A No-Lyse, No-Wash Approach to Characterizing Phagocyte Phenotype and Function in Whole Human Blood on the Attune® NxT Acoustic Focusing Cytometer

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Abstract

Analysis of biological samples in the most physiologic state with minimal sample preparation and manipulation is a key objective to any workflow. However, whole human blood samples generally require significant enrichment and manipulation to remove red blood cells for analysis of leukocytes on conventional flow cytometers. Sample manipulation such as wash/centrifugation and red blood cell lysis can result in sample loss and alter the phenotype of populations of interest. Acoustic focusing cytometry allows for high sample collection rates without compromising data integrity, thus allowing for the analysis of whole blood samples in a no-lyse, no-wash format. We have developed multiple no-lyse, no-wash assays to characterize phagocyte phenotype and function in human whole blood. Neutrophils are the most abundant leukocyte in human blood and their antimicrobial properties are essential for defending the host from infection. A primary antimicrobial mechanism utilized by neutrophils is phagocytosis followed by releasing bactericidal superoxides into the phagosome[1]. Here we utilize the rapid sample collection capabilities of the Attune® NxT Acoustic Focusing Cytometer to characterize phagocyte function in human whole blood with a pHrodo® BioParticles® phagocytosis/phagosome acidification assay and also a dihydrorhodamine 123 superoxide production assay, in a no-lyse, no wash format. Incubating whole blood with *E. coli* or *S. aureus* pHrodo® BioParticles® resulted in phagocytosis as shown by an increase in pHrodo® fluorescence. As expected, phagocytosis is primarily mediated by neutrophils and monocytes as shown by increased phagosome acidification in those cell types, however, there is a small phagocytic population within the lymphocyte gate. In addition, we are able to demonstrate monocyte maturation into dendritic cells based on upregulation of CD11c and HLA-DR with increasing doses of pHrodo® BioParticles®. These no-lyse, no-wash assays streamline workflows and limit artifacts introduced by sample manipulation and preparation steps.

