13-parameter immunophenotyping of human lysed whole blood with the the Attune NxT Flow Cytometer allows for the identification of B cells, NK cells, multiple T cell subsets and myeloid cells.

Immunophenotyping is the analysis of heterogeneous populations of cells within human blood for the purpose of identifying the presence and proportions of various cell populations. This is usually accomplished by using antibodies to detect specific antigens expressed by these cells. These antigens are usually functional membrane proteins involved in cell communication, adhesion or metabolism. Flow cytometry is the method of choice for identifying cells within complex populations, as it allows for multi parameter analysis of thousands to millions of cells in a short time. Applications of this technology are used both in basic research and in clinical laboratories.

The Attune™ NxT Flow Cytometer is available with up to 4 lasers and 16 detection chennels. All versions show excellent separation of cell populations into subsets for immunophenotyping. There is strong signal separation for more data clarity, and up to 14-color detection can be performed with the automated compensation module. This application note describes the use of the Attune NxT Flow Cytometer with 4 lasers and 13-color immunophenotyping analysis of stained human whole blood using a stain/lyse protocol. Lymphocyte, monocyte and granulocyte populations were distinguished with forward scatter (FSC) and side scatter (SSC); and monocyte, T cell, B cell and NK populations were identified using fluorescent antibodies against surface antigens specific for the different immunological populations.

Materials

- Human whole blood
- 96-well plates (optional)
- Flow cytometry tubes
- Gibco™ PBS (Cat. No. 10010-023)
- CD45 Pacific Orange™ (MHCD4530TR)
- CD8 Pacific Blue™ (Cat. No. MHCD0828TR).
- CD4 PerCP-Cy®5.5 (Cat. No. A15858).
- CD25 APC (Cat. No. A18616).
- CD19 Pacific Green™ (Cat.No. C11210).
- CD14 Qdot™ 705 (Cat No. Q22137)
- CD3 Alexa Fluor™ 700 (Cat. No. CD0329).
- HLA-DR PE-Cy®7 (Cat. No. A18558).
- CD62L APC Alexa Fluor™ 750 (Cat. No. MHCD62L27).
- CD33 PE-Cy®5 (Cat. No. A16215),
- CD45RA FITC (Cat. No. MHCD45RA01)
- CD56 PE (Cat. No. MHCD5604-4)
- Propidium iodide (Cat. No. P1304MP)
- AbC™ Total Antibody Compensation Bead Kit (Cat. No. A10497)
- ACK Lysing Buffer (Cat. No. A1049201)
- Countess™ II Automated Cell Counter (Cat. No. AMQAX1000)
- Countess™ Cell Counting Chamber Slide (Cat. No. C10228)
- Attune NxT Flow Cytometer (Cat. No. A24858)

Red blood cells were lysed from human whole blood, and white blood cells (WBCs) were surface stained with the antibodies listed above. The following protocol was used for sample preparation and analysis on the Attune NxT Flow Cytometer. Please see the user guide for detailed instructions on setting up an experiment and running samples.

Antibody labeling and red blood cell lysis

1. Turn on the instrument; run startup and performance test scripts as normal.
2. Create single-color compensation controls by labeling capture beads provided in the AbC Total Antibody Compensation Bead Kit (see "Compensation controls" section).
3. Collect blood into an appropriate anticoagulant (EDTA or heparin).
4. Dilute whole blood 1:10 in 1X ACK Lysing Buffer.
5. Incubate at room temperature for 30 minutes on a rotator.
6. Wash with PBS containing 1% BSA and 2mM sodium azide.
7. Following instrument user guide, count WBCs on the Countess II Automated Cell Counter.
8. Resuspend 10^6 cells in 50 µL PBS containing 1% BSA and 2mM sodium azide.
9. Add antibodies as indicated in the manufacturer's package insert to appropriately labeled tubes from step 8. In this case, 10 antibodies at 5µL each were added to the tube (total antibody volume of 50µL). The total volume including antibodies should be 100µL. Mix gently.
10. Incubate tubes for 15 minutes at room temperature (22–23°C) in the dark.
11. Centrifuge tubes for 5 minutes at 300 × g. Remove supernatant.
12. Resuspend the cells in all tubes in 1 mL of PBS or sheath fluid.
13. Analyze on a flow cytometer immediately, or store samples at 2–8°C in the dark and analyze within 24 hours.
14. Create a workspace on the Attune NxT Flow Cytometer software containing required plots (see "Data acquisition and gating strategy" section).
15. Load samples on the Attune NxT Flow Cytometer and acquire preferred number of events (e.g., 10,000).

