

# No-wash, no-lyse detection of phagocytic cells via a pHrodo BioParticles functional assay in human whole blood on the Attune NxT Flow Cytometer

## Introduction

Analysis of biological samples in the most physiologically relevant state with minimal sample preparation and manipulation is a key objective in many cell biology workflows. Normally, whole blood samples require time-consuming enrichment and manipulation in preparation for analysis on conventional hydrodynamic focusing flow cytometers. These manipulations can result in loss of rare cell types and undesirable phenotypic changes [1]. Acoustic focusing cytometry, introduced with the Applied Biosystems™ Attune™ Flow Cytometer, allows high sample collection rates without any loss in data resolution, thus eliminating the need for pre-acquisition enrichment and manipulation and helping to enable 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: 1) collection of a sufficient number of white blood cell events for statistically meaningful data, and 2) distinguishing white blood cells from red blood cells given the high probability of coincident red blood cell events. This application note outlines a strategy for a no-wash, no-lyse approach to a functional assay using Molecular Probes™ pHrodo™ BioParticles™ Conjugates and the Applied Biosystems™ Attune™ NxT Flow Cytometer to assess phagocyte function in human whole blood.



Phagocytic cells are a key component of the innate immune system, serving as a first line of defense against invading pathogens. Neutrophils are the most abundant white blood cells in humans and are often the first cell types recruited to the site of infection where they phagocytose and kill invading bacteria [2]. The significance of neutrophils as a first line of defense against infection is highlighted by certain diseases that result in a reduction of total neutrophil numbers or function, resulting in susceptibility to bacterial infection. There are additional phagocytic cells in blood, including monocytes and dendritic cells. Monocytes mature into cell types such as macrophages or inflammatory dendritic cells upon receiving various stimuli [3]. Dendritic cells are specialized antigen-presenting cells that bridge innate and adaptive immunity. Dendritic cells acquire bacterial antigens through phagocytosis and present these antigens to T cells of

the adaptive immune system, which are critical players in mediating protection against certain pathogens [4]. Phagocytosis can be detected using pHrodo BioParticles Conjugates—pH-sensitive reagents that fluoresce upon their ingestion into acidic phagosomes. Characterizing the functional capacity of these phagocytic cell types in a whole blood no-wash, no-lyse assay can save time and reduce the potential artifacts that are introduced in alternative protocols that require red blood cell lysis and multiple purification and wash steps.

## Materials

- Whole blood
- 96-well plates
- Flow cytometry tubes
- Gibco™ RPMI 1640 Medium (Cat. No. 11875119)
- Molecular Probes™ Vybrant™ DyeCycle™ stain (e.g., Ruby, Cat. No. V10309)\*
- pHrodo BioParticles Conjugates (choose one or more per experiment)
  - pHrodo Red *E. coli* BioParticles Conjugate (Cat. No. P35361)
  - pHrodo Red *S. aureus* BioParticles Conjugate (Cat. No. A10010)
  - pHrodo Green *E. coli* BioParticles Conjugate (Cat. No. P35366)
  - pHrodo Green *S. aureus* BioParticles Conjugate (Cat. No. P35367)
- Attune NxT Flow Cytometer

