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APPLICATION NOTE

Attune NxT Flow Cytometer

No-wash, no-lyse detection of leukocytes in human whole blood on the Attune NxT Flow Cytometer

Introduction

Standard methods for isolating and detecting leukocytes (white blood cells) in human whole blood are time-consuming and often involve significant manipulation and enrichment prior to analysis. These sample preparation steps can result in alterations in cell physiology and loss of cell types of interest [1]. Acoustic focusing cytometry, introduced with the Invitrogen™ Attune™ Flow Cytometer, allows high sample collection rates (up to 1,000 µL/min) without any loss in data resolution, thus eliminating the need for pre-acquisition enrichment and manipulation and helping to enable the detection of rare events in a timely manner.

In human whole blood, red blood cells outnumber white blood cells ~1,000-fold. This creates two hurdles in attempting to analyze whole blood samples without manipulation or enrichment: 1) collection of a sufficient number of white blood cell events for statistically meaningful data, and 2) differentiation of white blood cells from red blood cells given the high probability of coincident red blood cell events, which is clear when observing a conventional forward and side scatter profile of whole blood (Figure 1A). This application note outlines a strategy for the detection of white blood cells within whole blood using the Attune NxT Flow Cytometer.

We have developed three no-wash, no-lyse strategies for identifying leukocytes in whole human blood on the Attune NxT Flow Cytometer. One strategy exploits the difference in light-scattering properties between red

blood cells and leukocytes. Red blood cells contain hemoglobin, a molecule that readily absorbs violet laser (405 nm) light, whereas leukocytes do not (Figure 1B), resulting in a unique scatter pattern when observing human whole blood in the context of blue (488 nm) and violet (405 nm) side scatter (SSC) [2] (see Figure 1D). Inclusion of the Attune™ NxT No-Wash No-Lyse Filter Kit (Cat. No. 100022776) in the Attune NxT Flow Cytometer filter configuration allows simultaneous measurement of both blue and violet side scatter and the differentiation of red blood cells and leukocytes based on light-scattering properties alone (Figure 1D). This can be validated using fluorescently labeled antibodies that label CD45-expressing leukocytes or glycophorin A–expressing red blood cells (Figure 1C).

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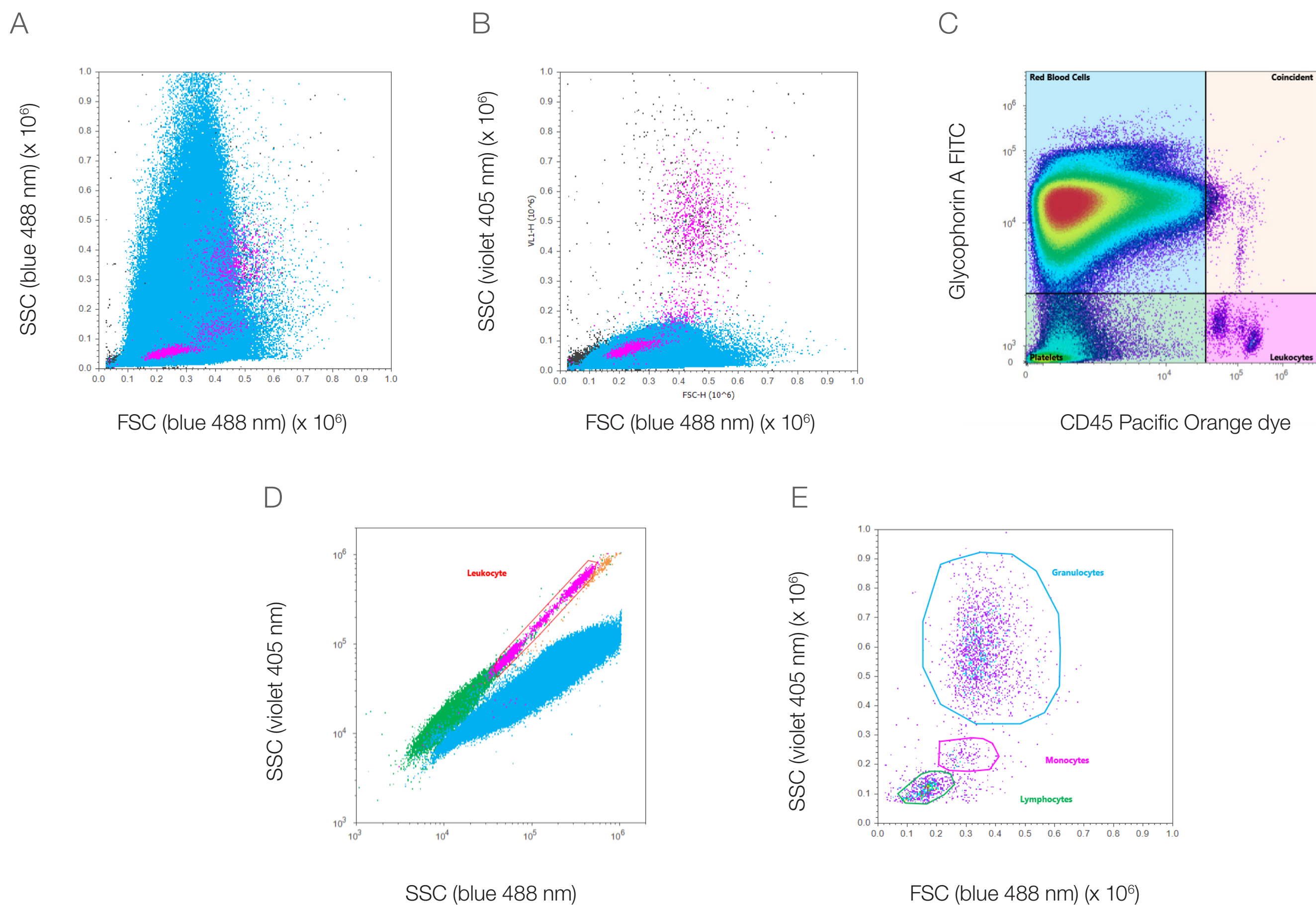

