.Live.Pop7.$CD3^+$ $CD56^+$ NKT cell	3550
	FlowSOM.Live.Pop5.$CD3^+$ $CD56^-$ T cell	24391
	FlowSOM.Live.Pop2.terminal NK	723
	FlowSOM.Live.Pop0.mature NK	8913
	FlowSOM.Live.Pop6.early NK	849
	Live	53833

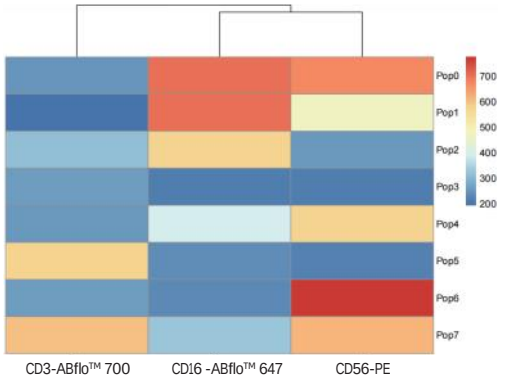
Automatic tSNE

Result : In the plot, the red color represents the Live cell population gated from lymphocytes, the blue color represents the early NK cells ($CD3^-CD56^+CD16^-$), the orange color represents the mature NK cells ($CD3^-CD56^+CD16^+$), the green color represents the terminal NK cells ($CD3^-CD56^-CD16^+$), the dark green color represents $CD3^+CD56^-$ T cells, and the brown color represents $CD3^+CD56^+$ NKT cells. The manual gating and FlowSOM automatic analysis showed consistent subtyping and cell counts.

(5) FlowSOM Clustering Analysis



Result : This plot shows the FlowSOM minimum spanning tree, with cells of the same background color grouped together. The size of the bubbles represents the population size, and the pie charts indicate the expression of corresponding marker colors. The larger the pie area, the higher the marker expression.



Result : This plot shows the FlowSOM heatmap, with color ranging from red to blue representing the intensity of marker expression. Pop0 represents the mature NK subset ($CD3^-CD16^+CD56^+$), Pop2 represents the terminal NK subset ($CD3^-CD16^+CD56^-$), Pop5 represents the $CD3^+CD56^-$ T cell subset, Pop6 represents the early NK subset ($CD3^-CD16^-CD56^+$), and Pop7 represents the $CD3^+CD56^+$ NKT cell subset.

Chapter 5: Common Issues and Solutions in Flow Cytometry Experiments

1. No or Weak Staining

Reason	Solution
Low expression level of the protein	For target proteins with low expression levels, it is advisable to use fluorescent dyes with high intensity in staining. Alternatively, indirect staining using primary and secondary antibodies can be attempted to enhance detection sensitivity.
Unclear expression of the target protein in the sample	Positive controls with clearly expressed target proteins can be used, while cells lacking expression of the target protein or knockout (KO) cells can serve as negative controls.
Lack of blocking agents during detection of secreted factors	For secreted proteins such as cytokines and chemokines, blocking agents such as monensin, brefeldin A, or a combination of both can be used to retain the factors inside the cells.
Improper choice of fixation and permeabilization reagents	When staining cytoplasmic and nuclear targets, appropriate fixation and permeabilization reagents should be used to ensure better detection of cytoplasmic and nuclear proteins.
Improper storage of antibodies	Strictly follow the recommended conditions for antibody storage as stated in the instructions.
Quenching of fluorescent dyes	Fluorescent-labeled antibodies and samples stained with fluorescent-labeled antibodies should be stored protected from light.
Improper antibody concentration	Optimal antibody concentration for the experiment can be determined through antibody titration.
Inadequate incubation time and temperature	Time and temperature for antibody incubation can be referred to the recommended conditions in the instructions, and appropriate gradient adjustments can be made based on the experiment.
Mismatch between primary and secondary antibodies	For indirect immunofluorescence staining, it is important to ensure proper matching between the fluorescent secondary antibody and the primary antibody.
Incorrect choice of filters	Check the excitation and emission wavelengths of the fluorescent dyes used to ensure the correct laser and filters are used for data acquisition.
Excessive compensation of data	Set compensation using single-stained controls and fluorescence minus one (FMO) controls for each experiment.
Incorrect gating of cell populations	Ensure proper gating of cell populations.

2. High Background or Nonspecific Staining

Reason	Solution
High autofluorescence	Cellular autofluorescence depends on cell type and may also be influenced by cell preparation methods. Use samples stained under the same stimulation conditions but without any reagents as controls for cellular autofluorescence.
Presence of dead cells	When performing surface staining on live cells, use live cell viability dyes (such as PI or 7-AAD) to gate out dead cells. However, when staining fixed cells, use fixable live cell viability dyes that can withstand fixation and be used for intracellular staining.
Insufficient Fc receptor (FcR) blocking	Use Fc receptor (FcR) blocking reagents or Fab fragments to mitigate the effects of FcR.
Excessive antibody concentration	Use the recommended antibody concentration as stated in the instructions, and titrate the antibody concentration based on the experimental conditions.
Prolonged antibody incubation time	The antibody incubation time can be referred to the manufacturer's recommended conditions, and appropriate gradient adjustments can be made based on the experiment.
Insufficient washing	Increase the number of washes after staining.
Inadequate compensation adjustment	Set compensation using single-stained controls and FMO controls for each experiment.

3. Other Abnormal Staining

Problem	Reason	Solution
False Positives	Presence of cell aggregates or dead cells in the analysis	Use single-cell gating and employ viability dyes to exclude cell aggregates and dead cells.
	Poor specificity of the antibody	Replace the antibody with a more specific one.
Signal from isotype control antibody is higher than the detection antibody	Incorrect concentration of the isotype control antibody	Ensure that the concentration of the isotype control antibody matches the detection antibody.
	Isotype control antibody is from a different manufacturer	Use an isotype control antibody from the same manufacturer as the detection antibody.

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