Application Note

Cell Analysis

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Sentinel Panel Design of 16-Color, 28-Markers for Immunophenotyping Peripheral Human Whole Blood

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Abstract

Flow cytometry is a powerful tool to analyze highly heterogeneous cells such as those in blood. However, when using traditional panel design, the number of markers that can be analyzed is limited to the number of available channels on the instrument and available fluorochromes. By using a sentinel panel design, in which antibodies can use shared fluorophores when they have unique cellular expression, a 16-color panel using 28 different antibodies can be designed for the three-laser Agilent NovoCyte Advanteon flow cytometer. The immunophenotyping panel presented in this application note comprehensively assesses the immune system in only a small amount of sample, covering 40 distinct cell populations in human peripheral blood including all major immune cell subsets and hematopoietic stem cells (HSCs).

Introduction

Significant advances in flow cytometry instrumentation and reagents have made complex multicolor experiments easier to achieve. However, when the number of target markers exceeds the number of detection channels and fluorochromes, these markers must be divided into different panels in separate sample tubes. This limits the data collected and increases experimental cost, sample use, and operation time. Additionally, if only a small number of cells are available, splitting them into multiple stains might not be a practical solution. One way to overcome this limitation and maximize the data obtained is by using a sentinel panel design. Planning a flow cytometry panel in this way takes advantage of differential marker expression on various cell types, using different antibodies coupled with the same fluorochrome that bind to different cellular subsets and can, therefore, be distinguished. A 16-color panel using 28 different antibodies was designed to examine 40 cell populations in human peripheral blood and acquired on a three-laser NovoCyte Advanteon. The assay requires only 150 µL of peripheral blood to identify and examine, in detail, all major immune cell subsets in human peripheral blood, including T cells, B cells, monocytes, dendritic cells (DCs), natural killer (NK) cells, neutrophils, eosinophils, and basophils, as well as hematopoietic stem cells (HSCs).

Designing the panel

Using a sentinel panel design can increase the total number of markers that can be examined simultaneously. With strict gating order, it is possible to differentiate between all antibodies and immune cell types. A diagram of cell populations that were examined, and the cell surface markers that were used to distinguish them, is outlined in Figure 1. Determining the hierarchical gating steps that are used to separate different cell types is crucial to select which antibodies can use a shared fluorochrome. Antibody titration is a key step for the optimization of multicolor flow cytometry. To get the best resolution, as determined by the stain index, the optimal amount of antibody is used to achieve the highest separation between negative and positive cells and minimize background. All antibodies that were used are listed in Table 1, including the clone information and amount added for each stain.

In a sentinel panel, the expression of each marker must be examined on all cell subsets to determine which antibodies can use the same fluorochrome. In Figure 2, all markers that are included in the panel are assessed for their expression in each cell subset. Analysis of cellular expression patterns is crucial to decide which antibodies can use a shared fluorochrome. To design the panel, the following sentinel panel design rules were followed:

- Markers that label distinct cell types use nonoverlapping fluorophores, such as CD3 to mark T cells or CD19 to mark B cells.
- Markers that label different cell types can use the same fluorochrome on different antibodies.
- Markers that are co-expressed on multiple cell types must have a unique fluorochrome.

Methods

Materials

- Agilent NovoCyte Advanteon flow cytometer
- Agilent NovoExpress software
- Table 1 outlines all commercial antibodies with catalog number, vendor, and quantity used in the immunophenotyping panel
- Agilent NovoLyse lysing solution
- Phosphate buffer saline, PBS (Genom Biomedical Technology GNM-14190)
- 8% paraformaldehyde in water (Electron Microscopy Sciences 157-8)

Immunophenotyping antibody stain of whole blood

1. Prepare the antibody cocktail listed in Table 1.
2. Spin down the antibody cocktail at 12,000 g for 5 minutes to remove fluorochrome aggregates.
3. Incubate 150 µL of fresh blood with antibodies for 15 minutes at RT.
4. Add 750 µL of NovoLyse for 10 minutes at RT.
5. Add 750 µL of PBS for 5 minutes at RT.
6. Wash twice with 1 mL of PBS, then resuspend cells with 150 µL of 1% PFA.
7. Acquire 100 µL of sample on the NovoCyte Advanteon flow cytometer.

