Multiparameter analysis of murine regulatory T cells and dendritic cells with the Attune NxT Flow Cytometer

Immunophenotyping is the analysis of heterogeneous populations of cells within mouse blood, splenocytes, and thymocytes 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 the 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 multiparameter 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 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 mouse splenocytes using a fixation and permeabilization protocol.

Materials and methods

- CD45R (B220) Rat Anti-Mouse mAb, Pacific Orange™ Conjugate (Cat. No. RM2630)
- CD3ε Hamster Anti-Mouse mAb, PerCP-Cy®5.5 Conjugate (Cat. No. A14784)
- CD25 Rat Anti-Mouse mAb, APC Conjugate (Cat. No. RM6005)
- CD45.2 Mouse Anti-Mouse mAb, APC-Cy®7 Conjugate (Cat. No. A18642)
- CD4 Rat Anti-Mouse mAb, Pacific Green™ Conjugate (Cat. No. C11207)
- CD8α Rat Anti-Mouse mAb, Alexa Fluor™ 700 Conjugate (Cat. No. MCD0829)
- CD11c Hamster Anti-Mouse mAb, PE-Cy®7 Conjugate (Cat. No. A15849)
- I-A/I-E (MHCII) Rat Anti-Mouse mAb, Pacific Blue™ Conjugate (Cat. No. A14901)
- CD11b Rat Anti-Mouse mAb, FITC Conjugate (Cat. No. RM2801)
- Foxp3 Anti-Mouse/Rat mAb, PE Conjugate (eBioscience Cat. No. 12-5773-80)
- Attune NxT Flow Cytometer (Cat. No. A24858)
- AbC™ Total Antibody Compensation Bead Kit (Cat. No. A10497)
- Foxp3 Transcription Factor Staining Buffer Kit (Cat. No. A25866A)
- Gibco™ PBS, pH 7.4 (Cat. No. 10010023)
- 96-well plates (optional)
- Flow cytometry tubes

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C57BL/6 splenocytes were stained with the cell surface antibodies listed above, followed by fixation and permeabilization using the Foxp3 Transcription Factor Staining Buffer Kit, a fixation and permeabilization kit designed to work with transcription factors, like Foxp3, and other intracellular markers. Intracellular staining was performed with Foxp3 Anti-Mouse/Rat mAb, PE Conjugate (eBioscience). The following protocol was used for multiparameter analysis on the Attune NxT Flow Cytometer. Please see the user guide for detailed instructions on experiment setup and running samples [1].

Antibody labeling

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. For each sample to be analyzed, pipette 100 µL (equivalent to 1 x 10^6 cells) of thoroughly mixed splenocytes into a conical tube.
4. Add antibodies as indicated in the manufacturer's package insert to appropriately labeled tubes from step 3. Mix gently.
5. Incubate tubes for 15 minutes at room temperature (22 ± 3°C) in the dark.
6. Centrifuge tubes for 5 minutes at 300 x *g*. Remove supernatant.
7. Resuspend the cells in 100 µL of PBS or sheath fluid.
8. Add 1 mL of 1X Fixation and Permeabilization Solution to the cells, and vortex briefly.
9. Incubate for 30–60 minutes at room temperature or 4°C.
10. Add 2 mL 1X Wash Buffer, and vortex briefly to wash the cells. Centrifuge the sample and decant the supernatant.
11. Resuspend sample in 100 µL of 1X Wash Buffer.
12. Add antibody specific to an intracellular protein (Foxp3 antibody, PE conjugate in this case) according to your standard protocol, and vortex briefly.
13. Incubate sample for 30–60 minutes at 4°C.
14. Add 2 mL 1X Wash Buffer, and vortex briefly to wash the cells. Centrifuge the sample and decant the supernatant.
15. Resuspend in an appropriate volume of flow cytometry staining buffer (PBS with 1% BSA and 2 mM sodium azide).
16. Create a workspace on the Attune NxT Flow Cytometer software containing required plots (see "Data acquisition and gating strategy" section).
17. 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 tube and mix well. Make sure to add the antibody directly into the bead suspension.
4. Incubate for 15 minutes at room temperature, protected from light.
5. Add 3 mL of PBS or other buffer to sample tubes. Centrifuge for 5 minutes at 250 x *g*.
6. Carefully remove the supernatant from tubes and resuspend the bead pellet by adding 0.5 mL of PBS or other buffer to sample tubes.
7. Add one drop of negative beads (Component B) to the tubes and mix well.
8. Vortex 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. Gate on the bead singlet population based on forward scatter (FSC) and side scatter (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