Acoustic Focusing Cytometry

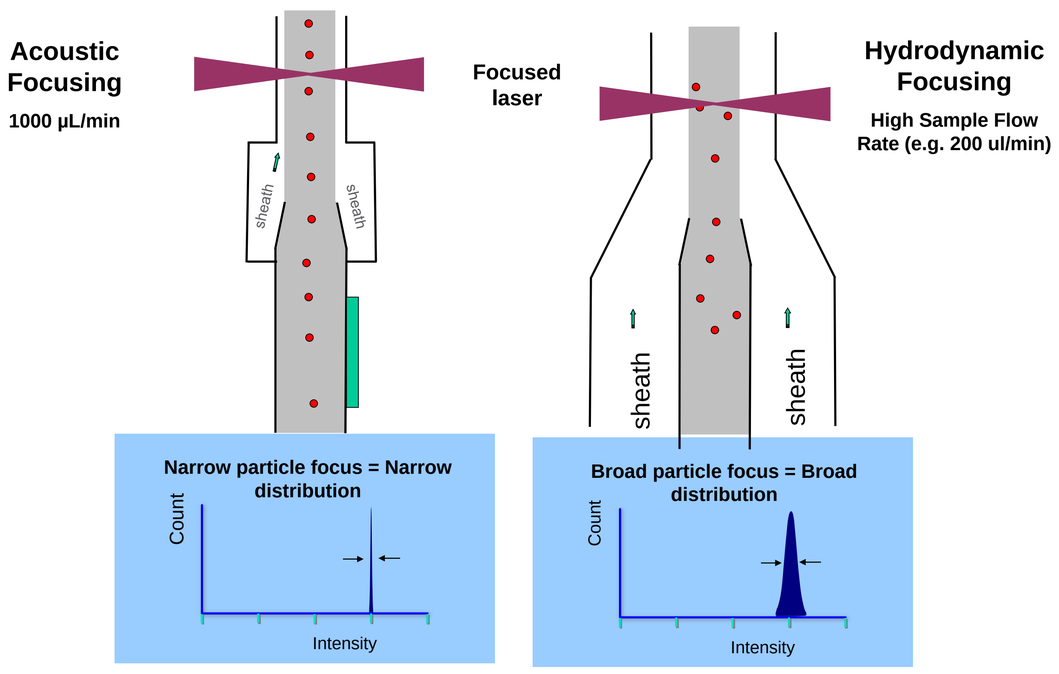

FIGURE 1. Acoustic focusing cytometers are able to achieve significantly higher sample collection rates compared to hydrodynamic instruments without any loss in data resolution. Standard hydrodynamic focusing flow cytometers are able to achieve sample collection rates up to ~200 µL/min, however this does result in a loss in data resolution capability due to events pass through the flow cell outside of the focal point of the laser. This results in an increase in CVs (right panel). Acoustic focusing allows for a sample collection rates up to 1000 µL/min without any loss in data resolution because the focusing occurs independently of the sample collection rate (left panel).

pHrodo® BioParticles® No-Lyse, No-Wash Experimental Approach

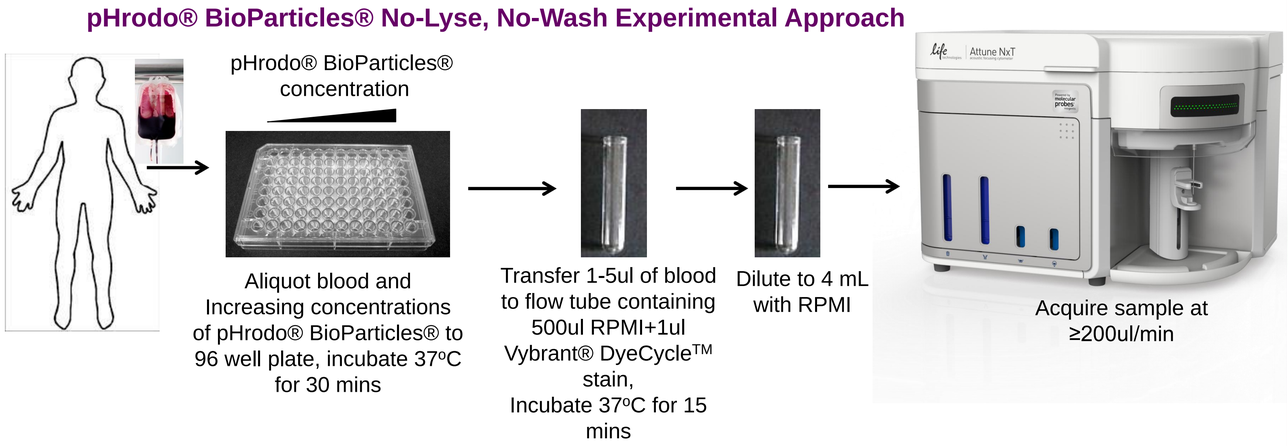

FIGURE 2. No-lyse, no-wash pHrodo® BioParticles® assay workflow. Human blood was aliquoted into 96 well plate and cultured with increasing concentrations of pHrodo® BioParticles® for 30 mins at 37ºC. After 30 mins 1 µL aliquots were then labeled with Vybrant® DyeCycle™ Violet for 15 mins at 37oC. Volume was brought up to 4 mL with Gibco® RPMI and then samples were acquired on the Attune NxT Acoustic Focusing Cytometer at sample collection rates of 200-500 µL/min.