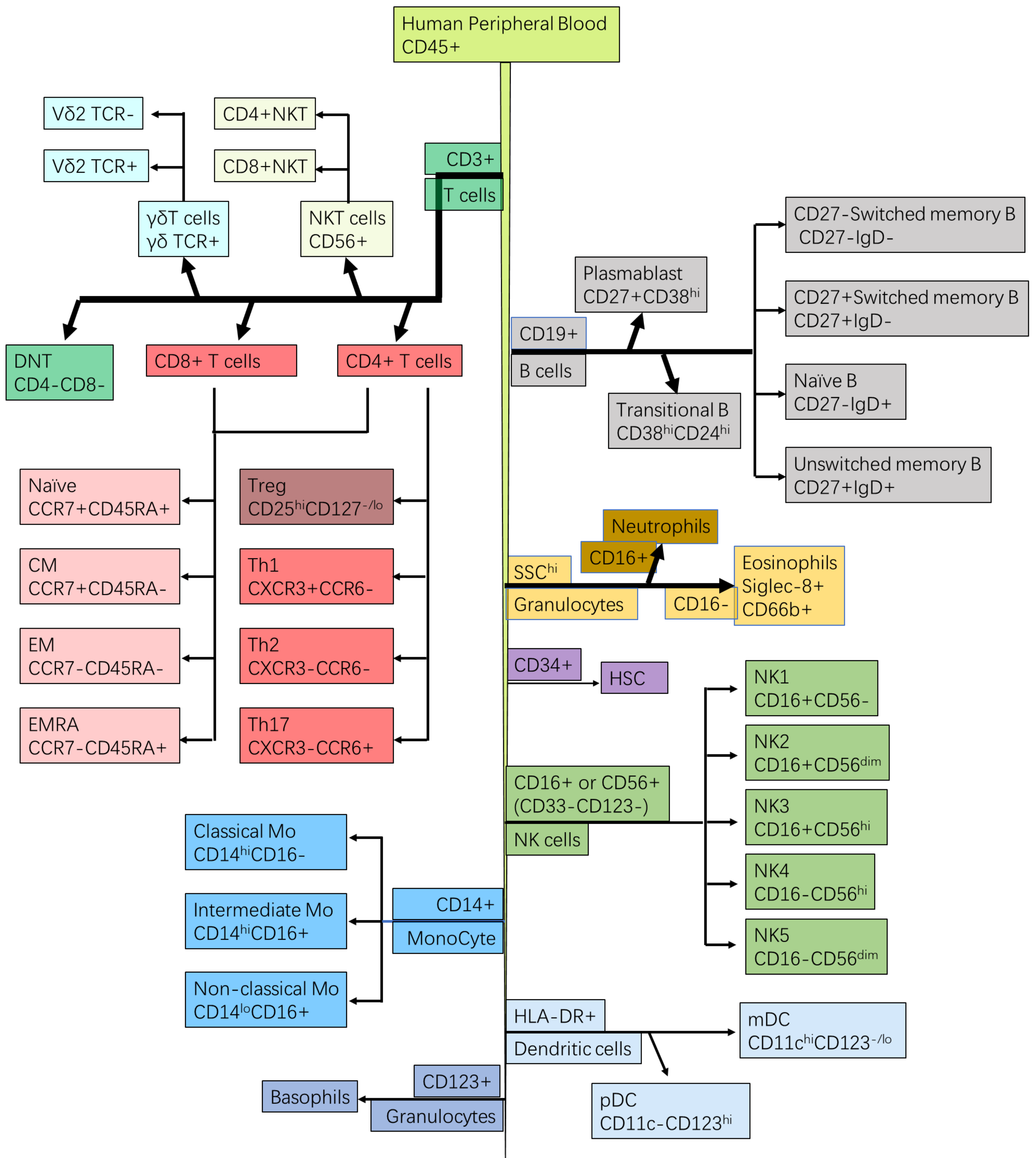

Figure 1. Schematic overview of the identified immune cell populations. The 16-color, 28-marker panel run on an Agilent NovoCyte Advanteon flow cytometer identifies 40 distinct subsets from hierarchical gating of major immune and stem cell populations in blood.

Table 1. List of all antibodies used for the 16-color, 28-marker panel. Table of antibodies used in the panel, corresponding detection channel on the Agilent NovoCyte Advanteon flow cytometer, clone id, concentration, and vendor information. All antibody concentrations were titrated to obtain optimal staining resolution.

