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Flow-cytometry-based protocols for human blood/marrow immunophenotyping with minimal sample perturbation

Laura G. Rico, Roser Salvia, Michael D. Ward, Jolene A. Bradford, Jordi Petriz

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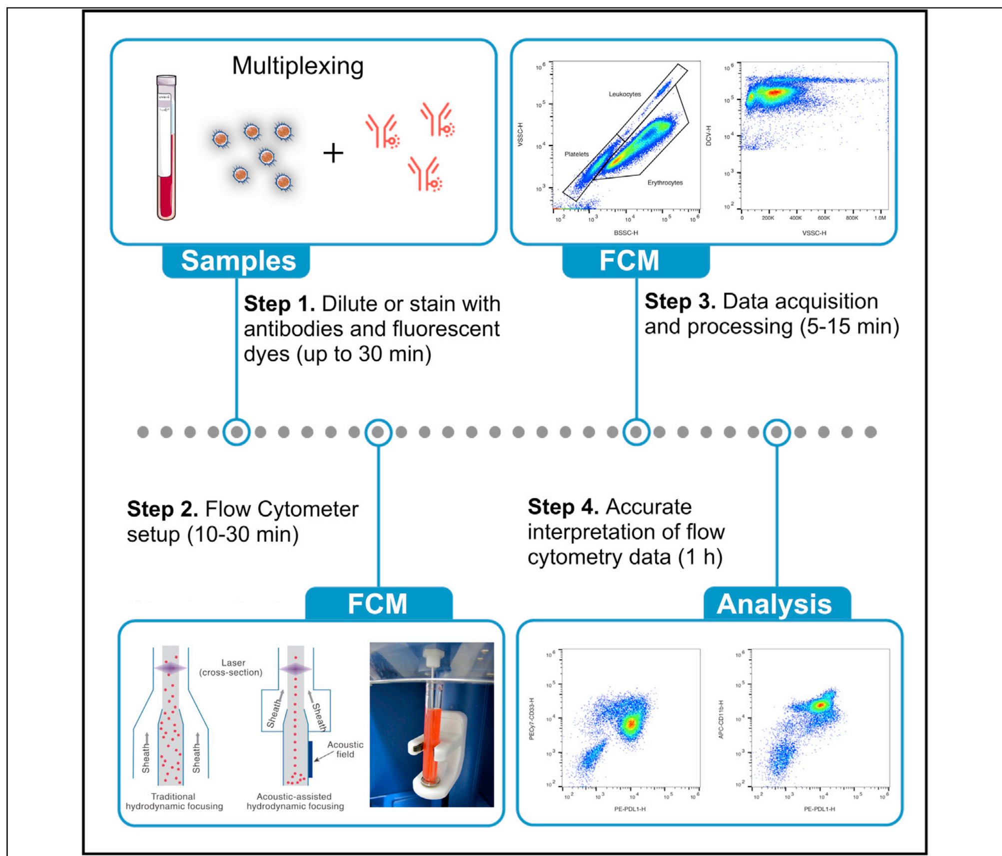

This protocol provides instructions to improve flow cytometry analysis of marrow/peripheral blood cells by avoiding erythrolytic solutions, density gradients, and washing steps. We describe two basic approaches for identifying cell surface antigens with minimal sample perturbation, which have been successfully used to identify healthy and pathologically rare cells. The greatest advantage of these approaches is that they minimize the unwanted effect caused by sample preparation, allowing for improved study of live cells at the point of analysis.

Highlights

Optimized protocol to identify cell surface antigens with minimal sample perturbation

Efficient identification of target cells avoiding artifacts from erythrolytic solutions

Suitable for the simultaneous phenotypic and functional analysis of blood/marrow cells

Detailed gating strategies to discriminate nucleated from non-nucleated cells

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SUMMARY

This protocol provides instructions to improve flow cytometry analysis of marrow/peripheral blood cells by avoiding erythrolytic solutions, density gradients, and washing steps. We describe two basic approaches for identifying cell surface antigens with minimal sample perturbation, which have been successfully used to identify healthy and pathologically rare cells. The greatest advantage of these approaches is that they minimize the unwanted effect caused by sample preparation, allowing for improved study of live cells at the point of analysis.
For complete details on the use and execution of this protocol, please refer to Petriz et al. (2018).

BEFORE YOU BEGIN

This protocol describes two basic flow cytometry-based methodologies for the quantitative analysis of cell surface expression markers on unlysed human whole blood and marrow specimens. This assay can be adapted to identify cell function in combination with cell surface immunophenotyping, even using mice and rats. Furthermore, this protocol can be adapted to fluorescence-activated cell sorting experiments, aimed at the isolation of rare and low abundance live cells in a much larger matrix of hematopoietic cells.

Peripheral blood/bone marrow samples were obtained from patients and healthy volunteers providing their written informed consent according to the protocol approved by the Ethical Committee of the Germans Trias i Pujol Hospital, in agreement with current Spanish legislation. Personal data management was in accordance with the General Data Protection Regulation (EU) 2016/679 (GDPR).

The assay uses monoclonal antibodies and fluorescent probes directly added to anti-coagulated fresh blood or marrow to both investigate cell immunophenotyping and function. After incubation, unlysed cell suspensions are directly diluted to achieve low coincidence with red blood cells (RBCs) and are acquired on a flow cytometer. Importantly, the method can be adapted to study different biomarkers with and without cell stimulation, provided protocols are optimized for cell preparation, staining conditions, flow cytometry settings and system configuration.

When designing an experiment, the investigator must consider that flow cytometry height data is generally more accurate than area data, owing to contributions in area from background coincidence or swarm detection from unlysed erythrocytes. Moreover, low background relative to signal

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is an important consideration for any parameter used for thresholding. Although forward scatter is the most common threshold parameter, fluorescence parameters chosen for thresholds will produce the highest separation possible from the background. We typically use fluorescence-based methods using cell-permeant DNA stains to provide a good compromise between performance and accurate manual gating, especially for highly concentrated cell products and pathological specimens. For example, viable DNA dyes, such as Vybrant™ DyeCycle™ Violet Stain or Hoechst 33342, can be used to detect nucleated cells in blood and in bone marrow, or to discriminate cell aggregates and debris, where scatter data is rapidly degraded with increasing event rate. Avoiding artifacts from sample manipulation, as well as the use of inaccurate light scatter triggering for small pathological cells or early apoptotic events as a consequence of cell shrinkage, is important. We also provide for this protocol a set of main figures illustrating the effect of red cell lysing reagents on cell loss and subsets, the effects on pulse parameter use and scatter degradation, choosing the appropriate threshold for event triggering, setting of threshold and voltage to exclude non-nucleated cells, as well as two representative polychromatic panels displaying color compensation.

Over the years we have successfully deployed this protocol using live and intact blood or marrow cells, such as those aimed at the study of healthy and pathological hematopoietic stem cells (Fornas et al., 2000), erythroid differentiation (Fornas et al., 2002), oxidative burst and neutrophil-platelet complexes (Avendaño et al., 2008), phagocytosis (Lecube et al., 2011), enzymatic activity (Rico et al., 2016, 2019; Bardina et al., 2020), reactive oxygen species production (Cossarizza et al., 2019), cell-mediated cytotoxicity (Rico et al., 2021a), or to the study changes in conformation of the extracellular domain of PD-L1 (Rico et al., 2021b).

Flow cytometer set-up

Timing: 30 min

1. Power on the flow cytometer.
2. Refill for all fluids to ensure all fluid lines are primed.
3. Ensure proper instrument performance and regular calibration of your instrument. The use of performance tracking beads will ensure accuracy and sensitivity of the instrumentation over time.
4. When possible, insert the filter components needed into appropriate locations to collect the violet side-scatter. This step must be done after running the performance test.
5. Create a new experiment or protocol.
6. Select the parameters for measurements. Select pulse height, area and width on different parameters.
7. Select histograms and dot plots needed for display.
8. Select logarithmic or linear plot scaling.
9. Select and adjust the threshold and parameter settings.
10. Draw regions and set gates to be used during data acquisition.

