Video Article

Enumeration of Major Peripheral Blood Leukocyte Populations for Multicenter Clinical Trials Using a Whole Blood Phenotyping Assay

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Abstract

Cryopreservation of peripheral blood leukocytes is widely used to preserve cells for immune response evaluations in clinical trials and offers many advantages for ease and standardization of immunological assessments, but detrimental effects of this process have been observed on some cell subsets, such as granulocytes, B cells, and dendritic cells [1-3]. Assaying fresh leukocytes gives a more accurate picture of the *in vivo* state of the cells, but is often difficult to perform in the context of large clinical trials. Fresh cell assays are dependent upon volunteer commitments and timeframes and, if time-consuming, their application can be impractical due to the working hours required of laboratory personnel. In addition, when trials are conducted at multiple centers, laboratories with the resources and training necessary to perform the assays may not be located in sufficient proximity to clinical sites. To address these issues, we have developed an 11-color antibody staining panel that can be used with Trucount tubes (Becton Dickinson; San Jose, CA) to phenotype and enumerate the major leukocyte populations within the peripheral blood, yielding more robust cell-type specific information than assays such as a complete blood count (CBC) or assays with commercially-available panels designed for Trucount tubes that stain for only a few cell types. The staining procedure is simple, requires only 100 µl of fresh whole blood, and takes approximately 45 minutes, making it feasible for standard blood-processing labs to perform. It is adapted from the BD Trucount tube technical data sheet (version 8/2010). The staining antibody cocktail can be prepared in advance in bulk at a central assay laboratory and shipped to the site processing labs. Stained tubes can be fixed and frozen for shipment to the central assay laboratory for multicolor flow cytometry analysis. The data generated from this staining panel can be used to track changes in leukocyte concentrations over time in relation to intervention and could easily be further developed to assess activation states of specific cell types of interest. In this report, we demonstrate the procedure used by blood-processing lab technicians to perform staining on fresh whole blood and the steps to analyze these stained samples at a central assay laboratory supporting a multicenter clinical trial. The video details the procedure as it is performed in the context of a clinical trial blood draw in the HIV Vaccine Trials Network (HVTN).

Video Link

The video component of this article can be found at https://www.jove.com/video/4302/

Protocol

Note: To protect the fluorophore-conjugated antibodies from light, perform all steps in a bio-safety cabinet with the light off.

1. Antibody Staining Panel Preparation

1. The antibody staining panel can be found in **Table 1**. Antibody concentration should be defined by titration with whole blood and using the same flow cytometry equipment and procedures that will be used to acquire the stained phenotyping samples.
2. Once appropriate staining titers are determined, combine all antibodies into a single mixture in a lock-cap tube. Add flow wash buffer (Dulbecco's PBS with 2% heat inactivated fetal bovine serum) to bring the total volume to 100 µl. Scale the mixture for the number of samples being stained. This mixture can be stored at 4 °C for up to eight weeks.

2. Staining

1. If the blood collected is to be used for other purposes in addition to this assay, set an aliquot aside while more time-sensitive procedures are performed on the remaining blood. The aliquot can be stored at room temperature for up to 4 hr after venipuncture without significant cell loss.
2. Verify that there is an intact bead pellet at the bottom of the Trucount tube and label the tube to identify the sample being stained. In HVTN clinical trials, the Laboratory Data Management System (Frontier Science and Technology Research Foundation; Amherst, NY) is used to label and track stained samples.