Laser	Filter	Specificity	Fluorochrome	Clone	Amount (µL)	Vendor
405	445/45	CD25	BV421	M-A251	1.25	BD
		CD11c	BV421	Bu15	0.625	Biolegend
	525/45	CD45	BV510	HI30	0.625	Biolegend
	586/20	CD3	BV570	UCHT1	2.5	Biolegend
	615/20	CD56	BV605	HCD56	0.625	Biolegend
	667/30	HLA-DR	BV650	L243	2.5	Biolegend
	695/40					
	725/40	CD27	BV711	O323	0.625	Biolegend
		CD14	BV711	MφP9	0.625	BD
	780/60	CCR7	BV785	G043H7	2.5	Biolegend
488	525/45	CD4	FITC	SK3	2.5	Agilent
		CD123	FITC	6H6	0.312	Biolegend
	586/20	CD34	PE	581	5	Agilent
		Vδ2 TCR	PE	B6	0.312	Biolegend
		CD24	PE	ML5	1.25	Biolegend
	615/20	CXCR3	PE-Dazzle594	G025H7	1.25	Biolegend
		CD64	PE-Dazzle594	10.1	1.25	Biolegend
	667/30	CD38	PE-Cy5	HIT2	0.625	BD
	695/40	CD8	PerCP-Cy5.5	SK1	10	BD
	725/40					
	780/60	γδ TCR	PE-Cy7	11F2	1.25	BD
		IgD	PE-Cy7	IA6-2	2.5	Biolegend
		CD33	PE-Cy7	WM53	1.25	Agilent
640	667/30	CD127	APC	A019D5	0.312	Biolegend
		CD19	APC	HIB19	10	Agilent
		Siglec-8	APC	7C9	0.312	Biolegend
	695/40					
	725/40	CCR6	APC-R700	11A9	1.25	BD
		CD16	AF700	3G8	2.5	Biolegend
	780/60	CD45RA	APC-Cy7	HI100	1.25	Biolegend
		CD66b	APC-Cy7	G10F5	5	Biolegend

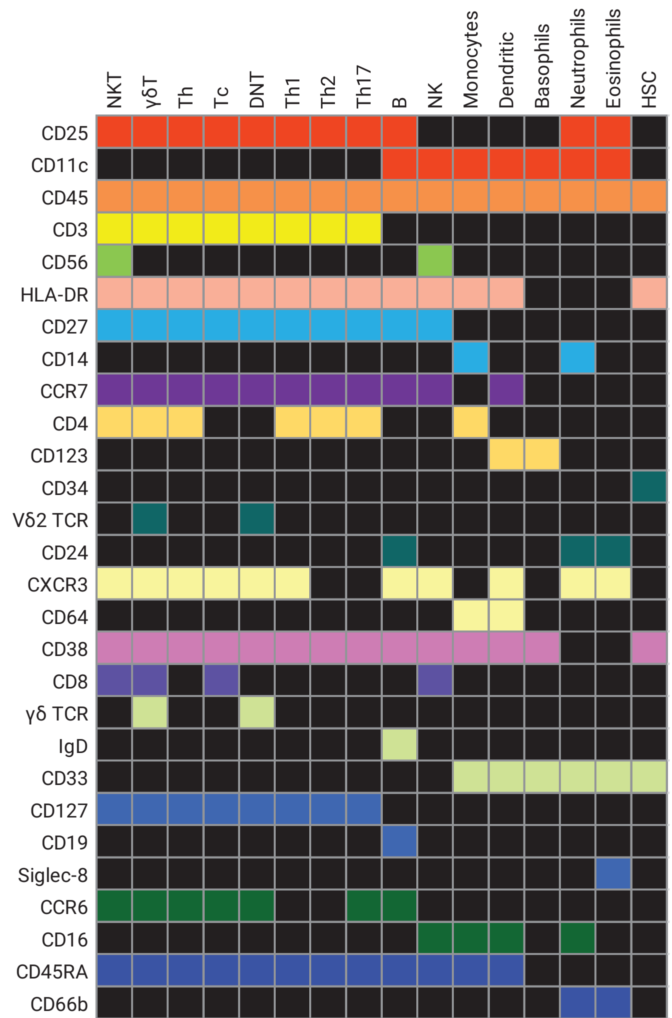

Figure 2. Expression of cell surface markers by distinct cell subsets. Color indicates that the surface antigen is expressed on the cell type; antibodies that use that same/similar fluorophore use the same color. If there is no detectable cell expression of the marker, it is displayed in black.

Results and discussion

A sample was stained with the antibody cocktail to demonstrate that all 40 cell populations can be analyzed simultaneously using a sentinel panel with a three-laser instrument.

