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J. Philip McCoy, Jr
Editor

Immuno-phenotyping

Methods and Protocols

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Series Editor
John M. Walker
School of Life and Medical Sciences
University of Hertfordshire
Hatfield, Hertfordshire, UK

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Immunophenotyping

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Edited by

J. Philip McCoy, Jr

Frederick, MD, USA

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Editor
J. Philip McCoy, Jr
Frederick, MD, USA

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Preface

Immunophenotyping has become more complex in recent years due to the increasing availability of new fluorochromes with novel spectral characteristics and the development of instrumentation with expanded capabilities. Propelled by technological advances in cytometry as well as the development of commercially available monoclonal antibodies, immunophenotyping of a multitude of cell types using flow cytometry has become commonplace in virtually all academic research centers, pharmaceutical companies, and clinical laboratories. Thirty-five to forty years ago, immunophenotyping was limited to one or two colors while today it is possible to construct panels consisting of several dozen fluorescent-conjugated markers. Similarly, analysis of flow cytometric immunophenotyping has developed beyond real time, direct observation of electronic signals represented by one- or two-parameter histograms viewed at the cytometer. Today, data acquired in list mode fcs format files allows for remote, retrospective, highly complex, and sometimes automated, data analysis performed independent of the cytometer and shared beyond the laboratory. Additionally, newer cytometric technologies such as imaging flow cytometry and mass cytometry permit immunophenotyping in conjunction with morphologic studies or with an expanded number of parameters, respectively. The following chapters will present a number of these topics as well as newer developments in the field of immunophenotyping.

Today, it is virtually impossible to present a complete and comprehensive compendium of all immunophenotyping methods and applications in one volume. Therefore, this text will present a representative collection of immunophenotypic methods and applications with examples of newer technologies and reagents used in the research and clinical environments. Basic methods in immunophenotyping such as construction of high-dimensional fluorescence and mass cytometry panels; fluorescence barcoding; and use of dried or lyophilized reagents will be presented first, followed by immunophenotyping examples of specific cell types often studied in research laboratories. The final chapters present immunophenotyping topics useful in the clinical laboratory, including a chapter on the critical role of quality control and immunophenotyping in the clinical environment.

I would like to thank each of the authors for volunteering to take the time to share their expertise for this volume. Without their hard work and dedication, it would not have been possible to share these methods with you.

Frederick, MD, USA — *J. Philip McCoy, Jr*

Contents

Contributors

Abdullah Alsuwaidan • *Division of Molecular and Genomic Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA, USA*

Kewal Asosingh • *Department of Inflammation and Immunity, Cleveland Clinic, Lerner Research Institute, Cleveland, OH, USA*

Gregory K. Behbehani • *Division of Hematology, Department of Internal Medicine, James Cancer Hospital, The Ohio State University, Columbus, OH, USA*

Angélique Biancotto • *Precision Immunology, Immunology and Inflammation Research Therapeutic Area, Cambridge, MA, USA*

Bonnie B. Blomberg • *Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL, USA; Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, Miami, FL, USA*

Raul Braylan • *Hematology Section, Department of Laboratory Medicine, Clinical Center, National Institutes of Health (NIH), Bethesda, MD, USA*

Jolene L. Cardinali • *Department of Pathology and Laboratory Medicine, Hartford Hospital, Hartford, CT, USA*

Erica L. Carpenter • *Division of Hematology and Oncology, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA*

Mingyi Chen • *Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX, USA*

Weina Chen • *Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX, USA*

Pradeep K. Dagur • *Flow Cytometry Core Facility, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA*

Alain Diaz • *Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL, USA*

Joseph A. DiGiuseppe • *Department of Pathology and Laboratory Medicine, Hartford Hospital, Hartford, CT, USA*

Anagha Divekar • *Department for Cellular Analysis, BioLegend, San Diego, CA, USA*

Giovanna Fantoni • *Center for Human Immunology, NIAID, NIH, Bethesda, MD, USA*

Daniela Frasca • *Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL, USA*

Franklin Fuda • *Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX, USA*

Pallavi Kanwar Galera • *Hematology Section, Department of Laboratory Medicine, Clinical Center, National Institutes of Health (NIH), Bethesda, MD, USA*

Valentina Giudice • *Department of Medicine, Surgery, and Dentistry, Scuola Medica Salernitana, University of Salerno, Baronissi, Italy*

