Detection of platelets in whole blood using the Attune NxT Flow Cytometer

Introduction

Platelets are non-nucleated cellular fragments that circulate in the peripheral blood. They derive from megakaryocytes and are critical for maintaining hemostasis. Alterations in platelet function are associated with a range of clinical conditions including Bernard–Soulier syndrome (BSS), Glanzmann's thrombasthenia, and storage pool disease [1]. Monitoring platelet number and function in blood via flow cytometry can provide useful diagnostic and prognostic information [2]. Thrombocytopenia, a condition defined by significant loss of platelets in circulating blood, can lead to excessive bleeding. Conversely, conditions characterized by excessive platelet activation can lead to thrombosis and associated cardiovascular complications including myocardial infarction and stroke [3]. In addition to their well-characterized role in hemostasis, platelets also function in regulating vascular endothelial permeability and leukocyte extravasation through the release of cytokines, growth factors, and proteases from storage granules [4]. A growing body of evidence suggests that platelets can directly modulate signaling to innate immune cells and mediate recognition of pathogens through their surface expression of toll-like receptor 4 (TLR4) [5]. Basic, translational, and clinical researchers are now studying the role of platelets beyond hemostasis and thrombosis, such as in areas of inflammation, immune response, wound healing, angiogenesis, metastasis, antibacterial activity, megakaryopoiesis, and platelet production, and in therapies, such as stem cell replacement and platelet transfusion [6].

Resting platelets, at 2–3 µm in diameter, are the smallest cellular component of peripheral blood. They have a concentration range of 1–3 x 10^8 platelets/mL in peripheral blood. Upon activation, platelets undergo rapid changes in cell surface receptor expression that lead to altered adhesive properties and changes in morphology that promote the formation of a platelet plug at the site of vascular disruption [7]. These properties can make the interrogation of platelets by flow cytometry challenging, especially in the context of light scatter detection. One of the most widely used markers to identify and study platelets via flow cytometry is CD41 (glycoprotein IIb), a member of the integrin family of receptors that mediate cell-matrix interactions [8].

Platelets are typically studied in platelet-rich plasma (PRP) where they are the dominant population, and can be identified by light scattering properties alone. However, the procedure to make PRP requires considerable sample manipulation that may affect platelet health and function, a significant drawback of this method. Other common methods for the study of platelets use techniques such as selective lysis to remove red blood cells (RBCs) from the sample. With lysis of RBCs by ammonium chloride or a similar osmotic treatment, discrimination of platelets by light scatter alone is confounded by the cellular debris from RBCs that overlaps with and obscures the platelet population. The treatment to remove the RBCs from the sample can also have a detrimental effect on platelet health and function. Detection of platelets in intact whole blood is desirable because minimal sample manipulation helps keep platelets in a healthy, inactivated state. However, resolving the platelet population poses a difficult challenge since RBCs outnumber platelets by more than an order of magnitude, and the broad scatter distribution produced by the nonspherical shape of RBCs can overlap the platelets.

This application note features two different methods for the detection of platelets in human blood using the Invitrogen™ Attune™ NxT Flow Cytometer. The first method (Method 1) employs the use of CD41 immunophenotyping in lysed whole blood. This method uses both a forward scatter (FSC) threshold and a fluorescence threshold in the detection of platelets. The second method (Method 2) presents a new technique with minimal sample processing for the detection of platelets in unprocessed whole blood by utilizing a dual-laser light scatter approach to distinguish platelets from RBCs and leukocytes. A dual FSC and side scatter (SSC) threshold, and a fluorescence threshold using phycoerythrin (PE) anti-CD41 antibody are both used for the detection of platelets. Using the latter method also presents the opportunity to identify the platelet population using the dual-laser light scatter technique in whole blood without using a marker such as CD41.

The default threshold channel for most commercial flow cytometers, including the Attune NxT Flow Cytometer, is FSC. The common practice of using FSC is largely a consequence of the utility of this channel in excluding small particles, including platelets and debris from white blood cell analysis. This feature is particularly useful in lysed blood where small particles from the RBC lysis greatly outnumber the cells to be analyzed, even after washing.

When using scatter thresholds for platelets or other small particles, it is often preferable to use SSC or a Boolean combination of SSC and FSC to differentiate the platelets from debris and instrument noise. In either case, care must be taken not to exclude cellular events from analysis. For small particles, FSC is a noisier parameter than SSC owing to high background autofluorescence, and a greater contribution from small particles in sheath fluid to the signal. With small scatter signals, this noise may be sufficient to make threshold setting with FSC alone more difficult.

