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One-step White Blood Cell Extracellular Staining Method for Flow Cytometry

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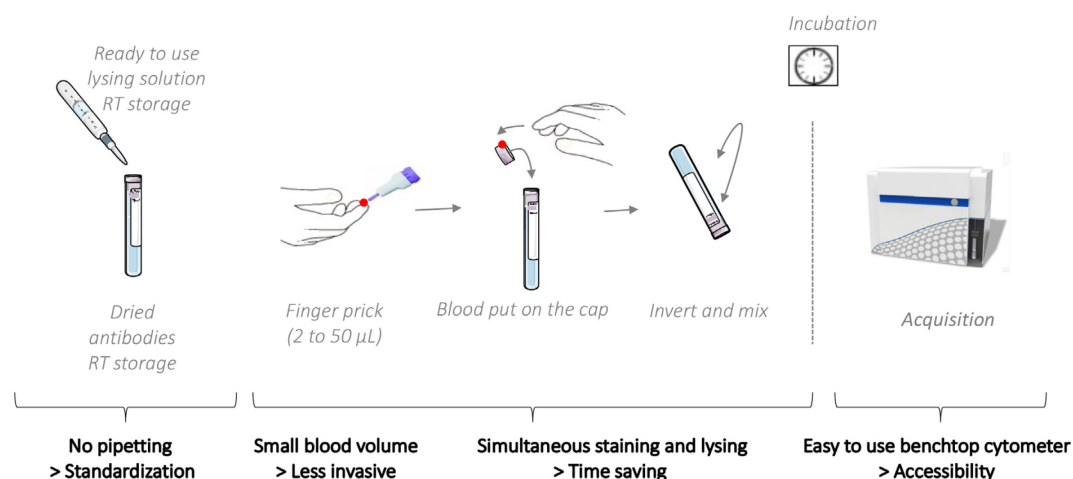
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[Abstract] Flow cytometry is a powerful analytical technique that is increasingly used in scientific investigations and healthcare; however, it requires time-consuming, multi-step sample procedures, which limits its use to specialized laboratories. In this study, we propose a new universal one-step method in which white blood cell staining and red blood cell lysis are carried out in a single step, using a gentle lysis solution mixed with fluorescent antibody conjugates or probes in a dry or liquid format. The blood sample may be obtained from a routine venipuncture or directly from a fingerprick, allowing for near-patient analysis. This procedure enables the analysis of common white blood cell markers as well as markers related to infections or sepsis. This simpler and faster protocol may help to democratize the use of flow cytometry in the research and medical fields.

Graphic abstract:



One-step White Blood Cell Extracellular Staining Method for Flow Cytometry.

Keywords: Blood, Leukocytes, Flow cytometry, One-step method, Point-of-care

[Background] Flow cytometry is increasingly used in scientific investigations and healthcare; however, it requires a time-consuming, multi-step sample preparation procedure, which limits its use to specialized laboratories. Most whole blood sample preparation protocols for extracellular staining consist of at least

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three steps: using a venous blood sample collected in an anticoagulated sampling tube, 50-100 μ l is pipetted into a reaction tube; as a second step, fluorescent antibodies are mixed and incubated with the blood to allow white blood cell staining; as a third step, the lysis solution is added to the reaction tube to allow for red blood cell removal; finally, an optional washing step is performed to reduce non-specific fluorescence.

Due to these multiple timed steps that need to be carried out in specialized laboratories, the use of flow cytometry is limited. It is, for example, not yet routinely used in the clinical field, even though the assessment of patient immune cells has proven helpful in the indication of potential disease activity (Brown and Wittwer, 2000). Some authors have tried to establish protocols with only one step, proposing to remove the lysis step; however, this method necessitates a dedicated flow cytometer, restricting its use (Petriz *et al.*, 2018).

