Autofluorescence subtraction by spectral flow cytometry

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Introduction

Cells have a natural level of fluorescence, called autofluorescence (AF), which is caused by the presence of fluorescent biological compounds within the cells. AF can result in the loss of signal resolution and the presence of false positive events.

Spectral flow cytometers can treat autofluorescence signal as a background or a "virtual" fluorochrome and extract it from the signal of fluorophore-labeled cells. The removal of AF can improve the unmixing accuracy and the signal resolution of fluorochromes in samples consisting of high AF signals.

Herein we compare the unmixed results with or without the subtraction of various AF across different samples. These samples contain highly homogenous or heterogeneous AF properties, including human blood and mouse lung cells.

Experimental

Mouse lung sample preparation

Mouse lungs were extensively perfused with HBSS through a right ventricle, cut into small pieces, and digested with enzymes. Homogenized lung cells were passed through 70-µm nylon mesh and red blood cells were lysed using RBC lysing buffer. Following that, cells were collected and stained by mixed antibodies in the table below. Dead cells were stained with Live/Dead Blue.

Specificity	Clone	Fluorochrome	Source	Catalog number
CD45	30-F11	PerCP	Biolegend	103129
Sca-1	D7	APC-Fire750	Biolegend	108146
EpCAM (CD326)	G8.8	eFluor450	Thermo fisher	48-5791-82
CD11b	M1/70	PE-Cy7	Biolegend	101215
Ly6G	1A8	BV605	Biolegend	127639
Siglec-F	E50-2440	PE-CF594	BD	562757
F4/80	BM8	Kiravia Blue520	Biolegend	123162
CD11c	N418	BUV805	BD	749038
CD103	2E7	PE	Biolegend	121405
MHC II	M5/114.15.2	BV785	Biolegend	107645
CD64	X54-5/7.1	APC	Biolegend	139306
CD24	M1/69	BV510	Biolegend	101831
Ly6C	HK1.4	BV711	Biolegend	128037
Live/Dead	NA	Live/Dead Blue	Thermo fisher	L34961

Human blood sample preparation

- CD-Chex Plus (stabilized human blood in a preservative medium, CAT#213323, Streck) were stained by mixed antibodies, CD45 Brilliant Violet 510, CD3 PerCP-Cy5.5, CD4 Brilliant Violet 421, CD8 Pacific Blue, CD16CD56 PE, and CD20 FITC. Subsequently, red blood cells were lysed. Afterward, the samples were washed and resuspended.
- Fresh human blood was lysed and washed without staining.

All samples were analyzed on a **five-laser NovoCyte Opteon flow cytometer**. All plots and spectra were generated by the NovoExpress (Opteon) software.