Note: For specific adjustment of the threshold and parameter settings, as well as the experiment workspace and gates to be used during data acquisition, please follow the Step-by-step method details. All details and description needed for every strategy are provided in this section.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies and stains		
PE-CD34 (clone 581). Working dilution: 0.2 mg/mL	Thermo Fisher Scientific	Cat#CD34-581-04
FITC-CD45 (clone HI30). Working dilution: 0.5 mg/mL	Thermo Fisher Scientific	Cat#11-0459-42

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Peripheral blood (aged 0 to 89, 37 males, 24 females)	Hospital Universitari Germans Trias i Pujol (HUGTiP)	http://www.hospitalgermanstrias.cat/
Mobilized peripheral blood (aged 13 to 68, 25 males, 17 females)	Hospital Universitari Germans Trias i Pujol (HUGTiP)	http://www.hospitalgermanstrias.cat/
Bone marrow (aged 26 to 91, 30 males, 13 females)	Hospital Universitari Germans Trias i Pujol (HUGTiP)	http://www.hospitalgermanstrias.cat/
Chemicals, peptides, and recombinant proteins		
7-AAD. Working dilution: 1 mg/mL	Thermo Fisher Scientific	Cat#A1310
Hanks' Balanced Salt Solution (HBSS)	Capricorn	Cat#HBSS2A
Bovine Serum Albumin (BSA)	Sigma-Aldrich	Cat#A9418
Sodium Azide (NaN_3)	Sigma-Aldrich	Cat#S2002
Critical commercial assays		
Vybrant™ DyeCycle™ Violet (DCV) Stain	Thermo Fisher Scientific	Cat#V35003
Vybrant™ DyeCycle™ Ruby (DCR) Stain	Thermo Fisher Scientific	Cat#V10309
Attune™ NxT Flow Cell Cleaning Solution	Thermo Fisher Scientific	Cat#A43635
Attune™ NxT No-Wash No-Lyse Filter Kit	Thermo Fisher Scientific	Cat#100022776
Software and algorithms		
FlowJo™ v10.7.2	Becton Dickinson & Co	n/a
Attune™ NxT Software Version 3.1.1162.1	Thermo Fisher Scientific	Cat#A25554
Deposited data		
Data sets supporting part of the current study	Rico et al. (2021a)	http://flowrepository.org ID# FR-FCM-Z2R8
Other		
12 × 72 mm polypropylene tubes	Sarstedt	Cat#55.526
Eppendorf tubes	Biosigma	Cat#390690
Pipette tips	Daslab	Cat#162001
Attune™ NxT Flow Cytometer	Thermo Fisher Scientific	Cat#A24858
BD Vacutainer Plastic K3EDTA tubes	Becton Dickinson & Co	Cat# 367836

MATERIALS AND EQUIPMENT

Flow cytometry

The flow cytometer used in this protocol was the Invitrogen™ Attune™ NxT Flow Cytometer, equipped with 4 lasers (405 nm Violet, 488 nm Blue, 561 nm Yellow, and 637 nm Red) and 14 fluorescent detectors. It combines acoustic focusing technology coupled with traditional hydrodynamic focusing. The acoustic focusing system complements the hydrodynamic focusing and confers a precise output, especially at high flow rates.

Traditional hydrodynamic focusing flow cytometers can also deploy this protocol successfully. Our preliminary research was done using three- and four-color single-laser bench-top analyzers, such as the EPICS XL system II analyzer, the FACSCan, followed by the FACSCalibur flow cytometers, and with an EPICS Elite and FACSVantage cell sorters for cell sorting experiments (Fornas et al., 2000, 2002).

Many flow cytometers and cell sorters can be configured to use the violet side scatter. Red blood cells contain hemoglobin, a molecule that readily absorbs violet laser (405 nm) light, whereas leukocytes do not (Ost et al., 1998), resulting in a unique scatter pattern when observing human whole blood in the context of blue (488 nm, BSSC) and violet (405 nm, VSSC) side scatter (Figure 1). Blue side scatter alone can also provide very good results, even for discrimination of subpopulations of nucleated erythroid cells during maturation (Fornas et al., 2002). If the cytometer of choice is not equipped with a violet laser, or alternatively, the filter configuration does not allow changes in the configuration optics, the blue side scatter can be collected and used to further categorize the cells of interest. Importantly, for whole blood, forward scatter and side scatter cannot be used to discriminate between erythrocytes and leukocytes owing to the different size of these populations (Figure 2).

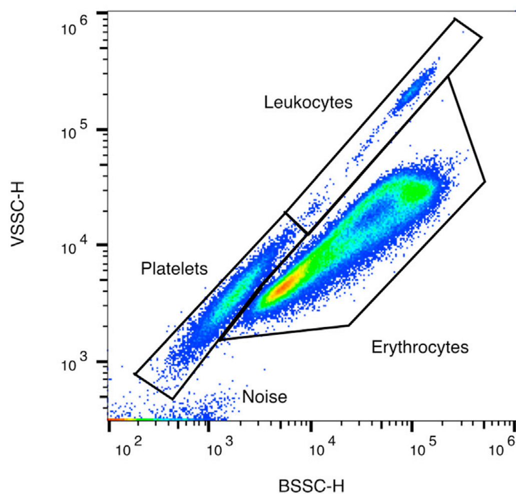

Figure 1. Dual blue and violet laser side scatter using unlysed whole blood
Erythrocytes, leukocytes, and platelets are separated on the basis of light scatter by plotting BSSC vs. VSSC. Hemoglobin absorption of light at 405 nm, reduces the erythrocyte 405 nm violet SSC signal, shifting the red blood cell population relative to leukocytes and platelets.

Examples of most common lasers and configuration optics are listed below:

- For Blue excitation:

Flow cytometer equipped with a blue laser operating at 488 nm

Forward Scatter (FSC): 488/10 band-pass filter.

- For Violet excitation:

Flow cytometer equipped with a violet laser operating at 405 nm.

Forward Scatter (FSC): 488/10 band-pass filter.

Side Scatter (SSC): 405/10 band-pass filter.

Note: Alternative lasers, detectors and filters configurations may be needed when combining conjugated antibodies and/or fluorescent probes when minimal sample perturbation methods are deployed (see Strategy 2: Exploiting the difference in fluorescence properties between leukocytes and erythrocytes).

Attune NxT acquisition software

Attune™ NxT Software 3.1.1162.1 ©2016 Thermo Fisher Scientific Inc is a flexible data acquisition and analysis package specifically used for acquisition on the Attune™ NxT Flow Cytometer.

Water bath kept at 37°C

Light-protected water bath kept exactly at 37°C. The temperature should be controlled by lab mercury thermometer or alternatively by digital thermometer.

HBA Medium: Hanks' Balanced Salt Solution supplemented with 1% BSA 0.1% Sodium Azide (500 mL final volume)		
Reagent	Final concentration	Amount
Bovine Serum Albumin (BSA)	150 μM	5 g
Sodium Azide (NaN_3)	15 mM	0.5 g
Hanks' Balanced Salt Solution	n/a	500 mL
Total	**n/a**	**500 mL**

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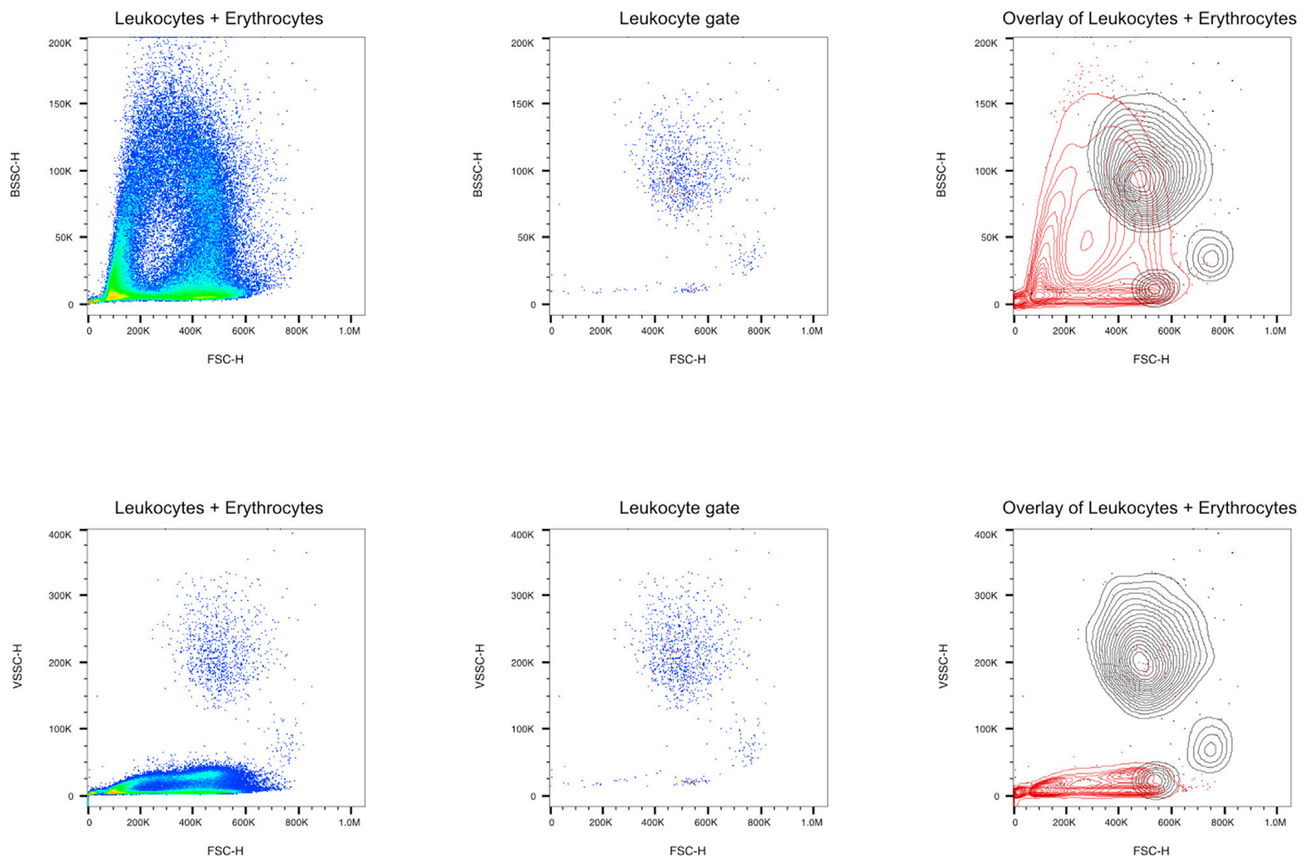

Figure 2. Scatter contribution of erythrocytes that are coincident with leukocytes

The upper row shows the contribution of erythrocytes that are coincident with RBCs when the blue side scatter is used, whereas the lower row shows how the contribution of coincident RBCs is minimized when the violet side scatter is selected. Nucleated cells were gated from a plot of side scatter versus DyeCycle Violet (DCV) fluorescence density plots to visualize nucleated and non-nucleated cells.