## Protocol

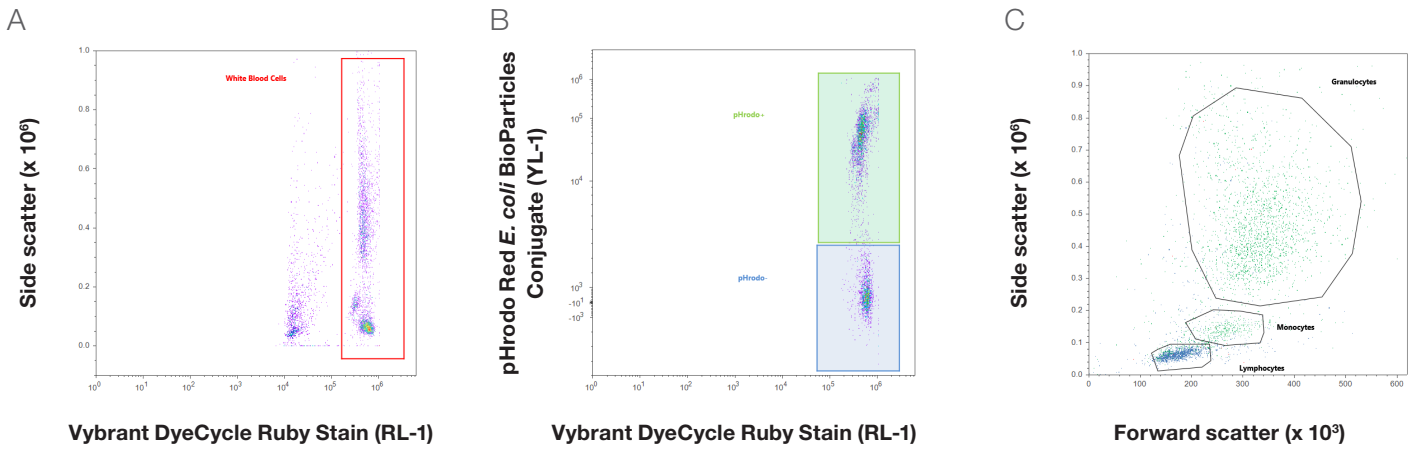
### Sample setup

1. Transfer 50  $\mu$ L aliquots of whole blood to each of two 96-well plates; the number of wells will depend on the number of assay conditions. One plate will be kept at 37°C and 5% CO<sub>2</sub> and the other plate at 4°C as a negative control.
2. Add the concentration indicated in the product insert of the pHrodo *E. coli* or *S. aureus* BioParticles Conjugate to the wells, being sure to include control wells with blood only and the pHrodo BioParticles Conjugate only.
3. Bring volumes up to 100  $\mu$ L per well with RPMI 1640 Medium; incubate one plate at 37°C and 5% CO<sub>2</sub> and the other plate at 4°C for 15–30 min.
4. Prepare flow cytometry tubes with 500  $\mu$ L of RPMI 1640 Medium and 1  $\mu$ L of Vybrant DyeCycle dye.
5. Transfer 1–5  $\mu$ L from each well of the 96-well plate into the tubes prepared in step 4; incubate all tubes for 15 min at 37°C and 5% CO<sub>2</sub>.
6. Dilute the samples to 4 mL with RPMI 1640 Medium in each flow cytometry tube.
7. Set up the Attune NxT Flow Cytometer (see the instrument setup protocol).
8. Acquire the samples on the Attune NxT Flow Cytometer.

### Instrument setup

1. Turn on the Attune NxT Flow Cytometer and run startup and performance tests.
2. Create a new experiment, and in the first sample workspace insert 3 dot plots. See Figure 1 for parameters and recommended gating strategy.
3. Use the blood-only control that is stained with your Vybrant DyeCycle dye of choice to set a threshold on the cells positive for Vybrant DyeCycle dye in plot A.

\*There are a variety of Vybrant DyeCycle dyes with multiple color options.

