Ten-parameter immunophenotyping of human lysed whole blood with the Attune NxT Flow Cytometer allows identification of 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 accomplished by using antibodies to detect specific antigens expressed by these cells. The antigens detected are usually functional membrane proteins involved in cell communication, adhesion, or metabolism. Multiparameter flow cytometry is a widely used method for identifying cell subsets within complex cellular systems. Applications of this technology are used both in basic research and clinical research laboratories.

The Invitrogen™ Attune™ NxT Flow Cytometer is available with up to 4 lasers and 16 detection channels. All configurations 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 for 4-laser and 10-color immunophenotyping analysis of stained human whole blood using a stain-and-lyse protocol.

Materials and methods

- CD8 Mouse Anti-Human mAb, Pacific Orange™ Conjugate (Cat. No. MHCD0830TR)
- CD4 Mouse Anti-Human mAb, PerCP-Cy®5.5 Conjugate (Cat. No. A15858)
- CD25 Mouse Anti-Human mAb, APC Conjugate (Cat. No. A18616)
- CD14 Mouse Anti-Human mAb, APC-Cy®7 Conjugate (Cat. No. A15453)
- CD19 Mouse Anti-Human mAb, Pacific Green™ Conjugate (Cat. No. C11210)
- CD3 Mouse Anti-Human mAb, Alexa Fluor™ 700 Conjugate (Cat. No. CD0329)
- HLA-DR Mouse Anti-Human mAb, PE-Cy®7 Conjugate (Cat. No. A18558)
- CD62L Mouse Anti-Human mAb, Pacific Blue™ Conjugate (Cat. No. MHCD62L28)
- CD33 Mouse Anti-Human mAb, FITC Conjugate (Cat. No. A16185)
- CD11c Mouse Anti-Human mAb, PE Conjugate (Cat. No. A18674)
- Attune NxT Flow Cytometer (Cat. No. A24858)
- 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 Slides (Cat. No. C10228)
- 96-well plates (optional)
- Flow cytometry tubes
- Gibco™ PBS, pH 7.4 (Cat. No. 10010023)

Red blood cells were lysed from human whole blood, and white blood cells (WBCs) were surface stained with the antibodies listed above. The following protocols were used for sample preparation and analysis on the Attune NxT Flow Cytomter. Please see the user guide for detailed instructions on setting up an experiment and running samples [1].

Protocols

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 min on a rotator.
6. Wash with PBS containing 1% BSA and 2 mM sodium azide.
7. Following the instrument user guide [2], count WBCs on the Countess II Automated Cell Counter.
8. Resuspend 106 cells in 50 µL PBS containing 1% BSA and 2 mM 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 5 µL each were added to the tube (total antibody volume of 50 µL). The total volume including antibodies should be 100 µL. Mix gently.
10. Incubate tubes for 15 minutes at room temperature (22 ± 3°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 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 Compensation capture beads (Component A) to each tube.
3. Add a pre-titrated amount of each mouse antibody conjugate to the AbC Total Compensation capture bead suspension in the designated tubes, and mix well. Make sure to add the antibody directly to the bead suspension.
4. Incubate for 15 minutes at room temperature, protected from light.
5. Add 3 mL 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 0.5 mL 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 by 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 and gating strategy

Samples were collected on the Attune NxT Flow Cytometer using the filters shown in Table 1. The gating strategy used in our multiparameter analysis is described in Figure 1.

Table 1. Filters used to detect each dye conjugate.

Dye	Excitation (nm)	Emission (nm)
Pacific Blue	405	440/50
Pacific Green	405	512/25
Pacific Orange	405	603/48
FITC	488	530/30
PerCP-Cy5.5	488	695/40
PE	561	585/16
PE-Cy7	561	780/60
APC	637	670/14
Alexa Fluor 700	637	720/30
APC-Cy7	637	780/60