Note: Stable under the following storage conditions: 4°C and maximum storage time of 4 weeks.

STEP-BY-STEP METHOD DETAILS

Strategy 1: Exploiting the difference in light-scattering properties between red blood cells and leukocytes

Timing: 35 min

Light scattering is commonly used to discriminate subpopulations of leukocytes by flow cytometry. By exploiting the differential scattering of red and white blood cells and simultaneously detecting side light scatter at 405 and 488 nm, it is possible to differentiate erythrocytes and leukocytes in diluted whole blood. This method is based in the differential light absorbance properties of oxyhemoglobin, with a maximum at 413.1 nm. As red blood cells exhibit a differential absorption of light at 405 nm and 488 nm, the dual scatter plot allows resolution of independent clusters of erythrocytes, platelets and leukocytes (Figure 1).

At 488 nm, the side light scatter of RBCs clearly dominates the whole scatter plot, making the discrimination of leukocytes impossible. At 405 nm, there is an apparent shrinkage of the erythrocyte cluster based on the differential light absorption of hemoglobin at this wavelength

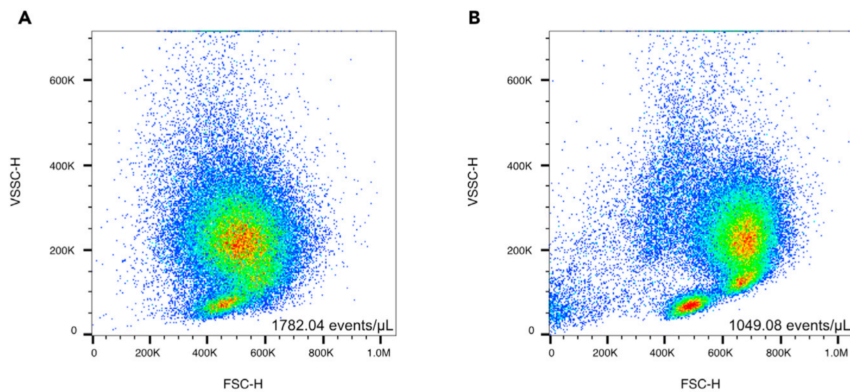

Figure 3. Effect of red cell lysing reagents on cell loss and subsets

Representative forward scatter (FSC) versus side scatter (SSC) density plots comparing the protocol used for minimal sample perturbation in (A) and the effect of lysing reagents in (B), showing alterations in the scatter measurements. Cell counts provided 1782.04 events/μL when using unlysed human peripheral blood (A) and 1049.08 events/μL after a 10 min ammonium chloride lysis protocol (B).

(Figure 2). Hence, the simultaneous detection of side light scatter at 405 and 488 nm the dual scatter plot allows discrimination of RBCs from leukocytes, avoiding the disadvantages of hemolysis, such as incomplete depletion of red blood cells, and potential damage and loss of leukocytes (Figure 3).

Keep in mind the need to plan carefully for the number of cells needed when analyzing a whole blood sample for rare cells, to ensure that a statistically acceptable number of cells can be analyzed from a diluted unlysed blood sample. Determining this number can start with a simple calculation based on the relative or absolute number of target cells (Cossarizza et al., 2019). Using this strategy, lower sample input rates up to 200 μL/min will minimize scatter degradation. Intact erythrocytes outnumber leukocytes up to three orders of magnitude. Expected counts after acquiring 15 μL of the above prepared dilution will approximately provide the following cell distribution: 100,000 to 150,000 erythrocytes, 1,000 to 1,500 platelets and 150 to 250 leukocytes. Even with this small number of leukocytes, scattered light will help to easily discern populations of lymphocytes, monocytes and granulocytes. Indicative percentage of erythrocytes, platelets and leukocytes using peripheral blood obtained from healthy donors (n=10) gave 94.54%–92.13% of erythrocytes, 7.66%–529% of platelets, and 0.30%–0.13% of leukocytes.

Flow cytometer set-up

Timing: 15 min

Note: The flow cytometer used in this study was the Attune NxT Flow Cytometer (Thermo Fisher) (see materials and equipment). Setting up a flow cytometer to detect violet side scatter (VSSC) is easy and requires changing of the optical configuration by physically moving the 405 and the 450 nm filters within the instrument. Please refer to the information supplied by the flow cytometer manufacturer and follow the specific section explaining the procedure you should follow to set up VSSC detection.

CRITICAL: A fixed optical assembly will not allow physically changing bandpass and dichroic filters. Fixed optical architectures without a VSSC channel will not allow VSSC detection even if your cytometer is equipped with a violet laser. If no alternative is possible for violet side scatter collection, the difference in fluorescence properties between leukocytes and erythrocytes can be exploited.

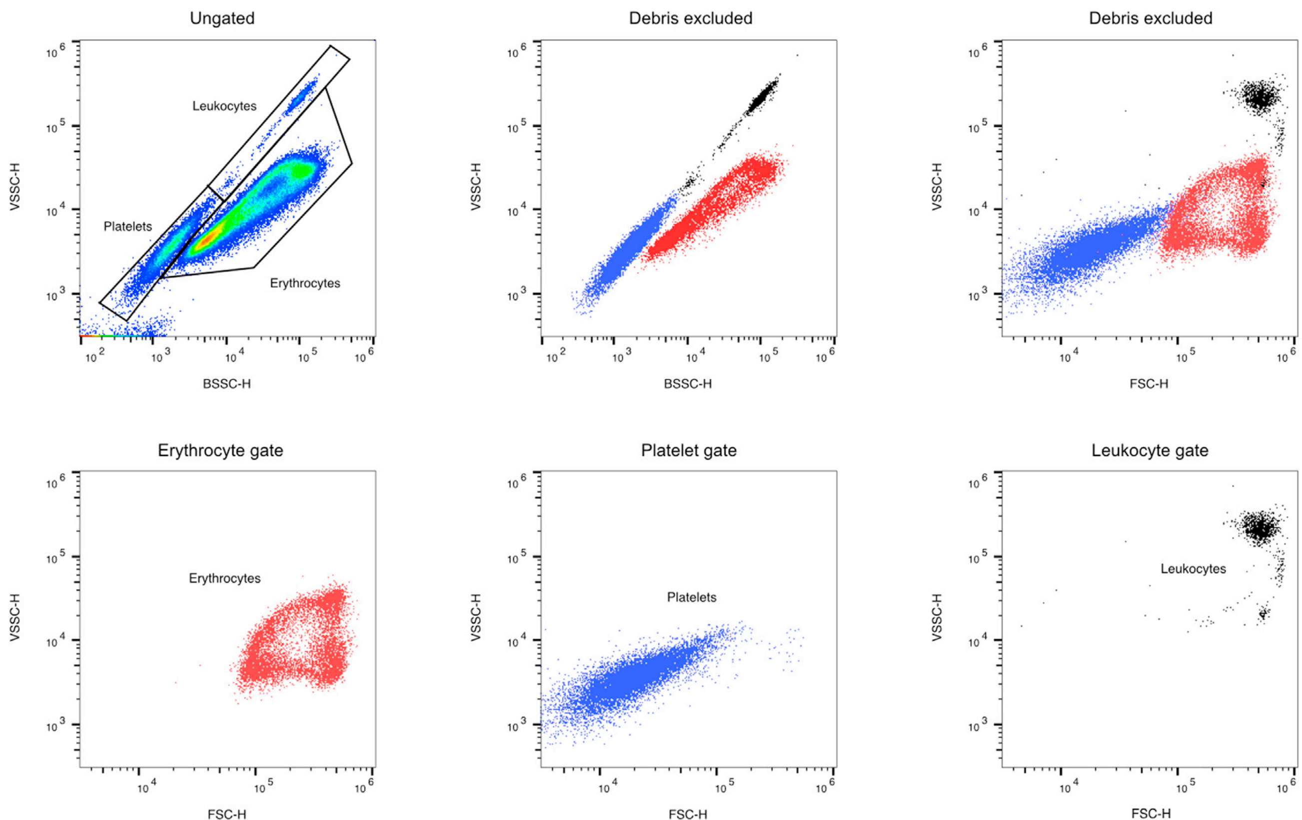

Figure 4. Gating strategy according to the target population of erythrocytes, platelets, and leukocytes

For the study of erythrocytes, gate the 488 nm BSSC-H vs. 405 nm VSSC and logarithmic display excluding platelets, leukocytes and noise. For the study of platelets, gate the 488 nm BSSC-H vs. 405 nm VSSC and logarithmic display including leukocytes and excluding erythrocytes. Use this gate to create a platelet differential scatterplot now excluding leukocytes. For the study of leukocytes, gate the 488 nm BSSC-H vs. 405 nm VSSC and logarithmic display including platelets and excluding erythrocytes. Use this gate to create a leukocyte differential scatterplot now excluding platelets.

