No-Lyse No-Wash Immunophenotyping Using Acoustic Cytometry

Jolene A Bradford and Rick Kerndt, Life Technologies, Eugene, Oregon, USA

Introduction: Immunophenotyping whole blood is a primary application in the study of white blood cell populations and their function. Red blood cells (RBCs) traditionally have been removed during sample preparation by lysis methods or by density-gradient selection, followed by washing steps to remove red blood cell fragments and platelets. This processing of whole blood can result in significant cell loss and damage thru inadvertent lysis, unintentional selection, or washing.[1] The application of a No-Lyse No-Wash protocol in human whole blood samples, where blood draw volumes are generally not as limited, benefit from a simpler streamlined protocol that also reduces the potential of losing the cells of interest. Mouse whole blood presents additional challenge due to the limited sample volume available (≤100 µL/day/animal), particularly in longitudinal studies.[2,3] Specifically, these small volumes limit the ability to perform multicolor immunophenotyping experiments with the required compensation and fluorescence-minus-one (FMO) controls.[4,5]

Methods previously describe using a No-lyse No-wash staining protocol which sacrifice light scatter resolution.[6] In conventional flow cytometry (which uses hydrodynamic focusing to center the cells in a fluid stream), accurate identification of some cell populations depends on high-resolution scatter data, so these No-lyse No-wash protocols are not feasible using a hydrodynamic instrument platform. In addition, the sample dilution required in No-lyse No-wash methods (to achieve low coincidence with red blood cells and platelets) generally dilutes the cell sample to such an extent that the time required to acquire sufficient events at flow rates available in these instruments is exceedingly long.

Methods: We describe a No-lyse No-wash method which takes advantage of the acoustic focusing technology offered by the Attune® Acoustic Cytometer. The Attune® cytometer aligns cells in the core stream using acoustic forces that are independent of the fluid stream. This allows a precise alignment of cells in the core and much higher throughput is possible than with traditional flow cytometry. Immunophenotyping panels are shown for both human and mouse whole blood. A total of 10µL whole blood is stained in a 50 µL total volume and then diluted 400-fold in PBS (2 mL final volume). A fluorescence threshold is used to distinguish the white blood cell population from the much more abundant red blood cell population.

In the mouse immunophenotyping example, CD45 fluorescence is used to threshold on white blood cells. For the human immunophenotyping example, a cell permeable nucleic acid stain, Vybrant® DyeCycle™ Ruby stain, is used to threshold on all nucleated cells. At the 400-fold dilution, the coincidence of the target population with red blood cells and platelets is reduced sufficiently so scatter signals (necessary for accurate differentiation of the white blood cell populations) are reliable. Acquisition times for such dilute samples are still completed in a reasonable 1–2 minutes, where a traditional hydrodynamic focusing requires 8–10 minutes per sample.

Conclusions: Significantly higher sample collection rates allow the Attune® Acoustic Cytometer with Violet/Blue laser option to deliver a No-lyse No-wash protocol to minimize cell loss and simplify sample preparation. Difficult-to-collect samples like mouse blood and bone marrow, thin-needle aspirates, or any sample with low cell yield can be stained and diluted without washing or performing RBC removal procedures. No sample loss occurs from sample preparation, and full panel testing is possible for all precious samples. All sample prep steps can be eliminated without compromise to the data.

Figure 1. Comparison of Acoustic and Hydrodynamic Focusing Methods

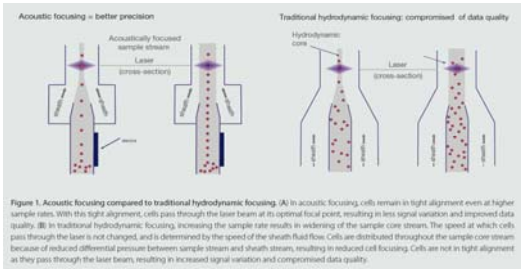

Figure 1. Acoustic focusing compared to traditional hydrodynamic focusing. (A) In acoustic focusing, cells remain in tight alignment even at higher sample rates. With this tight alignment, cells pass through the laser beam at its optimal focal point, resulting in less signal variation and improved data quality. (B) In traditional hydrodynamic focusing, increasing the sample rate results in widening of the sample core stream. The speed at which cells pass through the laser is not changed, and is determined by the speed of the sheath fluid flow. Cells are distributed throughout the sample core stream because of reduced differential pressure between sample stream and sheath stream, resulting in reduced cell focusing. Cells are not in tight alignment as they pass through the laser beam, resulting in increased signal variation and compromised data quality.

References:
1. Gratama JW, Menendez.P, KraanJ et al. (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. Hoff J (2000) Methods of blood collection in the mouse. *Lab Animal* 29:47-53.
3. Waynforth HB, Flecknell PS (1992) Experimental and Surgical Technique in the Rat. 2nd Ed. Academic Press Ltd.
4. M Roederer, *Compensation in Flow Cytometry*. Current Protoc. Cytometry (2002) 1.14.1-1.14.20
5. . Maecker, H. T., and Trotter, J. (2006) Flow cytometry controls, instrument setup, and the determination of positivity, *Cytometry A 69*, 1037-1042.
6. JL Weaver, *Phenotypic Analysis Using Very Small Volumes of Blood.* Curr. Protoc. Cytom. 54:6.30.1-6.30.8