Results and Discussions

The autofluorescence spectra of different samples ~~vary~~

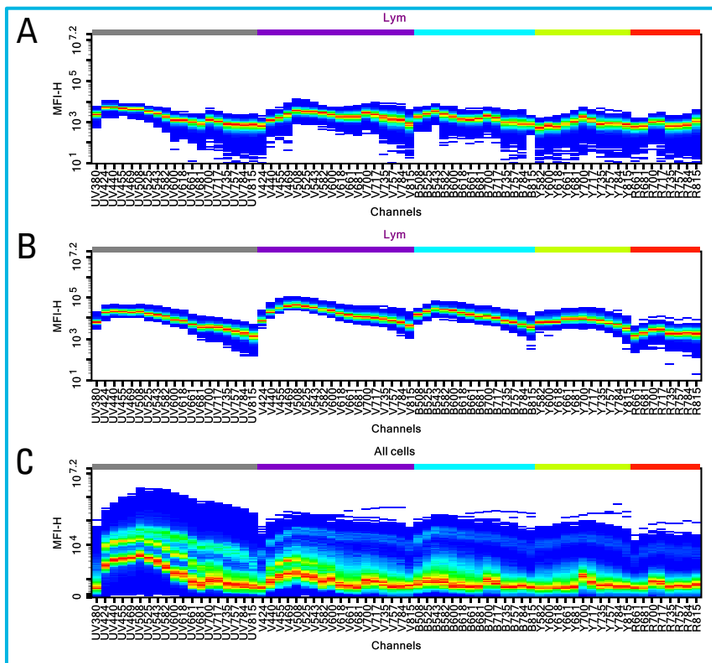

Figure 1. Density spectrum plots of unstained populations from different samples. The AFs of (A) lymphocytes from fresh human blood and (B) lymphocytes from stabilized human blood are homogenous, but the stabilized cells have much higher AF signals than those from fresh blood. There are heterogeneous and strong AF signals in (C) mouse lung cells.