Materials and methods

- Whole human peripheral blood from a healthy donor, collected in sodium citrate anticoagulant
- Modified HEPES-buffered Tyrode's solution; store in aliquots at –20°C; stable for up to 1 year at –20°C:
  - 10 mM HEPES buffer, pH 7.4
  - 137 mM NaCl
  - 2.8 mM KCl
  - 1 mM $MgCl_2$
  - 12 mM $NaHCO_3$
  - 0.4 mM Na_2HPO_4
  - 0.35% bovine serum albumin (BSA)
  - 5.5 mM glucose
- Invitrogen™ High-Yield Lyse solution (Cat. No. HYL-250)
- Gibco™ Earle's Balanced Salt Solution (EBSS), (Cat. No. 14155-063)
- Invitrogen™ mouse anti-human CD41-PE, clone VIPL3 (Cat. No. MHCD4104)
- Attune NxT Flow Cytometer, standard 4-laser configuration (Cat. No. A24858)
- Invitrogen™ Attune NxT No-Wash, No-Lyse Filter Kit (Cat. No. 100022776)
- 12 x 75 mm round-bottom tubes

Method 1: Lysed whole blood detection of CD41+ platelets (lyse/no-wash)

Sample processing and antibody labeling

1. Whole human blood was processed within 1 hour of collection to avoid potential artifacts due to platelet activation.
2. Whole blood was diluted 1:10 in modified HEPES-buffered Tyrode's solution and gently mixed by inversion.
3. 100 µL of the diluted whole blood was pipetted into round-bottom tubes at room temperature.
4. The mouse anti-human CD41-PE antibody conjugate was added according to the manufacturer's instructions and was mixed gently.
5. Samples were incubated for 20 minutes at room temperature, protected from light.
6. 2 mL of High-Yield Lyse solution was added to each tube, and samples were incubated for 10 minutes at room temperature to allow RBC lysis to proceed. This results in a 1:201 final dilution.
7. Samples were mixed and analyzed immediately on the Attune NxT Flow Cytometer.

Flow cytometry data acquisition

1. FSC and SSC data were collected using the standard 488 nm laser.
2. PE data were collected using an excitation of 561 nm and the YL1 detector with a bandpass of 585/16.
3. Three plots were used: FSC-H vs. SSC-H dot plot; CD41 fluorescence vs. SSC-H dot pot; and a single-parameter histogram of CD41 fluorescence. All plots used a logarithmic scale. A marker was placed on the CD41+ population in the histogram to include the positive PE events with the percent positive events displayed. Two regions were made in the SSC vs. CD41 fluorescence dot plot—one to include the PE-positive events labeled CD41+ and colored blue, and a second to include the PE-negative events that are not debris or instrument noise, and colored red. Color-backgating from this plot was used to display the CD41+ events (blue) and the white blood cell (WBC) events (red) on the FSC vs. SSC dot plot.
4. A 25 µL/min sample flow rate was used for collection with stop criteria set at 10,000 CD41+ events.
5. Samples were collected with a range of FSC thresholds and YL1 fluorescence thresholds, to define optimal conditions for acquisition.

Data analysis and results with the lyse/no-wash method

A sample labeled with anti-human CD41-PE antibody conjugate was first analyzed to identify positively labeled platelets, and adjust detector voltages and threshold settings. Threshold settings allow the user to set the minimum signal level to eliminate unwanted events in up to four detectors simultaneously. Multiple thresholds may also be combined using Boolean operators.

Refer to the Invitrogen™ Attune™ NxT Software User Guide (**Pub. No. 100024236**) for detailed instructions on the threshold settings. The FSC threshold was adjusted between 0.5 and 5.0 in an effort to distinguish platelets from debris and instrument noise (Figure 1). Using an FSC threshold alone was insufficient to distinguish platelets from debris by scatter. With the lower threshold setting, the FSC vs. SSC plot shows the platelet population overlayed with the debris population, which is distinct from the instrument noise and the WBCs. Increasing the FSC threshold did eliminate much of the instrument noise, yet the platelets still could not be distinguished from debris by scatter alone. Utilizing a two-parameter fluorescence vs. SSC plot, the CD41+ events are separated from the debris. WBCs are visible in both the scatter plot and the fluorescence vs. SSC plot, and in both cases, the WBCs are readily distinguished from the platelets and debris. The smaller WBC (lymphocyte) population may overlap the platelet population in the SSC parameter, but they are readily distinguished from platelets and debris in FSC.

A fluorescence threshold adjustment of between 0.1 and 5.0 was used to determine the optimal settings for eliminating unwanted nonfluorescence events, distinguishing CD41-PE labeling from debris and identifying the platelet population within the scatter plot (Figure 2). With a fluorescence threshold, ideally, the only signal detected comes from the fluorescence parameter and the full population is visible. The optimal fluorescence threshold setting in this example is 1.0. When using this fluorescence threshold setting, the CD41+ platelets and debris were distinguishable and the full fluorescent CD41 population is visible. At the lowest fluorescence threshold setting of 0.1, a significant amount of instrument noise and debris are visible. Increasing the fluorescence threshold to 0.2 partially eliminates instrument noise and debris. At the highest fluorescence threshold setting of 5.0, although the instrument noise and debris are eliminated, the platelet population is truncated.