1. Create a new experiment.
2. Select the parameters for measurements. Select pulse height for FSC, BSSC, and VSSC (Blue and Violet Side Scatter, respectively). Troubleshooting 1.
3. Select the parameters and an appropriate threshold or thresholds for event triggering. Threshold is selected low enough to capture all events of interests and to eliminate almost all noise and debris. Indicative thresholds on the Attune NxT will be around 1 to 3 × 1000 setting. Troubleshooting 1.

   Note: Although FSC is the most common threshold parameter for immunophenotyping, SSC and in particular VSSC will provide low background relative to signal, as the lower wavelength allows for better particle discrimination according to Mie theory (Bohren and Huffman, 1983). As shown in Figure 1, noise for the VSSC is lower when compared with BSSC. Figure 2 shows the reduced contribution of RBCs that are coincident with leukocytes, being minimized when the VSSC is used.

4. Design the experiment workspace with the following density/dot plots as shown in Figure 4:
   a. Use a dual wavelength scatterplot as a reference method to adjust the parameter settings and the appropriate threshold for triggering: 488 nm BSSC-H vs. 405 nm VSSC-H with logarithmic display.
   b. Use a 488 nm BSSC-H vs. FSC-H plot with logarithmic display identify platelets, erythrocytes and leukocytes. Also use a 405 nm VSSC-H vs. FSC-H density/dot plot.

c. Use a FSC-H vs. a 488 nm BSSC-H plot with linear display (WBC differential scatter plot) to identify leukocytes. Also use a FSC-H vs. a 405 nm VSSC-H plot with linear display, to better visualize the cell populations, especially when high leukocyte rates are needed.

Sample preparation

Timing: 5 min

5. Collect freshly drawn human blood anti-coagulated with EDTA, ACD or heparin. In our laboratory we commonly use K3EDTA tubes validated for routine hematological testing. Please keep in mind that not all anticoagulants are recommended for use in immunophenotyping or functional analysis. Process the samples and collect the data preferably as soon as possible after collection.

CRITICAL: In order to preserve cell function and cell viability, fresh whole blood should be analyzed immediately after collection, when possible. Alternatively analyze samples within two hours of collection. If blood samples cannot be processed within two hours, samples should be kept at room temperature (20°C–24°C) for up to 24 h. Using this strategy, lower sample input rates up to 200 μL/min will minimize scatter degradation. Intact erythrocytes outnumber leukocytes up to three orders of magnitude. Expected counts after acquiring 15 μL of the above prepared dilution will approximately provide the following cell distribution: 100,000 to 150,000 erythrocytes, 1,000 to 1,500 platelets and 150 to 250 leukocytes. Even with this small number of leukocytes, scattered light will help to easily discern populations of lymphocytes, monocytes and granulocytes.

6. Prepare a highly diluted sample of blood to determine the number of leukocytes. Dilute blood 1:500 by mixing 2 μL of freshly collected whole blood in 998 μL of HBSS using a 1.5 mL tube or a 12 × 75 mm polypropylene tube. Minimum number of cells required for analysis should be planned accordingly Poisson statistics.
7. Do not vortex. Carefully homogenize the cell suspension using a P1000 pipette. Wide-bore tips can be alternatively used for marrow processing.

Sample acquisition

Timing: 10 min

8. Run the sample at a low flow rate using a 488 nm BSSC-H vs. 405 nm VSSC-H plot in logarithmic scale and adjust both parameters.
9. Set threshold empirically for event triggering according to the population of interest. Threshold must be selected low enough to capture all events of interests as well as to eliminate almost all noise and debris.
10. Acquire the sample and collect the number of cells needed for analysis, using the gating strategy detailed below. Plan for event rates well below the Poisson 10% coincident rate of an instrument, where 90% of cells can be classified as singlets.

Note: Dual side scatter enables a rapid WBCs quantification of very small sample volume in highly diluted suspensions (1:500) when using VSSC thresholding. Lower volumetric throughput cytometers may need to reduce the theoretical dilution factors to achieve acceptable run times during acquisition.

Gating strategy

Timing: 5 min

11. Apply the following gating strategy according to the target population as shown in Figure 4:
    a. For the study of erythrocytes, gate the 488 nm BSSC-H vs. 405 nm VSSC and logarithmic display excluding platelets, leukocytes and noise.
    b. For the study of platelets, gate the 488 nm BSSC-H vs. 405 nm VSSC and logarithmic display including leukocytes and excluding erythrocytes. Use this gate to create a platelet differential scatterplot now excluding leukocytes.
    c. For the study of leukocytes, gate the 488 nm BSSC-H vs. 405 nm VSSC and logarithmic display including platelets and excluding erythrocytes. Use this gate to create a leukocyte differential scatterplot now excluding platelets.

Note: The differentiation of leukocytes and erythrocytes can be easily validated using fluorescent conjugates either to target specific antigen expressing cells or to combine with viable DNA stains. Moreover, the use of a fluorescent threshold will capture only fluorescent-labeled cells, and will dramatically increase throughput, even to identify rare cells. We have routinely used this method for CD34+ cell determination by flow cytometry analysis. Please see Strategy 2 for more information.

Strategy 2: Exploiting the difference in fluorescence properties between leukocytes and erythrocytes

Timing: 50 min

This strategy involves the use of fluorescent dyes and conjugated antibodies and is predicated on achieving a bright signal for identifying fluorescent triggered events. The method is based on the original strategy reported by Hallermann et al. (1964) in which acridine orange was used to show leukocyte staining in fresh human whole blood.

In this protocol, we describe a strategy for flow cytometric identification of leukocytes in which staining and analytical flow cytometry facilitate the detection and quantification of subpopulations of leukocytes as a reference for avoiding potential artifacts related to sample manipulation, such as those related to red cell lysing, centrifugation and washing steps, or alternatively in pathological specimens resistant to hemolysis. Target cells can be stained with viable DNA probes alone, or in combination with fluorescent conjugates and other fluorescent markers. Please note that some stains tend to be very bright with extensive spectral bleed across multiple channels, so this must be considered for panel design. Multi-laser excitation can often be leveraged to minimize color compensation. Importantly, all stains should be previously titrated to determine concentrations allowing the optimal fluorescence intensity and high signal to noise ratio. We recommend performing kinetic analysis as we previously described (Fornas et al., 2000), or alternately employing batteries of dilutions aimed at quantifying optimal concentrations.

The differentiation of leukocytes and erythrocytes can be easily validated using fluorescent conjugates and viable nucleic acid stains, either used to target CD45-expressing cells or living nucleated cells. The fluorescent threshold will then capture only CD45+ (Figure 5) or alternatively nucleated cells (Figures 6 and 7), and will contribute to dramatically increasing throughput, even allowing identification of rare cells. We have routinely used this method for CD34+ cell discrimination (Rico et al., 2019; Alvarez-Larran et al., 2002; Fornas et al., 2000). The identification of nucleated red blood cells can also be validated using fluorescent conjugates used to target CD45 negative cells, in combination with glycophorin A (GpA), a sialoglycoprotein expressed on human red blood cells and erythroid precursor cells, useful for red cell differentiation studies, as we previously described in Cytometry Part B (Fornas et al., 2002).

Cell-permeant DNA stains are available in several colors. We routinely use Vybrant DyeCycle Violet (DCV), and Hoechst 33342 with violet excitation. Vybrant DyeCycle Ruby (DCR) Stain can be used on