Alejandra Gomez-Cadena • *Department of Oncology UNIL CHUV, University of Lausanne, Epalinges, Switzerland*

Bruce Greig • *Hematopathology/Flow Cytometry, Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center, Nashville, TN, USA*

Andrea J. Illingworth • *Dahl-Chase Diagnostic Services, Bangor, ME, USA*

Camilla Jandus • *Department of Oncology UNIL CHUV, University of Lausanne, Epalinges, Switzerland*
Chunjie Jiang • *Hematology Section, Department of Laboratory Medicine, Clinical Center, National Institutes of Health (NIH), Bethesda, MD, USA*
Marc Langweiler • *Asheville, NC, USA*
Andreas Lundqvist • *Department of Oncology and Pathology, Karolinska Institutet, Stockholm, Sweden*
Holden T. Maecker • *Institute for Immunity, Transplantation, and Infection, Stanford University School of Medicine, Stanford, CA, USA*
Florian Mair • *Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA*
Iuri Marinov • *Institute of Hematology and Blood Transfusion, Prague 2, Czech Republic*
J. Philip McCoy, Jr • *Frederick, MD, USA*
Maria Romero • *Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL, USA*
Dalia A. Salem • *Laboratory of Pathology, CCR, NCI, NIH, Bethesda, MD, USA; Clinical Pathology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt*
Ankit Saxena • *Flow Cytometry Core Facility, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA*
Moen Sen • *Division of Hematology and Oncology, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA*
Janet Staats • *Department of Surgery, Duke University Medical Center, Durham, NC, USA; Duke Immune Profiling Core, Duke University Medical Center, Durham, NC, USA*
Maryalice Stetler-Stevenson • *Laboratory of Pathology, CCR, NCI, NIH, Bethesda, MD, USA*
D. Robert Sutherland • *University of Toronto, Toronto, ON, Canada*
Sara Trabanelli • *Department of Oncology UNIL CHUV, University of Lausanne, Epalinges, Switzerland*
Aaron J. Tyznik • *BD Biosciences, La Jolla, CA, USA*
Ling Wang • *BD Technologies and Innovation, Research Triangle Park, NC, USA*
Nicholas Wanner • *Department of Inflammation and Immunity, Cleveland Clinic, Lerner Research Institute, Cleveland, OH, USA*
Kristina Witt • *Department of Oncology and Pathology, Karolinska Institutet, Stockholm, Sweden*
Liping Yu • *Applied Cells, Inc., Santa Clara, CA, USA*
Chen Ziqing • *Department of Oncology and Pathology, Karolinska Institutet, Stockholm, Sweden*

Chapter 1

High-Dimensional Immunophenotyping with Fluorescence-Based Cytometry: A Practical Guidebook

Florian Mair and Aaron J. Tyznik

Abstract

Recent technological advances have greatly diversified the platforms that are available for high-dimensional single-cell immunophenotyping, including mass cytometry, single-cell RNA sequencing, and fluorescent-based flow cytometry. The latter is currently the most commonly used approach, and modern instrumentation allows for the measurement of up to 30 parameters, revealing deep insights into the complexity of the immune system.

Here, we provide a *practical guidebook for the successful design* and execution of complex fluorescence-based immunophenotyping panels. We address common misconceptions and caveats, and also discuss challenges that are associated with the quality control and analysis of these data sets.

Key words Immunology, High-dimensional, Fluorescence, Flow cytometry, Polychromatic, Panel design, Spillover spreading, SSM, Compensation, Staining

1 Introduction

Since its invention more than 50 years ago, flow cytometry has been a key tool for novel discoveries in the field of immunology [1, 2]. This has been driven primarily by the ability of flow cytometry to qualitatively and (within certain constraints) quantitatively measure multiple features on a single cell level. Given the diversity of immune cell subsets and their functional complexity, there is an increasing need to measure more targets per cell in order to accurately classify a given subtype while still being able to assess additional features of interest.

This need has fueled the development of novel cytometry platforms during the past decade, most notably mass cytometry (cytometry by time-of-flight, CyTOF) [3–5] and single-cell RNA sequencing (sc-RNAseq) [6–9]. Both of these approaches can provide previously unprecedented insight into the immune system, but come with their own set of limitations. Mass cytometry currently allows the measurement of up to 50 protein (or RNA) targets on

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millions of cells at a single cell level, but has a relatively slow throughput and does not allow for recovery of living cells via sorting [10]. Single-cell RNAseq increases the dimensionality of the possible measurements by almost two orders of magnitude (depending on the platform used), but the associated reagent and sequencing cost is currently prohibitive for analyzing more than tens of thousands of cells [9, 11]. For further information on these technologies, we direct the reader to some excellent reviews [5, 11, 12].

