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Life Sciences

CytoFLEX mosaic Spectral Detection Module Enables Enhanced Spectral Unmixing of White Blood Cell Populations by Extracting Multiple Autofluorescence Signatures

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Introduction

Cells exhibit an inherent fluorescence, known as autofluorescence (AF), due to various cellular components and metabolites that fluoresce upon excitation by specific wavelengths of light. Common endogenous fluorophores include reduced pyridine nucleotides (NADH), oxidized flavin coenzymes (FMN and FAD), vitamins, and proteins containing aromatic amino acids such as tryptophan, phenylalanine, and tyrosine.[1]

AF poses significant challenges in flow cytometry, particularly when analyzing complex biological samples containing multiple cell populations with distinct AF signatures. This interference can affect the precise detection of desired fluorescence signals, especially when studying low-abundance markers or identifying dim populations.

Additionally, in spectral flow cytometry, AF can lead to unmixing errors, compromising data reliability. By using the CytoFLEX mosaic Spectral Detection Module, users can extract fluorescent signatures from multiple (up to 10) distinct AF populations. Leveraging multiple distinct AF signatures allows for accurate unmixing and enhances the reliability of marker expression patterns. Conversely, failure to account for distinct AF or oversimplifying its extraction can result in erroneous interpretation of fluorescence signals.

In this study, we evaluated the impact of AF extraction on spectral unmixing in white blood cell populations and evaluated whether using multiple AF signatures further improved the unmixing. Peripheral blood leukocytes were selected as the sample as they are a common sample source and contain diverse cell types with variable AF properties.

The analysis was done using the CytoFLEX LX flow cytometer equipped with the CytoFLEX mosaic 88 Spectral Detection Module, which provides CytoFLEX LX with spectral capabilities. This approach emphasizes the importance of incorporating multi-AF extraction in experimental workflows to achieve accurate fluorescence signal resolution, thereby enhancing data reliability in immunophenotyping assays.

Instruments and Supplies

1. CytoFLEX LX Flow Cytometer C06779 (Beckman Coulter Inc., USA)
2. CytoFLEX mosaic 88 Spectral Detection Module U-V-B-Y-R-I (6 laser UV) (Beckman Coulter Inc., USA) **(Table 1)**
3. CytExpert for Spectral software, Version 1.0.0.49

4. Centrifuge (Andreas Hettich GmbH, Germany, Rotanta 460R)
5. Falcon 50-mL round bottom tubes (Merck, CLS352070)
6. Falcon 5-mL round bottom tubes (Corning, 352235)
7. 96-well plate (NUNC 249570)
8. Transfer pipette
9. Micropipettors
10. Pipette tips (1000 µL, 200 µL, 10 µL) (Greiner Sapphire 777355, 775355, 771354)
11. Automated cell counter (Countstar, China; Countstar Mira FL)
12. Miscellaneous: biological hazard container, aluminum foil, ice

The CytoFLEX mosaic Spectral Detection Module represents the next advancement in the CytoFLEX platform, enabling both CytoFLEX S* and LX instruments to operate in spectral mode in addition to their conventional mode. When paired with the CytoFLEX LX flow cytometer, the CytoFLEX mosaic 88 Spectral Detection Module allows operation in either spectral or conventional modes. Flow cytometry users can either choose to upgrade their exiting CytoFLEX LX or S instruments to include spectral functionality or opt for a bundled system that includes both the CytoFLEX analyzer and the CytoFLEX mosaic module.

The CytoFLEX mosaic is available in two configurations: the CytoFLEX mosaic 88, compatible with CytoFLEX LX flow cytometers (offering 88 detection channels), and the CytoFLEX mosaic 63, compatible with CytoFLEX S BRVY series (offering 63 detection channels). For this application note, we used the CytoFLEX mosaic 88, and specifications are mentioned in **Table 1**.

