First, do no harm

No-lyse, no-wash assays for the Attune™ NxT Acoustic Focusing Cytometer.

A key objective in many cell biology workflows is to maintain the cells' native characteristics throughout the analysis. Minimal sample preparation and manipulation helps to ensure that researchers are studying the natural state of the cell, and not an artifact of the enrichment or processing protocols. With its limited sample collection rates, however, a conventional hydrodynamic focusing cytometer restricts the volumes that can be analyzed in a timely and efficient manner, necessitating enrichment procedures that can result in phenotypic changes as well as loss of rare cell types [1].

In contrast, acoustic focusing cytometry allows high sample collection rates (up to 1,000 µL/min) without any loss in data resolution, thus reducing the need for pre-acquisition enrichment and allowing for detection of rare events in a heterogeneous population of cells. In addition, difficult-to-collect samples such as mouse blood and bone marrow or thin-needle aspirates can be stained and diluted without washing or performing red blood cell lysis.

Benefits of acoustic focusing for analyzing rare-cell populations

In human whole blood, red blood cells outnumber leukocytes (white blood cells) by ~1,000 to 1. This imbalance creates two hurdles when attempting to analyze leukocytes in whole blood samples without manipulation: (1) collecting a sufficient number of leukocyte events to have statistically meaningful data, and (2) distinguishing leukocytes from red blood cells given the high probability of coincident red blood cell events. Figure 1A shows a conventional 488 nm side scatter (SSC) vs. forward scatter (FSC) profile of whole blood in which the two cell types cannot be resolved; Figure 1B shows improved resolution of the two cell types when violet 405 nm SSC is used.

