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Application Note

Oct. 2024

A Comprehensive Guide to the Optimization of the Cell Surface Marker Staining Process for Spectral Flow Cytometry Cell Analysis

Background

The proper identification and classification of Cell Surface Markers is imperative for identifying and characterizing diverse cell populations within a heterogeneous sample. Spectral flow cytometry, with its ability to resolve overlapping fluorescence signals, has revolutionized the field by enabling the robust detection of a wider range of markers. Optimizing cell surface marker staining protocols is therefore crucial for unlocking the full potential of Spectral Cell Analysis in various research applications, including immunophenotyping, rare cell analysis, and uncovering novel cellular subsets. Antibody specificity, fluorophore selection, and staining conditions all significantly impact the quality and sensitivity of data acquisition. Suboptimal staining can lead to insufficient signal-to-noise ratios and inaccurate cell population identification.

Samples of Interest

Stabilized PBMCs

Cryopreserved PBMCs

Blocking Reagents

Monocyte Nonspecific Binding- blocking reagent

Nonspecific FC-antigen blocking reagent

Polymer-Dye aggregation blocking reagent

Instrument

Sony ID7000™ Spectral Cell Analyzer: Equipped with 4 Lasers (405nm, 488 nm, 561 nm, 638 nm)

ID7000™ Spectral Cell Analyzer

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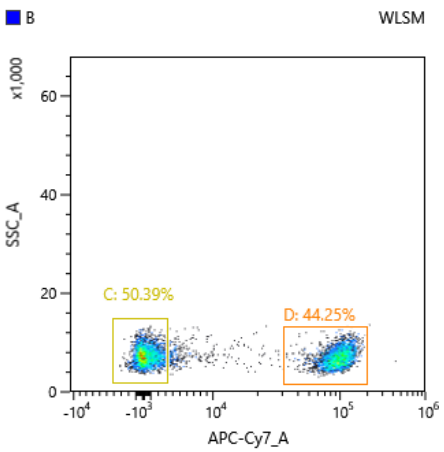
A significant challenge in flow cytometry is the non-specific binding of fluorescent dyes, particularly to monocytes. This phenomenon can lead to increased background signal, masking of specific signals from target antigens, and ultimately, inaccurate cell population identification. This phenomenon is not limited to cyanine-based dyes, but is commonly observed with fluorochromes such as PE-Cy5, PE-Cy7, PE-Dazzle 594, APC-Fire 750, APC-Cy7, and PerCP-Cy5.5.

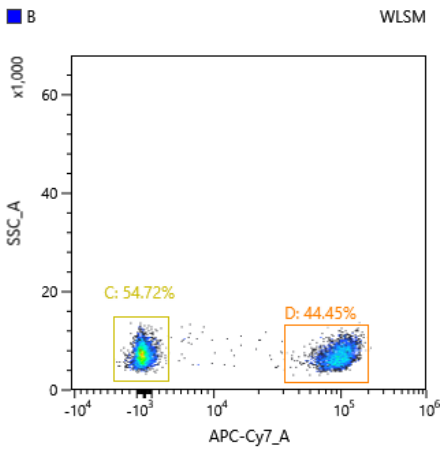
Another challenge in spectral flow cytometry involving human cells is Fc receptor (FcR)-mediated non-specific binding. This phenomenon is particularly relevant when working with cell types like neutrophils, monocytes, macrophages, B cells, natural killer (NK) cells, and certain T cell subsets. These cells express Fc receptors on their surface, which can bind to the Fc portion of antibodies used for staining, leading to unwanted background signal and inaccurate population identification.

To address this issue, a blocking agent specifically designed to block this non-specific interaction was employed. These reagents are typically formulated with human IgG, which competes with the antibodies of interest for binding to FcRs on the cells. This effectively blocks non-specific binding of the antibodies without interfering with their specific antigen recognition. Consequently, blocking reagents help ensure accurate staining and reliable identification of target cell populations.

Another consideration in spectral flow cytometry experiment design is the potential for polymer-based dye interactions that may cause the data to not be sufficiently spectrally unmixed. Unlike traditional small molecule fluorochromes, polymer-based dyes can exhibit a tendency to aggregate or interact with each other. This interaction can lead to inefficient energy transfer and a decrease in overall fluorescence intensity. This phenomenon can manifest as data that appears undercompensated, even after applying spectral unmixing.
When using multiple Polymer-based dyes, using a reagent that is meant to stop the Polymer-dye aggregation is recommended. However, in this case, the only Polymer-based dye was BV605, so there was no need for an extra buffering solution.