CytoFLEX Analyzer	CytoFLEX mosaic module	Channel (FSC/ SSC/FL)	FSC	355 nm (SSC/FL)	405 nm (SSC/FL)	488 nm (SSC/FL)	561 nm (SSC/FL)	638 nm (SSC/FL)	808 nm (SSC/FL)
CytoFLEX LX Instrument (UV355)	Spectral Detection Module, CytoFLEX mosaic 88	1/6/81	1	1/20	1/20	1/16	1/12	1/10	1/3

Table 1: Instrument Configuration

Reagents and Antibodies

1. Fluorochrome-labeled antibodies
2. Blood collection tubes with anticoagulant (sodium heparin)
3. Phosphate Buffered Saline (PBS) Buffer supplemented with 1% FCS
4. Red Blood Cell (RBC) lysis buffer (BioLegend, Cat. No. 420301)
5. Human TruStain FcX (BioLegend Cat. No. 422302)
6. True-Stain Monocyte Blocker (BioLegend Cat. No. 426102)
7. BD Horizon Brilliant Stain Buffer Plus (BD Biosciences 566349)
8. CytoFLEX Daily QC Fluorospheres (Beckman Coulter, Inc., C65719)
9. CytoFLEX Daily IR QC Fluorospheres (Beckman Coulter, Inc., C06147)
10. CytoFLEX sheath fluid (Beckman Coulter, Inc., B51503)
11. Flow cleaning agent (Beckman Coulter, Inc., A64669)

*Only valid for CytoFLEX S (V-B-Y-R) Series Flow Cytometer.

Marker	Fluorochrome	Clone	Company	Catalog	Dilution
CD66b	FITC	G10F5	BioLegend	305104	1:100
CD45	IR820	J33	Beckman Coulter	In development	1:20
CD14	APC-Fire750	63D3	BioLegend	367119	1:100
CD16	BUV496	3G8	BD Biosciences	612944	1:100
CD4	BV510	SK3	BD Biosciences	562971	1:100
HLR-DR	BV570	L243	BioLegend	307628	1:50
CD8	cFluor V547	SK1	Cytek Biosciences	R7-20064	1:50

Table 2: Antibody panel details

Methods

1. Specimen collection

a. Whole blood specimens were collected in tubes containing sodium heparin (NaHep) as an anticoagulant to prevent clotting and maintain sample integrity.

b. Prior to processing, the specimens can be stored at room temperature (RT) for up to 4 hours.

2. Sample preparation

- **Red blood cell lysis and washing**

a. RBCs were selectively lysed by incubating the sample with a 4:1 ratio of 1X RBC lysis buffer for 20 minutes on ice. Note: To ensure complete erythrocyte lysis, avoid contamination of the tube walls and caps with blood.

b. Following lysis, the remaining leukocytes were washed sequentially—first with 1X RBC lysis buffer to remove residual erythrocytes and then with FACS buffer (phosphate-buffered saline [PBS] supplemented with 1% fetal bovine serum [FBS]) to maintain cell viability and minimize non-specific binding.

- **Cell counting and staining**

a. White blood cells were counted, and 10^6 cells were resuspended in 100 µL of FACS buffer for staining in a 96-well plate.

b. Cells were incubated with antibodies on ice for 30 minutes. To reduce Fc receptor-mediated background staining TruStain FcX™ was added to the antibody cocktail, and to prevent aspecific binding of fluorophores to monocytes, True-Stain Monocyte Blocker™ was added. BD Horizon™ Brilliant Stain Buffer Plus was added to mitigate polymer-based fluorochrome interactions.

c. Samples were washed twice with 200 µL FACS buffer and resuspended in 100 µL FACS buffer for analysis.

d. Prepared samples remained stable for up to 5 hours when stored at 4°C prior to analysis.

- **Control preparation**

Single-stained controls were prepared using cells resuspended in the same buffer composition as the experimental samples. These controls were essential for unmixing.

Data Acquisition

1. Data acquisition on the CytoFLEX mosaic 88 Spectral Detection Module

a. Ensure the flow cytometer is properly aligned according to the manufacturer's recommendations—refer to CytoFLEX mosaic Spectral Detection Module IFU (D172052).

b. Run CytoFLEX Daily QC Fluorospheres (C65719, Beckman Coulter, Inc.) and CytoFLEX Daily IR QC Fluorospheres (C06147, Beckman Coulter, Inc.) to verify instrument alignment before the sample acquisition. Users are recommended to perform QC in the same manner as they would for CytoFLEX LX instruments. The only difference is that the QC report will check a significantly greater number of channels.

c. Acquire the samples by applying the standard assay settings to maintain consistency and accuracy during data collection.

d. Acquire at least **50,000 cells** for each single-stained control to ensure sufficient data for accurate unmixing and control validation.

e. Ensure at least **100,000 cells** were collected in the scatterplot for cellular analyses, providing a robust dataset for reliable statistical analysis and population identification.