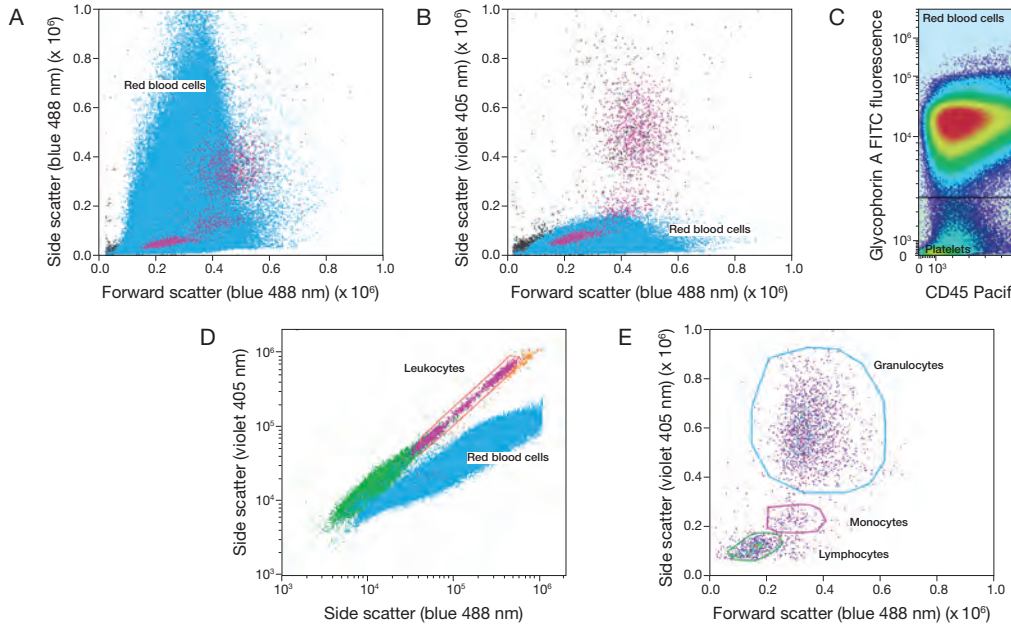

Figure 1. Identification of leukocytes in human whole blood using violet side scatter on the Attune™ NxT Acoustic Focusing Cytometer. (A) Using conventional blue 488 nm forward scatter (FSC) and side scatter (SSC) does not allow resolution of leukocytes in whole blood. Backgate analysis using fluorescently 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 SSC using the Attune™ NxT No-Wash No-Lyse Filter Kit (Cat. No. 100022776). **(C)** Backgate analysis using antibodies against the red blood cell marker glycophorin A (Cat. No. MHGLA014) and the leukocyte marker CD45 (Cat. No. MHCD4530TR) demonstrates the ease of identifying leukocytes in human whole blood, in contrast to the FSC and SSC analyses in **(A)** and **(B)**. **(D)** Using both violet and blue SSC allows identification of leukocytes in whole blood, which is corroborated by the backgate analysis using glycophorin A and CD45 labeling depicted in **(C)**, and demonstrates the different scatter properties of leukocytes and red blood cells when using violet SSC. **(E)** When leukocytes are gated based on violet light scatter properties, the three main leukocyte cell populations in human blood can be distinguished: lymphocytes, monocytes, and granulocytes.

Standard methods for isolating and detecting leukocytes in human whole blood are time consuming and often involve significant enrichment and manipulation prior to analysis. These sample preparation steps can result in alterations in cell physiology and loss of cell types of interest [1]. To overcome sample manipulation and enrichment artifacts and slow sample run times, we have developed three different no-lyse, no-wash protocols for identifying leukocytes in human whole blood on the Attune™ NxT Acoustic Focusing Cytometer, as well as a no-lyse, no-wash assay for phagocytic function.

Three no-lyse, no-wash protocols for leukocyte detection

The first leukocyte detection 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 for human whole blood with blue (488 nm) and violet (405 nm) SSC [2]. Inclusion of the Attune™ NxT No-Wash No-Lyse Filter Kit in the Attune™ NxT Acoustic Focusing Cytometer filter configuration allows simultaneous measurement of both blue and violet SSC and the differentiation of red blood cells and leukocytes based on light-scattering properties alone (Figure 1D), which we have validated using fluorescent antibodies that label CD45-expressing leukocytes or glycophorin A–expressing red blood cells (Figure 1C). The leukocytes can be further differentiated into granulocytes, monocytes, and lymphocytes by drawing a polygon gate around the leukocyte population and then plotting violet SSC vs. blue FSC (Figure 1E).

The next two no-lyse, no-wash strategies involve the use of fluorescent probes that are specific for markers expressed by red blood cells or leukocytes. For example, red blood cells express glycophorin A and leukocytes express CD45. Because the expression of these two markers is mutually exclusive, fluorophore-conjugated antibodies that recognize these two markers allow clear identification of red vs. white blood cells and exclusion of any red blood cell–coincident events (Figure 1C). Alternatively, the Vybrant™ DyeCycle™ dyes are cell-permeant nucleic acid stains that can be used to label nucleated cells. Because mature red blood cells are anucleate whereas leukocytes are nucleated, staining with one of the Vybrant™ DyeCycle™ dyes can distinguish red vs. white blood cells (Figure 2).

A no-lyse, no-wash assay for phagocytic function

We have also developed a no-lyse, no-wash functional assay using pHrodo™ *E. coli* BioParticles™ conjugate to assess phagocyte function in human whole blood using the Attune™ NxT cytometer. Phagocytic cells—such as neutrophils and monocytes—are key components of the innate immune system, serving as a critical line of defense against invading pathogens [3]. Monocytes mature into macrophages or inflammatory dendritic cells upon receiving various stimuli [4]. Characterizing the functional capacity of these phagocytic cell types in a whole blood no-lyse, no-wash assay saves time and reduces the potential artifacts that can be introduced during red blood cell lysis and multiple purification, centrifugation, and wash steps. Moreover, minimal sample processing and short acquisition times help keep cells healthy for multiplex functional assays. →