Results

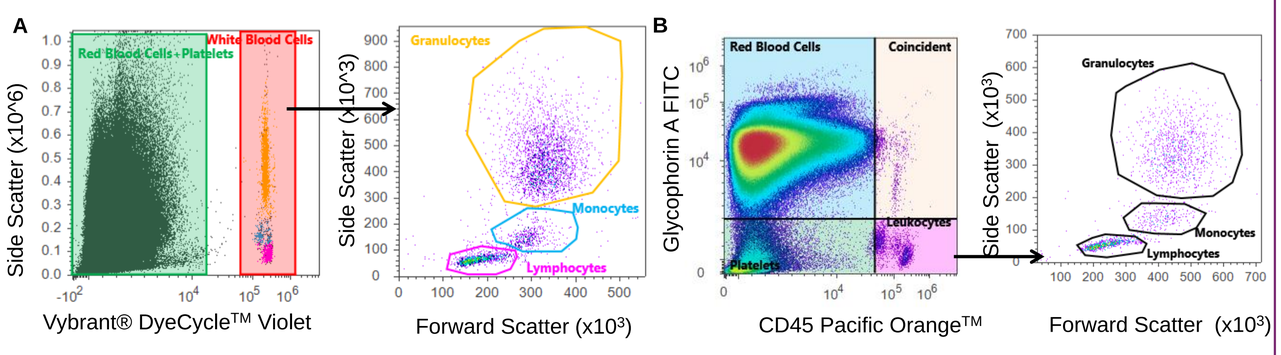

FIGURE 3. No-lyse, no-wash approaches to detecting leukocytes in whole human blood. Red blood cells outnumber white blood cells ~1000:1 making identification of white blood cells via flow cytometry difficult without significant sample manipulation and enrichment. The high sample collection rates enabled by acoustic focusing technology allows for the collection of large sample volumes in short period of time. A) Vybrant® DyeCycle™ stains identify live, nucleated cells in a human blood sample. Analyzing the forward and side scatter properties of the nucleated cells demonstrates the ability of the DyeCycle™ stain to identify leukocytes in whole blood.

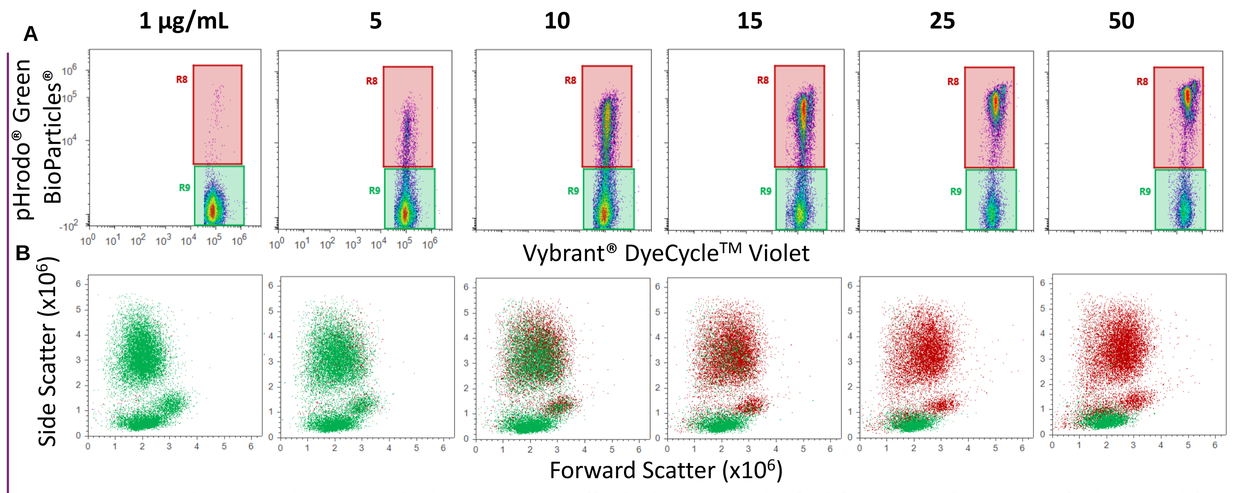

FIGURE 4. Characterization of phagocytic cells in human whole blood. Un-lysed whole human blood from healthy donors was cultured with increasing concentrations of pHrodo® Green *E. coli* BioParticles® for 30 mins at 37ºC. A 1-5 µL aliquot of cultured blood was then resuspended in 500 µL of Gibco® RPMI with 1 µL of Vybrant® DyeCycle™ Violet for 15 mins at 37ºC. Following incubation volume was increased to 4 mL by adding 3.5 mL of Gibco® RPMI to each tube. Data was then acquired on the Attune® NxT Acoustic Focusing Cytometer at a sample collection rate of 200 µL/min. A) Increasing frequencies of phagocytic cells present in whole human blood after culture with pHrodo® Green *E. coli* BioParticles® at 1 to 50 µg/mL. B) Backgate analysis of the pHrodo+ and pHrodo- populations in panel A. Phagocytic cells primarily lie within the high SSC granulocyte populations and intermediate SSC monocyte population. At the highest concentrations of BioParticles® there is a small population of phagocytic cells within the lymphocyte low SSC population.