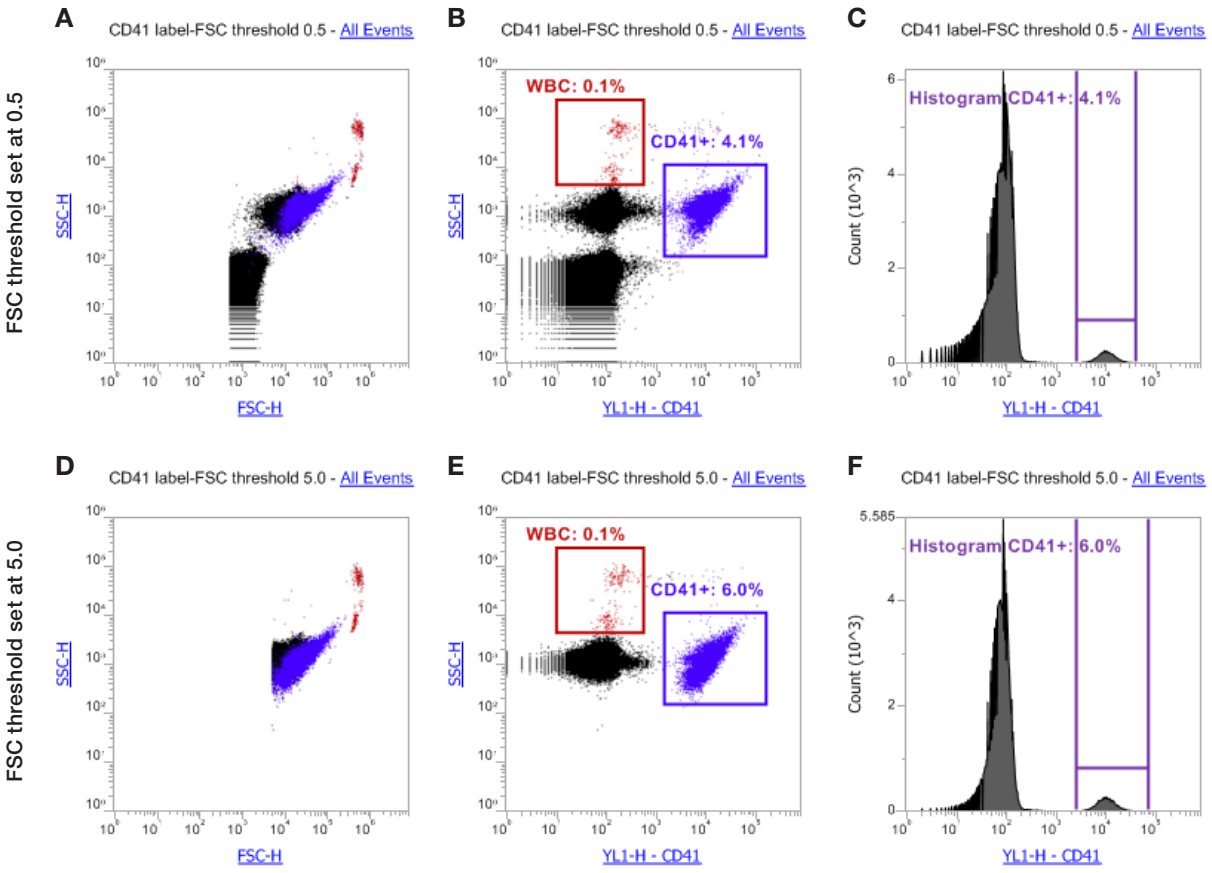

Figure 1. Platelet detection in lysed whole blood: use of FSC threshold. (**A**, **B**, and **C**) Accurate platelet detection is complicated by instrument and/or detector noise, and cellular debris when using a low FSC threshold value of 0.5. (**D**, **E**, and **F**) Alternatively, an increase of FSC threshold to 5.0 results in better resolution of the CD41+ platelet population as the instrument noise is eliminated from the analysis. However, even with this higher FSC threshold setting of 5.0, debris cannot completely be removed from analysis, and platelets cannot be resolved completely from debris using 488 nm light scatter alone.