3. Record the lot numbers and expiration dates of all reagents. Record the Trucount tube bead count number provided by the manufacturer on the bag of tubes; make sure that the lot number on the bag matches the lot number on the tube.
4. Use reverse pipetting to accurately pipette 100 µl of whole blood into the Trucount tube, just above the metal retainer. Avoid smearing blood down the side of the tube.
5. Using regular (forward) pipetting technique, pipette 100 µl of the mixed antibody staining panel (See **Table 1**) into the Trucount tube. Cap the tube and vortex at low speed for approximately 15 sec to mix. Visually inspect the tube to ensure that the bead pellet is fully dissolved.
6. Incubate the Trucount tube for 15 minutes at room temperature (15-30 °C) in the dark.
7. If necessary, dilute an aliquot of 10× FACS Lysing solution to 1X using diH_2O. Add 900 µl 1× FACS Lysing solution to the tube.
8. Cap the tube and vortex thoroughly at low speed for approximately 15 sec to mix. Push the cap down firmly into locking position on the tube and seal with laboratory film.
9. Store the tube at -65 °C to -95 °C until the sample is ready for shipment to the central analysis lab or for analysis in house. Samples are stable at this stage for at least four weeks. If shipping or assaying immediately, this step can be omitted.

3. Shipping

Note: The following instructions utilize an insulated shipping system from Saf-T-Pak, Inc. specifically designed for shipping category B exempt biological substances according to International Air and Transport Association (IATA) regulations. If analyzing the samples in the same location as the staining occurred, go to section 4.

1. Samples can be shipped immediately after staining or once they are frozen at -65 °C to -95 °C. Wrap each tube completely in foil and place in the stained specimen box. Place the stained specimen box inside a leakproof poly-bag with absorbent material.
2. Place the leakproof poly-bag and contents into a Tyvek bag and seal with as little air as possible in the bag.
3. Place the specimen package (specimen inside secondary packaging) inside the inner brown box.
4. Place the inner brown box inside the Styrofoam chest, securing it into the indentation to prevent shifting.
5. Fill the Styrofoam chest with dry ice (approximately 8 kg) and place the lid tightly on the chest.
6. Securely tape the shipping box and ship as Biological Substance, Category B (UN3373) with the proper Dry Ice (UN1845) markings; follow IATA PI-650 instructions.
7. Upon receipt, samples are stored at -65 °C to -95 °C until they are analyzed.

4. Thawing and Flow Cytometry Analysis

1. Remove the stained sample from the freezer to thaw at room temperature in the dark before collecting on the flow cytometer. If collecting data on more than one sample, standardize the process for all tubes by staggering thawing so that the tubes are not sitting at room temperature for more than 1 hr each.
2. Samples should be acquired using a four laser flow cytometer equipped with appropriate filters, such as the BD LSR II. Use standard cytometer calibration and fluorescence compensation methods for data collection [4].

Note: Do not set forward scatter or side scatter thresholds during collection [5]. Trucount beads can fall below the lowest possible threshold setting for these parameters causing a subset of beads to not be accounted for during analysis. If required by the instrument to set a threshold, set the lowest possible Am Cyan channel threshold. Because $CD45^+$ leukocytes stained with the panel will be Am Cyan positive, and Trucount beads also fluoresce in the Am Cyan channel, this should allow for all relevant data to be appropriately collected.

3. Vortex the sample tube for 5 sec before loading on the flow cytometer. Trucount beads fluoresce highly in many channels. Gate the beads during collection by finding the population that is highly double-positive for PE Cy5 and APC (the two colors that most easily distinguish the beads from the cells may vary depending on instrumentation). Select the bead gate as your stopping gate, and record data until at least 20,000 beads are acquired.
4. Analyze the data using appropriate software, such as FlowJo (Treestar; Ashland, OR). **Figure 1** shows the gating scheme utilized for analysis of different leukocyte populations from a representative control blood draw.

5. Trucount Calculations

1. Each Trucount tube contains a lyophilized pellet of fluorescent beads. After adding liquid to the tube and vortexing, the beads should become equally distributed throughout the sample. The number of beads in the pellet varies slightly by lot number and can be found on the storage bag for the tubes.
2. Gate the Trucount beads and cell populations as shown in **Figure 1** to determine event counts for each population. Comparing the number of events in the bead gate to the total number of beads originally in the tube will allow you to determine the ratio of sample collected, which can then be used to determine the absolute concentration (*i.e.*, cells/µl) for each population. The following equation can be used for this purpose: Cell concentration (# cells/µl whole blood)= [# population events/(# bead events/# total beads in pellet)]/100 µl.