Within this chapter, we focus on fluorescent-based flow cytometry as the most widely used platform for immunophenotyping. Briefly, fluorescent flow cytometers rely on multiple lasers as light sources to excite fluorescent molecules, which are usually coupled to antibodies that are bound to antigenic targets of interest on the samples being analyzed. In order to properly interrogate these cells, a fluidics system utilizes hydrodynamic focusing to linearly align the cells in a stream of liquid where they intercept the focused laser beams one cell at a time. Excitation of the fluorochrome results in the emission of photons that travel through an optical path consisting of filters and mirrors and are detected by a set of photon detectors, most commonly photon multiplier tubes (PMTs). In the past decade, incremental technical improvements have resulted in the latest generation of instruments, which allow the measurement of up to 30 parameters [13]. Present efforts in the field include the further development of spectral flow cytometry, which was originally developed at Purdue University and holds great potential for the future [14–16]. Instruments based on this principle measure the entire spectrum across the visible range and reconstruct individual fluorophore signals by spectral deconvolution algorithms. This approach will most likely increase the number of measurable fluorescent parameters beyond 40 and promises several additional advantages such as autofluorescence unmixing. However, spectral flow cytometers are not very widely available yet, and thus within the scope of this chapter we focus on "conventional" fluorescent panel design.

Designing fluorescent panels to produce high-quality and reliable data for 15–30 parameters requires significant effort and thus far has not been automated due to the wide range of available instrumentation and the varying biological targets between different research questions. Here, we provide a practical step-by-step guidebook for designing high-dimensional fluorescent panels and focus on common challenges. An overview of the process is outlined in Fig. 1, and from start to finish a 30-parameter panel can successfully be established in a 3–6 week time frame (given that all reagents are commercially available).

Of note, an important consideration for any high-dimensional assay is the subsequent analysis of the resulting data. Manual gating, while still the gold standard in the field and required for quality control of the data, is often insufficient to fully explore high-

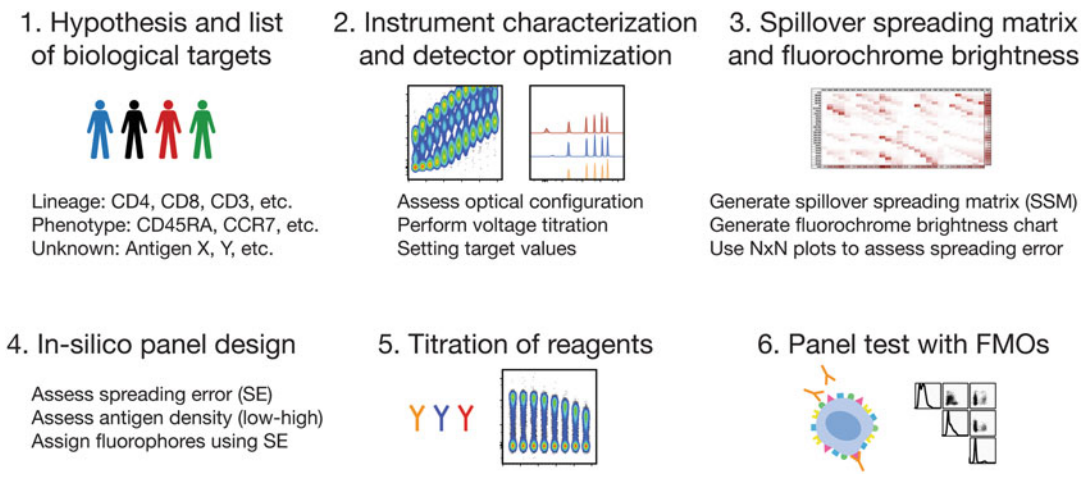

Fig. 1 Overview of the typical steps required for a high-dimensional immunophenotyping experiment. Each of the six steps is described in detail in Subheadings 3.1 through 3.7

dimensional datasets [17]. An exhaustive discussion of this topic is beyond the scope of this chapter, and for further information we direct the reader to the following reviews on computational analysis methods for flow cytometry data [18–20], and some key papers describing the comparison of computational methods to traditional manual gating [21, 22]. Furthermore, for a comprehensive review of the current state-of-the art on many cytometry-related topics, we recommend the "Guidelines on the use of flow cytometry and cell sorting in immunological studies" [23].