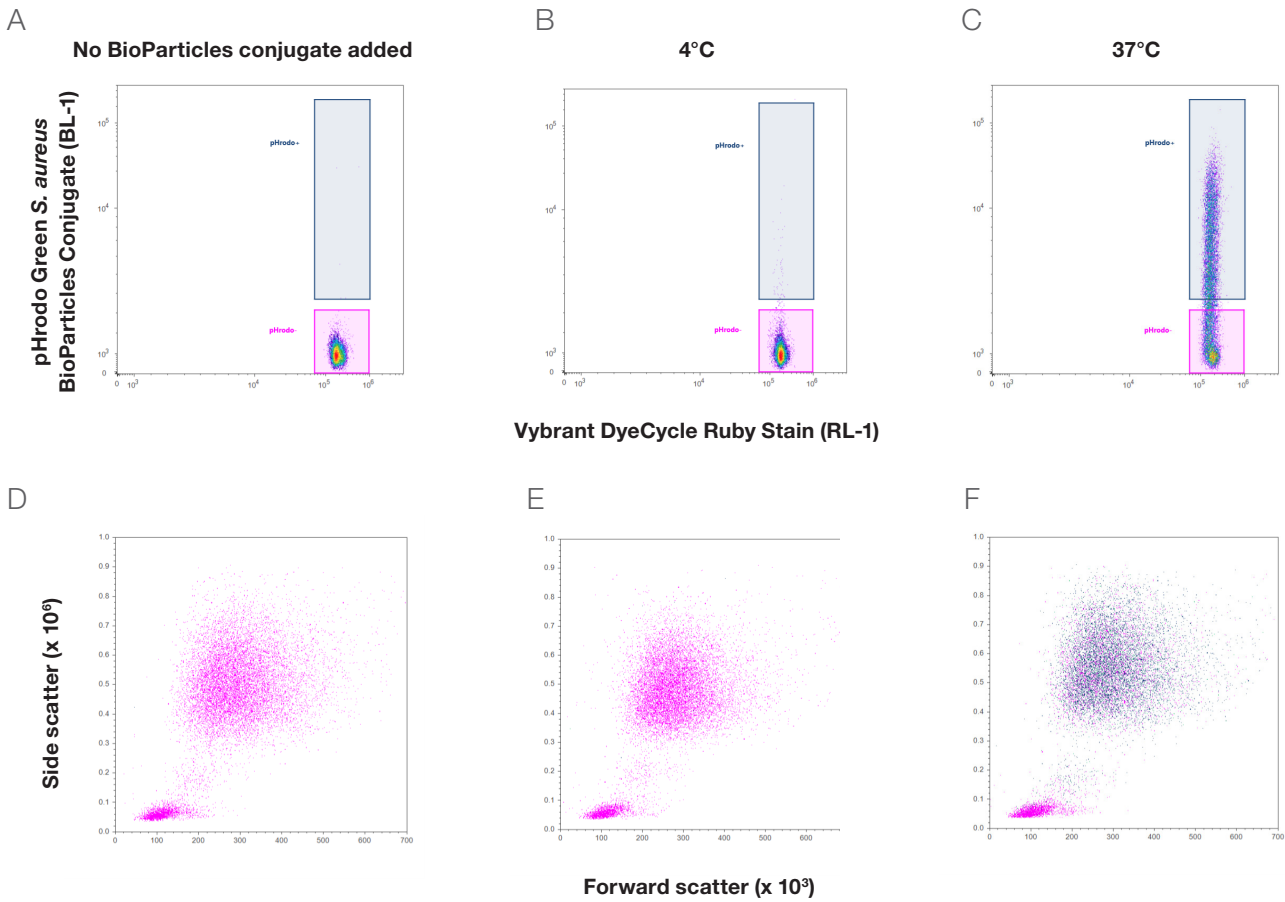


**Figure 1. Gating strategy for identifying phagocytes using Vybrant DyeCycle Ruby Stain and pHrodo BioParticles Conjugates in a whole blood no-wash, no-lyse assay on the Attune NxT Flow Cytometer.** Human whole blood was incubated with 15 µg/mL pHrodo Red *E. coli* BioParticles Conjugate for 30 min at 37°C and 5% CO<sub>2</sub>, diluted, and then labeled with Vybrant DyeCycle Ruby Stain for 15 min at 37°C and 5% CO<sub>2</sub>. **(A)** Threshold set based on the Vybrant DyeCycle Ruby Stain signal, which labels nucleated white blood cells. **(B)** pHrodo Red *E. coli* BioParticles Conjugate signal in phagocytic white blood cells. Backgating on cells that are positive and negative for pHrodo BioParticles Conjugate in a scatter plot **(C)** demonstrates which cell populations in human whole blood are phagocytic.