In this study, we propose a new universal method in which white blood cell staining and red blood cell lysis are carried out in a single step. The lysis solution is stable at room temperature (RT) and does not interfere with the staining owing to its composition, which comprises a substrate that is specifically cleaved by an enzyme uniquely present in RBC membranes (van Agthoven, 2007). White cells are maintained at neutral pH under isotonic conditions and are therefore not affected. The lysis solution can include low-dose formaldehyde (0.05%) to stabilize markers of interest (Bourgoin *et al.*, 2020c).

The fine titration of the conjugates and their direct dilution in the lysis solution enable the final washing step to be eliminated while maintaining low background levels. Using the new Dried Unitized Reagent Assays (DURA) Innovations technology, the conjugates can be dried, which enables RT storage and removes the need for pipetting, thereby supporting better standardization. The blood sample may be of venous origin or from a fingerprick, allowing for less invasive sampling suitable for a point-of-care setting. This approach is possible thanks to an excess volume of lysis solution, which permits efficient RBC lysis in up to 50 μ l of blood. The conjugate quantities have also been adjusted to stain white blood cells contained in 2-50 μ l blood, with a small quantity of blood no longer being a limitation because 2 μ l blood contains an average of 10,000 leukocytes. As a 100-cell subpopulation is usually considered statistically representative, using 2 μ l of blood allows for analysis of subpopulations as low as 1%, which is sufficient for most routine applications.

Since management of patients with infections in the Emergency Department (ED) is challenging for practitioners, we developed a panel that enables rapid patient triage in the ED using flow cytometry. It has been shown that monitoring the expression of CD169 on monocytes (mCD169), CD64 on neutrophils (nCD64), and HLA-DR on monocytes (mHLA-DR) by flow cytometry can be indicative of viral or bacterial infection, or sepsis, respectively. We therefore established a panel consisting of antibodies targeting these three markers and evaluated the one-step method in subjects with infection and septic conditions by measuring the expression of the three infection-related markers (Bourgoin *et al.*, 2019a, 2019b, 2020a, 2020b, 2020c, and 2021; Bedin *et al.*, 2020; Michel *et al.*, 2020).

Many other applications can be envisioned in fields where flow cytometry is routinely performed, such as measuring T, B, and NK cell proportions, detecting leukemias and lymphomas, and enumerating CD34⁺ stem cells for transplantation.

Materials and Reagents

1. Regular flow cytometry 5-ml test tubes, polypropylene or polystyrene (12 × 75 mm), or 1.4-ml microtubes (e.g., from Micronic), or deep-well plates (any vendor).
2. Lysis solution (Beckman Coulter, VersaLyse™, catalog number: IM3648, store at 18-24°C, 2-year shelf-life)
3. Fixative solution (IOtest®3 10×; Beckman Coulter, catalog number: A07800, store at 2-8°C, 1-year shelf-life)
4. Antibody cocktail (IOtest Myeloid Activation CD169-PE (Phycoerythrin)/HLA-DR-APC (Allophycocyanin)/CD64-PB (Pacific-Blue) Antibody Cocktail from Beckman Coulter, catalog number: C63854, store at 2-8°C, 1-year shelf-life)
5. Easy batch preparation of the lysis buffer (see Recipes)
6. Batch preparation of the staining and lysis buffer for n tests (see Recipes)

Equipment

1. Pipettes (Gilson, PIPETMAN®, catalog numbers: FA10003M [2-20 µl] and FA10006M [100-1000 µl])
2. 3-laser, 10-color cytometer (Beckman Coulter, Navios, catalog number: B47904) or 3-laser 13-color cytometer (Beckman Coulter, CytoFLEX, catalog number: B53000). Any other 3-laser cytometer can be used (e.g., Becton Dickinson FACSCanto, Cytex Aurora)

Software

1. Kaluza Software version 2.1 (Beckman Coulter, <https://www.beckman.fr/flow-cytometry/software/kaluza>)

Note: Any other flow cytometry software can be used (e.g., Becton Dickinson Flowjo and Cytex SpectroFlo).

