No-lyse no-wash new strategy for CD34+ for absolute cell counting without beads

Laura G. Rico [a], Jolene Bradford [b], Mike D. Ward [b], and Jordi Petriz [a]

Josep Carreras Leukaemia Research Institute, Barcelona, Spain [a] / Thermo Fisher Scientific, Eugene, OR, USA [b]

Josep Carreras LEUKAEMIA Research Institute

ABSTRACT

We have developed a no-wash, no-lyse strategy for identifying leukocytes in whole human blood using acoustic cytometry with the Invitrogen™ Attune™ NxT Flow Cytometer. The strategy exploits the difference in light-scattering properties between red blood cells and leukocytes/platelets. Standard methods for isolating and detecting leukocytes in whole blood involve significant manipulation/enrichment, these sample preparation methods can result in alterations in cell physiology and potential cell loss. (1) The novel protocol presented here is ideally suited to study the numbers of circulating CD34+ in whole blood. Further modification including a fluorescent threshold in conjunction with Violet Side Scatter is also described.

INTRODUCTION

The antigen recognized by the CD34 antibody is a cell surface marker widely used to both identify and isolate hematopoietic progenitor cells. Although this marker was initially identified as an antigen expressed in hematopoietic progenitors cells, it has also been used to detect CD34+ leukemic cells, vascular endothelial cells, muscle satellite cells and epidermal precursors (2).

Flow cytometry has classically been used to detect CD34+ cells. First assays consisted of using indirect immunofluorescence techniques, red-cell lysing procedures, and centrifugation and washing steps. CD34+ counts were obtained using a dual-platform technique, also known as the Milan Protocol (3). Since then, different methodologies have been described for improved detection of CD34+ cells. Using mutiparametric flow cytometry and, in agreement with the International Society of Hematotherapy and Graft Engineering (ISHAGE), CD34+ cells are routinely counted in combination with CD45 staining (4)

BACKGROUND

This strategy exploits the difference in light-scattering properties between red blood cells and leukocytes. Red blood cells contain hemoglobin, a molecule that readily absorbs violet laser (405 nm) light, whereas leukocytes and platelets/debris do not, resulting in a unique and reproducible scatter pattern when observing human whole blood in the context of blue (488 nm) and violet (405 nm) side scatter (SSC). Inclusion of the Attune NxT No-Wash No-Lyse Filter Kit (Cat. No. 100022776) in the Attune NxT Flow Cytometer filter configuration allows simultaneous measurement of both blue and violet side scatter and the differentiation of red blood cells and leukocytes based on light-scattering properties alone (Figure 1). The Attune NxT Flow Cytometer uses a volumetric sample and sheath fluid delivery system, which gives accurate cell concentration data without adding count beads.

Light-scattering properties of whole blood

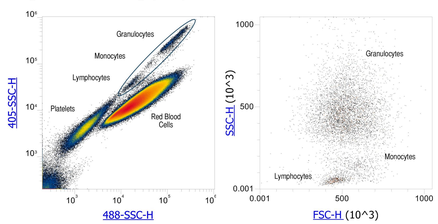

Figure 1. Identification of leukocytes in human whole blood using violet side scatter on the Attune NxT Flow Cytometer. Leukocytes are outnumbered by red blood cells ~700-fold in whole blood and generally require enrichment by red blood cell lysis or gradient centrifugation prior to analysis (5). The rapid sample collection rates and inclusion of the Attune NxT No-Wash No-Lyse Filter Kit on the Attune NxT Flow Cytometer allow identification of leukocytes by scatter properties alone. Resolution of leukocytes from red blood cells in whole blood is improved by incorporating violet 405 nm side scatter. Using both violet and blue side scatter allows identification of leukocytes in whole blood (A). Using a gate in figure 1A as the parent gate the three main leukocyte cell populations in human blood are identified using blue 488 nm forward and side scatter: lymphocytes, monocytes, and granulocytes (B).

The Attune NxT cytometer is quickly and easily configured to collect violet side scatter by changing dichroic and emission blades. This protocol is ideally suited to study the numbers of whole blood cells and it avoids lysis and centrifugation steps, which can result in unwanted damage to leukocytes and CD34+ cells (Figure 2).

MATERIALS AND METHODS

Samples
Human blood samples were obtained from samples taken for clinical testing purposes not related to this poster. Steady state peripheral blood, mobilized peripheral blood, and bone marrow specimens were used, and we provide detailed information about how to use the Attune NxT flow cytometer for the analysis of hematopoietic progenitor cells in whole blood. All samples were processed immediately after collection.