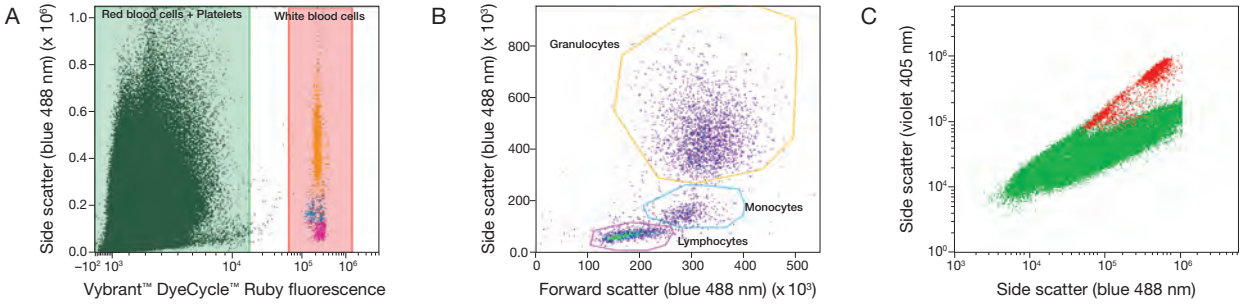

Figure 2. Identification of leukocytes in human whole blood using Vybrant™ DyeCycle™ dyes and the Attune™ NxT Acoustic Focusing Cytometer. The cell membrane–permeant Vybrant™ DyeCycle™ dyes label live nucleated cells, thus allowing identification of leukocytes in human whole blood. **(A)** Human whole blood was labeled with Vybrant™ DyeCycle™ Ruby Stain (Cat. No. V10309) to identify leukocytes by DNA staining. **(B)** Analysis of the blue forward scatter (FSC) and side scatter (SSC) properties of the Vybrant™ DyeCycle™ Ruby Stain–labeled cells discriminates the three main leukocyte populations in human blood: lymphocytes, monocytes, and granulocytes. **(C)** Use of the Attune™ NxT No-Wash No-Lyse Filter Kit (Cat. No. 100022776) with violet differential scatter confirms the ability of Vybrant™ DyeCycle™ Ruby Stain to identify leukocytes in a whole blood sample.