Procedure

1. Prepare the lysis buffer (wear common Personal Protective Equipment).
Dilute 1:200 the fixative solution in the lysis solution under chemical hood protection and mix.
2. Perform the lysis–staining step.
3. In a tube containing 500 µl lysis buffer and 10 µl conjugate panel, add 2-50 µl blood sample (venous or capillary), mix, and incubate for 15 min in the dark at RT (18-25°C).
4. Acquire the data on a 3-laser cytometer (then dispose of the waste in containers dedicated to biohazard waste).

Data analysis

A. Acquiring data on the flow cytometer

Unstained cells are used to set the parameters of the flow cytometer.

1. Turn on the flow cytometer.
2. Open the acquisition software and create four dot plots:
 - a. Side Scatter (SSC) on the x-axis and Forward Scatter (FSC) on the y-axis.
 - b. Channel dedicated to PE (FL2 on Navios) detection on the x-axis and SSC on the y-axis.
 - c. Channel dedicated to APC (FL6 on Navios) detection on the x-axis and SSC on the y-axis.
 - d. Channel dedicated to PB (FL9 on Navios) detection on the x-axis and SSC on the y-axis.
3. Set the voltages/gains for the SSC-FSC plot such that most of the cell population is in the middle of the graph (Figure 1a). The discriminant/threshold should be set up in such a way that minimum debris is acquired.
4. Set all the compensations to 0.
5. Set the voltages for FL9, FL2, and FL6 such that the lymphocyte mean or median fluorescence intensity is around 0.3 (Figure 1b).

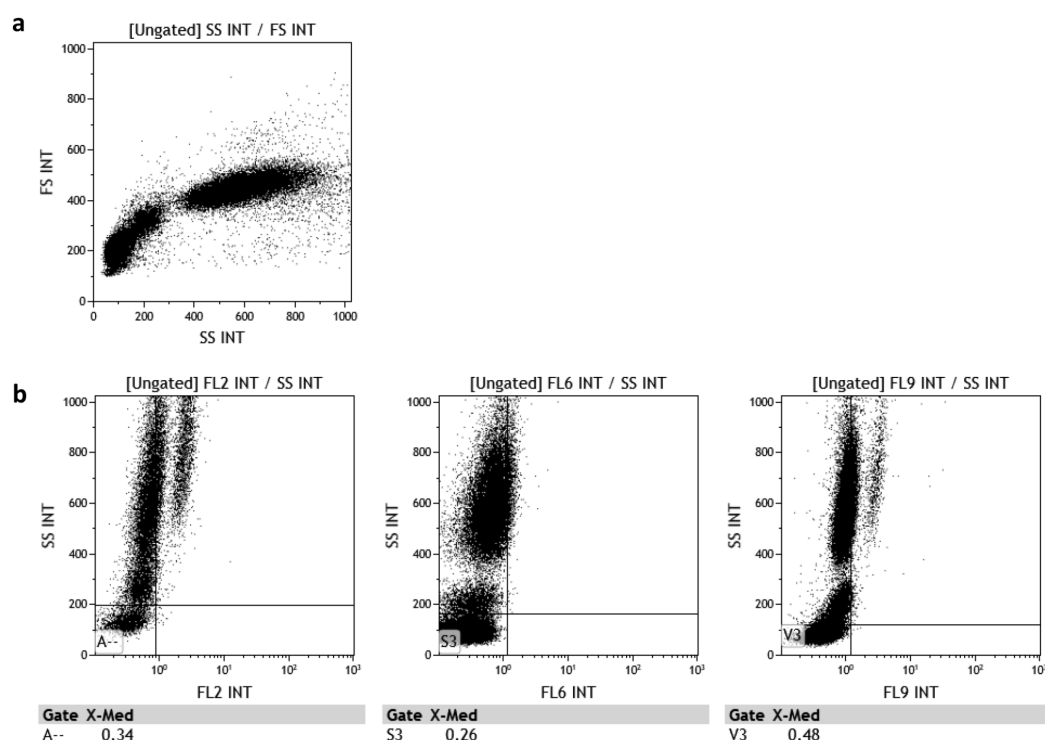


Figure 1. Flow cytometry parameters on (a) SSC-FSC plot and (b) FL2-, FL6-, and FL9-SSC plots