Figure 1. Identification of leukocytes in human whole blood using violet side scatter on the Attune NxT Flow Cytometer. Leukocytes are outnumbered by red blood cells ~1,000-fold in whole blood and generally require enrichment by red blood cell lysis or gradient centrifugation prior to analysis. The rapid sample collection rates and inclusion of the Attune NxT No-Wash No-Lyse Filter Kit on the Attune NxT Flow Cytometer allow identification of leukocytes by scatter properties alone. **(A)** Using conventional blue 488 nm forward and side scatter does not allow resolution of leukocytes in whole blood. Backgate analysis using fluorescently labeled antibodies specific for leukocytes (pink) and red blood cells (blue) demonstrates this problem. **(B)** Resolution of leukocytes from red blood cells in whole blood is improved by incorporating violet 405 nm side scatter. **(C)** Backgate analysis using antibodies against the red blood cell marker glycophorin A and the leukocyte marker CD45 demonstrates the ease of identifying leukocytes in human whole blood, as opposed to in A and B. **(D)** Using both violet and blue side scatter allows identification of leukocytes in whole blood. This is corroborated by the backgate analysis done using the glycophorin A and CD45 labeling depicted in C, which demonstrates the different scatter properties of leukocytes and red blood cells when using violet side scatter. **(E)** When leukocytes are gated based on violet light scatter properties, the three main leukocyte cell populations in human blood are present: lymphocytes, monocytes, and granulocytes.

The second no-wash, no-lyse strategy involves the use of fluorescent probes or conjugated antibodies that are specific for markers expressed by red blood cells or leukocytes. For example, red blood cells express glycophorin A and white blood cells express CD45. The expression of these two markers is mutually exclusive, allowing clear identification of red vs. white blood cells and exclusion of any red blood cell coincident events (Figure 2A). A third approach is to use one of the Vybrant™ DyeCycle™ series of dyes to label nucleated cells, because mature red blood cells are anucleate (Figure 3A). Necessary materials and suggested workflows for both the violet scatter and fluorescent trigger approaches to the no-wash, no-lyse identification of leukocytes in human whole blood are included here.