Materials
Mouse anti-Human CD34 R-PE Conjugate (CD3458104)
Mouse anti-Human CD45 FITC Conjugate (MHCD4501)
Hanks' Balanced Salt Solution (HBSS), no calcium, no magnesium, no phenol red (14175-095)
Attune NxT No-Wash No-Lyse Filter Kit (100022776)
Attune NxT Flow Cytometer 4 Laser Configuration (A24858)
AbC™ Total Compensation Bead Kit (A10513)

Sample Preparation
EDTA anticoagulated whole blood containing 5 x 10^5 cells were prepared for antibody staining. Samples were incubated for 20 min at room temperature in the dark, in the presence of PE-CD34 and FITC-CD45 antibodies (Life Technologies). Hank's balanced salt solution (Life Technologies) supplemented with 1% bovine serum albumin (BSA) and 0.1% NaN_3 (Sigma) was then added to the pre-stained whole blood samples, in a final volume of 100 µL. 900 µL HBBS was added to the labeled whole blood. This represents a 1:10 dilution. Compensation samples were prepared with only one of the colors of the experiment; there should be one compensation control for each other. Samples were immediately acquired on the Attune NxT Flow Cytometer.

Instrument Configuration
Install the Attune NxT No-Wash No-Lyse Filter Kit (Cat. No. 100022776) in the optical bench of the instrument (Figure 2) To use the filter kit, remove the 440/50 bandpass filter in VL1 slot 1 and place the 405/10 bandpass filter that is placed in the VL1 slot 1in slot 1. Remove the 495 Dichroic Longpass (DLP) filter in a lot A the 415DLP. The Blank filter in slot 1A is switched with the 417LP filter in slot 0. SSC was detected using both blue laser 488/10 Bandpass and violet laser 405/10 bandpass filters.

Configuration to collect violet side scatter

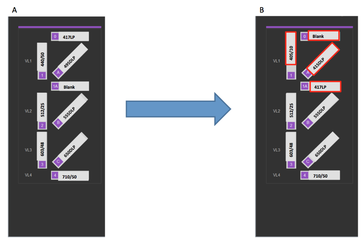

Figure 2. Use of the Attune NxT No-Wash No-Lyse Filter Kit. The standard configuration for the 405 nm violet laser optical filter block is shown in (A) and the same optical filter block using the No-Wash No-Lyse Filter Kit shown in (B), with changes outlined in red. To use the filter kit, remove the 440/50 bandpass filter in VL1 slot 1 and place the 405/10 bandpass filter that is placed in the VL1 slot 1in slot 1. Remove the 495 Dichroic Longpass (DLP) filter in a lot A the 415DLP. The Blank filter in slot 1A is switched with the 417LP filter in slot 0.

• FITC was detected with the blue laser 488 nm excitation and a 530/30 Bandpass filter in the BL1 detector in all configuration.

• PE was detected in the first example using the blue laser 488 nm excitation using a 590/40 bandpass in BL2. The approximate compensation settings were: BL1- %BL2, 7.29; BL2-%BL1, 22.02.

• FITC and PE fluorescence are displayed in logarithmic scale.

Sample acquisition using fluorescent threshold with the VSSC method

• Labeled and diluted blood samples were introduced to the Sample Injection Port (SIP) to initiate data acquisition (100 µL/min).
• CD45 fluorescent threshold levels were set empirically using a VSSC vs. CD45-FITC dual parameter plot to eliminate from detection the large amounts of red blood cells that are found in unlysed whole blood. A proper threshold is shown in Figure 3A. In this example the CD45 threshold values on the Attune NxT are set at 0,3 x 1000, and this setting also excludes from analysis non-nucleated cells and debris. This value is adjusted while acquiring data and observing the position of the CD45+ cells on the bivariate dot plots such that all the nucleated blood cells were on scale with the least amount of debris appearing in these plots. Other specimens, such as marrow or cord blood may appear with different scatter properties and minor variations in fluorescence intensities .
• In order to have a statistically significant count of the CD34+ cells a minimum of 100 CD34 events should be counted, accordingly to ISHAGE guidelines (Figure 3).