2 Materials

2.1 Instrument and QC Particles

Flow cytometer.

Computer with analysis software capable of reading FCS files.

Hard-dyed reference beads (e.g., Spherotech Ultra Rainbow beads, #URCP-38-2F).

Instrument QC-particles if required (see instrument-specific instructions).

2.2 Reagents and Plastic Ware

Fluorochrome-conjugated antibodies (various vendors).

Fc-blocking reagent (various vendors).

Fixable live/dead reagent (depending on the panel, various vendors).

Compensation particles (e.g., BD Biosciences, anti-rat compensation beads #552844 and anti-mouse compensation beads #552843, or anti-mouse Plus beads #560497).

Brilliant staining buffer (various vendors).

PFA-based fixative (various vendors, or freshly prepared 2% PFA solution).

Optional: fix/perm reagent for intracellular or intranuclear targets.

PBS.

Fetal bovine serum (FBS).

96-well round-bottom plates.

5-ml polystyrene tubes.

2.3 Solutions to Prepare

Stain/wash buffer: add 10 ml of heat-inactivated FBS to a 500 ml bottle PBS.

Fc-Block/viability dye solution: add 25 μl of Fc-blocking reagent and 1 μl of reconstituted live/dead reagent to 500 μl of PBS immediately prior to use. Titration of the live/dead reagent is recommended.

3 Methods

3.1 Generating Your Hypothesis and List of Biological Targets

Any immunophenotyping experiment should start with a biological hypothesis and a list of targets that are to be included in the experiment. Typically, this will comprise two types of target antigens: the so-called lineage markers, which are used to delineate canonical immune cell populations (such as CD3 for T cells), and the markers of interest, which can either have a known expression pattern that changes under experimental conditions (such as CD69 as an early activation marker for T cells), or a potentially unknown expression pattern.

As a first step, we recommend compiling a table that lists all target antigens, their expected expression pattern (continual or variable), expression type (bimodal, continuum), and expression level (low to high) as well as expected coexpression patterns, that is, which markers are expected to be expressed on the same cell types. An example for such a table with some human T cell-centric markers is given in Fig. 2. Furthermore, it is helpful to include reagent availability in this table, that is, which fluorophore–antibody conjugates are available from which vendor for a given target. Having this information at hand simplifies the actual panel design process, which will be discussed in detail in Subheading 3.4. The antigen targets listed within this chapter are based on human leukocytes, but the same principles apply for any other cell type such as murine leukocytes.

1. Define the cellular populations that are relevant for the planned experiment (e.g., $CD4^+$ T cells, $CD8^+$ T cells, and $CD56^+$ NK cells).

Example list of target populations and target antigens of interest			
Cellular population	**CD4+ T cells**	**CD8+ T cells**	**CD56+ NK cells**
Required lineage markers	CD45, CD3, CD4	CD45, CD3, CD8	CD45, CD56, CD16
Targets, high priority	CD45RO, CD45RA, CCR7, CD127, CD25, OX40	CD45RO, CD45RA, CCR7, CD127, CD25, OX40	CD161
Targets, intermediate priority	PD1, CD28, CD27	PD1, CD28, CD27	

Example list of target antigen features			
Target	**CD3**	**CD4**	**CD16**
Clone	UCHT1	RPA-T4	3GA
Category	Lineage marker	Lineage marker	Lineage marker / Fc-receptor
Expression known?	Yes	Yes	Yes
Cell types expressed	All T cells	CD4+ T cells, intermediate on monocytes	NK cells, myeloid cells
Expression pattern	Bimodal	Bimodal	Usually bimodal
Expression level	High	High	Usually high
Conjugates available	Company A all fluorophores, Company B PE-Cy5.5, Company C all fluorophores except BUV	Company A all fluorophores, Company B PE-Cy5.5, Company C all fluorophores except BUV	Company A: all fluorophores except BUV496, BUV661 and BUV805
Other notes	Other clones available: OKT3, HIT3A	Other clones available: SK3, OKT4	Other clones available: B73.1
Target	**CD45RA**	**PD1**	**OX40 (CD134)**
Clone	HI100	MIH4	ACT-35
Category	T cell phenotyping	T cell phenotyping	T cell phenotyping
Expression known?	Yes	No	No
Cell types expressed	T cells, unkown on other cells	T cells, unkown on other cells	T cells, unkown on other cells
Expression pattern	Continous pattern	Inducible, continous pattern	Inducible, continuous pattern
Expression level	High	Dim to intermediate	Dim to intermediate
Conjugates available	Company A: all fluorophores, Company C all fluorophores except BUV	Company A: all fluorophores except BUV496, BUV661, BUV737 and BUV805, Company C all fluorophores except BUV	Company A: BV421-BV786, all PE-conjugates, FITC and AF647. Company C: all APC and PE-conjugates, BV421, BV510, BV605, BV711
Other notes	N.A	Test other clone: EH12.2H7	TNF receptor family member, needs to be titrated on activated PBMCs. Alternative clone L106