6. Save the protocol.
7. Acquire the first stained sample using a high flow rate ($\sim 1 \mu\text{l/s}$) without any compensation. Make sure to acquire at least 500 monocytes for a robust analysis (usually 30 to 90 s).

8. On the SSC-FSC plot, draw a leukocyte gate, avoiding debris (Figure 2a).
9. On the SSC-FL9 (CD64-PB) plot, draw the lymphocyte, monocyte, and neutrophil gates (Figure 2b).
10. CD169 and HLA-DR expression levels should be assessed on monocytes. CD64 expression levels should be assessed on neutrophils (Figure 2b and 2c).

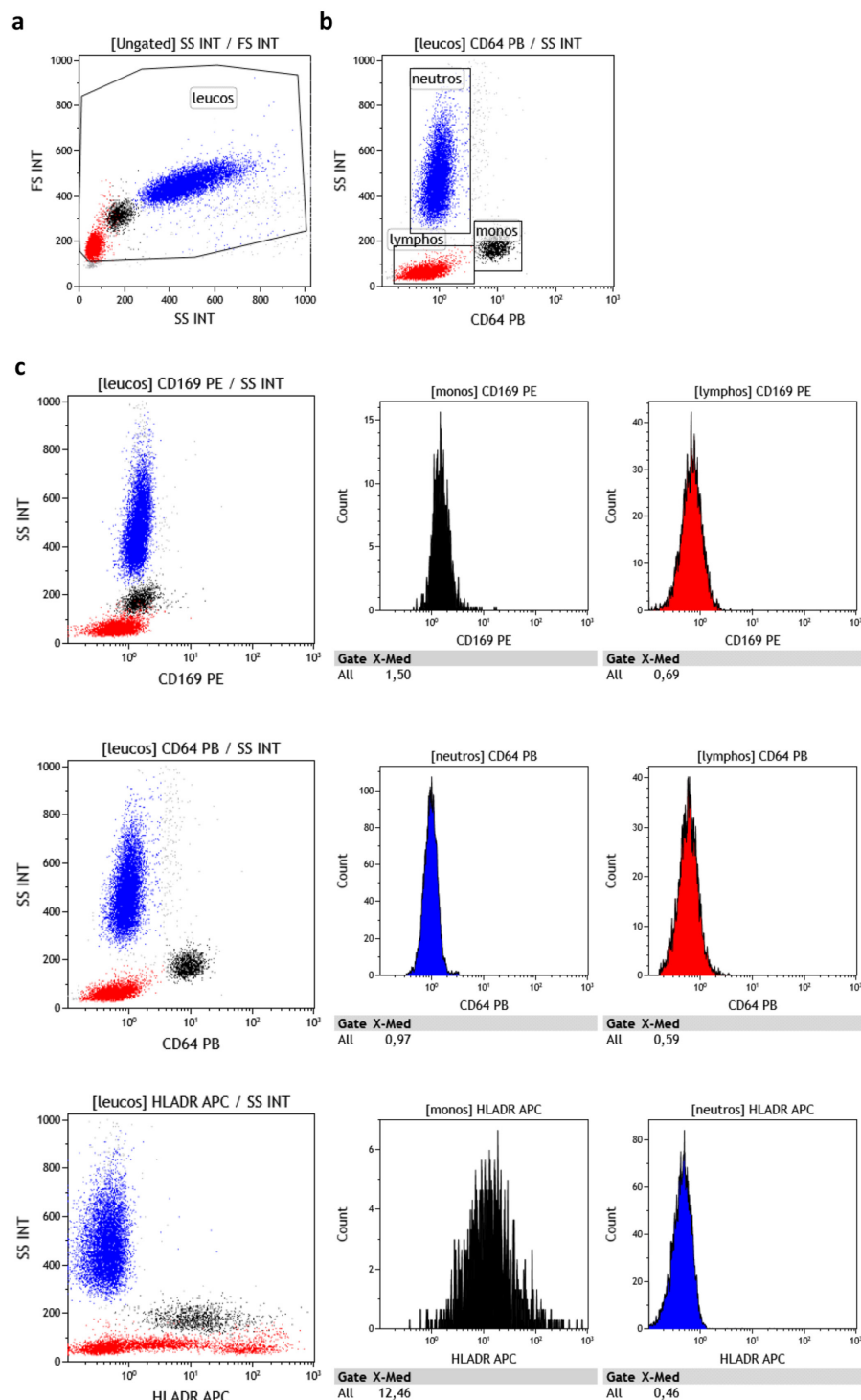


Figure 2. Data analysis procedure. (a and b) Gating strategy of lymphocytes, monocytes, and

neutrophils. (c) CD169, CD64, and HLA-DR expression level assessment strategy.

11. Marker levels should be expressed as the Mean of Fluorescence Intensity, Median of Fluorescence Intensity, or Signal-to-noise. Signal-to-noise is calculated as follows: CD169 expression levels on monocytes should be divided by CD169 expression levels on lymphocytes; CD64 expression levels on neutrophils should be divided by CD64 expression levels on lymphocytes; and HLA-DR expression levels on monocytes should be divided by HLA-DR expression levels on neutrophils (Figure 3).
12. Results are considered as follows: High mCD169 expression is indicative of viral stimuli, high nCD64 expression is indicative of bacterial stimuli, high mHLA-DR expression is indicative of infectious stimuli, and low mHLA-DR expression is indicative of immune exhaustion (Figure 3).