Materials

- Whole blood collected into heparinized tubes
- 96-well plates (optional)
- Flow cytometry tubes
- Gibco™ PBS, pH 7.4 (Cat. No. 10010023)
- Attune No-Wash No-Lyse Filter Kit (Cat. No. 100022776)*
- Anti-CD45 antibody (e.g., Cat. No. MHCD4530TR)†
- Anti–glycophorin A antibody (e.g., Cat. No. MHGLA014)†
- Gibco™ RPMI 1640 Medium (Cat. No. 11875119)‡

- Vybrant DyeCycle stain (e.g., Ruby, Cat. No. V10309)‡
- Attune NxT Flow Cytometer

No-wash, no-lyse protocols

Violet side scatter

1. Turn on the instrument; run startup and performance test scripts as normal.
2. Insert the Attune NxT No-Wash No-Lyse Filter Kit components into appropriate locations. VL-1 will serve as your violet side scatter channel (this step must be done after running the performance test).
3. Create a new experiment and a sample workspace containing two dot plots. See Figures 1D and 1E for the recommended gating strategy.
   - Plot A: blue SSC (x-axis) vs. violet SSC (y-axis), both in log scale
   - Plot B: blue FSC (x-axis) vs. violet SSC (y-axis), both in linear scale
4. Pipet 1 µL of blood into 4 mL of PBS, and acquire the sample (without recording) on the Attune NxT Flow Cytometer at a sample collection rate of ≥200 µL/min. Adjust the scatter voltages (blue FSC, SSC, and VL-1 for violet SSC) so that the blood populations appear similar to those seen in Figure 1D.
5. Once optimal instrument settings have been determined, acquire and record data from the sample at a collection rate of ≥200 µL/min.
6. Gate on leukocytes by drawing a polygon gate around the leukocyte population found on the diagonal of plot A (example shown in Figure 1D).
7. Using the leukocyte parent gate, three white blood cell populations can be differentiated from red blood cells in the sample by scatter properties. Figure 1E depicts the lymphocyte, monocyte, and granulocyte populations using this gating strategy.

* For use with violet side scatter approach.
† For use with antibody labeling approach.
‡ For use with Vybrant DyeCycle dye labeling approach.

Antibody labeling

1. Turn on the instrument; run startup and performance test scripts as normal.
2. Create at least 4 samples by pipetting 100 µL of whole blood into sample vessels (microcentrifuge tubes, flow cytometry tubes, or wells within a 96-well plate). Reserve one sample as an unstained control. Additional control samples will be required if more than two conjugated antibodies are used to label the sample.
3. To identify all leukocytes within the sample, label cells in one tube using an antibody directed against the surface marker CD45.
4. To identify red blood cells within the sample, label cells in one tube using an antibody directed against the surface marker glycophorin A.
5. To differentiate white blood cells from red blood cells within a single sample, label cells in the last tube using both the anti-CD45 and anti–glycophorin A antibody conjugates.
6. Incubate all labeling reactions for 20–30 min at room temperature, protected from light. An example of this approach is shown in Figure 2A, in which anti-CD45 Pacific Orange™ conjugate and anti–glycophorin A FITC conjugate were used to label the two cell types. Single-color controls should be included to adjust instrument voltages and compensation (when needed) prior to sample acquisition.
7. Create a workspace on the Attune NxT Flow Cytometer software containing two dot plots as indicated in Figures 2A and 2B:
   - Plot A: CD45 (x-axis) vs. glycophorin A (y-axis)
   - Plot B: blue FSC (x-axis) vs. blue SSC (y-axis)

   Additional plots may be inserted into the workspace as necessary if additional antibody conjugates were used to label cells within the sample.
8. After the 20–30 min incubation, pipet 1 µL of each antibody-labeled sample into 4 mL of PBS.
9. Select one of the single-color control samples and acquire (without recording) at a sample collection rate of ≥200 µL/min on the Attune NxT Flow Cytometer. Adjust PMT voltages such that the glycophorin A–labeled cells and CD45-labeled cells are on scale using the unstained and single-color controls prepared above.