Conclusion

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

Reference

1. Attune NxT Flow Cytometer User Guide. Pub. No. 100024235, Rev. A.

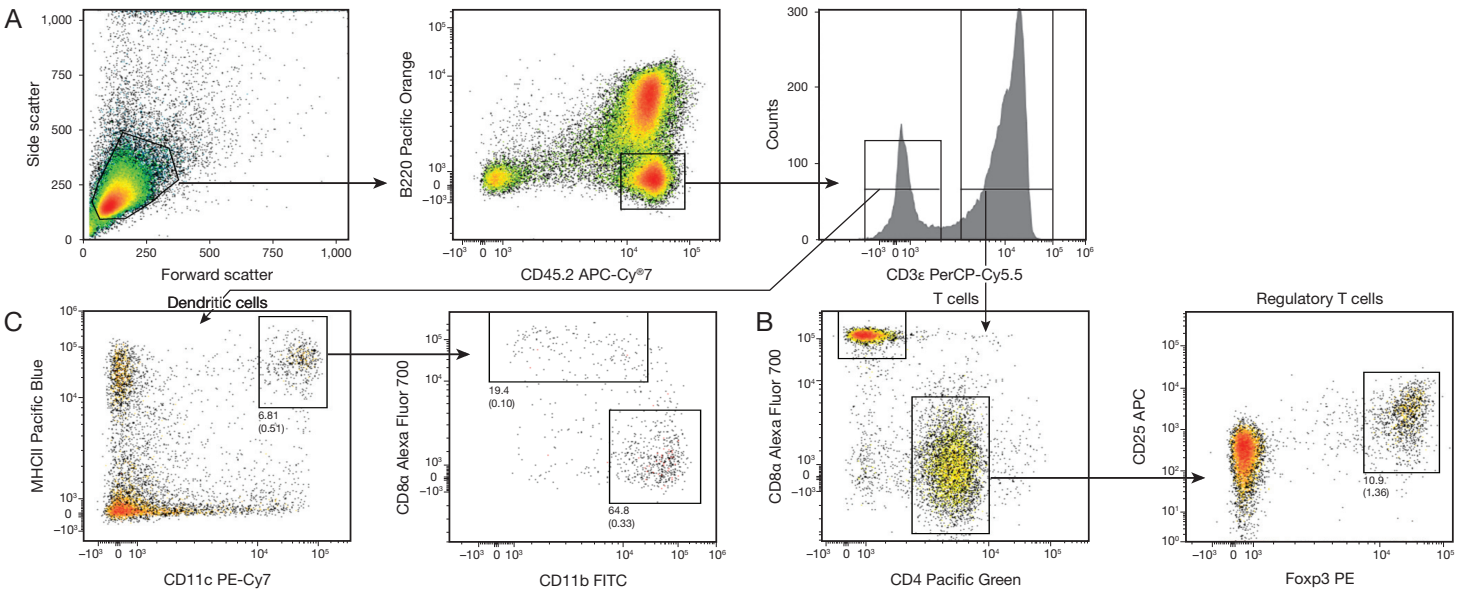

Figure 1. Multiparameter analysis of murine regulatory T cells and dendritic cells with the Attune NxT Flow Cytometer. (A) Lymphocytes were gated on FSC/SSC parameters, and B220-expressing B cells were omitted from subsequent analysis. Within the $B220^-$, $CD45.2^+$ gate, T cells were analyzed based on their expression of CD3ε. **(B)** T cells can be separated into two populations based on expression of the co-receptors CD4 or CD8α. Within the $CD4^+$ T cells there is a subpopulation of suppressive regulatory T cells that express the transcription factor Foxp3 and the cell surface marker CD25 (IL-2Rα). **(C)** Within the $CD3^-$ gate there is a rare population of $CD11c^+$, $MHCII^+$ dendritic cells, which are professional antigen-presenting cells that play a role in activating T cells. Splenic dendritic cells can be subdivided further into $CD11b^+$ and $CD8α^+$ dendritic cell subsets. Values noted are percentage of the parent gate (top number, no parentheses) or percentage of the FSC/SSC lymphocyte gate (bottom number, in parentheses).

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