Detection of murine regulatory T cells on the Attune™ NxT Flow Cytometer

CD4+ CD25+ regulatory T cells are a specialized subpopulation of T cells that act to maintain homeostasis within the immune system by suppressing the immune response of other cells. This is an important "self-check" built into the immune system to prevent excessive reactions. Regulatory T cells come in many forms, with the most understood being those that express CD4, CD25, and Foxp3 (CD4+ CD25+ regulatory T cells). Recent advances in the characterization of this cell population have firmly established their critical role in regulating the immune response. Interest in regulatory T cells has been accelerated by evidence from experimental mouse and human models demonstrating that the immunosuppressive potential of these cells can be utilized in research associated with autoimmunity, infectious agents, and cancer [1].

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 3-color immunophenotyping analysis of stained mouse splenocytes 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.

Materials and methods

- CD25 Rat Anti-Mouse mAb, APC Conjugate (Cat. No. RM6005)
- CD45.2 Mouse Anti-Mouse mAb, APC-Cy®7 Conjugate (Cat. No. A18642, optional)
- CD4 Rat Anti-Mouse mAb, Pacific Orange™ Conjugate (Cat. No. MCD0430)
- Foxp3 Rat Anti-Mouse 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

C57BL/6 splenocytes were surface stained with the CD25, CD45.2, and CD4 antibodies listed above, followed by fixation and permeabilization using the Foxp3 Transcription Factor Staining Buffer Kit and intracellular staining with Foxp3 Rat Anti-Mouse mAb, PE Conjugate or isotype control. The following protocol was used for sample analysis on the Attune NxT Flow Cytometer. Please see the user guide for detailed instructions on setting up an experiment and running samples [2].

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 the "Compensation controls" section).
3. For each sample to be analyzed, pipet 100 µL (equivalent to 1 x 10^6 cells) of thoroughly mixed splenocytes into a flow cytometry tube.
4. Add antibodies as indicated in the manufacturer's package insert to appropriately labeled tubes from step 3. Mix gently.
5. Incubate all tubes for 15 minutes at room temperature (22 ± 3°C) in the dark.
6. Centrifuge tubes for 5 minutes at 300 × *g*. Remove supernatant.
7. Resuspend the cells in all tubes in 100 µL of PBS or sheath fluid.
8. Add 1 mL of 1X fixation and permeabilization solution from the Foxp3 Transcription Factor Staining Buffer Kit.
9. Incubate for 30–60 minutes at room temperature or 4°C.
10. Add 2 mL of 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 for intracellular protein (e.g., Foxp3 antibody, PE conjugate) according to your standard protocol, and vortex briefly.
13. Incubate sample for 30–60 minutes at 4°C.
14. Add 2 mL of 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.
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 fluorochrome-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 deposit 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 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 and gating strategy

Samples were collected on the Attune NxT Flow Cytometer using 405 nm excitation and the 603/48 nm bandpass emission filter to detect Pacific Orange™ dye, 561 nm excitation and the 585/16 nm bandpass emission filter to detect PE, and 637 nm excitation and the 670/14 nm bandpass emission filter to detect APC. The gating strategy used in our analysis is described in Figure 1.

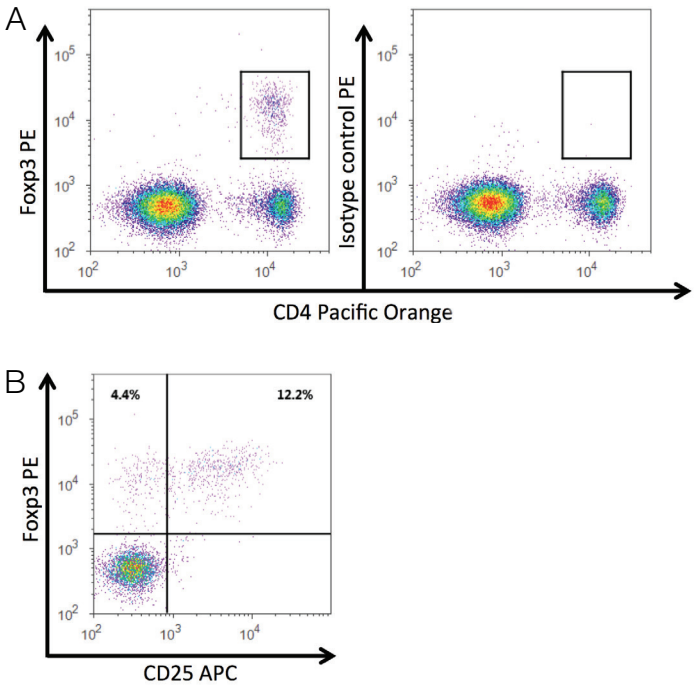

Figure 1. Detection of murine regulatory T cells on the Attune NxT Flow Cytometer. (A) Bivariate dot plot depicting the $CD4^+$ $Foxp3^+$ regulatory T cell population (gated) present in mouse spleen (left panel) compared to isotype control (right panel). Cells were gated on lymphocytes based on FSC/SSC profile. **(B)** $CD4^+$ T cells were gated and analyzed for CD25 and Foxp3 expression. The majority of murine regulatory T cells co-express the transcription factor Foxp3 and the cell surface marker CD25.

Conclusions

The Attune NxT Flow Cytometer with 4 lasers and 14 colors shows excellent cell population resolution for mouse regulatory T cells consisting of both surface and intracellular markers. This approach can be used to facilitate immunophenotyping studies of regulatory T cells in a variety of sample types.

References

1. Andersen MH (2014) Potential roles of self-reactive T cells in autoimmunity: lessons from cancer immunology. *Immunol Res* 60:156–164.
2. Attune NxT Flow Cytometer User Guide. Pub. No. 100024235, Rev. A.

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