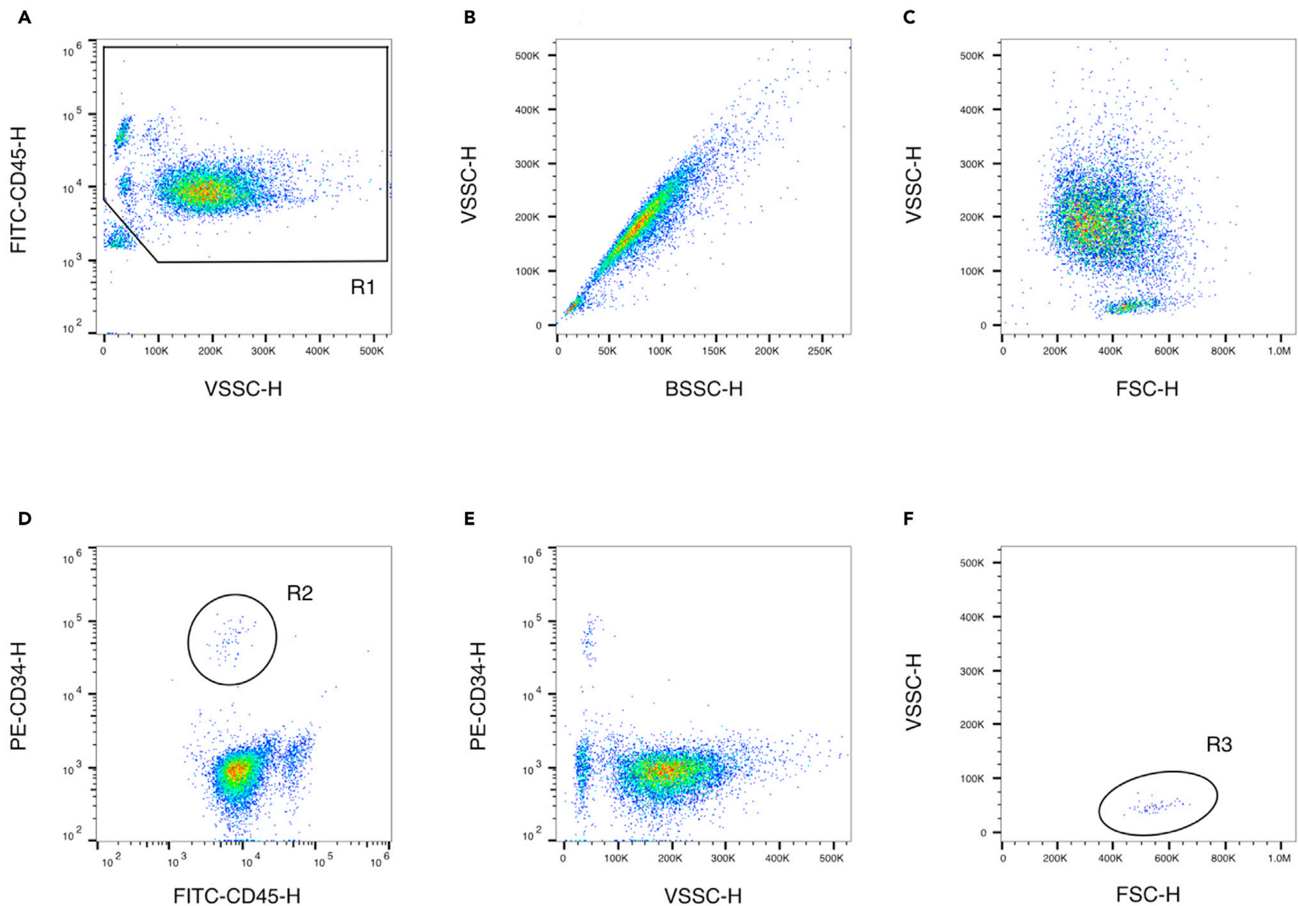

Figure 5. CD34+ progenitor counting in mobilized peripheral blood

Threshold was set on FITC-CD45 fluorescence (A) to discriminate erythrocytes, platelets and debris. CD45+ events were subsequently gated on BSSC vs. VSSC (B), FSC vs. SSC (C), FITC-CD45 vs. PE-CD34 (D) and VSSC vs. PE-CD34 (E). Region (R) 2 comprehends CD34+ progenitor cells, which are further gated in VSCC vs. FSC (F) and selected in R3.

a 488 nm or 635 nm red laser. Green and Orange DyeCycle stains can also be used and adapted for multicolor panel design.

Flow cytometer set-up

Timing: 10 min

12. Create a new experiment or protocol.
13. When possible, insert the filter components needed into appropriate locations to collect the violet side-scatter. This step must be done after running the performance test.
14. Select the parameters for measurements. FSC, 405 nm VSSC, 488 nm BSSC, VL2 (Vybrant DyeCycle™ Violet, DCV), BL1 (FITC-CD45), YL1 (PE-CD34), YL3 (7-AAD). Select pulse height, area and width on different parameters.
15. Insert the filter components needed into appropriate locations to collect the following signals:
    a. Use a 440/50 band-pass filter and 405 nm excitation to collect DCV fluorescence.
    b. Use a 530/30 band-pass filter and 488 nm excitation to collect FITC
    c. Use a 585/16 band-pass filter and 561 nm excitation to collect PE
    d. Use a 695/40 band-pass filter and 561 nm excitation to collect 7-AAD fluorescence

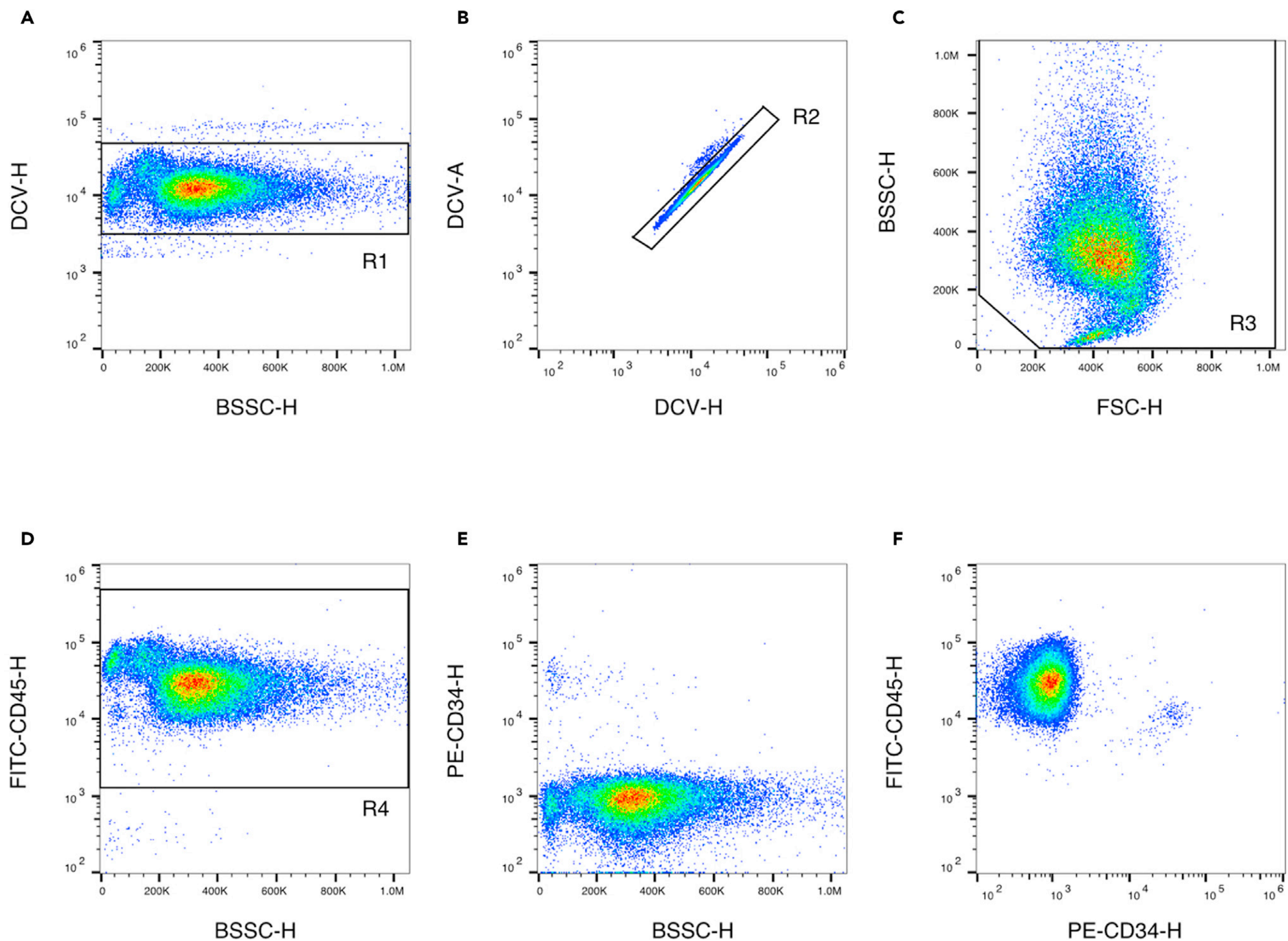

Figure 6. CD34+ progenitor counting in mobilized peripheral blood involving the use of DNA fluorescent stains

Boolean gating strategy was performed following the ISHAGE guidelines for CD34 and CD45 staining. Region (R) 1 was set in a BSSC vs. DCV-H dot-plot (A) to include nucleated cell events and applied in a DCV-H vs. DCV-A dot-plot (B) for doublet discrimination. R2 including single cells was applied in a FSC-H vs. BSSC-H dot-plot (C) to gate leukocytes (R3). R3 was gated in a BSSC vs. FITC-CD45 dot-plot (D) to select CD45+ events in R4, which was applied to display CD34+ in a PE-CD34-H vs. BSSC-H dot plot (E) and in a PE-CD34-H vs. FITC-CD45-H dot plot (F).