Fig. 2 Example table with target antigen information. This table lists a selection of antigens for a human T cell centric panel and the anticipated coexpression patterns. Researchers should adopt this table to fit their own needs. Alternatively, one can draw a gating tree instead of using a table. If available, it is helpful to assess common expression patterns in preliminary experiments or in public data sets such as those included in published OMIPs

2. List the lineage markers required for the reliable identification of all canonical cell populations (e.g., CD45/CD3/CD4 for $CD4^+$ T cells).
3. Define the target antigens of interest (e.g., for measuring the phenotype of naïve vs. effector T cells: CCR7, CD45RO, CD45RA, CD25, CD127, etc.).
4. List the expression patterns according to the following categories: bimodal, continuous, unknown.
5. List the expected expression level for each target antigen according to the following categories: low, medium, high.
6. Optional: perform an Internet search for reagent availability for all targets and list the corresponding catalog numbers and vendors.
7. Save and print this table for future reference.

3.2 Instrument Characterization and Detector Gain Optimization

For most researchers, the instrument available is an institute-specific fixed constant and not necessarily subject to change. However, it is critical to familiarize oneself with the given instrument configuration and the available optical detectors [24], and perform an optimization of the detector gains. The latter is a necessity that

stems from the fact that photon multiplier tubes (PMTs), which are still the most commonly used detector type, show significant model-to-model variation, and thus each detector will require a different voltage gain to deliver optimal resolution (i.e., optimal signal-to-noise ratio). A more extensive discussion on the factors describing the performance of a given PMT can be found in the following reference [25]. Of note, avalanche photodiodes (APDs) which are better suited for the detection of long-wavelength signals [26] are becoming more popular but are beyond the scope of this chapter.

There are several methods available to perform detector optimization: for instruments from BD Biosciences, the Cytometry Setup and Tracking (CS&T) module is commonly used to generate consistent settings and track cytometer performance (*see* vendor specific CS&T application guide). This system relies on a set of hard-dyed dim, medium, and bright beads (CS&T beads). During initial setup, voltages are determined such that the signal of the dim beads is placed 10 times above the robust standard deviation (rSD) of the electronic noise. Later on, the signal of the bright beads is used to set target values, which are being matched during daily quality control (QC) by modification of the detector voltages. Often times, users utilize PMT voltage settings from the CS&T application for setting and tracking detector gains. However, as CS&T was developed at a time when the selection of available fluorophores was limiting, the CS&T routine might deliver unnecessarily high baseline voltages for some detectors excited off the violet, blue, and ultraviolet laser, thereby pushing signals from cells off scale. Additionally, the rSD of the dim bead may not be the same as the autofluorescence of a biological sample and correlation to optimal PMT voltage settings may not be accurate. As a result, researchers may need to lower or, in some instances, increase their detector gains manually, making the CS&T method of setting baseline voltages suboptimal.

Another approach is based on using unstained cells and setting the voltages in such a way that the rSD of the cells equals 2.5 times the rSD of the electronic noise, which ensures that dim signals are pulled out of the electronic noise [27]. Finally, there are two approaches that utilize a voltage titration experiment using either the peak 2 of 8-peak rainbow beads (called the second peak method, mimicking a dim signal) or single-stained samples (using cells or antibody capture beads).

Here, we will describe a simplified voltage titration (voltration) experiment using cells stained at saturating levels with a single antibody clone (e.g., anti-CD4 or anti-CD8) conjugated to all fluorophores that can be measured on a given instrument [28]. Of note, the same data can be used to generate a relative fluorochrome brightness chart, which will be useful in Subheading 3.4 for panel design. This experiment needs to be carried out only

once for any cytometer unless the optical configuration, that is, lasers, filters, or detectors are changed. A representative analysis of two typical voltage titrations is shown in Fig. 3a, b, and two fluorochrome brightness charts with a classification of fluorochromes into four broad categories is shown in Fig. 3c.