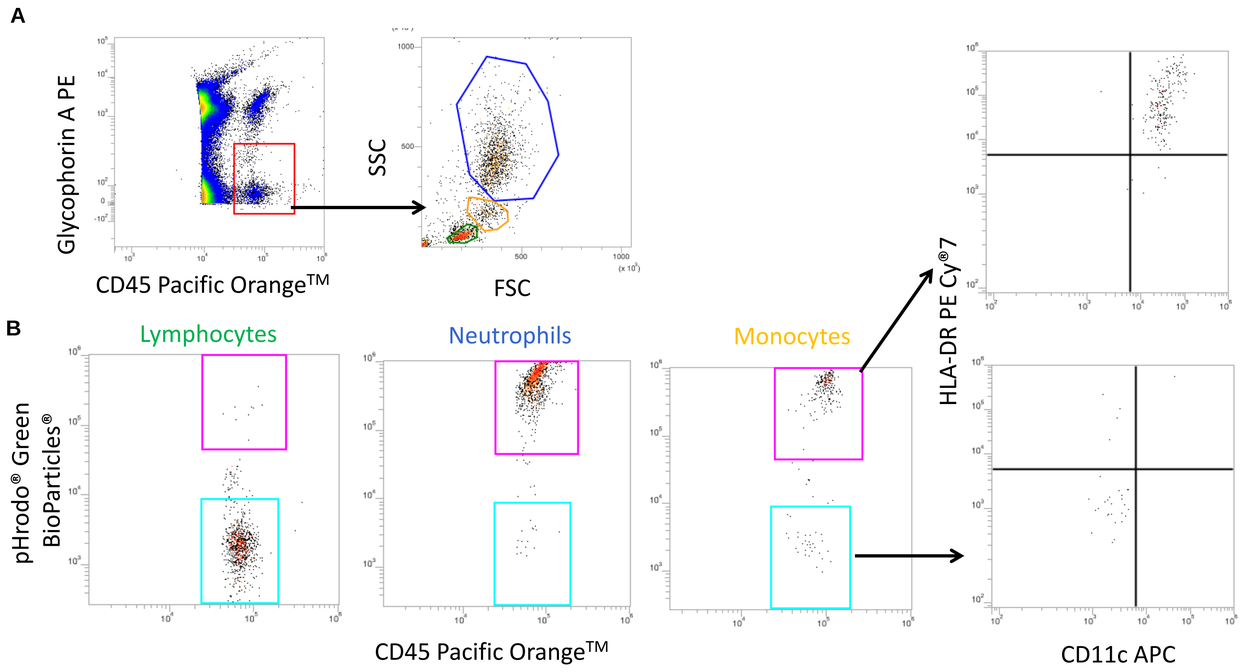

FIGURE 5. Monocyte maturation in a whole blood no-lyse, no-wash Assay using pHrodo® BioParticles®. Blood was cultured with 15 µg/mL pHrodo® Green *S. aureus* BioParticles® for 30 mins at 37ºC and then in the presence of fluorochrome conjugated antibodies against Glycophorin A, CD45, CD11c and HLA-DR for 20 mins at room temperature. 1 µL of culture blood was diluted in 4 mL of PBS. A) Identification of white CD45+Glycophorin A- white blood cells. B) Characterization of phagocytic capacity of lymphocytes, neutrophils, and monocytes based on phagosome acidification. Monocytes are highly phagocytic with the vast majority of monocytes, identified based on scatter properties, demonstrating phagosome acidification. Furthermore, pHrodo+ monocytes have a dendritic cell-like phenotype expressing CD11c and HLA-DR, whereas pHrodo- monocytes are primarily CD11c^{LO}HLA-DR^{LO}.

