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Mouse immunophenotyping

A no-lyse, no-wash, and no-cell loss method using the Attune® Acoustic Focusing Cytometer

Immunophenotyping mouse whole blood presents a challenge due to the limited sample volume available (≤100 µL/day/animal) [1,2], particularly in longitudinal studies. Specifically, these small volumes limit the ability to perform multicolor phenotyping experiments with the required compensation and fluorescence-minus-one (FMO) controls [3]. Methods previously described using a no-lyse, no-wash staining protocol for small sample volumes sacrifice light scatter resolution [4]. In traditional flow cytometry (which uses hydrodynamic focusing to orient the cells in the flow stream), accurately identifying some cell populations depends on high-resolution scatter data, so no-lyse, no-wash protocols are not feasible on this type of platform. Moreover, 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 the flow rates available in those instruments is inordinately long.

This application note describes a no-lyse, no-wash method that takes advantage of the acoustic focusing technology offered by the Attune® Acoustic Focusing 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 than is possible with traditional flow cytometry. In this application, 5 µL of mouse blood is stained in a 50 µL total volume and then diluted 400-fold in PBS (2 mL final volume). To keep data set sizes manageable, a fluorescence threshold (CD45) is used to distinguish the white blood cell population from the much more abundant red blood cell population. In addition, at this dilution, the coincidence of the target population with red blood cells and platelets is reduced sufficiently so that scatter signals (necessary for accurate differentiation of the granulocyte and lymphocyte populations) are reliable. Acquisition times for such dilute samples are still completed in a reasonable 1–2 minutes, whereas a traditional hydrodynamic focusing cytometer requires 8–10 minutes per sample.

This method delivers additional time savings by reducing the number of sample preparation steps and eliminating lysis and wash steps to avoid sample loss.

Basic protocol

Materials

- Rat anti-mouse CD45 FITC (Invitrogen Cat. No. MCD4501)
- Directly labeled anti-mouse antibodies
- Anti-mouse CD16/32 (Invitrogen Cat. No. MFCR00)
- Attune® Performance Tracking Beads (Applied Biosystems Cat. No. 4449754)
- Mouse peripheral blood [1]
- PBS + 1% BSA + 2 mM NaN_3, pH 7.2 (PBS-BSA)
- PBS
- Anticoagulant-coated collection tubes or 0.5 M EDTA, or 140 USP units/mL sodium heparin, or 3.8% w/v sodium citrate
- 12 x 75 mm tubes or other flow cytometry tubes
- Attune® Acoustic Focusing Cytometer
- AbC™ Anti-Rat/Hamster Bead Kit (Invitrogen Cat. No. A10389)

Titration of antibodies

Titrate all antibody conjugates using the following staining protocol to determine optimal staining concentration. Antibody conjugates may be used at the manufacturer's recommended staining concentration with the AbC™ Anti-Rat/Hamster Bead Kit (see *Protocol notes* on preparing antibodies, page 3). For multi-color testing, premix antibody conjugates in 1X PBS-BSA to provide the final antibody mixture in a 45 µL total volume.

Prepare blood

1. Collect peripheral blood following Institutional Animal Care and Use Committee (IACUC) acceptable practices [1]. If an anticoagulant-coated collection device is not used, then add 1/10 the volume of anticoagulant (0.5 M EDTA, or 3.8% w/v sodium citrate, or 140 USP units/mL sodium heparin) to the whole blood and mix well.
2. Block Fc binding receptors by pretreating with 0.1 µg of CD16/CD32 per 10 µL of whole blood and incubating for a minimum of 10 minutes prior to antibody labeling.

Staining protocol

1. Pipet antibody conjugates into labeled sample tubes; the volume should be 45 µL.
2. Add 5 µL of anticoagulated mouse whole blood to the antibody solution and mix well.
3. Incubate protected from light for 30 minutes (or reagent manufacturer's recommendation).
4. Add 2 mL PBS to the tubes immediately prior to loading on the Attune® Acoustic Focusing Cytometer.

