Application Note

Cell Analysis

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Immunophenotypic Analysis of Human Blood Leukocyte Subsets Using a 13-Color Antibody Panel on the Agilent NovoCyte System

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Introduction

Identification and analysis of specific subpopulations of cells is one of the many essential uses of flow cytometry, especially in terms of its ability to multiplex and identify several markers in one sample.[1] Immunophenotyping of blood samples is one of the more popular uses of multicolor flow cytometry. In certain applications, using multiple markers simultaneously for immunophenotyping is more powerful and efficient than using multiple samples with fewer markers. However, to accomplish this task, a powerful flow cytometer is needed; one that has the ability to detect and analyze the plethora of colors used in such an experiment. Here, we demonstrate immunophenotypic analysis of a human blood sample using 13 fluorescent markers on the Agilent NovoCyte 3000, a compact benchtop flow cytometer equipped with three lasers.

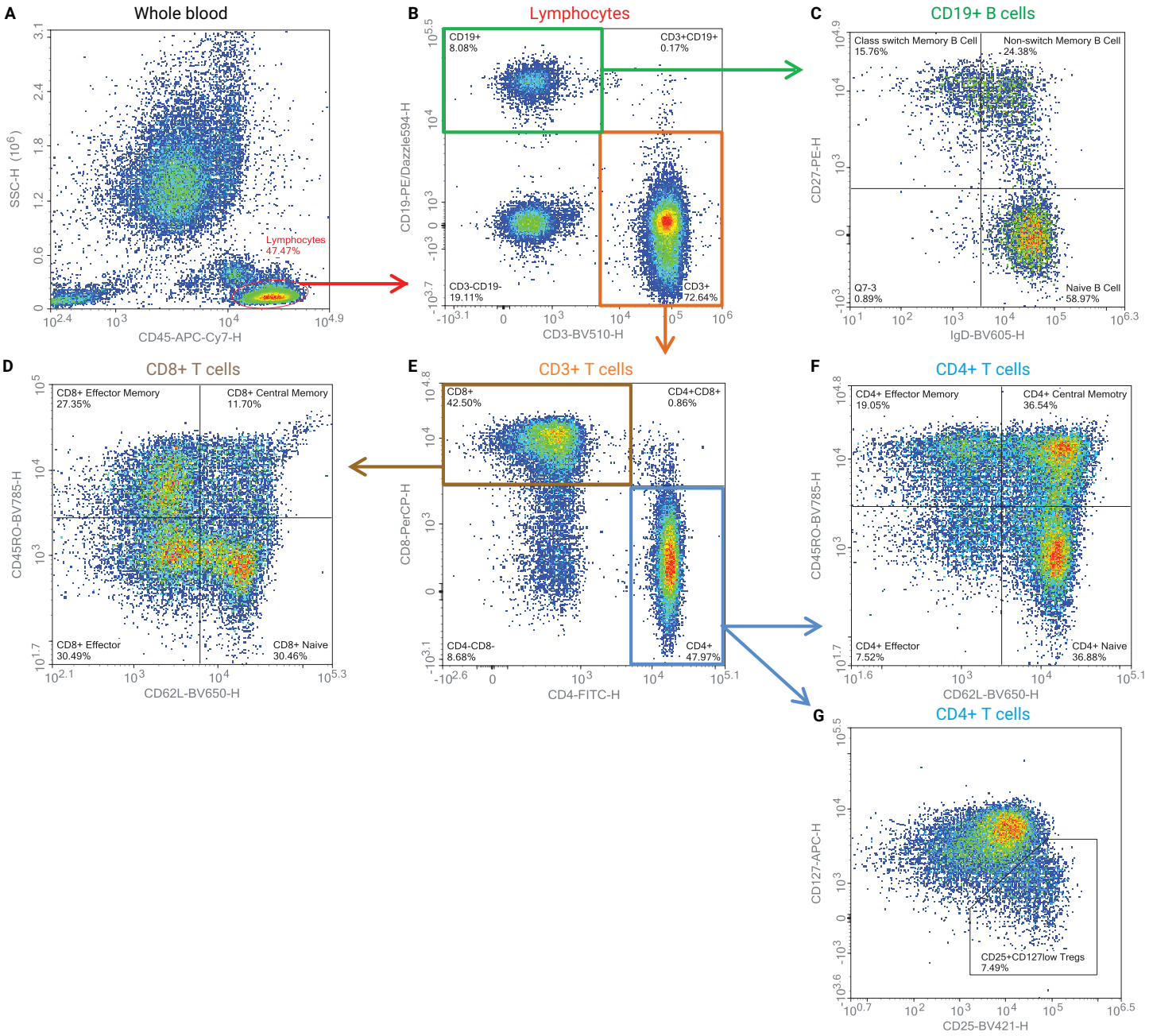

Figure 1. Immunophenotyping of T and B lymphocytes. A) Lymphocytes that were identified from their CD45+ staining profile, were separated into either B) CD3+ T cells or CD19+ B cells. C) Subsets of B cells were classified according to their IgD and CD27 staining. E) T cells were classified as either CD4+ or CD8+ T cells and further subclassified based on their (D and F) CD62L and CD45RO staining. G) In the CD4+ T cell population, T regulatory cells (Tregs) were also identified using CD25 and CD127.

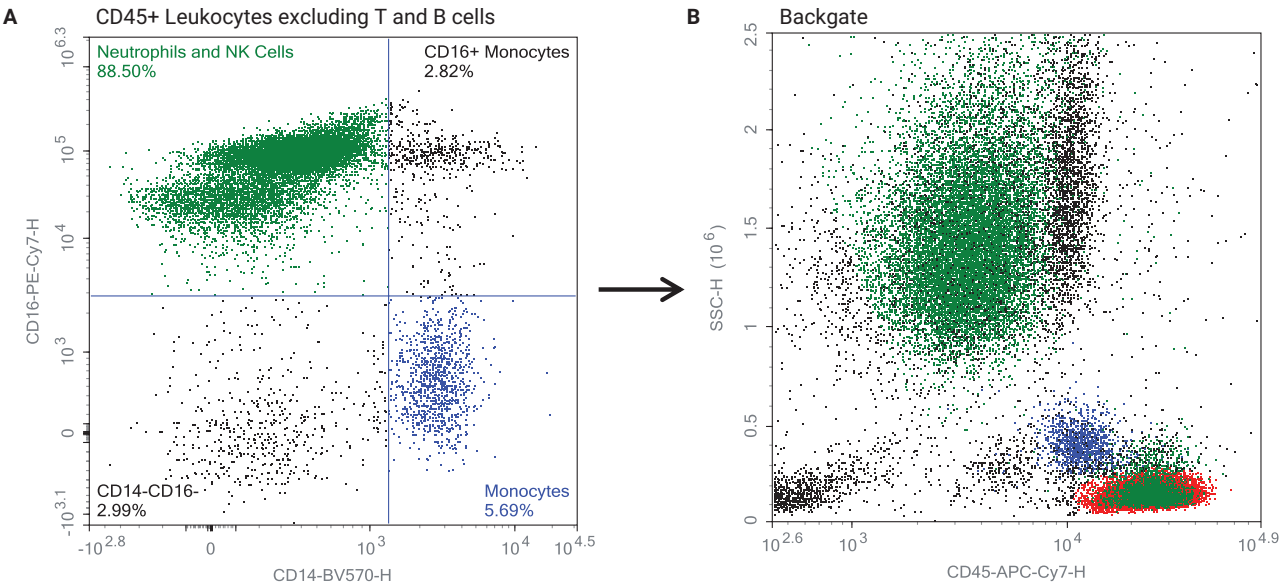

Figure 2. Immunophenotyping lymphocyte and nonlymphocyte subsets. A) Neutrophils and NK cells were detected by analyzing the leukocytes (CD45+), excluding T and B lymphocytes for CD16+/CD14- staining. Monocytes were detected when analyzing the same population, but identified by the CD14+ only stain or CD14+/CD16+ double positive staining. B) Selected populations from A were backgated to CD45 versus SSC.