4. The blood-only control should be negative for the pHrodo BioParticles Conjugate signal; adjust the corresponding channel's voltage to reflect this in plot B.
5. Draw a gate around the population negative for the pHrodo BioParticles Conjugate, and another gate about where the population positive for the pHrodo BioParticles Conjugate will lie, as shown in plot B.
6. In plot C, adjust the forward scatter and side scatter voltages such that the three main leukocyte populations are evident: lymphocytes, monocytes, and granulocytes.
7. To include both cell scatter profiles in plot C that are positive and negative for the pHrodo BioParticles Conjugate, first ensure that the parent gate for plot C is the Vybrant DyeCycle dye gate drawn in plot A. Right click on plot C, select Back Gates, and then select the pHrodo BioParticles Conjugate gates and negative gates that were drawn in plot B. The colors of the populations in plot C should match the colors of the gates in plot B.
8. Run a positive control sample that contains blood, pHrodo BioParticles Conjugate, and a Vybrant DyeCycle dye, and make appropriate adjustments to gates.
9. Acquire samples at collection rates of  $\geq 200$  µL/min.

## Results and conclusions

Whole blood was incubated with various concentrations of pHrodo BioParticles Conjugate for 15–30 min followed by labeling with Vybrant DyeCycle Ruby Stain. Phagocytic cells that take up pHrodo BioParticles Conjugates into their acidic phagosomes acquire a positive signal; this allows comparison of cells that have undergone phagocytosis with those that have not (Figures 1B, 1C). The no-lyse, no-wash pHrodo BioParticles phagocytosis assays are highly specific,



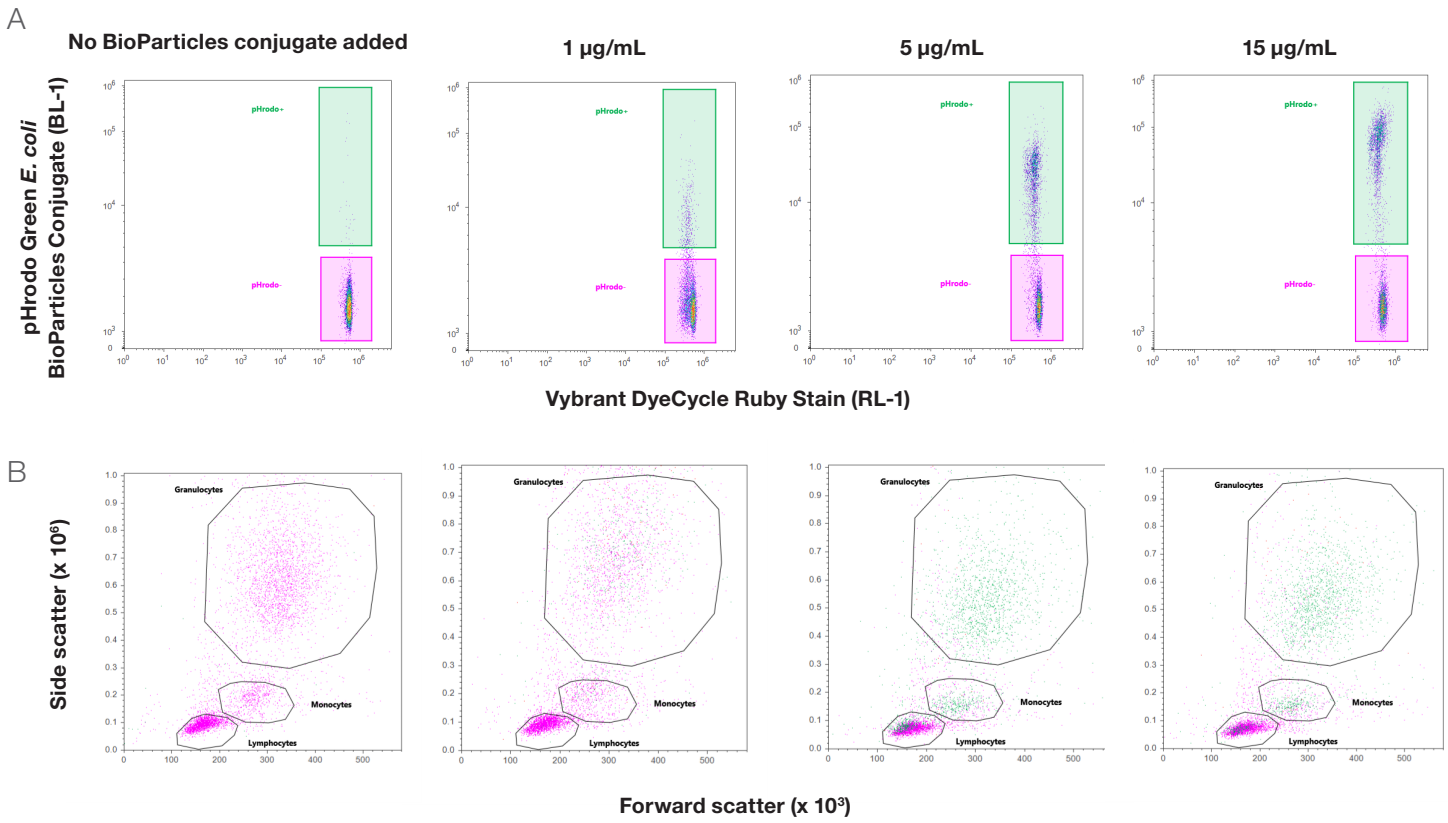
**Figure 2. A no-wash, no-lyse assay for identifying phagocytes in whole human blood on the Attune NxT Flow Cytometer.** Whole blood was incubated with or without 5  $\mu\text{g}/\text{mL}$  pHrodo Green *S. aureus* BioParticles Conjugate for 30 min at 4°C or 37°C and 5%  $\text{CO}_2$ , diluted, and then labeled with Vybrant DyeCycle Ruby Stain for 15 min at 37°C and 5%  $\text{CO}_2$ . There is very little background fluorescence present when whole blood is cultured with 5  $\mu\text{g}/\text{mL}$  pHrodo Green *S. aureus* BioParticles Conjugate at 4°C (**B, E**) compared to the strong signal seen at 37°C (**C, F**), demonstrating the specificity and efficacy of this reagent in a whole blood no-lyse, no-wash assay.