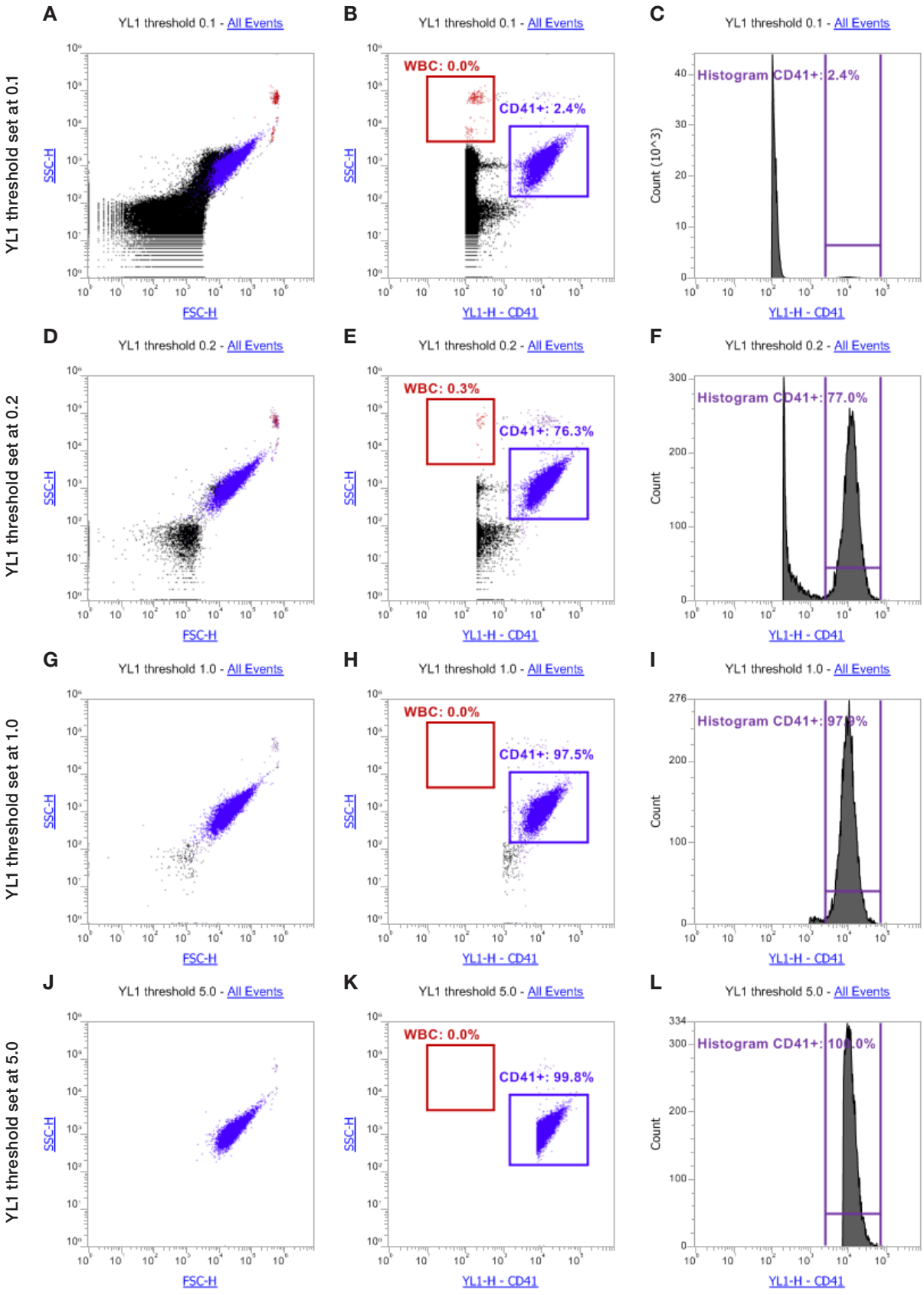

Figure 2. Platelet detection in lysed whole blood: use of fluorescence threshold. (**A**, **B**, and **C**) At the lowest YL1 threshold setting of 0.1, a significant amount of debris and instrument noise are detected in all three plots. (**D**, **E**, and **F**) By increasing the YL1 threshold value to 0.2, the debris can be excluded from the analysis. This allows for the data collection from only the CD41+ population. (**G**, **H**, and **I**) An optimal YL1 threshold of 1.0 eliminates the instrument noise and most of the debris, so the main population visible is the fluorescent CD41 platelets. (**J**, **K**, and **L**) Increasing the YL1 threshold further to a setting of 5.0 has the effect of truncating the platelet population, and is set too high.

Method 2: Intact whole blood detection of CD41+ platelets (no-lyse/no-wash)

Sample processing and antibody labeling

1. Whole human blood was processed within 1 hour of collection to avoid any potential artifacts due to platelet activation.
2. 100 µL of undiluted whole blood was added to 12 x 75 mm round-bottom tubes.
3. The mouse anti-human CD41-PE antibody conjugate was added to the samples according to the manufacturer's instructions and samples were incubated for 20 minutes at room temperature, protected from light.
4. Earle's Balanced Salt Solution (EBSS) was added to give three different final dilutions of 1:40, 1:400, and 1:4,000 before analysis on the Attune NxT Flow Cytometer.
5. Samples were mixed and analyzed immediately on the Attune NxT Flow Cytometer equipped with the Attune NxT No-Wash, No-Lyse Filter Kit.

Flow cytometry data acquisition

1. The Attune NxT No-Wash, No-Lyse Filter Kit was installed (Figure 3) to allow dual-laser light scatter detection using both 405 nm and 488 nm laser excitation.
   a. Hemoglobin in RBCs readily absorbs light at 405 nm, whereas leukocytes and platelets do not.
   b. This property allows resolution of the platelet and WBC populations away from the intact RBCs by light scatter alone.
2. FSC and blue laser SSC (488 nm–SSC) data were collected from the 488 nm laser, and violet laser SSC (405 nm–SSC) data were collected from the 405 nm laser. PE data were collected using an excitation of 561 nm and the YL1 detector with a bandpass of 585/16.
3. Gating strategy used three plots: blue 488 nm SSC-H vs. violet 405 nm laser SSC-H dot plots (Figure 4), and single-parameter histograms of CD41 fluorescence (Figure 5). All plots used logarithmic display. Regions were made in the 488 nm–SSC vs. 405 nm–SSC dot plot, to encompass the RBC, WBC, platelet, and instrument noise populations. A marker was placed on the CD41+ population in the histogram to include the positive PE events, with the percent positive events displayed. Color-backgating from this plot was used to display the CD41+ events on the scatter plot showing the locations of PE-positive events.
4. A 25 µL/min flow rate was used for collection.
5. Samples were collected with dual FSC and SSC thresholds and YL1 fluorescence thresholds, to confirm that the putative population identified by scatter was composed of CD41+ platelets.
6. The window extension (WE) setting was adjusted between 0 and –20 to further improve data precision for smaller events such as platelets. Refer to the Attune NxT Software User Guide, "Advanced Settings" section (**Pub. No. 100024236**) for detailed instructions on how to properly adjust the WE setting.