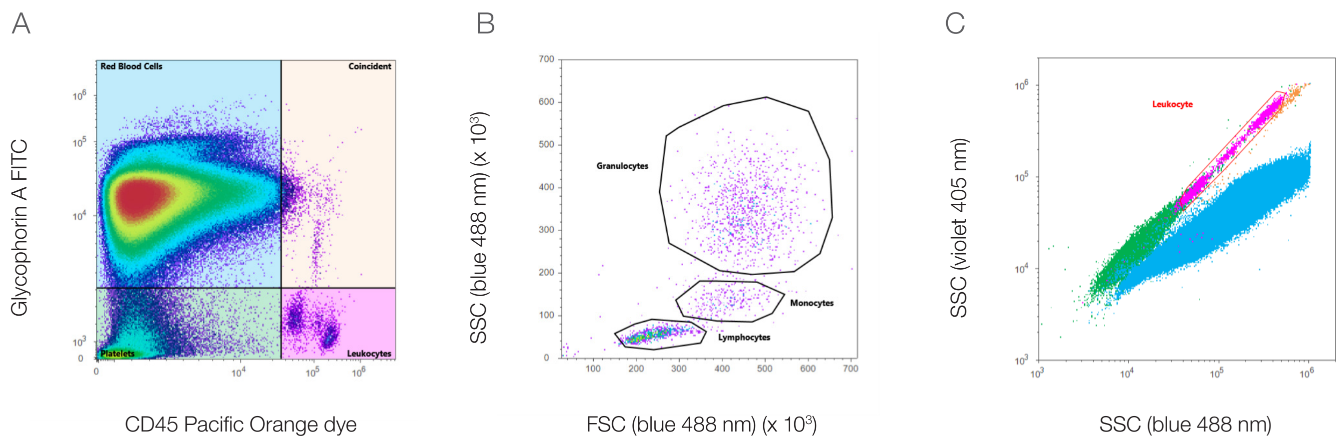

Figure 2. Fluoroescent conjugated antibody approach to identifying leukocytes in whole human blood using the Attune NxT Flow Cytometer. An alternative to the approach depicted in Figure 1 to identify leukocytes in human whole blood is using a fluorescent trigger. **(A)** CD45-expressing leukocytes can be targeted with fluorescently labeled antibodies, in this case anti-CD45 Pacific Orange conjugate. Anti–glycophorin A FITC conjugate is used to eliminate red blood cell coincident events. **(B)** Gating on CD45$^+$, glycophorin A$^-$ cells allows identification of the three primary leukocyte populations in human blood (lymphocytes, monocytes, and granulocytes) using blue forward and side scatter while avoiding CD45$^+$, glycophorin A$^+$ coincident events. **(C)** Combining violet side scatter and backgating analysis demonstrates the accuracy of the fluorescent antibody approach to no-wash, no-lyse applications with human whole blood.

10. While recording, acquire each of the samples at a sample collection rate of ≥200 µL/min until a total number of events that is statistically meaningful for your experiment is collected. After acquiring the sample, view results of the dual-stained sample using the CD45 vs. glycophrin A plot. The CD45$^+$, glycophorin A$^-$ cells (bottom right quadrant, Figure 2A) are the leukocytes within the sample. Using this region as a parent gate, a blue FSC vs. blue SSC dot plot can be used to visualize the three standard white blood cell populations (lymphocytes, monocytes, and granulocytes) based upon their scatter properties (Figure 2B). Violet differential scatter can be used to validate that leukocytes are being analyzed using the antibody labeling approach (Figure 2C).

Vybrant DyeCycle dye labeling

1. Turn on the instrument; run startup and performance test scripts as normal.
2. Pipet 1 µL of whole blood into two flow cytometry tubes containing 500 µL of Gibco RPMI 1640 Medium.
3. Pipet 1 µL of Vybrant DyeCycle dye of choice (Ruby, Green, Orange, or Violet) into one of the tubes from step 2 and incubate the sample for 30 min at 37°C, protected from light. Vybrant DyeCycle Ruby Stain is used as an example in Figure 3.
4. Set up a workspace on the Attune NxT Flow Cytometer software with two dot plots:
   – Plot A: RL-1 (x-axis) vs. blue SSC (y-axis)
   – Plot B: blue FSC (x-axis) vs. blue SSC (y-axis)
5. Following the incubation, add 3.5 mL of Gibco RPMI 1640 Medium to the tube for a total volume of 4 mL.
6. Select the single-color control sample and acquire (without recording) at a sample collection rate of ≥200 µL/min on the Attune NxT Flow Cytometer. Adjust PMT voltages such that the Vybrant DyeCycle dye–labeled cells are on a scale similar to that shown in Figure 3A.
7. While recording, acquire samples on the Attune NxT Flow Cytometer at a sample collection rate of ≥200 µL/min.
8. Vybrant DyeCycle dye–labeled cells should show significant separation from unlabeled cells, as shown in Figure 3A. Gating on the Vybrant DyeCycle dye–labeled cells and assessing their violet side scatter properties relative to Vybrant DyeCycle dye–negative cells demonstrates that leukocytes label with the dye. Furthermore, Vybrant DyeCycle Ruby–labeled cells gated in Figure 3A contain the three standard white blood cell scatter populations (lymphocytes, monocytes, and granulocytes) as shown in Figure 3B, and are readily distinguished from red blood cells when using violet and blue scatter (Figure 3C).