Compensation controls

Prepare single-color compensation samples using the AbC™ Anti-Rat/Hamster Bead Kit.

1. Add one drop of AbC™ Anti-Rat/Hamster Capture Beads (Component A) to a labeled sample tube for each antibody conjugate included in the panel.
2. Add the recommended amount of each rat or hamster antibody conjugate to the AbC™ Anti-Rat/Hamster Capture Beads.
3. Incubate for 15 minutes at room temperature, protected from light.
4. Add 3 mL PBS and centrifuge for 5 minutes at 200 x *g*.
5. Carefully remove the supernatant and resuspend the bead pellet by adding 1 mL PBS.
6. Prepare one AbC™ Anti-Rat/Hamster Control Beads (Component B) sample by adding 1 drop to a labeled sample tube along with 1 mL PBS.

Data collection

Verify instrument performance with the Attune® Performance Tracking Beads.

1. Create a new experiment using instrument settings optimized for no-lyse, no-wash staining.
2. In the experiment browser, right-click Compensation and select Compensation setup.
3. In the Compensation setup dialog box, select to compensate on height and select the parameters required for your panel. Note: Pulse height is used for both scatter and fluorescence signals, as it provides lower measurement standard deviations than pulse area (see *Protocol notes* on instrument optimization, page 4).
4. There should be one single-color compensation sample matched to each parameter selected.
5. Click OK to create the compensation control samples.
6. Select the workspace for your specimen sample by double-clicking on the first tube for the specimen in the experiment browser.
7. Create a bivariate plot of the CD45$^+$ threshold channel vs. SSC and additional plots as needed for data analysis.
8. Place a CD45$^+$ polygon gate around the CD45-positive population (Figure 1).
9. Optimize the fluorescence threshold to include all of the lymphocyte and granulocyte populations.
10. Optimize the remaining fluorescence PMT voltage levels.
11. Once instrument optimizations are complete, select the first compensation control tube.
12. Enable forward scatter threshold for the compensation controls by changing the FSC threshold logic to "or".
13. Beads require a forward scatter threshold. You may also need to adjust the forward and side scatter voltages to place the bead singlets on scale. Do not, however, change fluorescence PMT voltages from those optimized for your cells. Compensation controls and the stained cell panel must be run at the same fluorescence voltages to obtain a valid compensation matrix.
14. Set the recording conditions to collect 5,000 R1 events, acquisition volume to 200 µL, and flow rate to 200 µL/min.
15. Run the Component B compensation control, adjust the R1 gate to include only bead singlets, and record the data.
16. Copy the R1 gate to the remaining compensation controls and run each tube in order.
17. After completing the compensation controls, return the FSC and SSC PMT voltages to values optimized for cells (if changed) and reset the FSC threshold logic back to 'Ignore' to threshold the whole blood only on fluorescence.
18. Select your first sample tube of stained cells.
19. Set the collection rate to 500 µL/min acquisition volume and recording criteria to obtain the desired events (we recommend starting with 600 µL volume for 10,000 CD45$^+$ events).
20. Proceed with collecting data for samples.

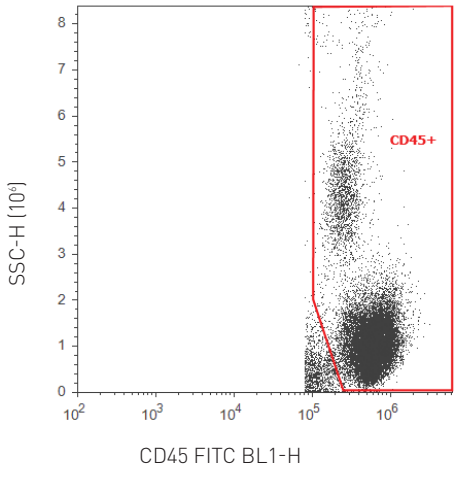

Figure 1. Dot plot of CD45 FITC (channel BL1-H, 488 laser, filter 530/30BP) vs. side scatter (channel SSH-H, violet laser, filter 405/10BP) showing both the threshold level and CD45+ gate. Threshold was set below the level of granulocyte CD45 FITC fluorescence. The CD45$^+$ polygon gate was drawn to include lymphocyte and granulocyte populations and to exclude lower-fluorescence cellular debris.