2. Spectral unmixing

Spectral unmixing mathematically separates these overlapping signals to assign the correct fluorochrome to each marker. For spectral unmixing using the CytoFLEX mosaic Spectral Detection module, follow these steps:

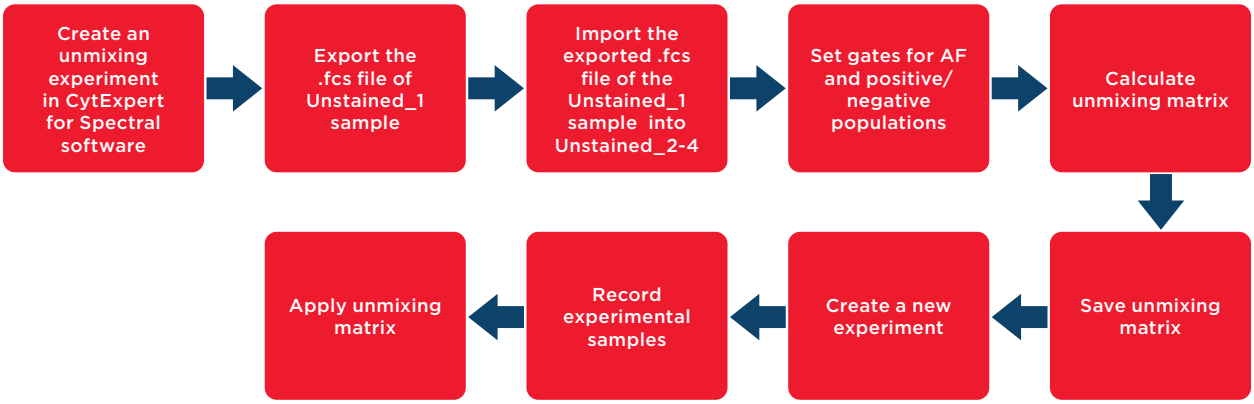

Figure 1: The process of spectral unmixing and autofluorescence (AF) extraction followed in this experiment using the CytoFLEX mosaic Spectral Detection Module.

a. Open a new unmixing experiment in the CytExpert for Spectral software and add the required number of fluorochromes and four unstained populations. **(Figure 1)**

b. Acquire cells for single-stained controls. The unstained sample needs to be recorded only once. Please be aware that unmixing is possible with as little as 200 positive events, but measuring more events improves the accuracy of unmixing.

c. Export the .fcs file of the measured unstained sample.

d. Import the .fcs file of the unstained sample into the other unstained wells.

e. Set positive and negative gates for your single-stained controls and set gates for AF cell population in each of the unstained wells (see **Figure 3**). Positive gates should be set on the cells with the highest intensity.

f. Calculate and save the unmixing matrix. Matrices with different numbers of AF signatures can either be calculated by setting the unstained to **Unmixing only** to ensure an AF signature was not taken into account during unmixing. Alternatively, the matrix with 4 AF can be saved into the library and unmixing matrices with different numbers of AF signatures can be created by importing only the desired AF signatures.

   a. Note: CytExpert for Spectral software automatically creates each NxN plot and performs quality checks to ensure the data is well unmixed. If required, users can review normalized signatures, stain indices and NxN plots, address any warnings, adjust the gating as necessary, and unmix again if required.

g. Create a new experiment in the CytExpert for Spectral software and record samples.

h. Apply calculated unmixing matrix to the sample .fcs files.

i. Define the gated populations as shown in **Figure 3** and **Table 3** .