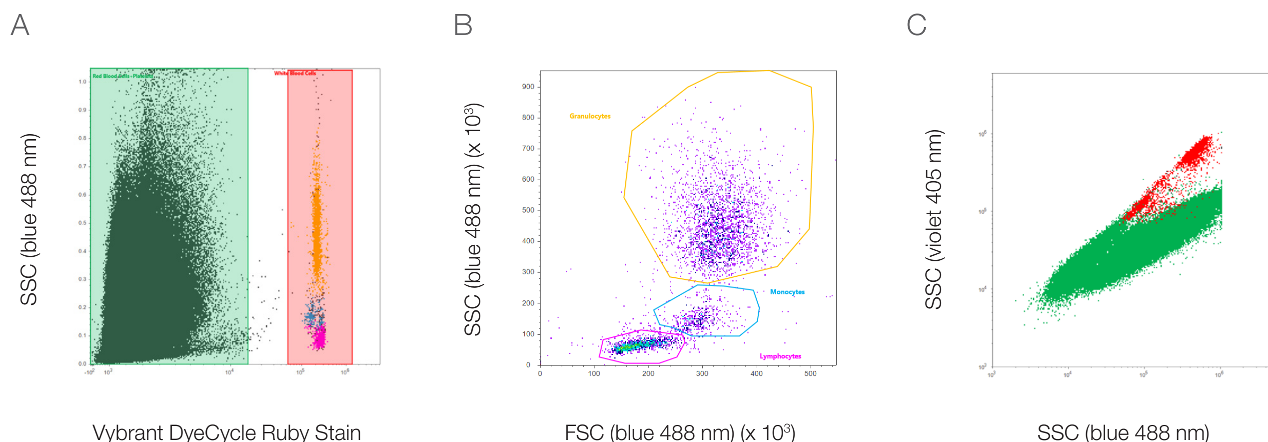

Figure 3. Identification of leukocytes in whole human blood using Vybrant DyeCycle dyes on the Attune NxT Flow Cytometer. The cell membrane–permeant Vybrant DyeCycle dyes label live nucleated cells, thus allowing identification of leukocytes in whole human blood. **(A)** Human whole blood labeled with Vybrant DyeCycle Ruby Stain to identify leukocytes by DNA staining. **(B)** Analysis of the blue forward and side light-scattering properties of the Vybrant DyeCycle Ruby–labeled cells demonstrates the ability of the dye to identify the three main leukocyte populations in human blood: lymphocytes, monocytes, and granulocytes. **(C)** Use of the Attune NxT No-Wash No-Lyse Filter Kit confirms the ability of Vybrant DyeCycle Ruby Stain to identify leukocytes in a whole blood sample.

Conclusion

We have developed three no-wash, no-lyse strategies for identifying leukocytes in human whole blood using the Attune NxT Flow Cytometer. Use of these fluorescent labeling or violet differential scatter strategies helps save time and reduces the incidence of artifacts introduced through sample preparation. It is possible to use any of the above discussed approaches as a stand-alone method for identifying leukocytes in human whole blood for further analysis.

References

1. Gratama JW, Menendez P, Kraan J, Orfao A (2000) Loss of CD34(+) hematopoietic progenitor cells due to washing can be reduced by the use of fixative-free erythrocyte lysing reagents. *J Immunol Methods* 239:13–23.
2. Ost V, Neukammer V, Rinneberg H (1998) Flow cytometric differentiation of erythrocytes and leukocytes in dilute whole blood by light scattering. *Cytometry* 32:191–197.

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