6. Representative Results

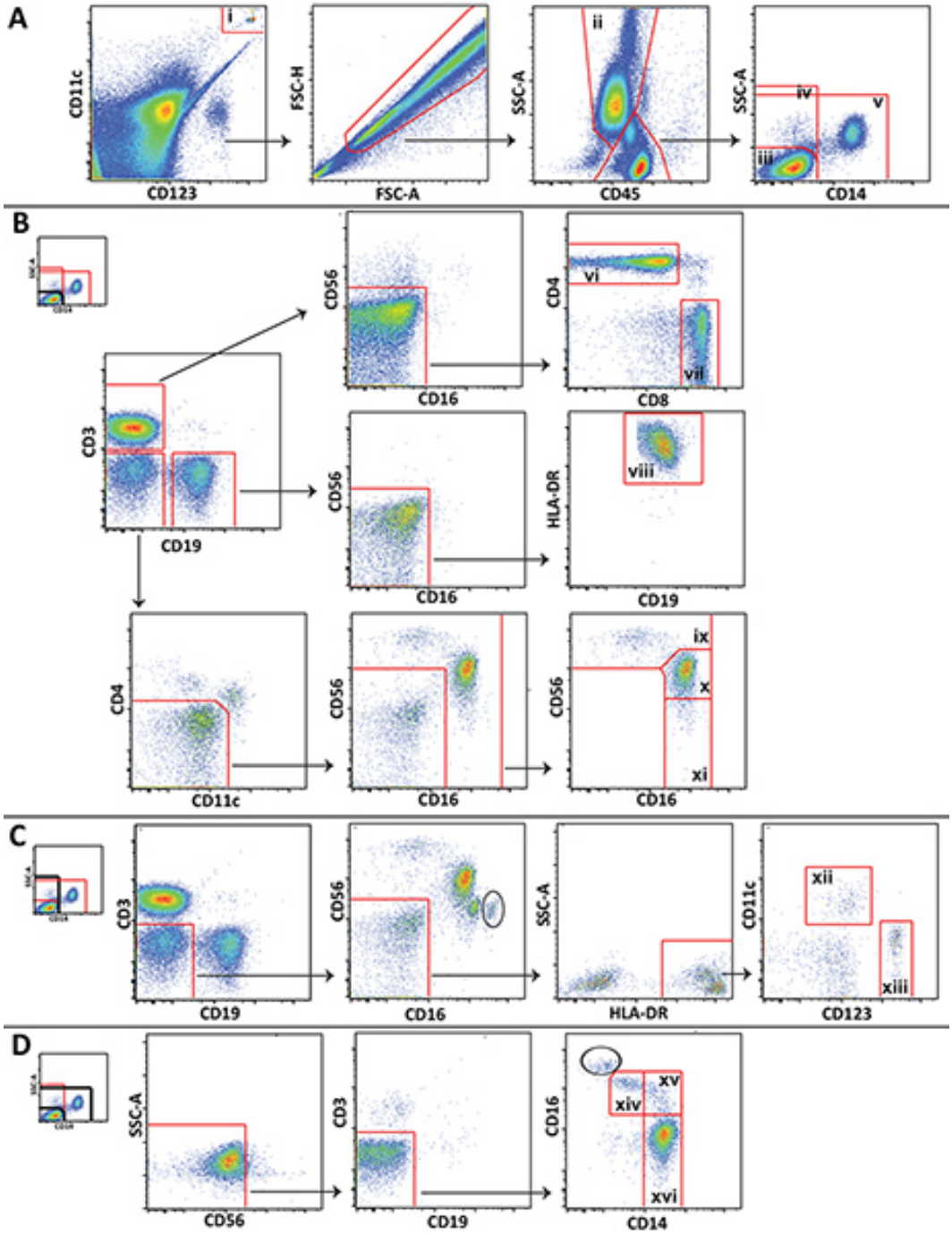

Figure 1. Gating scheme utilized for analysis of major leukocyte populations showing representative data from a healthy volunteer. A) Trucount beads (i) are gated and excluded from cells. Granulocytes (ii) are delineated and lympohcytes and monocytes are divided into 3 popluations: CD14 negative lymphocytes (iii), all CD14 negative cells (iv), and non-lymphocytes (v). B) CD14 negative lymphocytes are gated to distinguish CD4+ T cells (vi), CD8+ T cells (vii), B cells (viii), CD56 bright NK cells (ix), CD56 dim NK cells (x), and CD56 negative NK cells (xi). C) All CD14 negative cells are gated to distinguish myeloid (xii) and plasmacytoid (xiii) dendritic cells. D) Non-lympocytes are gated to distinguish non-classical (xiv), intermediate (xv), and classical (xvi) monocytes. Click here to view larger figure.

More specific cell subsets that are not shown in **Figure 1** (*e.g.*, NKT cells or neutrophils) can also be distinguished using the panel we present, and the gating scheme can be expanded or modified to meet specific study needs. Certain gating steps shown are unique to this method. Of particular note, an inclusion gate and exclusion gate are drawn around the Trucount beads and placed on top of one another, one to gate the beads for counting, and one to exclude the beads from the cellular analysis (**Figure 1A**). Also, because lymphocytes, monocytes, and granulocytes are not as easily distinguished in whole blood as they are in peripheral blood mononuclear cells by forward scatter and side scatter, gating these cells using CD45 expression and side scatter is often necessary (**Figure 1A**). Contaminating granulocytes (circled) that could not

be separated from lymphocytes and monocytes using CD45 and side scatter are distinguishable in some plots by their high CD16 expression (**Figure 1C** and **Figure 1D**). The number of contaminating granulocytes is usually small, and they do not interfere with monocyte and NK cell gating.

Discussion

In this report, we present a bead-based method for enumerating leukocyte populations in fresh whole blood by flow cytometry and cover the parameters necessary for its use in a multicenter clinical trial with centralized sample analysis. This method builds on and optimizes the BD Trucount protocol and enables its reliable use in a multicenter clinical trial setting. The staining assay is simple and takes approximately 45 minutes to perform, making it feasible for blood processing laboratory technicians to perform