16. Set the threshold in CD45 to target only CD45+ cells (Figure 5) or alternatively in DCV to capture nucleated cells (Figures 6 and 7). Indicative thresholds on the Attune NxT will be around 0.5 to 2 × 1000 setting.
17. Design the experiment workspace with the following density/dot plots:
    a. For CD45 thresholding:
        i. BSSC-H vs. VSSC-H with linear scale to display CD45+ cells
        ii. FSC-H vs. VSSC-H with linear scale to display leukocytes
        iii. CD34 PE-H vs. CD45 FITC-H with logarithmic scale to display progenitor cells
        iv. VSSC-H vs. CD34 PE-H with linear and logarithmic and scale respectively to display progenitor cells
        v. FSC-H vs. VSSC-H with linear scale to display leukocytes CD34+ cells
        vi. VSSC-H vs. 7-AAD-H with linear and logarithmic and scale respectively to display necrotic cells
    b. For DCV thresholding:
        i. VSSC-H vs. DCV-H with linear and logarithmic and scale respectively to discard non-nucleated cells.

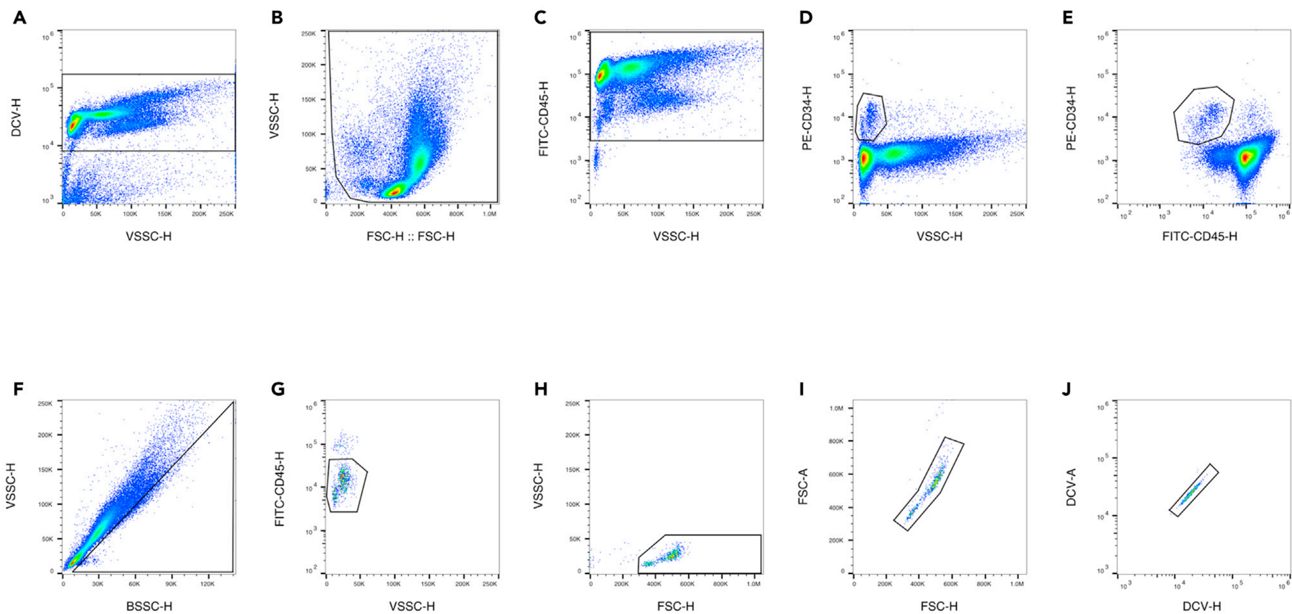

Figure 7. CD34+ progenitor counting in mobilized peripheral blood by light scatter and fluorescent singlet discrimination
Threshold was set on a DCV-H vs VSSC-H dot plot (A) and DCV+ events were subsequently gated on scatter (B), CD45 events (C), and CD34 events (D). CD45 and CD34 fluorescence was plotted to identify CD34 positive cells (E). Blue vs. Violet SSC was used to exclude coincident events (F). Violet SCC vs. CD45 fluorescence was used to select CD34+ events in R3 (G) and subsequently gated on light scatter (H), on light scatter singlets (I) and on fluorescent singlets (J).

ii. FSC-H vs. VSSC-H with linear scale to display leukocytes
iii. DCV-H vs. DCV-A with logarithmic scale to select singlets
iv. VSSC-H vs. CD34 PE-H with linear and logarithmic and scale respectively to display progenitor cells
v. VSSC-H vs. 7-AAD-H with linear and logarithmic and scale respectively to display necrotic cells
vi. CD34 PE-H vs. CD45 FITC-H with logarithmic scale to display progenitor cells
vii. FSC-H vs. VSSC-H with linear scale to display leukocytes CD34+ cells

Note: The ISHAGE strategy guidelines (Sutherland et al., 1996) use a sequential gating strategy to identify target cell populations. According to the ISHAGE protocol, single platform CD34+ counts are obtained after CD34 antibody staining, CD45 antibody staining, and 7-AAD to exclude necrotic cells.

Sample preparation

Timing: 30 min

18. Collect freshly drawn whole blood samples with EDTA-anticoagulant tubes. Prepare the sample and perform the assay within 24 h after sample collection.

CRITICAL: In order to preserve cell function and viability, freshly drawn whole blood should be analyzed immediately after collection. If sample cannot be processed immediately, it should be stored at room temperature for no longer than 24 h.

19. Incubate 50 μL of EDTA anti-coagulated mobilized whole blood for 20 min at room temperature (20°C–24°C) in the dark, with PE-CD34 and FITC-CD45 antibodies, according to the

manufacturer's recommendations (see key resources table). The suggested use of these reagents is 10 μL per million cells in 100 μL volume.

20. Add HBSS supplemented with 1% bovine serum albumin (BSA) and 0.1% sodium azide (NaN_3) (HBA) (see materials and equipment) to obtain a final volume of 100 μL.
21. Dilute the labeled blood 1:10 by adding 900 μL of HBBS for a total 1:20 dilution.
22. Add the DNA stain of choice (in this case DCV) to the cell suspension at a final concentration of 10 μM.
23. Incubate the mixture in the dark at room temperature (20°C–24°C) for 5 min.
24. Homogenize the sample gently using a P1000 pipette.

Sample acquisition

Timing: 5 min

25. Run the sample at 25 μL/min and display the VSSC-H vs. FSC-H plot and the CD34 PE-H vs. CD45 FITC-H plot, both in logarithmic scale. Troubleshooting 2 and 3.

Note: The relative background for unlysed samples can also be decreased by using a side scatter threshold to produce the highest separation from the background. White blood cells can also be separated with a CD45 threshold and applied for CD34 counting without loss of positive events.

26. Set the threshold empirically on the DNA stain used while observing which populations are excluded. Troubleshooting 4.
27. Adjust the different voltages and color compensation values for improved signal to noise ratio.
28. Run the sample to collect the desired events using the gating strategy detailed below.

Gating strategy

Timing: 5 min

29. Set a gate on a VSSC-H vs. DCV-H plot to discard non-nucleated cells
30. Set a gate on a FSC-H vs. VSSC-H plot to select nucleated cells
31. Set a gate on a DCV-H vs. DCV-A plot to select singlets
32. Set a gate on a VSSC-H vs. CD45 FITC-H plot to select leukocytes
33. Set a gate on a VSSC-H vs. CD34 FITC-H plot to select CD34+ events
34. Set a gate on a CD34 PE-H vs. CD45 FITC-H plot to select CD45low progenitor cells.
35. Show preservation of scatter phenotype on a FSC-H vs. VSSC-H plot.

Note: The accuracy of the height (H) parameter is better preserved when unlysed blood is used and is more accurate than area (A). For scatter measurements, VSSC is also more accurate than BSSC. Figures 5 and 6 summarize the gating strategy used to target CD45 expressing cells or living nucleated cells, respectively. Figure 7 shows CD34+ progenitor counting in mobilized peripheral blood by light scatter and fluorescent singlet discrimination.

EXPECTED OUTCOMES

This method has been previously compared with other established methods and it has been applied to peripheral blood and marrow of humans (Fornas et al., 2000; Petriz et al., 2018; Rico et al., 2021a, 2021b) and rats (Núñez- Espinosa et al., 2015, 2016). It avoids erythrocyte lysing procedures, washing steps and peripheral blood mononuclear cell density separation. Critically important for a minimal perturbation assay is selecting the height parameter (H) because it will be more accurate than the area parameter (A) due to significant contributions to the area pulse from the erythrocytes on the scattered light. Importantly, VSSC is more accurate than BSSC (Figure 8).