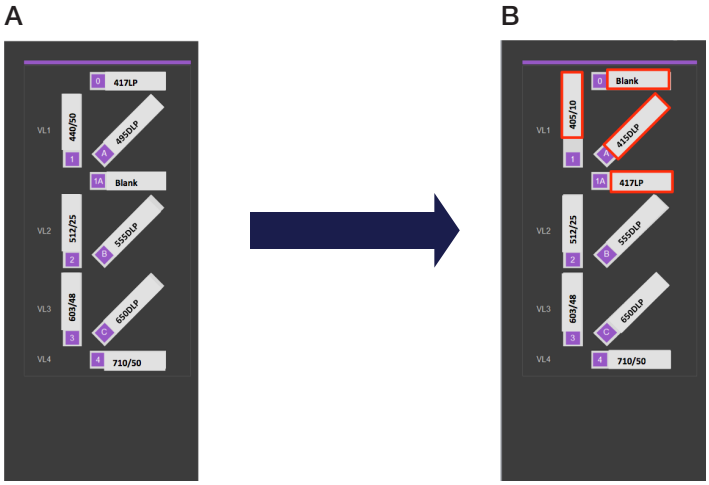

Figure 3. Installation of the Attune NxT No-Wash No-Lyse Filter Kit. (**A**) The standard configuration for the 405 nm violet laser optical filter block and the same optical filter block using the (**B**) Attune NxT No-Wash No-Lyse Filter Kit are shown, with changes outlined in red. To use the filter kit, remove the 440/50 bandpass filter in VL1 slot 1 and replace with the 405/10 bandpass filter. Remove the 495 dichroic longpass (DLP) filter from slot A and replace with the 415 DLP. The blank filter in slot 1A is switched with the 417 LP filter in slot 0.

Data analysis and results with intact whole blood method (no-lyse/no-wash)

A recently described method [9] for resolving leukocytes and platelets from RBCs by light scatter properties alone was assessed for the ability to further resolve platelets, WBCs, RBCs, and debris in whole blood. This method exploits the differences in light-scattering properties between RBCs and leukocytes. Hemoglobin in RBCs readily absorbs 405 nm laser light, whereas leukocytes and platelets do not, resulting in a reproducible scatter profile when analyzing human whole blood with blue (488 nm) and violet (405 nm) side scatter (Figure 4). Using this approach, a distinct platelet population can be discerned.