it and to freeze and ship the samples to a central assay lab for analysis. The assay requires only 100 µl of whole blood, which eliminates the need for time- and cost-intensive PBMC processing and uses far less volume than standard phenotyping assays. In addition, by using fresh whole blood, this assay more accurately depicts the *in vivo* state of the cells and eliminates the detrimental effects of cryopreservation observed on some cell types [1-3]. This staining method produces accurate measurement of cell concentrations, but with the added benefit of distinguishing significantly more cell types than CBC and other counting methods. The key to obtaining accurate and consistent results is to carefully standardize critical steps in the protocol and to utilize a documentation system that keeps track of all the steps so that any anomalies that are discovered can be traced to the source of the error.

There are multiple critical steps throughout the process that influence the quality of the data acquired from this assay. First, standardization of the treatment and handling of blood from the time it is drawn to the time it is stained is important, similar to what has been previously reported for PBMC processing [6]. We obtain consistent data when blood is stored in the collection tubes at the clinical site and transported to the processing labs at ambient temperature, staining is performed within 4 hr of blood draw, and the stained samples are then shipped on dry ice to the central assay lab. In addition, by making the antibody cocktail at the central lab and sending it to the site processing labs, we ensure consistency between sites and can test each cocktail on a control blood draw before it is used to stain trial samples. It is important that the central assay lab standardize this process. Therefore we recommend that once the constituent antibodies are titrated to optimize the separation between the positive and negative populations [7], the same lot of antibody be used for the staining of all samples in a clinical trial to maintain consistent staining levels for each marker. If this is not possible (*e.g.*, lack of availability from an antibody manufacturer), each new lot of antibody should be appropriately titrated to ensure that the flow cytometry data will be comparable to that obtained using the previous lot. The central lab and the site processing lab must take great care to ship and store the antibody cocktail at 4 °C and avoid light exposure to minimize degradation that can occur, especially with tandem dyes [8-9].