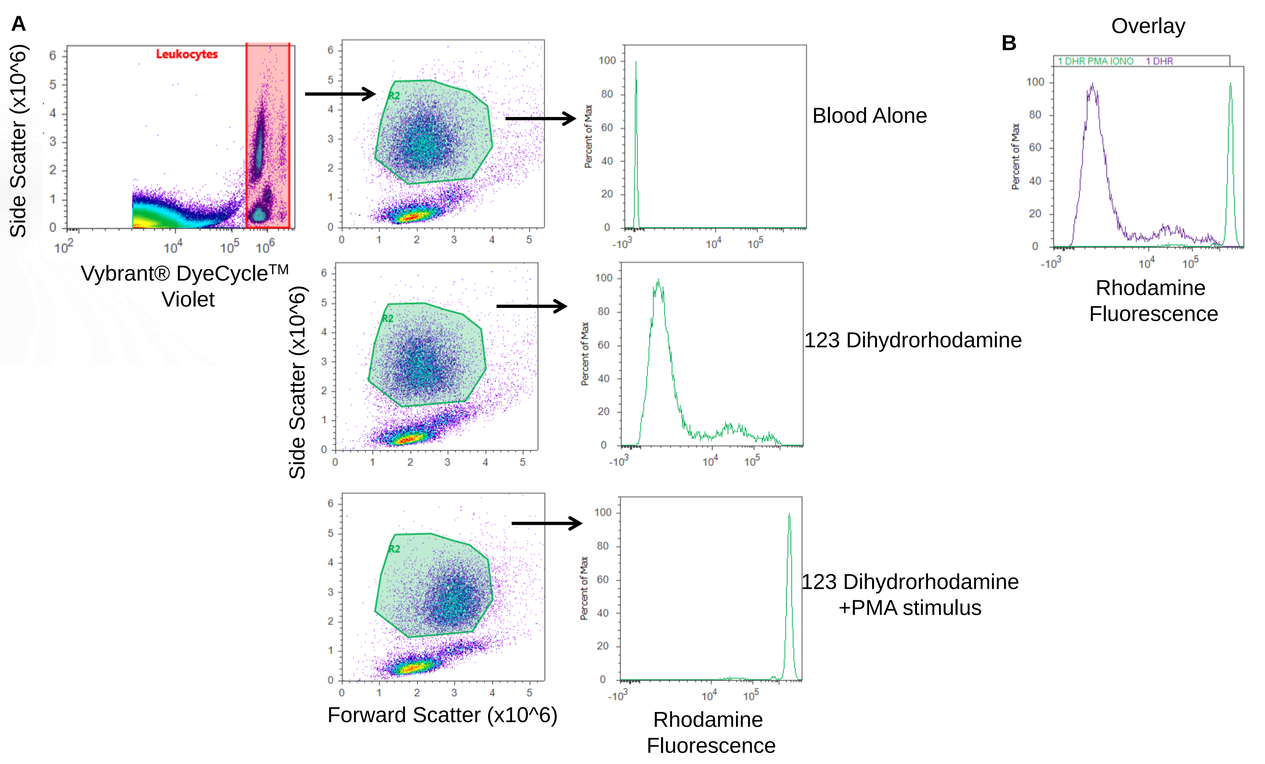

FIGURE 6. No-lyse, no-wash detection of superoxide production by granulocytes in response to stimulation with PMA. 1 uL of whole blood was labeled with 123 dihydrorhodamine for 15 mins at 37ºC and then labeled with Vybrant® DyeCycle™ Violet in the presence of PMA for 15 mins at 37ºC in 500 uL Gibco® RPMI. Samples were then diluted to 4 mL with RPMI and analyzed for superoxide production as measured by an increase in Rhodamine fluorescence. A) PMA stimulated neutrophils have significant rhodamine signal due to superoxide production, B) compared to control treated cells.

Conclusions

Flow cytometry-based analysis of white blood cells generally requires multiple enrichment and wash steps prior to analysis due to the abundance of red blood cells. No-lyse, no-wash assays simplify this work flow by eliminating the need for red blood cell lysis or washing prior to acquisition. In addition, this approach is compatible with functional assays, such as pHrodo® BioParticles® and immunophenotyping approaches.

References

1. Vowells *et al*., *Journal of Immunological Methods* 1995. 178(1):89-97

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