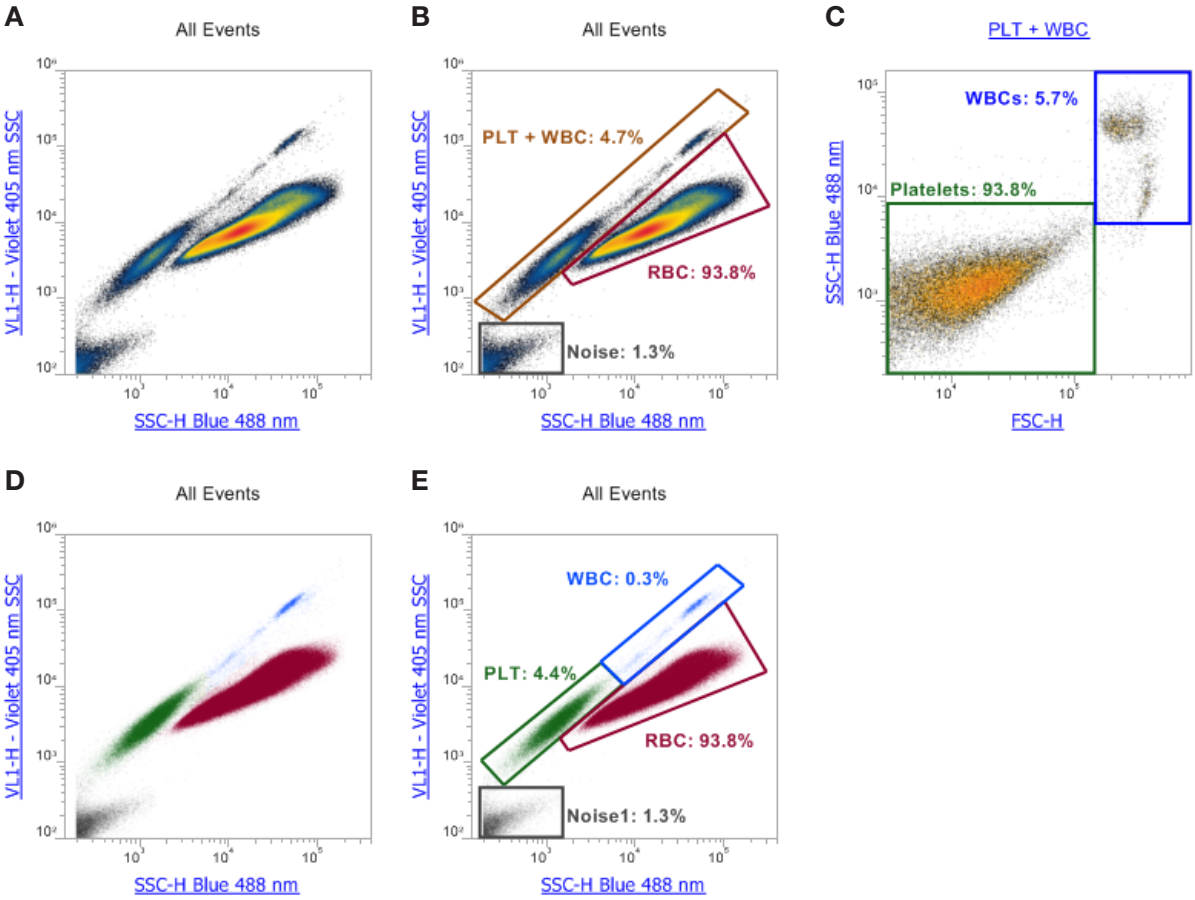

Figure 4. Dual laser blue (488 nm) and violet (405 nm) laser SSC with intact whole blood (no-lyse/no-wash). (**A**, **B**) RBCs, WBCs, and platelets are separated on the basis of light scatter only by using a combination of blue and violet laser SSC analysis. Hemoglobin in RBCs readily absorbs light at 405 nm, shifting the RBC population to the right by reducing the SSC for RBCs in the violet SSC channel relative to leukocytes and platelets. Dual FSC and SSC threshold is set low enough to show instrument noise, ensuring the full platelet population is visualized. (**C**) Using the gate that includes WBCs and platelets, a standard plot of FSC vs. 488 nm SSC can be used to distinguish the platelet population from the WBCs with regions created around the two populations. (**D**) Using color-backgating on the same plot as previously shown in (**A**), the RBC population is colored red, the platelet population is colored green, and the WBC population is colored blue, while the noise is black. The three main WBC populations of lymphocytes, monocytes, and granulocytes can be distinguished. (**E**) Placing regions around the RBC, WBC, and platelet populations show the dominant cell type in whole blood is the RBC, while the WBC and the platelets are relatively rare events.

To avoid excessive coincidence and sufficiently resolve the platelet population away from RBCs, whole blood must be diluted significantly. The labeled whole blood sample was diluted 40-fold, 400-fold, and 4,000-fold in isotonic physiological saline solution (EBSS), to observe the effect of dilution on coincident events and to determine optimal dilution for collection (Figure 5). Using the marker placed on the positive PE population in the histogram to color-backgate on the differential scatter plot shows positive PE fluorescence in all three cell populations at the 40-fold and 400-fold dilutions, indicating that many platelet cells are coincident with RBCs. A 4,000-fold dilution of the sample, combined with using a low flow rate (25 µL/min) for data collection reduces the number of particles or cells passing through the interrogation point simultaneously, allowing for more accurate population counts. If lower dilutions are required, in combination with minimal coincidence, the WE setting can be reduced in the advanced threshold settings window. This is commonly adjusted between –10 to –20 for small particles like platelets for which it can significantly reduce coincident events and improve data precision. This is particularly useful for whole blood where the red blood cell population is significantly larger and more numerous than the target platelet population.