as shown by the lack of signal when phagocytosis is inhibited at 4°C (Figure 2, middle column). Furthermore, neutrophils (a highly phagocytic cell type) are the primary cell type that acquires a positive pHrodo dye signal, as expected (blue dots in Figure 2C and 2F).

A dose response to pHrodo Green *E. coli* BioParticles Conjugate elucidates the presence of multiple phagocyte subpopulations within the three major leukocyte scatter populations (Figure 3). At concentrations of 5  $\mu\text{g}/\text{mL}$  pHrodo Green *E. coli* BioParticles Conjugate or less, neutrophils and monocytes appear to be the primary phagocytic cell types. As the concentration of pHrodo Green *E. coli* BioParticles Conjugate increases, greater frequencies

of neutrophils and monocytes show a positive pHrodo signal, and a population within the standard lymphocyte gate acquires a positive pHrodo signal; these are likely dendritic cells (Figure 3, far right column).

With a 4-laser Attune NxT Flow Cytometer, this assay could be further multiplexed to identify specific cell types and changes in activation state due to phagocytic activity. Additional functional probes could be included to detect oxidative burst, a critical microbicidal function in phagocytes. The Attune NxT Flow Cytometer coupled with Molecular Probes reagents allows analysis of biological samples with no manipulation and minimal preparation.



**Figure 3. A pHrodo Green *E. coli* BioParticles Conjugate dose response reveals multiple phagocytic cell types in a human whole blood no-wash, no-lyse assay.** Whole blood was incubated with 1, 5, or 15  $\mu\text{g/mL}$  pHrodo Green *E. coli* BioParticles Conjugate or left untreated for 30 min at 37°C and 5%  $\text{CO}_2$ , diluted, and then labeled with Vybrant DyeCycle Ruby Stain for 15 min at 37°C and 5%  $\text{CO}_2$ . **(A)** As the concentration of pHrodo BioParticles Conjugate increases, the frequency of phagocytic cells that are positive for pHrodo dye increases along with a shift in green fluorescence MFI at the higher concentrations of pHrodo BioParticles Conjugate. **(B)** At 15  $\mu\text{g/mL}$  of the pHrodo BioParticles Conjugate, it is evident that there are subpopulations within the granulocyte, monocyte, and lymphocyte gates that are actively phagocytosing pHrodo BioParticles Conjugate.

## References

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2. Segal AW (2005) How neutrophils kill microbes. *Annu Rev Immunol* 23:197–223.
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