Autofluorescence Extraction on the CytoFLEX mosaic Spectral Detection Module

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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 light1. AF poses significant challenges in flow cytometry, particularly when analyzing complex biological samples containing multiple cell populations with distinct AF signatures. In this study, we evaluated the impact of AF extraction using CytoFLEX LX flow cytometer equipped with the CytoFLEX mosaic Spectral Detection Module on spectral unmixing in white blood cell populations. We also assessed whether using multiple AF signatures further improved the unmixing process.

METHODS

Blood specimens were collected with sodium heparin as anticoagulant, and red blood cells were lysed to collect leukocytes. Leukocytes were stained with antibodies (CD66b FITC, CD45 IR820, CD14 APC-Fire750, CD16 BUV496, CD4 BV510, HLA-DR BV570, CD8 cFluorV547), incubated for 30 minutes on ice, and washed twice with FACS buffer. Data acquisition was done on a CytoFLEX LX Flow Cytometer equipped with a CytoFLEX mosaic 88 Spectral Detection Module, collecting 50,000 cells for single-cell controls and 100,000 of fully stained samples for analyses. Data was processed using CytExpert for Spectral software to perform spectral unmixing and define gated populations.

RESULTS

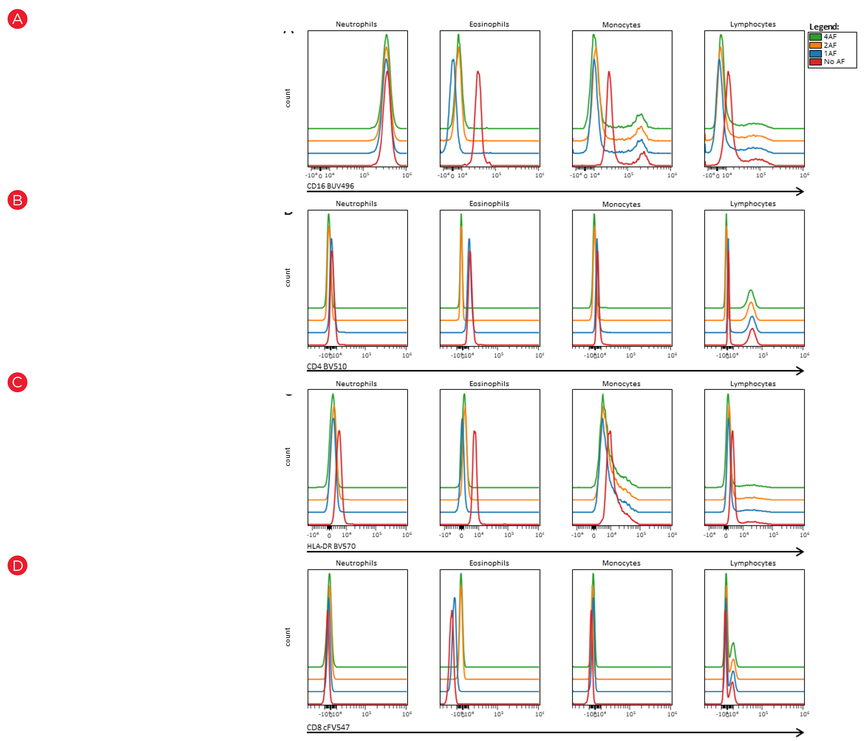

Figure 1: A-D Histograms of cell surface marker expression in different cell populations (neutrophils, eosinophils, monocytes, lymphocytes) on the same file with 0, 1, 2 or 4 AF signatures taken into account in the unmixing matrix. Red (No AF) represents data without AF extraction, Blue (1 AF) represents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

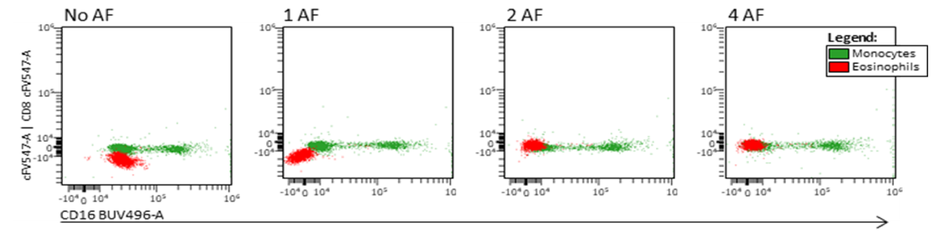

Figure 2: 2D plots demonstrating eosinophils appearing negative for CD16 and CD8 when unmixing with 0 or 1 (neutrophil) AF signature. Monocytes were added to the plot as a reference. After adding the eosinophil AF signature to the unmixing (2AF), eosinophils appear on the same area of the plot as classical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

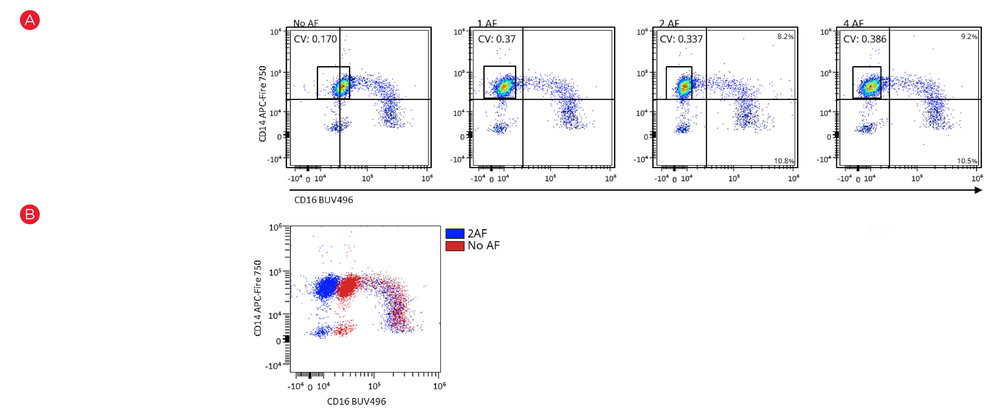

Figure 3: CD16 and CD14 expression of monocytes analyzed with a different number of AF signatures in the unmixing matrix. CV indicates the cumulative variance of the classical monocyte population in the rectangular gate. This gate is adjusted for each unmixing, while quartile gates remain the same for 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 Spectral Detection 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.

ACKNOWLEDGEMENT

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

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