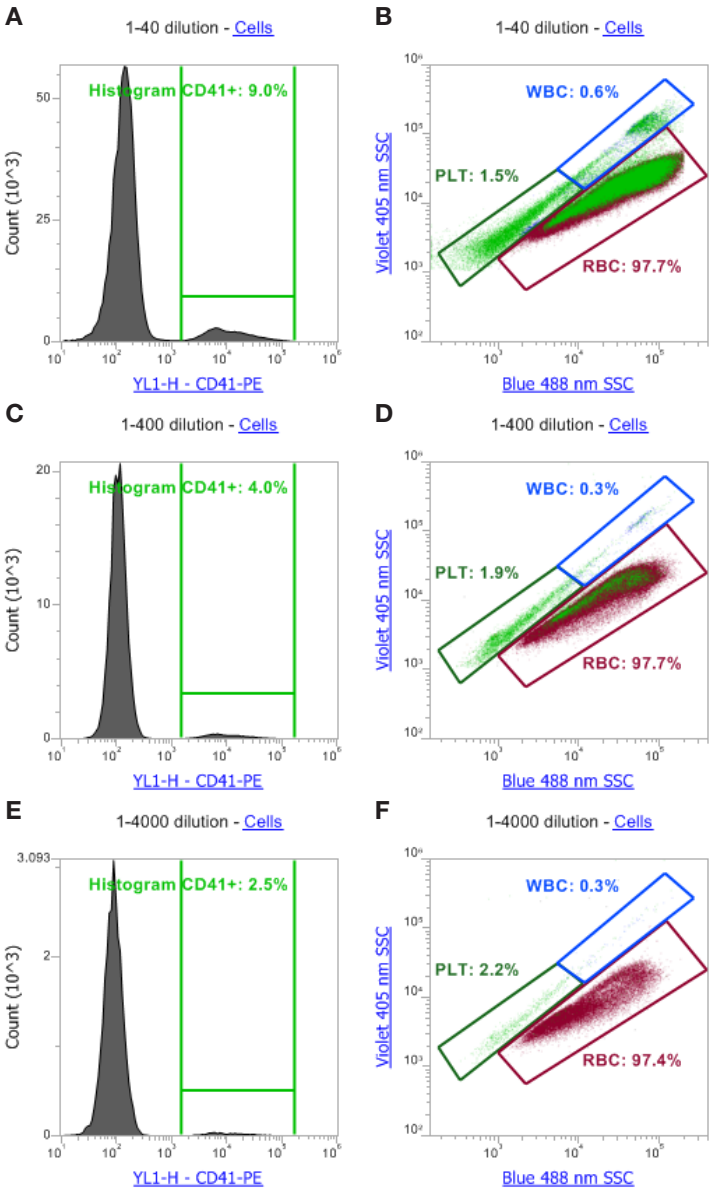

Figure 5. Platelet detection with intact whole blood: use of FSC threshold. Using a dual SSC + FSC threshold, three different dilutions of whole blood were collected: (**A**, **B**) 40-fold, (**C**, **D**) 400-fold, and (**E**, **F**) 4,000-fold. All plots use a gate around the total cell population to eliminate the noise events. The PE-positive events identified in the histogram and backgated on the scatter plots (light green) reveal significant coincident events with the 40-fold and 400-fold dilutions. The single-parameter CD41 histogram plot thus over-reports the percent CD41-positive at the 40-fold and 400-fold dilutions. At the 4,000-fold dilution, the results demonstrate minimal coincidence.

The application of a fluorescence threshold on the YL1 detector allowed for the confirmation of the discrete population of CD41+ platelets. This has the effect of eliminating the nonfluorescence events from the collection (Figure 6). Finally, because platelets are smaller than either RBCs or leukocytes, a WE setting of –20 was applied to allow for greater resolution of subcellular particles. Using the default WE setting of 0, some CD41+ events are coincident with the RBC population; applying a WE setting of –20 effectively narrows the detection width and reduces the coincident detection of RBCs and platelets.