Materials and methods

Whole, human blood was stained with the antibody cocktail detailed in Table 1 for 30 minutes at room temperature in the dark. To remove red blood cells, 2 mL of 1X RBC lysis buffer (Agilent) was added to blood samples with gentle vortexing and incubated for 15 minutes. Samples were then washed, fixed in 1% paraformaldehyde, and run on the NovoCyte flow cytometer. Color compensation was calculated automatically using the autocompensation feature after acquiring single-color compensation bead samples. Fluorescence minus one (FMO) controls were used to ensure accurate gating.

Table 1. 13-color antibody panel.

Laser	FL channel on NovoCyte	Format	Specificity
405 nm	VL1	BV421	CD25
	VL2	BV510	CD3
	VL3	BV570	CD14
	VL4	BV605	IgD
	VL5	BV650	CD62L
	VL6	BV785	CD45RO
488 nm	BL1	FITC	CD4
	BL2	PE	CD27
	BL3	PE/Dazzle 594	CD19
	BL4	PerCP	CD8
	BL5	PE-Cy7	CD16
640 nm	RL1	APC	CD127
	RL2	APC-Cy7	CD45

Results and discussion

Optimizing antibody concentration and including proper controls are critical components for obtaining quality data. In this staining, subsets of T cells, B cells, and other leukocytes were identified and analyzed using the NovoExpress acquisition and analysis software. Through light scattering and CD45 expression, the major leukocyte subsets in the blood, lymphocytes, monocytes, and granulocytes can easily be discriminated. Lymphocytes have the smallest side scatter (SSC) and the highest expression of CD45 (Figure 1A).

Lymphocytes consist of T, B, and NK cells, B cells were first identified by expression of CD19 (Figure 1B). B cell status was assessed by expression of CD27 and IgD to determine the frequencies of naïve B cells (IgD+ CD27-), class-switched memory B cells (IgD- CD27+), and nonclass switched memory B cells (IgD+ CD27+). T cells were selected by the expression of CD3, thereafter identified as CD4 T or CD8 T cells. T cell differentiation status was further assessed by the expression of CD45RO and CD62L to determine the frequencies of naïve (CD45RO-CD62L+), effector (CD45RO-CD62L-), effector memory (CD45RO+CD62L-), and central memory (CD45RO+CD62L+) CD4 and CD8 T cells.

A subset of T cells that are known to suppress T cell responses, regulatory T cells, can be identified by their high expression of IL-2 receptor (CD25) and low expression of CD127 compared to other CD4 T cells and make up 5 to 10% of the total CD4 T cell population. In this sample, regulatory T cells are 7.49% of the total CD4 T cell population. Next, a monocyte was identified by CD14 expression, while neutrophils and NK cells were determined by the expression of CD16 (Figure 2). To distinguish between CD14+ neutrophils and NK cells, we show here a backgate of the SSC/CD45 plot that first identified the lymphocyte population (Figure 2B). Since NK, cells are part of the lymphocyte population, these CD16+ cells have high expression of CD45 and low SSC while the neutrophil population has much higher granularity and can easily be identified by high SSC levels. Therefore, using this 12-color panel, the major leukocyte population frequencies can be assessed, as well as in-depth analysis of T and B cell differentiation.

Reference

1. Maecker, T. H.; McCoy, J. P.; Nussenblatt, R. *Nature Immunol.* **2012** Feb 17, *12(3)*, 191–200.

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RA.5334722222
DE.3093981481

Printed in the USA, February 2, 2021
5994-1763EN

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