Compensation controls

1. Completely resuspend the AbC Total Antibody Compensation capture beads (Component A) and negative beads (Component B) by gently vortexing for 10 seconds before use.
2. Label a sample tube for each fluorophore-conjugated antibody you are using, and add 1 drop of AbC Total Antibody Compensation capture beads (Component A) to each tube.
3. Add a pre-titrated amount of each mouse antibody conjugate to the AbC Total Antibody Compensation capture bead suspension in the designated tube, and mix well. Make sure to dispense the antibody directly into the bead suspension.
4. Incubate for 15 minutes at room temperature, protected from light.
5. Add 3mL of PBS or other buffer to the sample tubes. Centrifuge for 5 minutes at 250 × g.
6. Carefully remove the supernatant from the tubes, and resuspend the bead pellets by adding 0.5mL of PBS or other buffer to the sample tubes.
7. Add one drop of negative beads (Component B) to the tubes, and mix well.

8. Vortex the tubes before analyzing using flow cytometry. You may briefly sonicate to increase the percentage of singlet beads, if necessary.

9. Perform manual or automatic compensation according to the preferred procedure for the flow cytometer in use. Gate on the bead singlet population based on FCS and SSC characteristics.

Data acquisition

See table for bandpass filters, lasers and channels used on the Attune NxT cytometer to acquire the samples. Antigen ranking is also included, where 1 is high expression, 2 is medium expression and 3 is low expression of the antigen.

Laser	Excitation (nm)	Emission filter (nm)	Channel	Fluorophore	Marker	Cat. No.	Antigen ranking
Violet	405	440/50	VL-1	Pacific Blue	CD8	MHCD0828TR	1
		512/25	VL-2	Pacific Green	CD19	C11210	2
		603/48	VL-3	Pacific Orange	CD45	MHCD4530TR	1
		710/50	VL-4	Qdot 705	CD14	Q22137	1
Blue	488	530/30	BL-1	FITC	CD45RA	MHCD45RA01	1
		590/40	BL-2	Propidium Iodide	N?A[†]	P1304MP	1
		695/40	BL-3	PerCP-Cy®5.5	CD4	A15858	1
Yellow	561	585/16	YL-1	PE	CD56	MHCD5604-4	3
		620/15	YL-2				
		695/40	YL-3	PE-Cy5	CD33	A16215	2
		780/60	YL-4	PE-Cy7	HLA-DR	A18558	2
Red	637	670/14	RL-1	APC	CD25	A18616	3
		720/30	RL-2	Alexa Fluor 700	CD3	CD0329	1
		780/60	RL-3	APC-Alexa Fluor 750	CD62L	MHCD62L27	2

[†]NA = not applicable

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Gating strategy

Dead cells were excluded from the analysis by gating on live cells in a dot plot (Figure 1A). CD45-positive cells were gated on to select the leukocyte population from the lysed whole blood (Figure 1B). Lymphocytes and monocytes were gated based on forward and side scatter profiles (Figure 1C). Monocytes are found above lymphocytes based on scatter profiles and express both CD14 and CD33 (Figure 1D). Within the lymphocyte gate T cells can be isolated based on their expression of CD3 (Figure 1F) and further subdivided into CD4 (T helper cells) and CD8 (Cytotoxic T cells) subpopulations (Figure 1G). B cells can be further characterized by HLA-DR and CD45RA expression (Figure 1E). In addition, regulatory T cells express CD4 and CD25 (Figure 1J). CD62L identifies naïve (TN) CD4 and CD8 T cells, whereas HLA-DR is expressed by activated T cells (TA) (Figure 1H and Figure 1K). NK cells can be identified as they lack B cell (CD19) and T cell (CD3) markers and express CD56 (Figure 1I).