Data analysis

1. Gate on events expressing CD45 in a CD45 FITC vs. SSC dot plot.
2. Derive daughter plots from CD45$^+$ gating to analyze the target populations.

5-color example

In the following example, whole mouse (BALB/CJ) blood was stained with a 5-color panel comprising 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) conjugates. Titer for these five direct conjugates ranged from 0.008 µg to 0.125 µg per test. This five-color staining example required five FMO control samples and one 5-color panel sample tube. The FMO controls were used to both fine-tune compensation levels and determine appropriate quadrant marker placement for the panel.

Two density plots were created to identify the four subpopulations—B lymphocytes, T lymphocytes, monocytes, and granulocytes—from the 5-color panel. First, a daughter plot is created from the CD45$^+$ gate for CD3 PE-Cy®5 vs. CD45R Pacific Blue™ conjugate (Figure 2A). Quadrant markers are set from the FMO controls minus the CD3 PE-Cy®5 dye and minus CD45R Pacific Blue™ dye to identify the CD45R$^+$ (B lymphocyte) and CD3e$^+$ (T lymphocyte) populations. Next, a second daughter density plot is created from the negative cell population CD3$^-$ CD45R$^-$ (non-B, non-T cells) to identify the granulocyte and monocyte population expression of GR-1 and CD11b.

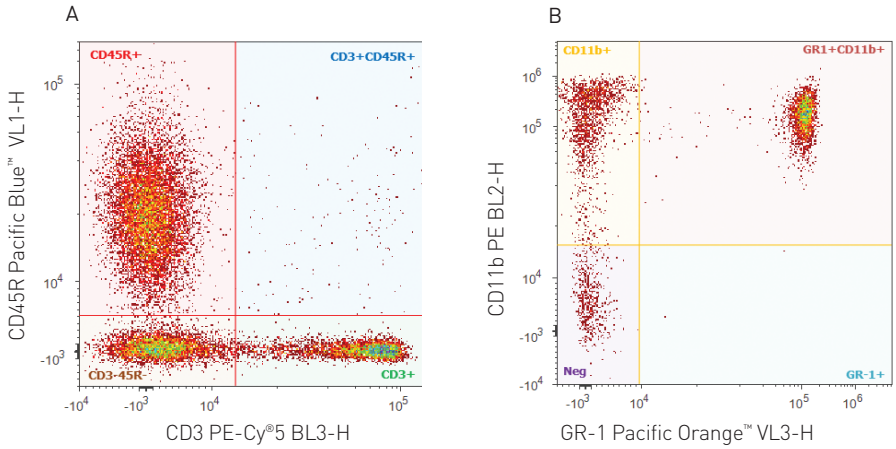

Figure 2. Density plot analysis of 5-color panel with (A) a daughter plot of CD45$^+$ gate showing the B cells stained with rat anti-mouse CD45R Pacific Blue™ conjugate (parameter VL1-H, violet laser, filter 450/40 nm BP) and T cells stained with hamster anti-mouse CD3 PE-Cy®5 (parameter BL3-H, blue laser, filter 530/30 nm BP). A second **(B)** density dot plot, a daughter of the CD3$^-$ CD45R$^-$ gate, showing the double-positive granulocytes stained with rat anti-mouse CD11b PE (parameter BL2-H, blue laser, 575/24 nm BP filter) and rat anti-mouse GR-1 Pacific Orange™ conjugate (parameter VL3-H, violet laser, 603/48 nm BP filter). The CD11b$^+$ GR1$^-$ population represents phagocytes (monocytes, macrophages, and any circulating dendritic cells).