Population	Identification
Neutrophils	$CD45^+VSSC^{high}FSC^{high}CD14^-CD66b^+CD16^+$
Eosinophils	$CD45^+VSSC^{high}FSC^{high}CD14^-CD66b^+CD16^-$
Monocytes	$CD45+VSSC^{mid}FSC^{high}$
Classical monocytes	$CD45+VSSC^{mid}FSC^{high}$ $CD14^{++}CD16^-$
Intermediate monocytes	$CD45^+VSSC^{mid}FSC^{high}$ $CD14^{++}CD16^+$
Non-classical monocytes	$CD45^+VSSC^{mid}FSC^{high}$ $CD14^+CD16^+$
Lymphocytes	$CD45^+VSSC^{low}FSC^{low}$

Table 3: Populations of various cells and their identification

Data Analysis

Spectral unmixing

Samples were acquired using a 6-laser, deep-UV CytoFLEX LX flow cytometer with the CytoFLEX mosaic 88 Spectral Detection Module. During unmixing, 4 different AF populations could be identified using the CytExpert for Spectral software **(Figure 2A-D)**.

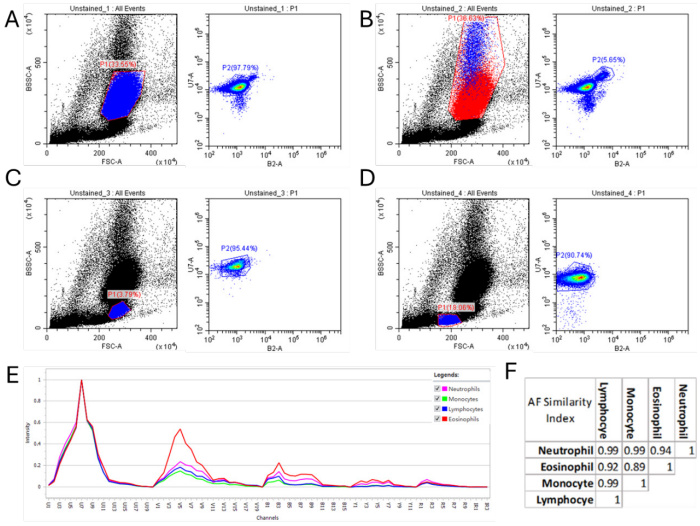

Figure 2. AF extraction. Gating strategy of AF extracted from (A) neutrophils, (B) eosinophils, (C) monocytes and (D) lymphocytes. Unstained populations were gated on FSC/BSSC and UV7/B3 dot plots. Blue populations in the FSC/BSSC are populations selected for AF extraction in UV7/B3 plots. (E) Normalized spectra across all detectors and (F) similarity indices of AF signatures as calculated by the CytExpert for Spectral software.

When calculating the unmixing matrix, the AF signatures of neutrophils, monocytes and lymphocytes **(Figure 2E)** had a high similarity (>0.98, see **Figure 2F**). This meant that most here likely only two AF signatures were necessary for optimal unmixing. From these highly similar signatures, the neutrophil signature was retained because this was the brightest autofluorescent population.

Gating strategy

White blood cells were first gated as CD45+ singlets. Based on FSC-A (Forward Scatter Area) vs. VSSC-A (Violet Side Scatter Area), lymphocyte, monocyte and granulocyte gates were set **(Figure 3)**. Granulocytes were further gated as CD14- and CD66b+ and separated into eosinophils (low or negative for CD16) and neutrophils (high CD16 expression) based on CD16 expression. Monocytes could be separated into classical, intermediate and non-classical monocytes based on CD14 and CD16 expression. (Classical Monocytes (~73.4%): CD14++ CD16−, Intermediate Monocytes (~9.2%): CD14++ CD16+, Non-Classical Monocytes (~9.9%): CD14+ CD16+)