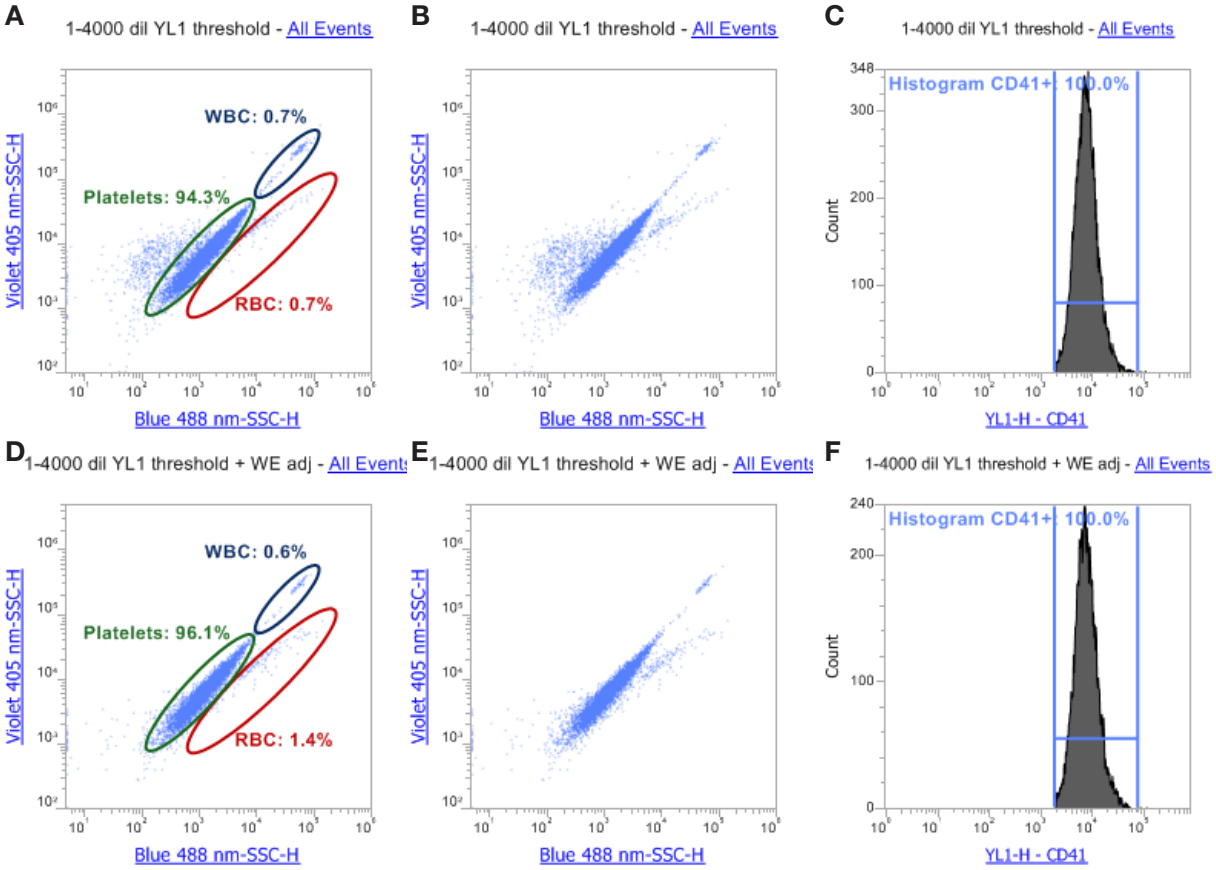

Figure 6. Platelet detection with intact whole blood: use of a fluorescence threshold. (**A**, **B**, and **C**) Applying a fluorescence threshold to the 4,000-fold dilution of labeled whole blood eliminated all nonfluorescence events from the acquisition. (**D**, **E**, and **F**) Decreasing the WE setting to –20 further eliminates coincident events.

Tips for using the dual SSC method for analysis of intact whole blood:

- Use log/log plots for differential blue/violet side scatter.
- Use height parameter for scatter to maximize separation of RBCs from WBCs and platelets.
- Define scatter gates and thresholds at lower event rates (<8,000) before increasing event rates for recording of data. Once thresholds have been set, they should not be changed during an experiment, or data integrity may be compromised.
- Choose a high signal-to-noise ratio for the fluorophore labels to set the fluorescence threshold.
- Use minimal sample manipulation to minimize platelet activation.
- Optimization of sample dilution and flow rate will help minimize coincident events.
- When using a FSC threshold, be sure to include the granulocyte population, which may have a lower FSC compared to lysed blood.
- Mouse RBCs are smaller than human RBCs; there may be some overlap of the platelet and RBC populations in the dual SSC plot when analyzing mouse cells.

Discussion

In the analysis of lysed whole blood, the results obtained indicate that inclusion of a fluorescently labeled antibody to detect a platelet marker, such as anti–CD41-PE, is required for unambiguous detection of platelets. Masked by the presence of RBC debris following ammonium chloride lysis, platelets cannot be distinguished by light scatter alone. While instrument noise and smaller cellular debris can be reduced by increasing the FSC threshold setting, the application of a fluorescence threshold is a more effective approach to discriminate CD41+ platelets from cellular debris in lysed whole blood samples. As the data demonstrate, any scatter threshold for platelets with lysed, unwashed whole blood can be problematic. For this analysis or any small particle analysis in lysed blood, a fluorescence threshold that detects only fluorescently labeled events is often required for best results.

When analyzing intact whole blood samples, platelets can effectively be identified by light scatter alone using the Attune NxT No-Wash No-Lyse Filter kit in conjunction with dual 405/488 nm laser excitation. RBCs can be shifted away from the platelet population due to the strong absorption of hemoglobin with violet 405 nm excitation. Using both FSC and SSC thresholds, distinct populations of RBCs, WBCs, and platelets can be identified in whole blood by light scatter alone. The optimal dilution of the labeled whole blood at 4,000-fold dilution demonstrated a minimized rate of coincidence. The use of a fluorescence threshold confirms that this population, defined by light scatter, is indeed composed of CD41+ platelets. A minor population of apparent CD41+ events is detected outside of the platelet region. Reducing the pulse width by applying a negative WE setting further enables accurate detection of platelets by reducing coincidence detection. The ability to distinguish the platelet population in intact whole blood without sample manipulation presents an improved method for the study of platelets.

All cytometers are governed by Poisson statistics, which predict the probability of a given number of cells being intercepted by the laser beam simultaneously. While increasing the sample concentration results in higher sample throughput, it also increases the probability of a coincident event, defined as more than one cell present in the laser beam at one time. For both methods, it is recommended to define scatter gates and thresholds at an event rate of <8,000 events/sec before increasing the rates for data collection. This serves to minimize coincidence for improved accuracy.

Conclusions

The Attune NxT Flow Cytometer, along with the Attune NxT No-Wash, No-Lyse Filter Kit for violet laser SSC detection, offers a robust assay for detecting platelets in whole blood without sample manipulation. The Attune NxT Flow Cytometer's acoustic focusing technology empowers research with unmatched speed (up to 10 times faster than the traditional cytometers), thereby greatly reducing the assay time. With fast detection speed, rare-event detection is simplified; and the use of cost-saving, no-wash/no-lyse techniques is enabled.

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