Method for counting CD34+ cells without beads

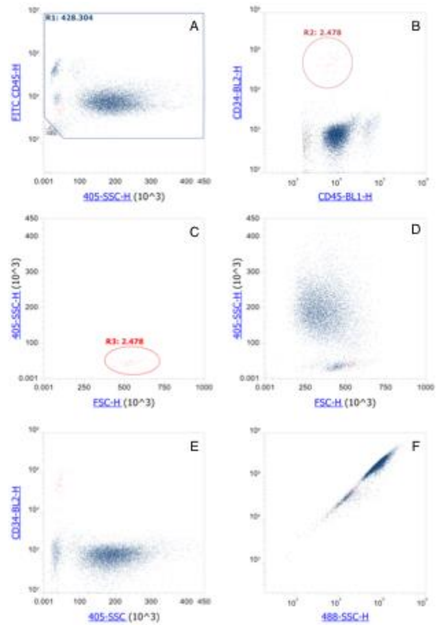

Figure 3. Single-platform assay for absolute CD34+ cells without beads. Analysis of human CD34+ cells using the Attune NxT cytometer configuration using the 405 nm and 488 nm lasers. Utilizing a fluoresecent threshold on CD45-FITC, the CD45-stained cells appear on the bivariate plots as a region of dots clustered in three main populations, consisting of lymphocytes, monocytes and polymorphonuclear cells. RBCs have been eliminated by the dual SSC technique combined with CD45 threshold A V-SSC versus CD45 dot plot (A) is used to discriminate CD45+ cells from debris. A polygonal gate R1 was drawn to enclose the CD45-positive cells, and subsequets bivariate plots were generated based on this gate. CD34+CD45 dim cells are shown in R2 (B). Forward scatter vs. violet side scatter plot is used again to record at least 100 CD34+ cells (R3). This plot is gated to remove debris, then gated to only include CD45+/CD34+ cells (C). D and E plots can also be used to remove debris and non-specific events, whereas CD45+ events are shown in F. Note that red blood cells are excluded as a consequence of an acquisition threshold set in CD45 fluorescence.

COMPENSATION GUIDELINES

• Single color compensation controls should be used with all multicolor experiments in flow cytometry.

• Compensation controls need to be at least as bright as the sample they apply to. Antibody capture beads are ideally suited for this.

• Background fluorescence should be the same for the positive and negative control populations for any given parameter.

• The compensation color must be matched to the experimental color.

• Match fluorophores by brightness (values from the stain index) to density of the antigens—try to match brightest fluorophores with lowest-expressed antigens (PE-CD34), and least bright fluorophores with highest-expressed antigens (FITC-CD45).

• If multiple lasers (spatially separated) are present, spread fluorophores across the lasers to minimize spillover.

• Know your instrument configuration—pick fluorophores that work with your instrument's optical configuration and design panels to maximize use of multilaser instruments.

RESULTS

The CD45-stained cells appeared on the bivariate plots vs. SSC as a region of dots clustered in three main populations, consisting of lymphocytes, monocytes and polymorphonuclear cells. The gating strategy is represented in Figure 3. SSC versus CD45 dot plot (2A) was used to discriminate CD45+ nucleated cells from erythrocytes and debris. A polygonal gate was drawn to enclose the CD45 positive cells (R1), and subsequent bivariate plots were generated based on this gate. Plot 2B shows CD45 vs, CD34, and an oval gate (R2) is drawn around the CD34+ cells. Forward scatter vs. side scatter plots were used again to record at least 100 CD34+ cells (R3).

CONCLUSIONS

This no-lyse/no-wash procedure has several advantages:

- Provides a streamlined sample preparation protocol that avoids cell lysis and centrifugation steps.
- Eliminates the variability when using an assayed counting bead.
- Contributes to a higher level of standardization
- Allows for calculation of absolute numbers of CD34+ cells using the concentration statistic with the Attune NxT Flow Cytometer
- An alternative approach (not presented here) uses Vybrant® DyeCycle™ Violet stain (DCV), in no-lyse no-wash protocols. DCV can be excited with violet lasers and can be used for simultaneous staining with antibodies. A fluorescent threshold based on the DCV fluorescence is used to include only nucleated cells in the analysis.
- The PE may be detected using the yellow laser 561 nm excitation. By moving PE to the yellow laser, there is no spectral overlap between FITC and PE, eliminating the need to perform compensation and thus simplifying the experimental design and instrument set up.

This No-lyse No-wash strategy exploits the difference in light-scattering properties between red blood cells and leukocytes. It may be combined with a fluorescent threshold technique. This protocol is ideally suited to study the numbers of circulating CD34+ in whole blood.

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