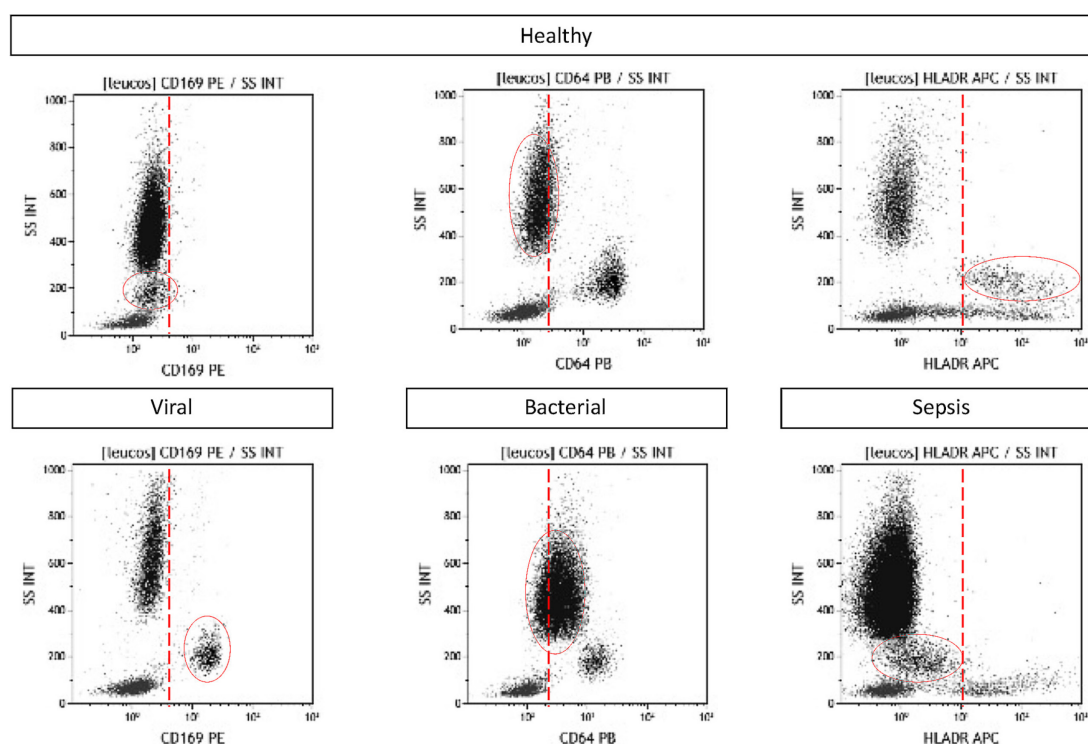


Figure 3. mCD169, nCD64, and mHLA-DR expression assessment in whole blood using the one-step method. Examples are given for one healthy volunteer, one virus-infected patient, one bacteria-infected patient, and one patient with sepsis.

13. More research will be necessary to precisely set the thresholds in each laboratory setting, but as an indication, previous evaluations have shown that thresholds should be close to 3 for the CD169 and CD64 indexes.
14. Further analysis can be performed using software such as Kaluza (re-analysis, other gating and auto-gating, other histograms or dot plots, coloring, compensation adjustments, refined percentages and fluorescent values, overlay and merge data sets, batch analysis for higher throughput, etc.).

Notes

Depending on the target marker, application, and required stability, the method should be adapted in each laboratory setting. For example, and only for indication and not as an established performance, it has been observed in some applications that:

1. Blood sample can be venous or capillary.
2. Blood can be anticoagulated with EDTA or heparin.
3. Thanks to the gentle lysis reagent and added fixative, blood can be processed up to 3-4 days after sampling when stored at RT or 2-8°C. Moreover, samples can be analyzed on the cytometer up to 3 days after processing when stored at 2-8°C.
4. The lysis solution can include 0.05% fixative solution.
5. The lysis buffer (lysis solution containing fixative solution) can be stored for up to one month at RT.
6. The targeted marker can be any membrane marker.
7. Antibodies can be liquid or dried depending on product availability.

WARNING:

- a. If a liquid cocktail is used, an antibody volume higher than 100 µl could impact lysis efficiency.*