Conclusion

The Attune NxT Flow Cytometer with 4 lasers and 14 colors shows excellent cell population resolution for 13-color human lymphocyte immunophenotyping experiments on lysed human whole blood. Designing multicolor panels is greatly improved with choices of reagents for 4 spatially separated lasers and 14 color choices.

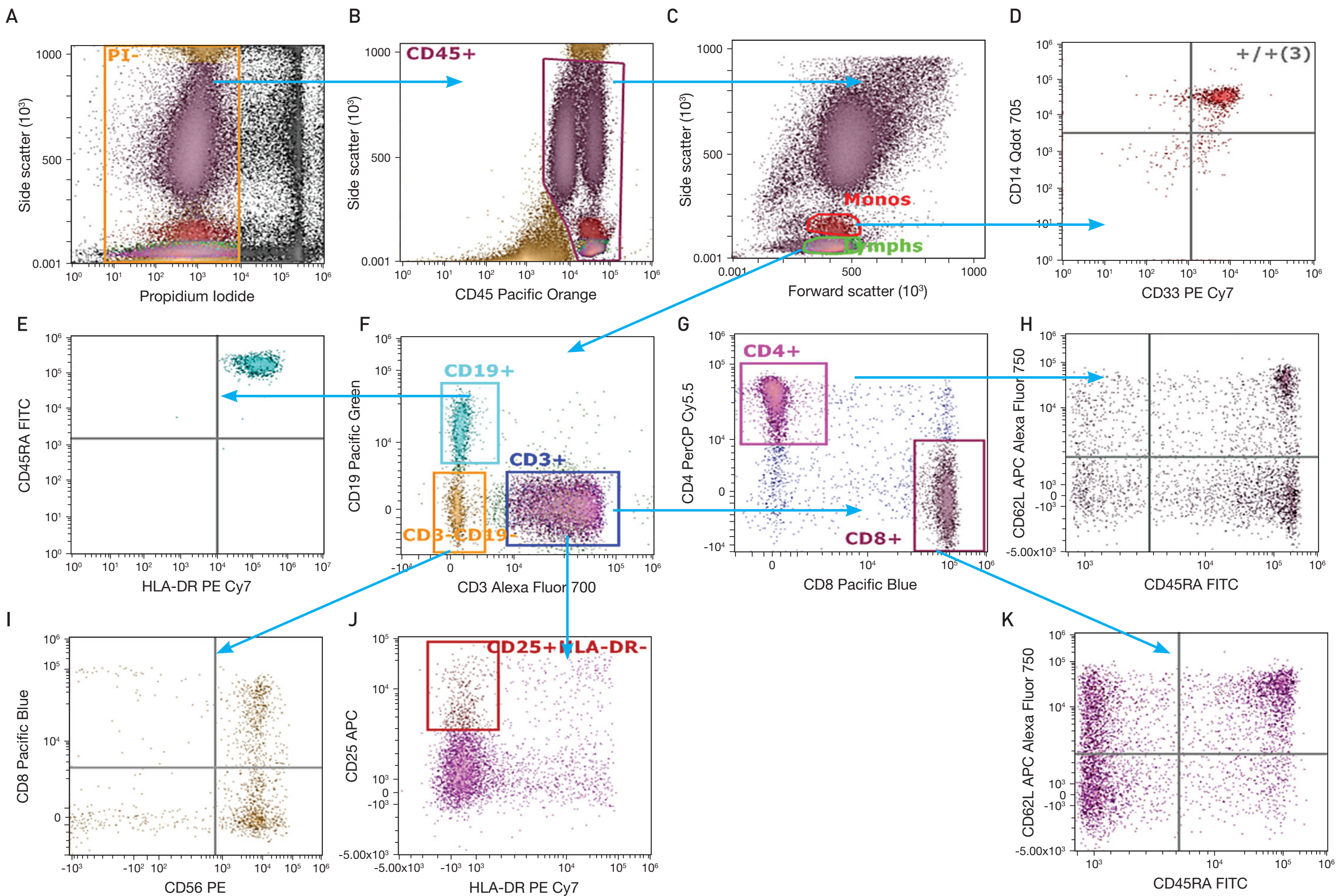

Figure 1. Gating strategy.

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