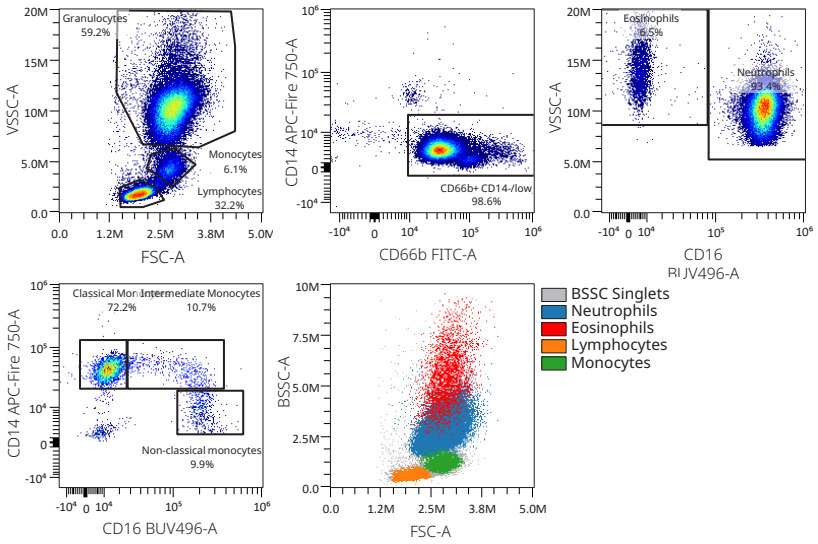

Figure 3. Gating strategy on sample unmixed with 2 AF signatures. When plotting each population on an FSC/BSSC 2D plot, their distinct FSC/BSSC profiles can be clearly observed.

Results

Histograms of the same data unmixed with a different number of AF signatures in the unmixing matrix illustrate the impact of AF on marker expression across different immune cell populations (neutrophils, eosinophils, monocytes, and lymphocytes) **(Figure 4)**. Data are shown without AF extraction to demonstrate the importance of AF extraction. The experiment revealed the following observations regarding autofluorescence (AF) signatures and cell population characterization:

First, the experiment shows how AF extraction improves the quality of flow cytometry data:

Without AF extraction (0 AF), aberrant expression patterns are observed for all markers coupled to fluorochromes that emit in the same range as the AF (BUV496, BV510, BV570 and cF547). All cell populations appeared positive for CD16, CD4 and HLA-DR, which is inconsistent with expected biological expression patterns. Only neutrophils and subsets of lymphocytes and monocytes should be CD16 positive, only a subpopulation of lymphocytes should be CD4 positive and only monocytes, and a small (activated) subpopulation of lymphocytes should be HLA-DR positive. Interestingly, CD14 expression appeared normal without AF extraction, probably because the emission spectrum of APC-Fire 780 is very different from the AF emission spectra. The impact of AF-induced false positivity was most pronounced in eosinophils, followed by monocytes, with lymphocytes exhibiting a comparatively milder shift.

Eosinophils exhibit distinct autofluorescent properties due to the presence of intracellular granules containing autofluorescent molecules such as flavoproteins.[2] The AF spectrum of eosinophils is clearly different from that of the other cell types **(see Figure 2)** and has a profound effect on the unmixing results of this cell type. Not only do eosinophils appear positive for CD4, CD16 and HLA-DR without AF extraction, they appear to have a negative fluorescence for CD8. Furthermore, this effect of negative CD8 expression is even worse when AF extraction is performed with the AF spectrum obtained from neutrophils. To a lesser extent, this is also visible for CD16, which appears slightly negative after extraction of 1 AF signature. This phenomenon suggests that autofluorescence interference does not always manifest as an artificial increase in fluorescence intensity; rather, it can also lead to spectral distortion that results in a relative decrease in signal intensity for specific markers. This can be displayed more clearly in 2D plots **(Figure 5)**.

Hence, we observe that the addition of a single AF signature (the neutrophil signature) to the unmixing removes the autofluorescence from neutrophil, monocyte and lymphocyte populations, but does not remove the aberrant expression on eosinophils. For these cells, addition of a second AF signature to the unmixing was required to remove the artifact. This highlights the importance of using multiple AF signatures when unmixing.