Human Whole Blood with No-Lyse No-Wash

- Human blood is used in this example, but this technique may be used with any mammalian blood
- 10µL whole blood is stained in a 50 µL total volume of antibody cocktail, and then diluted 400 fold (2 mL final volume)
- A cell-permeant DNA stain, Vybrant® DyeCycle™ Ruby, was added to label all nucleated cells
- A fluorescence threshold based on the Vybrant® DyeCycle™ Ruby fluorescence was used to select the nucleated cell population from the non-nucleated red blood cells
- The antibody cocktail includes mouse anti-human monoclonal antibody conjugates: CD3 FITC, CD56 PE, CD8 Pacific Blue™, CD4 V500, and CD19 QDot® 605
- To set compensation, AbC™ anti-mouse Compensation Beads were used for single color controls
- Fluorescence-Minus-One (FMO) controls are used to determine appropriate marker placement
- Data was acquired on the Attune® Acoustic Cytometer with Violet/Blue laser option using a collection rate of 500 µL/minute

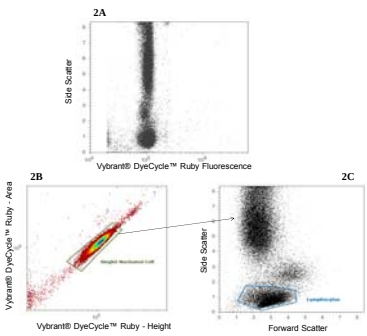

Figure 2: Threshold and Gating Strategy

2A. Dot plot of Vybrant® DyeCycle™ Ruby Stain vs Side Scatter showing the fluorescence threshold level on human peripheral whole blood. The flourescence threshold is optimized to eliminate all non-nucleated cells and debris.

2B. Height vs. Area density plot of Vybrant® DyeCycle™ Ruby fluorescence with a polygon gate selecting singlet nucleated cells. This gate is used for further gating in 1C.

2C: A daughter dot plot of the singlet nucleated cell gate gated in 1B is used to look at forward vs. side scatter and create a polygon gate around the lymphocyte population, used for further analysis of subpopulations.

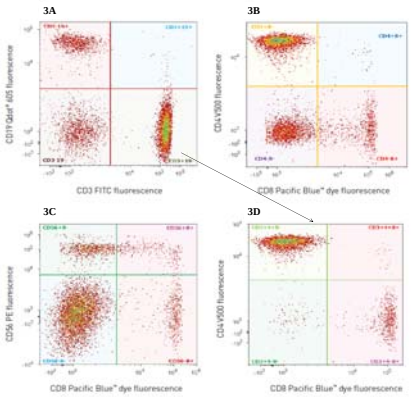

Figure 3. Immunophenotyping

3A. T and B cell populations with CD3 FITC vs. CD19 Qdot® 605 bivarient density plot. Gating is on singlet lymphocytes.

3B. T-helper (CD4 V500) and T-cytotoxic (CD8 Pacific Blue™) cell subpopulations in a bivarient density plot. Gating is on singlet lymphocytes.

3C. Natural killer (NK) (CD56 PE) and T-cytotoxic (CD8 Pacific Blue™) population subsets in a bivarient density plot. Gating is on singlet lymphocytes.

3D. The CD3+CD19- population from plot 2B is used to provide gating for this bivarient density plot with CD8 Pacific Blue™ and CD4 V500.

Mouse Whole Blood with No-Lyse No-Wash

- Mouse (BALB/CJ) whole blood was used in this example, but this technique may be used with any mammalian blood
- 10µL whole blood is stained in a 50 µL total volume of antibody conjugates and then diluted 400 fold (2 ml final volume)
- A 5 color panel included rat anti-mouse CD45 FITC (pan-leukocyte), rat anti-mouse CD11b PE (monocytes, granulocytes, macrophages, dendritic), hamster anti-mouse CD3e PE-Cy® 5 (T cells), rat anti-mouse CD45R Pacific Blue™ (B220, B cells), and rat anti-mouse GR-1 (Ly-6C/G, granulocytes) direct conjugates were used
- To set compensation, AbC™ anti-Rat/Hamster Compensation Beads were used for single color controls
- A fluorescence threshold based on the CD45-FITC fluorescence was used to select the CD45+ population for analysis
- Fluorescence-Minus-One (FMO) controls were used to determine appropriate marker placement
- Data was acquired on the Attune® Acoustic Cytometer with Violet/Blue laser option using a collection rate of 500µL/minute

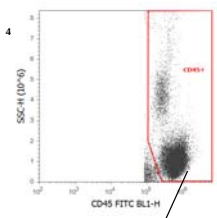

Figure 4. Dot plot of CD45 FITC vs side scatter showing the threshold level and CD45+ gate. Threshold was set below the level of granulocyte CD45 FITC fluorescence. A CD45+ polygon gate was drawn to include lymphocyte and granulocyte populations and the exclude the lower cellular debris fluorescence.

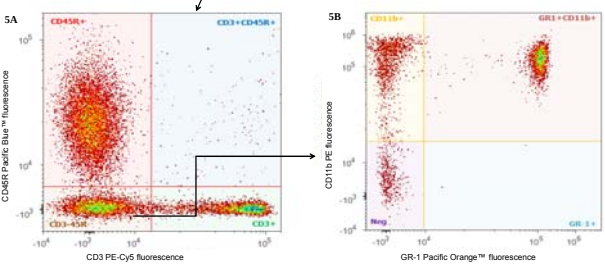

Figure 5. Immunophenotyping based on CD45+ fluorescence threshold

5A. A daughter plot of CD45+ gate showing the B cells stained with rat anti-mouse CD45R Pacific Blue™ and T cells stained with hamster anti-mouse CD3 PE-Cy® 5.

5B. A second density dot plot, a daughter of the CD3-CD45R- population, shows the double-positive granulocytes stained with rat anti-mouse CD11b PE and rat anti-mouse GR-1 Pacific Orange™ dyes. The CD11b+ GR1- population represents phagocytic cells (monocytes, macrophages, and any circulating dendritic cells).