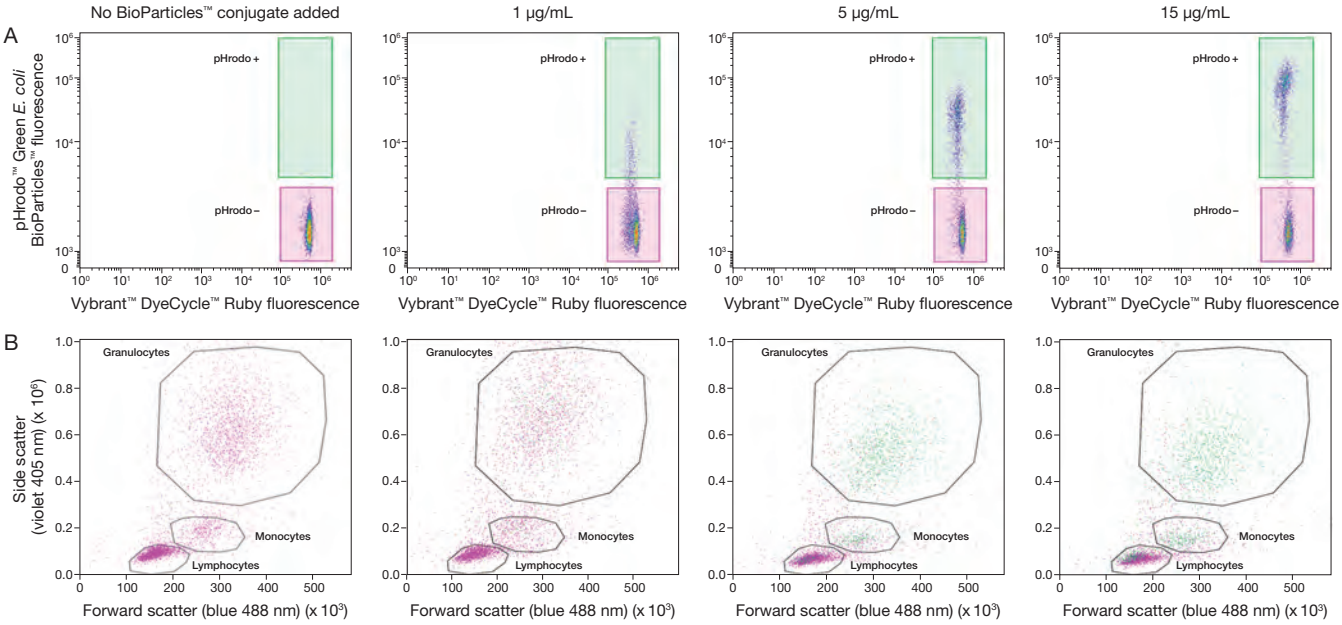

Figure 3. Dose-response plots for pHrodo™ Green *E. coli* BioParticles™ conjugate show multiple phagocytic cell types in a human whole blood no-lyse, no-wash assay. Whole blood was incubated with 1, 5, or 15 µg/mL pHrodo™ Green *E. coli* BioParticles™ Conjugate (Cat. No. P35366) or left untreated for 30 min at 37°C and 5% CO_2, diluted, and then labeled with Vybrant™ DyeCycle™ Ruby Stain for 15 min at 37°C and 5% CO_2. **(A)** As the concentration of pHrodo™ Green BioParticles™ conjugate increases, the frequency of phagocytic cells that are positive for pHrodo™ dye increases along with a shift in green-fluorescent mean fluorescence intensity (MFI) at the higher concentrations of pHrodo™ Green BioParticles™ conjugate. **(B)** At 15 µg/mL pHrodo™ Green BioParticles™ conjugate, it is evident that there are subpopulations within the granulocyte, monocyte, and lymphocyte gates that have actively phagocytosed the pHrodo™ conjugate (green data points).

In our no-lyse, no-wash phagocytic assay, we incubated whole blood with pHrodo™ Green *E. coli* BioParticles™ conjugate for 15 to 30 min, and then labeled cells with Vybrant™ DyeCycle™ Ruby Stain, a cell-permeant nucleic acid dye. pHrodo™ BioParticles™ conjugates are killed bacteria (*E. coli* or *S. aureus*) or yeast zymosan (from *S. cerevisiae*) labeled with pHrodo™ dyes—pH sensors that are nonfluorescent at neutral pH and exhibit increasing fluorescence as pH becomes more acidic. Phagocytic cells that have internalized the pHrodo™ Green or pHrodo™ Red BioParticles™ conjugates into phagosomes (pH ~5) fluoresce green or red, respectively, and this internalization is inhibited at 4°C.

Figure 3A shows that as the concentration of pHrodo™ BioParticles™ conjugate increases, the frequency of phagocytic cells that are positive for pHrodo™ dye increases. Furthermore, the dose-response data coupled with violet SSC vs. blue FSC gating shows the three major leukocyte subpopulations—granulocytes, monocytes, and lymphocytes (Figure 3B). At concentrations of ≤5 µg/mL pHrodo™ BioParticles™ conjugate, neutrophils (within the granulocyte population) and monocytes appear to be the primary phagocytic cell types. As the concentration of pHrodo™ BioParticles™ conjugate increases, greater frequencies of neutrophils and monocytes show a positive pHrodo™ signal, as does a population within the standard lymphocyte gate, which are likely dendritic cells.

Multiplexing with the Attune™ cytometer

With the 4-laser, 16–detection channel Attune™ NxT Acoustic Focusing Cytometer, these no-lyse, no-wash assays can be multiplexed with Molecular Probes™ reagents to identify specific cell types as well as changes in cell function. Learn about applications for the Attune™ NxT cytometer at **thermofisher.com/attuneappnotesbp71**. ■

References

1. Gratama JW, Menendez P, Kraan J et al. (2000) *J Immunol Methods* 239:13–23.
2. Ost V, Neukammer V, Rinneberg H (1998) *Cytometry* 32:191–197.
3. Segal AW (2005) *Annu Rev Immunol* 23:197–223.
4. Tacke F, Randolph GJ (2006) *Immunobiology* 211:609–618.

Product	Quantity	Cat. No.
pHrodo™ Green *E. coli* BioParticles™ Conjugate	5 x 2 mg	P35366
pHrodo™ Green *E. coli* BioParticles™ Phagocytosis Kit	1 kit	P35381
pHrodo™ Green *S. aureus* BioParticles™ Phagocytosis Kit	1 kit	P35382
pHrodo™ Red *E. coli* BioParticles™ Phagocytosis Kit	1 kit	A10025
Vybrant™ DyeCycle™ Green Stain	400 µL	V35004
Vybrant™ DyeCycle™ Orange Stain	400 µL	V35005
Vybrant™ DyeCycle™ Ruby Stain	100 assays	V10309
Vybrant™ DyeCycle™ Violet Stain	200 µL	V35003