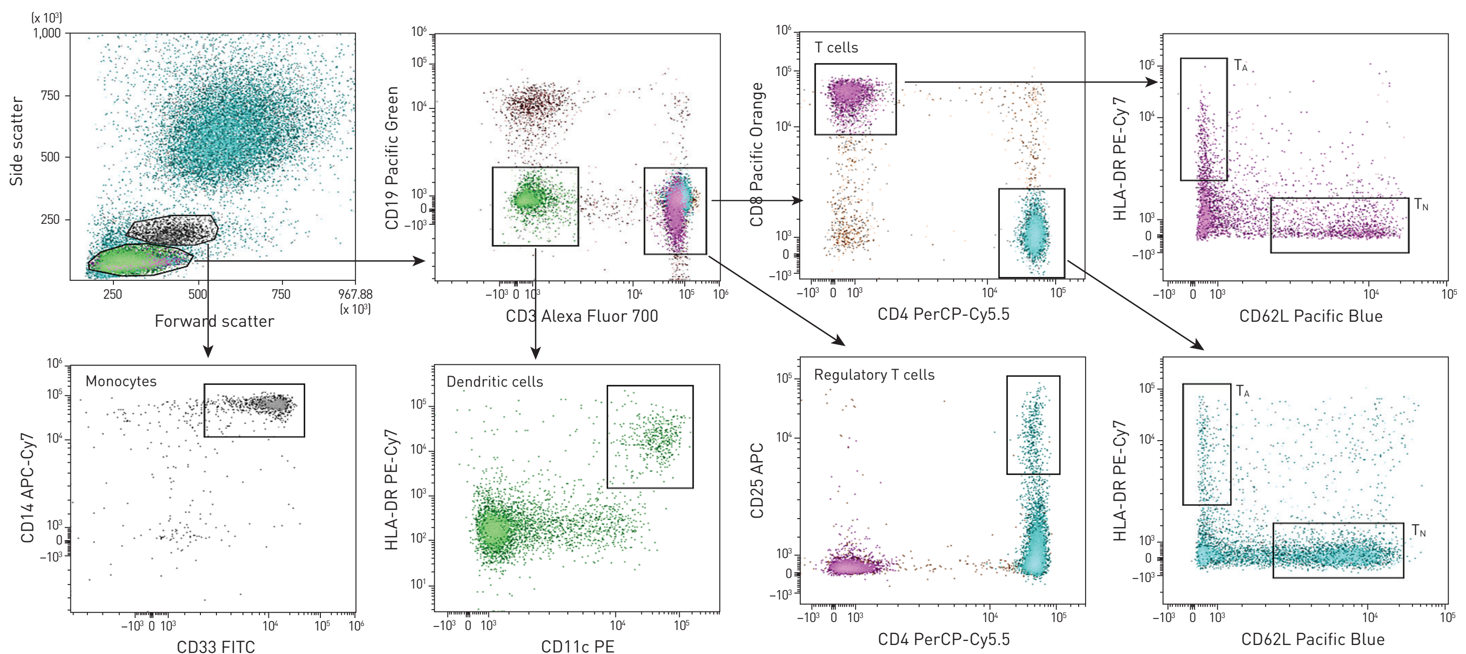

Figure 1. Ten-parameter immunophenotyping of human PBMCs with the Attune NxT Flow Cytometer. Lymphocytes and monocytes were gated based on forward and side scatter profiles. Within the lymphocyte gate, T cells can be isolated based on their expression of CD3 and further subdivided into CD4 and CD8 subpopulations. In addition, regulatory T cells (mediators of dominant peripheral tolerance) express CD4 and CD25. CD62L identifies naive (T_N) $CD4^+$ and $CD8^+$ T cells, whereas HLA-DR is expressed by activated T cells (T_A). Conventional dendritic cells found in peripheral blood are generally negative for T and B cell lineage markers and co-express the integrin CD11c and HLA-DR. Monocytes fall just above lymphocytes based on scatter profile and express both CD14 and CD33.

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Conclusion

The Attune NxT Flow Cytometer with 4 lasers and 14 colors shows excellent cell population resolution for 10-color human lymphocyte immunophenotyping experiments. Designing multicolor panels is greatly improved with choices of reagents for 4 spatially separated lasers and 14 colors.

References

1. Attune NxT Acoustic Focusing Cytometer User Guide. Pub. No. 100024235, Rev. A.
2. Countess II and Countess II FL Automated Cell Counters, Pub. No. MAN0010644, Rev. B.

Find out more at **thermofisher.com/attune**

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