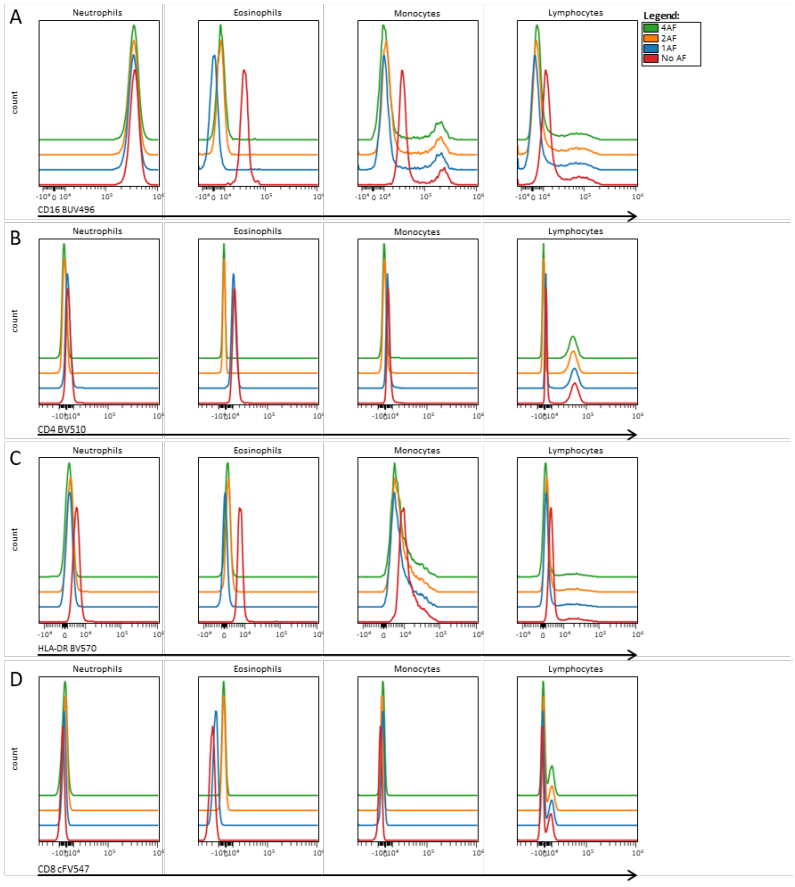

Figure 4. A-D Histograms of cell surface marker expression in different cell populations (neutrophils, eosinophils, monocytes, lymphocytes) on the same file with 0, 1, 2 or 4 AF signatures taken into account in the unmixing matrix. Red (No AF) represents data without AF extraction, Blue (1 AF) represents data after removal of one autofluorescence signature, Orange (1 AF) represents data after removal of two autofluorescence signatures, Green (2 AF), represents data after removal of four autofluorescence signatures.

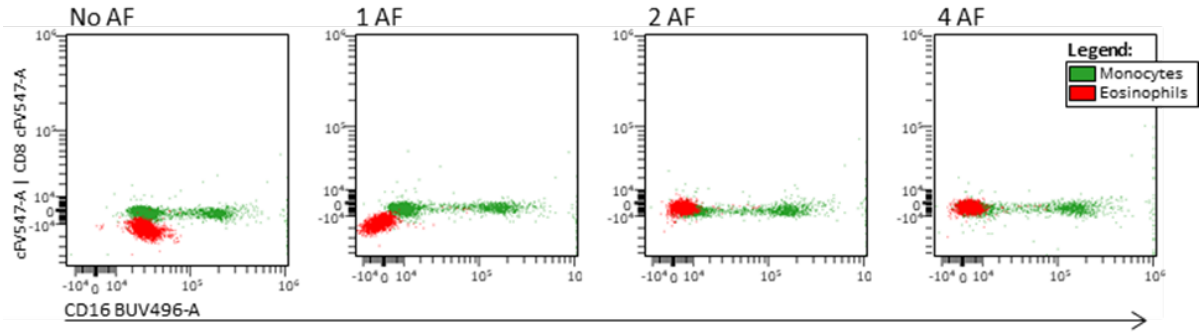

Figure 5. 2D plots demonstrating eosinophils appearing negative for CD16 and CD8 when unmixing with 0 or 1 (neutrophil) AF signature. Monocytes were added to the plot as a reference. After adding the eosinophil AF signature to the unmixing (2AF), eosinophils appear on the same area of the plot as classical monocytes.

Using all 4 AF signatures did not have much impact on overall unmixing

As stated before, the AF signatures of neutrophils, monocytes and lymphocytes have a high similarity (>0.98, **Figure 2**). In this case it is advisable to use the brightest signal (neutrophils). However, to demonstrate the effect of using too many AF signatures, data were also unmixed using all 4 AF signatures. In the histograms **(Figure 4)**, using all 4 AF signatures does not appear to improve data quality for any of the markers compared to using 2 AF signatures.

However, an effect is observed in the gating of the three monocyte subsets **(Figure 6)**. Especially the CD16- classical monocyte population appears to display more spread in CD16 after addition of a 4th AF signature. This is apparent by an observed increase in the size of the population and reflected in an increase of the cumulative variance (CV) from 0.337 to 0.386. This observation suggests that while multiple AF signatures improve the overall accuracy of spectral unmixing, excessive AF correction may introduce variability affecting data quality. Although the effect is small in this case, it may become more problematic in more complex panels. These findings highlight the importance of optimizing the number of AF signatures to balance AF removal while preserving true biological variation.