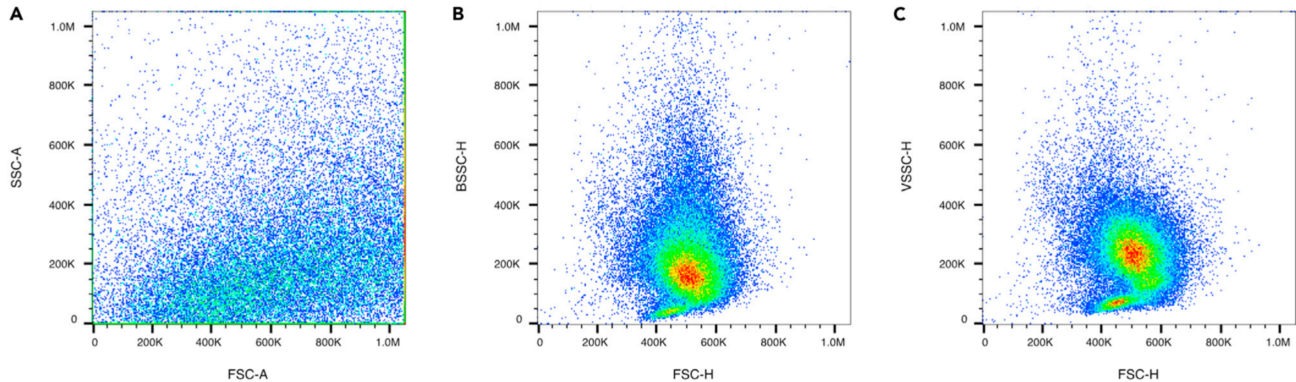

Figure 8. Flow cytometry height (H) data is generally more accurate than area (A) data

Forward scatter (FSC) versus side scatter (SSC) density plots indicating the high contribution in area from background coincidence or swarm detection from unlysed red blood cells when FSC-A vs. SSC-A are used to display leukocytes in (A). FSC-H vs. BSSC-H in (B) and FSC-H vs. VSSC-H in (C) displays accurate leukocyte scatter profiles when the Height parameter is used.

When exploiting the difference in light-scattering properties between red blood cells and leukocytes (Strategy 1), the investigator should be able to visualize the data as shown in Figure 1, following flow cytometry analysis. The first plot is used to display BSSC and VSSC height in logarithmic scale. Dual side scatter analysis will provide information about whole blood populations. Debris in the bottom left corner is gated out. Hemoglobin absorption of light at 405 nm, reduces the RBCs 405 nm violet SSC signal, shifting the RBC population relative to WBCs and platelets. Platelets and WBCs are gated to display lymphocytes, monocytes and neutrophils using a forward scatter and side scatter height plot.

The differentiation of leukocytes and erythrocytes can be easily validated using fluorescent conjugates either to target specific antigen expressing cells or to combine with viable DNA stains. Moreover, the use of a fluorescent threshold will capture only fluorescent-labeled cells, and will dramatically increase throughput, even for rare cell identification and early apoptotic events (Figure 9). We have routinely used this method for CD34+ cell determination by flow cytometry analysis.

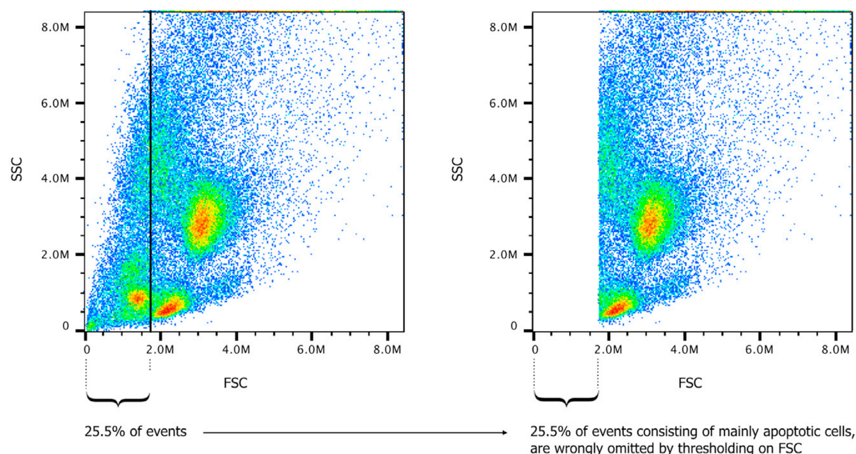

Figure 9. Effect of choosing an appropriate threshold for event triggering

Unlysed whole blood cell data obtained with a fluorescence threshold enables analysis of low forward scatter. Reduced FSC is an indicator of cell death as a consequence of cell shrinkage, and loss of cell membrane integrity have impact in decreased refractive index of cells. In general, granulocytes are the most affected cells, whereas populations of apoptotic lymphocytes and monocytes can also be discerned, globally representing more than 25% of total events as displayed here.

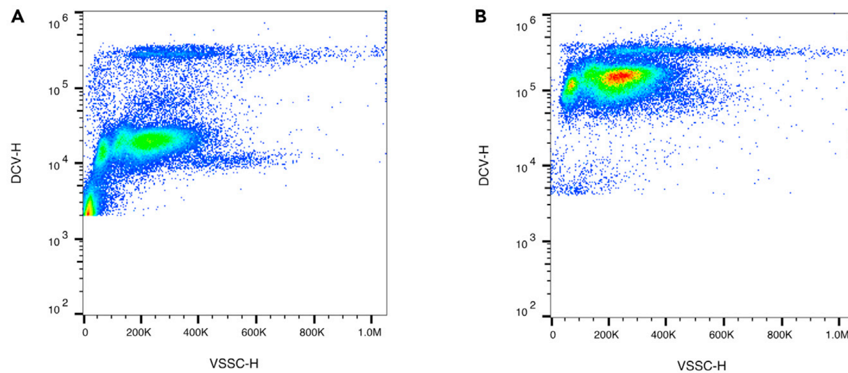

Figure 10. Setting of threshold and voltage used to exclude non-nucleated cells
Side scatter versus DyeCycle Violet (DCV) fluorescence density plots display inappropriate thresholding, low DCV fluorescence intensity, and poor signal to noise ratio (A). Note that the setting of threshold is higher in (B), allowing an optimal separation from non-nucleated cells and debris. The DCV voltage was also set such that all DCV+ events were captured on scale and adjusted to encompass the nucleated cell population and instrument background noise.

Please read Strategy 2 for more information. Figures 5, 6, and 7 show how to analyze human progenitor cells in unlysed whole blood. Representative plots summarize how this configuration can be used to collect the violet side scatter and CD45 triggering to count CD34+ cells in mobilized peripheral blood (Figure 5). Note that platelets and RBCs are not displayed in the classical BSSC and VSSC height plot, because an additional trigger is used for CD45+/low events. After incubation, cells were acquired using the lowest possible sample rate (12.5 events/μL) for 3 min, giving a total approximate yield of 10,000 CD45+ and 50 CD34+ events.

Figures 6 and 7 show representative plots about how to exploit the difference in fluorescence properties between leukocytes and erythrocytes. In this specific case, DNA viable stains are used to discriminate nucleated from non-nucleated cells. The concentration of the nucleic acid dye used is critical for optimal separation and signal to noise ratio (Figure 10). Importantly, when diluting samples, the diluent should then also contain the same staining concentration of dye used to maintain equilibrium. For the evaluation of this methodology, preparation of dyes, labeling strategies with antibodies and probes may need optimization.

LIMITATIONS

This method is designed for analysis of nucleated blood cells in unlysed whole blood. This protocol is not suitable for intracellular staining. This method may be unsuitable for analysis at higher sample rates if dilutions are not prepared accordingly. Assisted acoustic cytometers will provide high resolution when compared with classical hydrodynamic cytometers. Some nucleic acid stains can exhibit spillover into other channels and must be compensated for accurate data measurements (Figures 11 and 12). Potential clogging issues can emerge when using marrow specimens, and samples can be easily filtered using a 50 μM nylon mesh. Some fluorescent dyes can be especially sticky and its convenient to perform a deep flow cell cleaning. Hellmanex® III is a liquid alkaline concentrate for highly effective cleaning of cuvettes made of glass and quartz glass and other sensitive optical parts. Alternatively, Contrad™ 70, a liquid detergent concentrate phosphate-free, chlorine-free, and biodegradable can be used to effective cleaning cuvettes and tubing.

TROUBLESHOOTING

Problem 1

Scattered light is inadequate to discriminate erythrocytes (step 2, 3).