Performing the voltage titration experiment (unique to instrument):

1. Obtain antibodies for a given clone (typically targeting a lineage marker such as CD4) conjugated to all different fluorophores that can be measured on the available instrument.
2. Obtain cells (typically cryopreserved PBMCs or murine splenocytes).
3. Prepare antibody dilutions of every conjugate at a saturating titer in stain buffer (*see* Subheading 3.5).
4. Aliquot 500,000 cells in as many wells as required into a 96-well plate.
5. Spin plate at 400 × *g* for 5 min. Flick supernatant, dry the top of the plate by carefully placing it on a paper towel and draining remaining liquid.
6. Add 100 μl of a single antibody dilution per well, resuspend, and incubate for 20 min at RT in the dark.
7. Add 200 μl of stain buffer to all wells, centrifuge plate at 400 × *g* for 5 min, flick supernatant, and dry the top of the plate by carefully placing it on a paper towel. Repeat this wash for a total of two washes.
8. Optional: add 100 μl of fixative to each well, resuspend, and incubate for 20 min at RT in the dark (*see* **Note 1**).
9. Add 200 μl of stain buffer to all wells, centrifuge plate at 400 × *g* for 5 min, flick supernatant, and dry the top of the plate by carefully placing it on a paper towel.
10. Resuspend in 200 μl of stain buffer and store in the dark at 4 °C until acquisition.
11. Perform flow cytometer startup and daily QC as required per instrument-specific instructions.
12. Load one of the single-stained samples, and adjust the voltage of the respective detector in 30 V increments from 200 V to 700 V. Record 10,000 events for every given detector voltage setting and name the tubes accordingly (BV421_200V, BV421_230V, BV421_260V, etc.).
13. Disregard samples where the MFI of the positive signal has exceeded detector linearity or is no longer on scale as erroneous values may be reported.
14. Repeat this step for all single-stained samples.