Protocol notes

Preparing antibodies

Successful results with this no-lyse, no-wash method depend on testing titrated, directly conjugated antibodies with this staining method prior to preparing mixed antibody cocktails. Indirect staining is not compatible with the no-wash staining protocol, and only the use of directly labeled or Zenon® labeled antibodies is recommended. Antibody conjugates must be titered in a single color following this method, and optimal titer selected where there is maximal separation of positive cells with minimal background of the negative population.

The use of antibody capture beads as a compensation control is highly recommended. This not only saves valuable sample but is also critical in cases where the targeted population is rare for the marker requiring compensation. It is not necessary to titrate the conjugated antibodies on the AbC™ Anti-Rat/Hamster Beads separately from the mouse blood titration. You may use conjugated antibodies at the manufacturer's recommended amount for cell staining. However, you can save on reagent consumption by titrating conjugates antibodies on AbC™ Anti-Rat/Hamster Beads. The correct titer will provide a fluorescence median intensity of the AbC™ Anti-Rat/Hamster Beads at least as bright as the stained whole blood [1]. Do not expect the titer determined for staining mouse blood to be appropriate for staining AbC™ Anti-Rat/Hamster Beads.

Though the example in this application note uses CD45 as the threshold to select all white blood cells, it may be more practical to use the major cell type included in your panel as the threshold parameter. For example, using a CD3 threshold for T cells or CD19 for B cells rather than CD45 would save an additional parameter for subtyping in multicolor experiments. When selecting a dye as a threshold, it is recommended to use one such as FITC, Alexa Fluor® 488 dye, or Pacific Blue™ dye, which have minimal spillover from other dyes into their channels.

Instrument setting optimization

Optimization of instrument PMT voltages and threshold values should be done during the antibody titration and panel development. As these optimal instrument settings are determined, save the settings in a .gis instrument settings file named for your panel. This settings file can then be loaded as your default instrument settings when creating new experiments. Loading predefined instrument settings at the start of your experiment will save valuable time and sample.

The recommended flow setting during data recording is a standard collection rate of 500 µL/min. Setting a slower collection rate of 200 µL/min will provide more time to observe changes while adjusting thresholds and PMT voltages.

Select pulse height in your parameter configuration. In this application pulse height will provide better scatter and fluorescence precision than pulse area. The use of fluorescence threshold along with collecting pulse height data minimizes the effect of coincidence on the cells of interest. Note that compensation must be configured with pulse height selected to apply to pulse height fluorescence parameters.

Optimize the fluorescence threshold to separate $CD45^+$ events from cellular debris (Figure 1). The granulocytes will have a slightly lower CD45 expression than lymphocytes; be sure to set the threshold below granulocyte staining by viewing the CD45 vs. SSC dot plot. Adjust fluorescence PMT voltages so that cell autofluorescence is well above system noise by placing the autofluorescence around a median fluorescence of 10^3. Use a sample labeled with only CD45 FITC (or your thresholding conjugate) so you can evaluate cellular autofluorescence for the other fluorescence parameters. Next, verify that all stained cells for each of the conjugated antibodies remain on scale at titer. Where events become off-scale you should lower PMT voltages or reconsider what dyes are being used for your targets. In multicolor staining use the brightest dyes (e.g., R-phycoerythrin, aka PE) for the lowest-expressing targets. Save the optimized settings and use these in creating an experimental template to simplify instrument setup when running your study samples.

Acquisition volumes

White blood cell (WBC) counts in mice vary between stains, anesthesia, and where the sample is drawn [5]. You will have to determine the exact acquisition volume based on desired sample conditions and event count. Typically, 5 µL of whole blood will provide at a minimum 2 acquisitions for 10,000 $CD45^+$ events, with acquisition times of approximately 1 minute. A good starting point is to use a 600 µL acquisition volume and 500 µL/min collection rate.

References

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