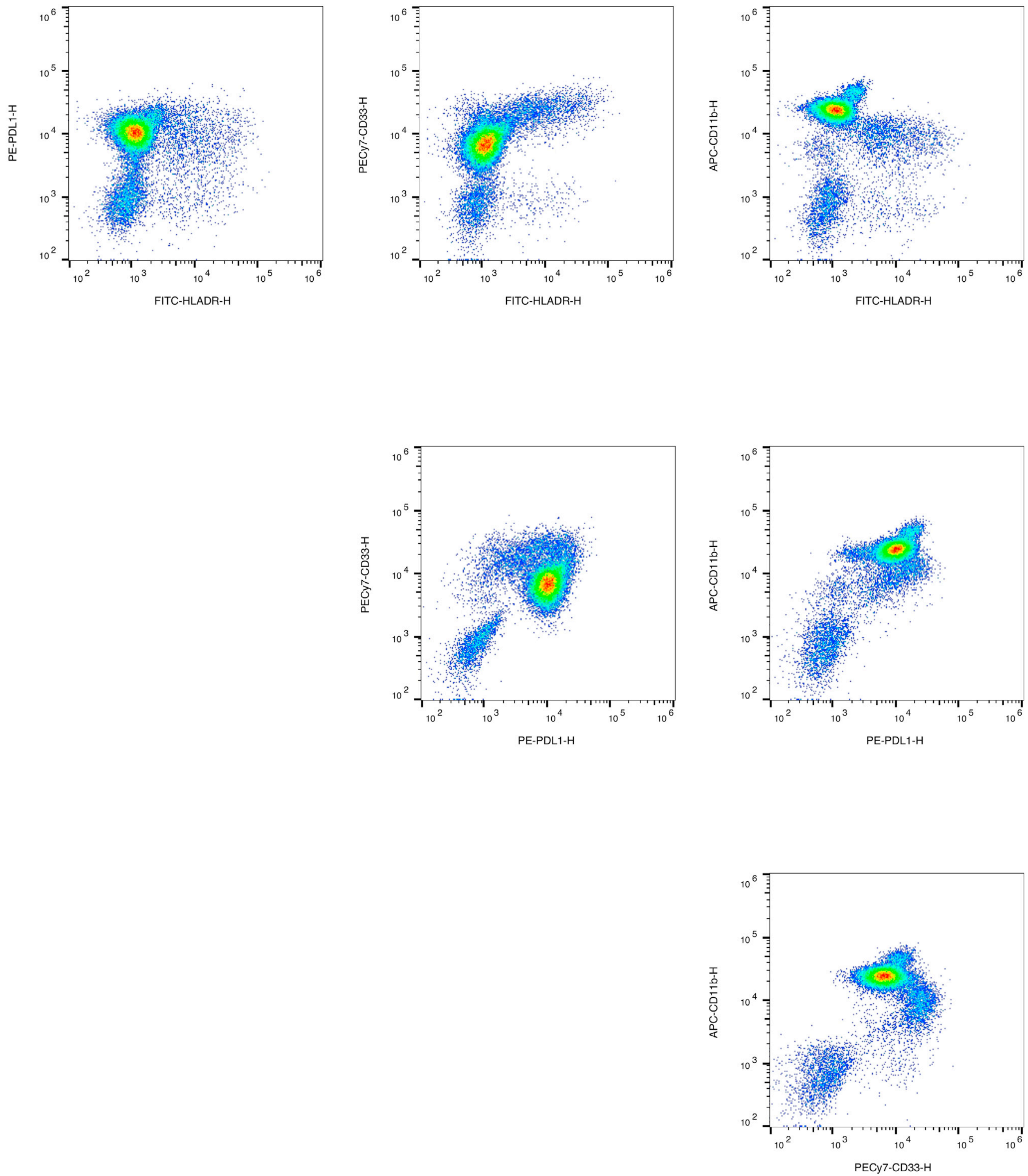

Figure 11. Spectral compensation for flow cytometry with minimal perturbation assays

Nucleated cells were gated from a plot of side scatter versus DyeCycle Violet (DCV) fluorescence density plots to visualize non-transformed compensated data.

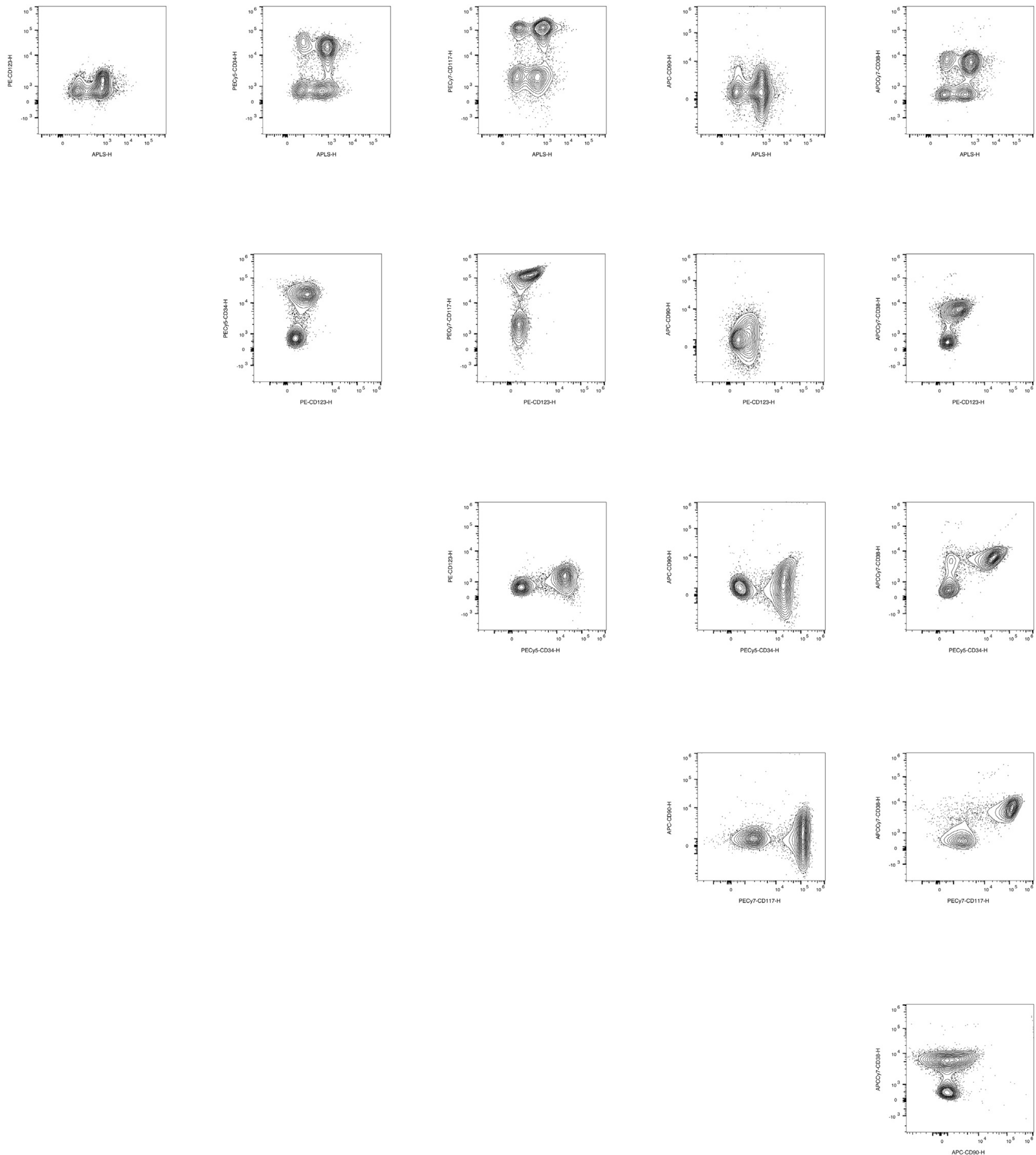

Figure 12. Spectral compensation for flow cytometry with minimal perturbation assays

Nucleated cells were gated from a plot of side scatter versus DyeCycle Violet (DCV) fluorescence contour plots to visualize transformed compensated data.

Potential solution

Possible reasons are that sample rate is too high and is affected by RBC coincidence. Alternatively, Area pulse is used, instead of Height pulse. Since light scatter degradation increases with event rate.

Decrease sample rate or dilute the sample as a potential solution. Moreover, Height is more accurate than Area data, and H pulse data should be used instead the Area pulse.

Problem 2

No fluorescent signal is detected from nucleated cells (step 25).

Potential solution

Possible reasons are that the laser and PMT settings are incompatible with the nucleic acid stain or the fluorescent probe used, or alternatively that a low PMT voltage for the specific fluorescence channel is used. Increase or decrease fluorescence-triggered throughput will help to verify nucleated cells signal. If background Is too high, ensure the proper instrument settings are loaded prior to acquisition as a potential solution. Use suitable positive and negative controls to optimize settings for every fluorochrome. Choose appropriate threshold for event triggering. Verify dye concentration, staining time, temperature and cell counting. Dilution reduces background.

Problem 3

Nucleic acid dye or fluorescent conjugate/probe staining is weak (step 25).

Potential solution

A possible reason is that samples are compromised or aged. Use freshly collected specimens and exclude necrotic cells as a potential solution.

Problem 4

Potential problems may occur when there is a false positive signal due to spillover in one of the fluorescent channels. Using minimal perturbation assays, these problems can be avoided using color compensation and control samples (step 26).

Potential solution

Color compensation can be performed as described by the manufacturer, using the same software alternatives provided, such as those calculated and applied using the compensation matrix. Open the compensation setup dialog. Please note that when exploiting the difference in fluorescence properties between leukocytes and erythrocytes, unstained controls should include a nucleic acid stain. First of all, set the threshold and appropriate voltage for the nucleic acid stain, and later adjust the voltages for the unstained controls. The compensation matrix will be calculated as new compensation controls are recorded. Then repeat the same process for each of the single stained controls.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jordi Petriz (jpetriz@carrerasresearch.org).

Materials availability

This study did not generate new unique reagents. Please contact Dr. Petriz (jpetriz@carrerasresearch.org) to inquire about access to other materials in this manuscript.

Data and code availability

This study did not generate any new code. The data sets supporting part of the current study have been deposited in a public repository (http://flowrepository.org; ID#FR-FCM-Z2R8; Rico et al., 2021a). All data is available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

L.G.R., R.S., M.D.W., J.A.B., and J.P. developed the protocols used in the study and wrote the manuscript.

DECLARATION OF INTERESTS

M.D.W. and J.A.B. are employees of Thermo Fisher Scientific, which is in the business of selling flow cytometers and flow cytometry reagents. The rest of the authors declare no competing interests.

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