 - b. If a commercial DURAClone format is used, the lysis volume should be increased to 1 ml (for 2-50 µl blood) or 2 ml (for 50-100 µl blood as per IFU).*
8. Antibodies can be premixed with the lysis buffer up to a few days prior.

Recipes

Note: To be adapted depending on each application; performances are not established.

1. Easy batch preparation of lysis buffer
One bottle Versalyse solution (100 ml)
500 µl fixative solution (10×)
Mix and store at RT
2. Batch preparation of the staining and lysis buffer for n tests
Pipet (n+1) × 500 µl lysis buffer
Add (n+1) × 10 µl myeloid activation cocktail (or any other panel at its optimal dose)
Mix well and distribute 510 µl per reaction tube, store in the dark
Tube is now ready to receive the blood sample

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research reagents used in the flow cytometry experiments and participation of the four employees: IAB, PB, JMB, and FM. The method described was modified from 'VersaLyse and the mechanism of ammonium chloride lysis' (van Agthoven, 2007) and resulted in recent publications (Bourgoin *et al.*, 2019a, 2019b, 2020a, 2020b, 2020c, and 2021; Bedin *et al.*, 2020; Michel *et al.*, 2020).

Competing interests

There are no conflicts of interest or competing interests.

Ethics

All enrolled patients provided informed consent, and the procedures followed were in accordance with the Helsinki Declaration. Routine care of the subjects was not modified; analyses were performed on anonymized left-over blood, and all data collected in the study were part of routine clinical practice and retrieved from subject records. Results of this study had no influence on subject management.

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