Hierarchical gating was used to gate all major immune cell types followed by additional gating of cellular subsets, cell differentiation, and activation status (Figure 3). Initial gating was performed on forward scatter height (FSC-H) and forward scatter area (FSC-A) to remove doublets followed by gating the white blood cells by expression of CD45. T cells were separated from the total CD45 positive population by expression of CD3. Subsequent gating

Figure 3. Gating of 16-color, 28-parameter stain on the three-laser Agilent NovoCyte Advanteon flow cytometer of peripheral human whole blood. An antibody cocktail of all 28 antibodies was added to 150 µL of peripheral human blood and acquired on the Agilent NovoCyte Advanteon flow cytometer. Following acquisition, the data was analyzed using Agilent NovoExpress software. All antibodies used are listed in Table 1.

of CD3+T cells identified T cell subsets, CD4, CD8, Double Negative (DN), Vδ2+ and Vδ2- γδT cells. Several differentiation states of T cells were identified including naïve, effector, effector memory, and central memory T population using the expression patterns of CD45RA and CCR7. Regulatory T cells were identified within the CD4 T cell population through the high expression of CD25 and low levels of CD127. Th subsets were also identified using CCR6 and CXCR3 expression to determine the frequency of Th1, Th2, and Th17 cells. Finally, T cell activation status was determined by the expression of CD38, HLA-DR, CD25, CD27, and CD127. By expanding the total numbers of parameters that can be assessed, an in-depth analysis of T cell subsets, differentiation, and activation was achievable.

Next, out of the CD3 negative population of cells, B cells were identified by expression of CD19. Plasmablast cells were further identified via CD27 and CD38 expression, and transitional B cells were labeled via CD24 and CD38 expression. The double staining of B lymphocytes for CD27 and IgD made it possible to determine the percentages of naïve B cells (IgD+CD27–), CD27 negative switched memory B cells (IgD-CD27–), CD27 positive switched memory B cells (IgD-CD27+, Sm B), and unswitched memory B cells (IgD+CD27+, Um B).

The population of cells that were not T or B cells was analyzed to identify other major subsets of the immune cells. NK cells were identified by the lack of expression of CD33 and CD123, followed by the expression of CD16 and CD56 to identify five NK cell subsets (CD16-CD56^{hi}, CD16-CD56^{dim}, CD16+CD56^{hi}, CD16+CD56^{dim}, and CD16+CD56-). The activation and maturation status of NK cell subsets was further analyzed by expression of CD38, HLA-DR, CD8, and CD45RA. Next, neutrophils were identified as CD16+ SSC^{hi}, while CD16-SSC^{hi} cells were confirmed as eosinophils through the expression of Siglec-8. Both neutrophil and eosinophil activation were measured by expression of CD66b. Monocytes were identified by CD14 and further divided into classical, intermediate, and nonclassical monocytes by the expression of CD14 and CD16. Monocyte activation was measured by the expression of CD38, HLA-DR, CD64, and CD45RA. DC cells were identified as HLA-DR+, and the DC subsets, pDC, and mDC, were identified through differential expression levels of CD11c and CD123. Finally, CD123+ cells were identified as basophils. Besides the major immune cell subsets, the frequencies of hematopoietic stem cell populations were determined by the expression of CD34 and a dim signal from CD45.

Altogether, every major immune cell subset as well as stem cell population was determined on a three-laser NovoCyte Advanteon by using a sentinel panel. To verify the accuracy of antibody staining, fluorescence minus one (FMO) controls were used to assess the separation of markers expressed at low levels such as CD34 PE, Siglec-8 APC, CD66b APC-Cy7, CCR6 APC-R700, CD33 PE-Cy7, CD123 FITC, and CD11c BV421 (Figure 4, not all shown). The results confirmed that even the dim markers that were included in this panel were easily discriminated from negative samples. In conclusion, using a sentinel panel, it is possible to do an in-depth analysis of immune and stem cells in the blood.