No blocking reagent used



Monocyte-Specific Nonspecific Binding Blocking Reagent Used

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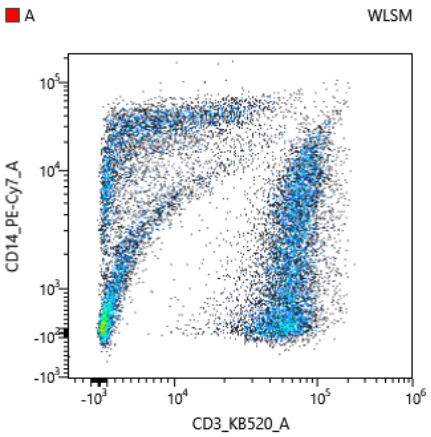
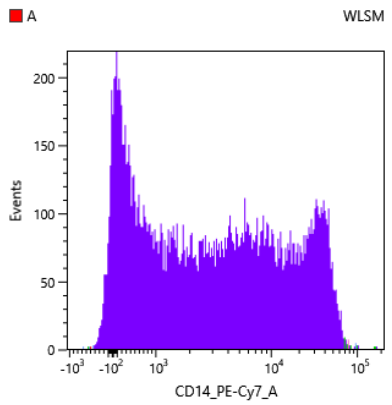
Panel Design

Marker	Fluorochrome
CD3	KB520
CD4	PE
CD8	APC
CD14	PE-Cy7
CD16	PE-Dazzle 594
CD19	BV605

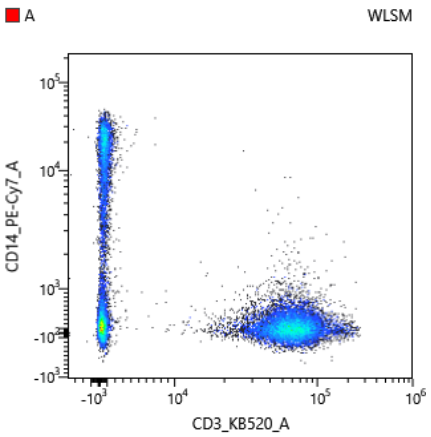
Results of Cryopreserved PBMCs staining

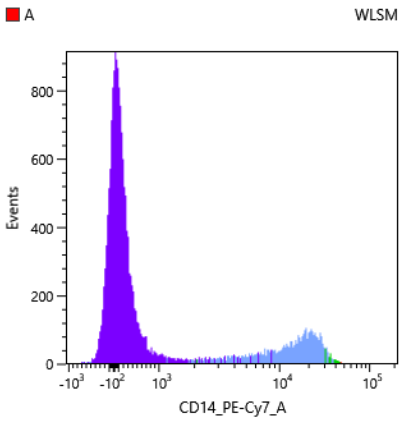
No blocking Reagent Used





Blocker Combination Used*





*A combination of a monocyte-specific nonspecific binding blocker and an Fc-Receptor-Specific Blocker was used.

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Materials

- PBMCs (Cryopreserved)
- Culture Medium (RPMI, DMEM, etc)
- DNase
- Cell Staining Buffer (PBS supplemented by 2mM EDTA and 0.5% BSA)
- Blocking Reagents:
  - True-Stain Monocyte Blocker (Bio-Legend 426102)
  - CellBlox™ Blocking Buffer (Thermofisher B001T03F01)

PBMC Recovery Protocol

1. Warm Culture Medium to 37 C
2. Gently swirl the PBMCs in a water bath of 37 C (*Keep it within 2 minutes)
3. Place the PBMCs in 10 mL of Culture Medium
4. Centrifuge (at Room temperature- 500g for 10 mins)
5. Discard the supernatant
6. Suspend the pellet in 10 mL of fresh culture medium
7. **Let the cells rest in the incubator for 1 hour**

   ★Letting the freshly awoken PBMCs rest in the incubator prior to staining greatly enhances the recovery of the cells, resulting in a higher cell viability and the proper expression profile.
8. Centrifuge (at RT- 500g for 10 mins)
9. Discard the supernatant
10. Gently tap to see if there are white clumping sections to check the cells
11. If Clumps are present: dissolve the clump by adding DNase
    - Add 5uL of the DNase onto the pellet and remaining medium
    - Check to see if the clumps dissolve, little by little
12. Resuspend in 1 mL of cell staining buffer
13. Cell Count
14. Centrifuge and dilute to get a concentration of 10^6 cells/mL
15. Resuspend in the appropriate volume of Cell staining buffer or Polymer-Based Dye buffer (in the case of using more than one Polymer-based Dye) in a tube.

*Final Immersion Volume prior to staining: <u>100 uL cell buffer solution/1uL of Antibody</u>

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PBMC Staining Protocol

Fully-Stained sample:

1. Add 5 uL of appropriate blockers as designated in the pre-staining preparations above into an empty 1.5 mL tube.
2. Add the appropriate volume (100uL) of Cell/Buffer Solution to the desired tube.
   - Lightly tap to mix the solution- be careful not to add bubbles (signaling protein damage)
   - Let the cell/blocker solution incubate for 10 minutes prior to staining
3. Add the appropriate amount of antibodies to the cell/buffer.
4. Incubate for around 30 minutes at 4 C, in a dark area away from any light.
5. Wash as desired prior to sample acquisition.

Referenced Literature

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- Jahrsdörfer B, Blackwell SE, Weiner GJ. Phosphorothyoate oligodeoxynucleotides block nonspecific binding of Cy5 conjugates to monocytes. J Immunol Methods 2005;297:259–263.
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- Biburger M, Trenkwald I, Nimmerjahn F. Three blocks are not enough—Blocking of the murine IgG receptor Fc γ RIV is crucial for proper characterization of cells by FACS analysis. Eur J Immunol 2015; 45:2694–2697.

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