Results and Discussions

Autofluorescence subtraction from mouse lung cells

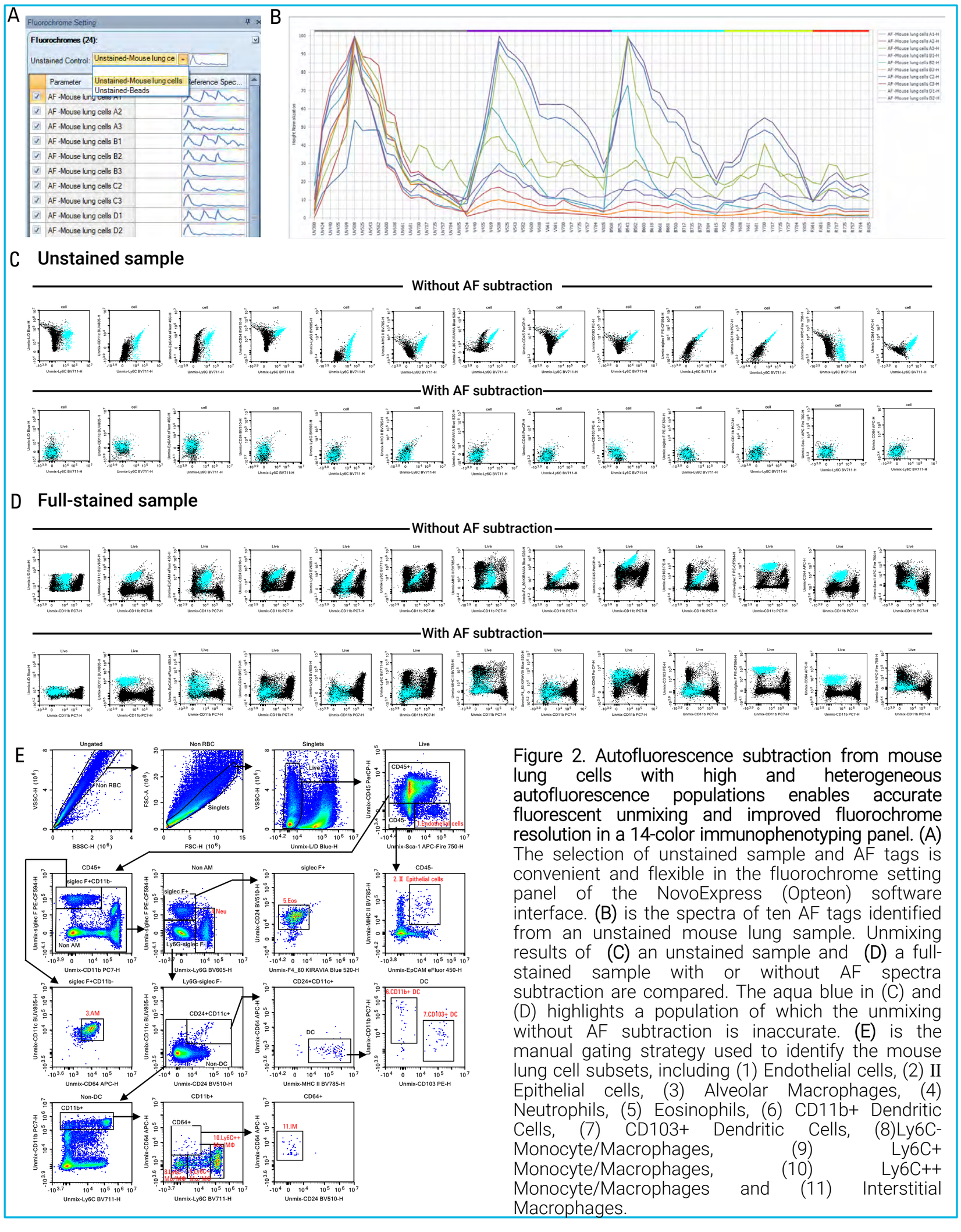

Figure 2. Autofluorescence subtraction from mouse lung cells with high and heterogeneous autofluorescence populations enables accurate fluorescent unmixing and improved fluorochrome resolution in a 14-color immunophenotyping panel. (A) The selection of unstained sample and AF tags is convenient and flexible in the fluorochrome setting panel of the NovoExpress (Opteon) software interface. (B) is the spectra of ten AF tags identified from an unstained mouse lung sample. Unmixing results of (C) an unstained sample and (D) a full-stained sample with or without AF spectra subtraction are compared. The aqua blue in (C) and (D) highlights a population of which the unmixing without AF subtraction is inaccurate. (E) is the manual gating strategy used to identify the mouse lung cell subsets, including (1) Endothelial cells, (2) II Epithelial cells, (3) Alveolar Macrophages, (4) Neutrophils, (5) Eosinophils, (6) CD11b+ Dendritic Cells, (7) CD103+ Dendritic Cells, (8)Ly6C- Monocyte/Macrophages, (9) Ly6C+ Monocyte/Macrophages, (10) Ly6C++ Monocyte/Macrophages and (11) Interstitial Macrophages.

Unmixed results with or without AF subtraction in a 6C Panel

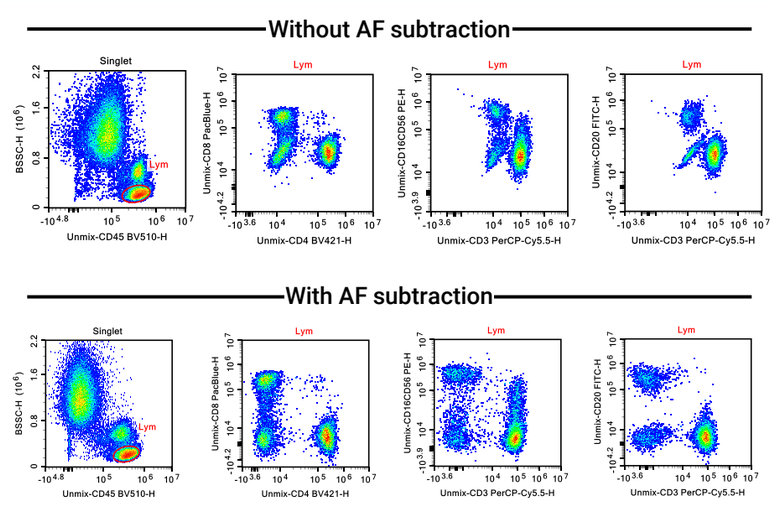

Figure 3. Autofluorescence extraction from stabilized human blood with high homogenous autofluorescence signals significantly improves fluorochrome resolution in a 6-color immunophenotyping panel.

Conclusions

- There is a high autofluorescent signal diversity in flow cytometry samples, not only in signal intensity but also in spectrum signature.
- The removal of autofluorescence can improve the signal resolution and unmixing accuracy.
- The combination of the NovoCyte Opteon spectral flow cytometer and NovoExpress (Opteon) software enables convenient and accurate autofluorescence subtraction.

References

[1] Surre, J., Saint-Ruf, C., Collin, V. et al. Strong increase in the autofluorescence of cells signals struggle for survival. Sci Rep 8, 12088 (2018).