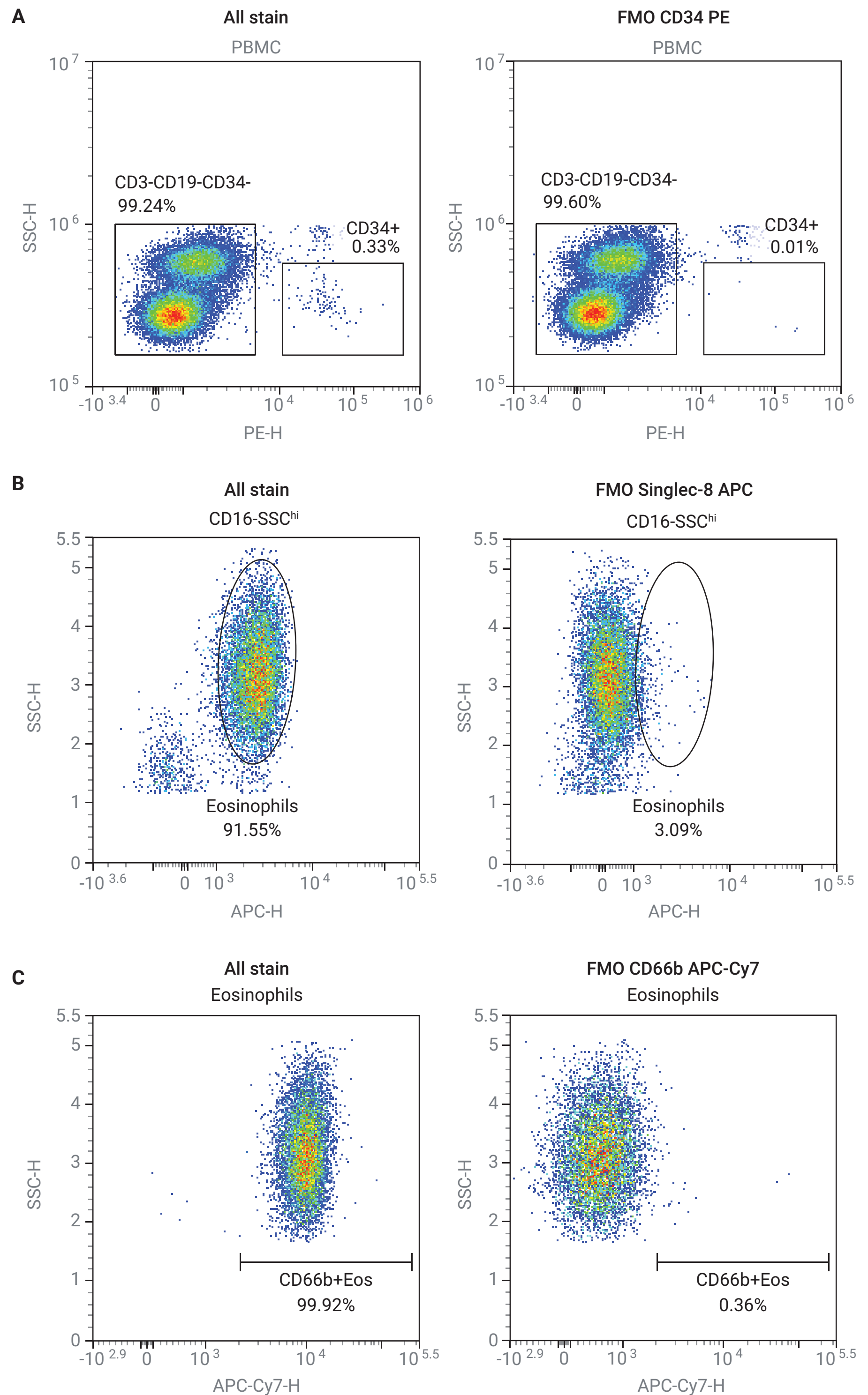

Figure 4. Evaluation of staining resolution on selected dim markers. Sample was stained either with all antibodies in the panel or without one antibody of the panel, the fluorescence minus one (FMO) control. FMO control samples were prepared for CD34 PE (A), Siglec-8 APC (B), and CD66b APC-7 (C). Sample stained with all antibodies is on the left panel and the FMO control is on the right.

Conclusion

In this application note, grouping multiple antibodies labeled with the same fluorochrome is described to enable deeper immunophenotyping of cell populations without splitting samples into multiple smaller panels. This 16-color panel includes 28 commonly used immune cell surface and activation markers to separate peripheral human whole blood into 40 unique populations using a three-laser NovoCyte Advanteon. Each cell population can be easily distinguished, while simultaneously analyzing the activation and differentiation states of these cells. The number of parameters that can be measured is not limited to the number of channels in the instrument. Therefore, by using a strategically designed panel, it is possible to push flow cytometric analysis beyond the number of detection channels and increase the number of distinct cell populations that be examined in a small amount of sample.

References

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