The accuracy of the data acquired with this assay depends on several critical steps during the staining procedure and sample acquisition on the flow cytometer. Of particular note, accurate reverse pipetting of the blood into the Trucount tube is essential; a small difference in blood volume could have a large effect on the calculated concentrations of cell subsets. Vortexing at low speed after adding the antibody cocktail and BD FACS lyse to thoroughly mix the reagents is also important. Similarly, vortexing thoroughly to dispense the beads evenly throughout the sample before acquisition is essential for accurate counting [10]. Slight variations in analysis settings on the flow cytometer can also have a large impact on the data acquired, so careful attention should be paid to the calibration and setup of the cytometer with daily use[4].

It should also be noted that while preliminary data suggests that freezing the stained samples does not appear to affect the cell concentrations obtained (data not shown), this has yet to be tested thoroughly. Although this is a potential concern, comparisons between samples can still be made with confidence as long as all samples are handled in a consistent manner.

Careful sample tracking throughout staining and analysis is particularly important, especially when hundreds of samples are processed in the context of a clinical trial. Once the data is acquired and analysis begun, it is critical to standardize analysis gates as much as possible between samples. Some variability in the fluorescence of certain populations may occur, especially between different volunteers, but standard gate locations should be set that are applicable to as many samples as possible. Any shifting of the gates from the standard location should be documented and taken into account when determining if changes in cell count occur between samples.

This staining method was optimized to enumerate the major leukocyte subsets using one antibody staining panel, but the procedure and panel could be easily modified to meet specific study needs. Contrary to the Trucount technical data sheet procedure, which suggests using 50 µl of blood, the method we present uses 100 µl. By using a larger blood volume, more events can be acquired during analysis, which is useful when studying cell populations with relatively low concentrations in the peripheral blood, such as dendritic cells. Although previous studies have enumerated dendritic cell populations via Trucount using 50 µl and 100 µl blood volumes [11-12], reports indicate that reasonable precision is dependent on acquiring at least 400 positive population events, and this can often be difficult with dendritic cells when using only 50 µl of blood [13]. The blood volume used can be further adjusted to meet the analysis requirements for specific cell types or staining panels. Similarly, the staining panel could be modified to focus on certain cell types, thus allowing for the enumeration of more specific cell subsets not currently distinguished by the panel shown here (*e.g.*, additional NK cell subsets or regulatory T cells). The use of a single panel simplifies the staining protocol and reduces errors associated with multiple panels, such as mislabeling of stained samples. Although the use of multiple panels increases the costs and potential for error, separate panels tailored to specific cell types allow for more detailed phenotyping and/or the inclusion of activation markers, and their use could be easily implemented in multicenter clinical trials, taking into account the considerations previously discussed.

Marker	Fluorophore	Company	Clone
CD45	Am Cyan	BD Biosciences	2D1
CD3	FITC	BD Biosciences	SK7
CD8	PerCP Cy5.5	BD Biosciences	SK1
CD4	Ax700	BD Biosciences	RPA-T4
HLA-DR	ECD	Beckman Coulter	Immu-357

CD14	v450	BD Biosciences	MφP9
CD19	PE	BD Biosciences	HIB19
CD16	APC-H7	BD Biosciences	3G8
CD56	PE Cy7	BD Biosciences	NCAM16.2
CD11c	APC	BD Biosciences	B-ly6
CD123	PE Cy5	BD Biosciences	9F5

Table 1. Antibody staining panel to determine all major peripheral blood leukocyte populations.

Disclosures

No conflicts of interest declared.

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