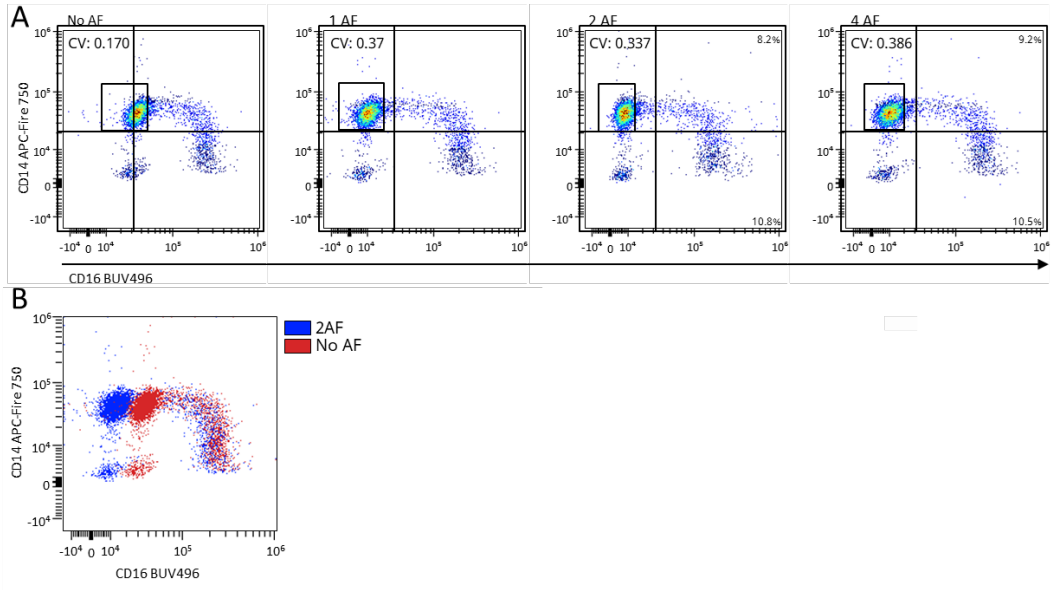

Figure 6. CD16 and CD14 expression of monocytes gated as shown in Figure 2 with a different number of AF signatures in the unmixing matrix. **CV** indicates the cumulative variance of the classical monocyte population in the rectangular gate. This gate is adjusted for each unmixing, while quartile gates remain the same for all plots.

Conclusion

This study demonstrates the critical role of multiple autofluorescence (AF) signatures in improving AF extraction using the CytoFLEX mosaic Spectral Detection Module, particularly in complex cell populations. By optimizing AF correction, false CD16 positivity in eosinophils, monocytes, and lymphocytes was reduced while preserving true biological signals. Notably, balancing AF extraction is essential to prevent artificial signal variation, as observed in classical monocytes. The CytoFLEX mosaic module provides the ability to extract up to 10 autofluorescence signatures which could be highly beneficial for managing AF in complex biological samples. Overall, these findings highlight the utility of the CytoFLEX mosaic Spectral Detection Module in enhancing the accuracy and resolution of immunophenotyping, reinforcing its value for high-precision spectral flow cytometry.

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Abbreviations

Abbreviation	Full Form
AF	Autofluorescence
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Saline
RBC	Red Blood Cell
FSC	Forward Scatter
SSC	Side Scatter
APC	Allophycocyanin
CD	Cluster of Differentiation
BUV	Brilliant Ultraviolet
BV	Brilliant Violet
FITC	Fluorescein isothiocyanate
PN	Part Number
QC	Quality Control
UV	Ultraviolet
U-V-B-Y-R-I	UV-Violet-blue-Yellow Green-Red-IR

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1. Monici, M. 2005. “Cell and Tissue Autofluorescence Research and Diagnostic Applications.” Biotechnology Annual Review 11: 227-256.

2. Buchwalow, I. et al. 2018. Identification of autofluorescent cells in human angioimmunoblastic T-cell lymphoma. Histochem Cell Biol 149, 169–177

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