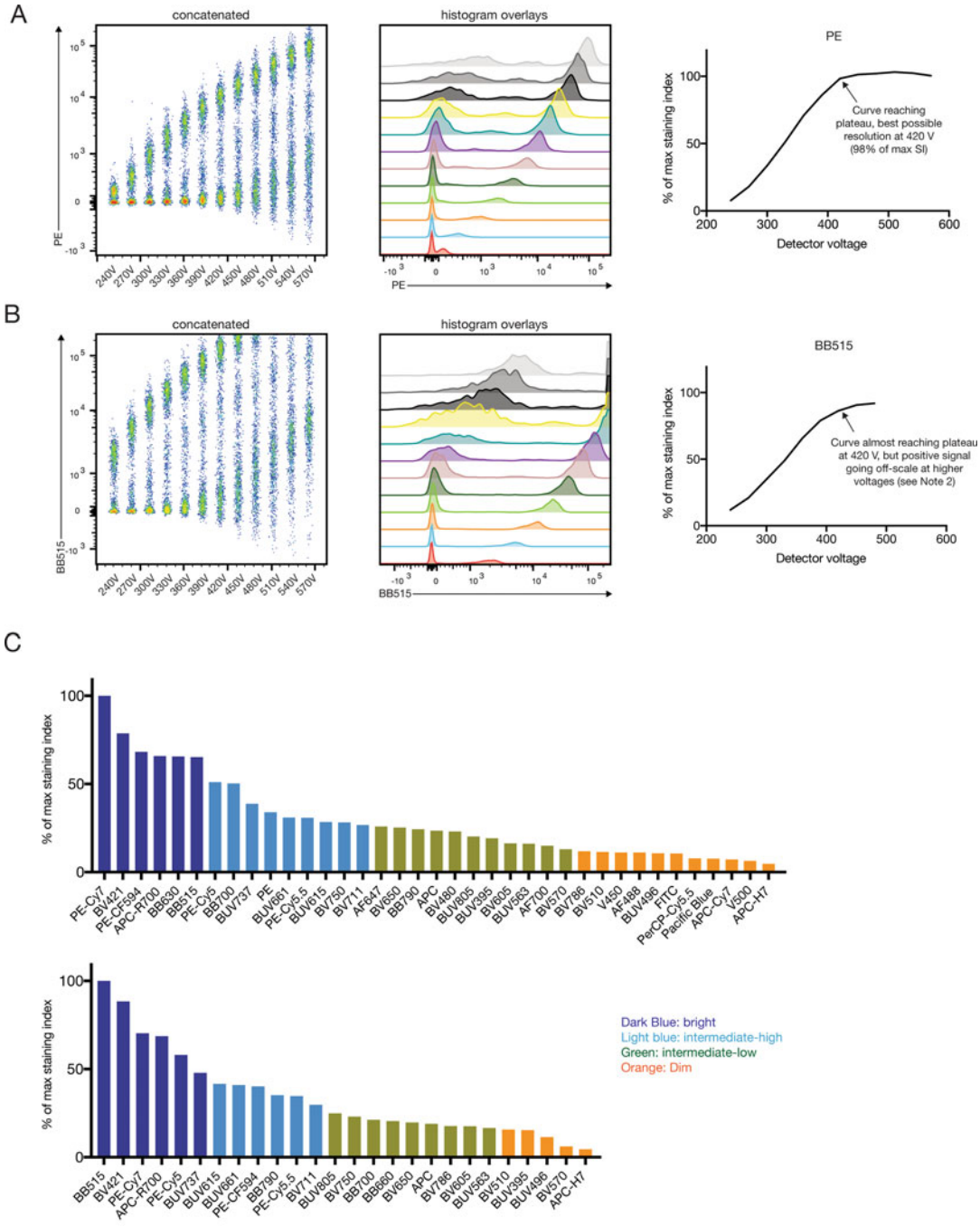

Fig. 3 Examples for a voltage titration experiment and a fluorochrome brightness chart. (**a**) and (**b**) Two representative voltage titration experiments are shown as concatenated pseudocolor plots (left) and as histogram overlays (right). Staining indices have been calculated as described in Subheading 3.2. For CD4-PE the plateau of the stain index is reached around 420 V, indicating that this is the minimal voltage required to deliver optimal resolution. If the positive population exceeds the upper end of the scale as is the case for CD4-BB515, *see* **Note 2**. (**c**) Two representative fluorochrome brightness charts generated on two independent commercially available high dimensional flow cytometers. As each instrument is unique in laser power and detection optics, it is best to characterize the relative fluorochrome brightness for each individual instrument. However, assigning fluorochromes to four broad categories will allow some generalization for relative brightness (e.g., APC-H7 tandems being at the lower end of the scale, or BV421 and PE-tandem dyes being at the upper end of the scale)

15. Export data in FCS3.1 file format and load into a flow cytometry data analysis program.
16. For a given single-stained control, draw gates delineating singlets, and subsequently the positive peak and the negative peak for the corresponding fluorophore.
17. Optional: concatenate the individual FCS files for a given single-stained control into a single FCS-file, or generate histogram overlays to obtain a visual representation of the voltration experiment.
18. Repeat this step for all single-stained samples.
19. Calculate the median fluorescence intensity (MFI) of both the positive and the negative peak, as well as the rSD for the negative peak.
20. Calculate the staining index (SI) for each sample by using the following formula: $SI = MFI(pos) - MFI(neg)/(2 \times rSD)$.
21. Using a spreadsheet program, generate a plot showing the SI on the *y*-axis relative to the voltage on the *x*-axis.
22. For each detector, pick the voltage on which the SI starts to reach a plateau, which is the minimal voltage delivering the best possible resolution for a given detector. As an alternative visualization, plot the SI as a percentage of maximum SI on the *y*-axis relative to the voltage on the *x*-axis (also *see* **Note 2**).
23. Once the optimal baseline PMT voltages have been determined, record a sample of appropriate reference beads (e.g., Spherotech Ultra Rainbow Beads) as described in **steps 29–34**. *See* **Note 3** for examples of reference particles.

Create relative fluorochrome brightness ranking chart unique to the instrument

24. Identify the single-stained samples acquired to reach the optimal PMT voltage for any given detector.
25. Calculate the median fluorescence intensity (MFI) of both the positive and the negative peak, as well as the rSD for the negative peak.
26. Calculate the staining index (SI) for each fluorophore by using the following formula: $SI = MFI(pos) - MFI(neg)/(2 \times rSD)$.
27. Using a spreadsheet program, generate a plot showing the SI for each fluorophore and arrange them by decreasing values. Alternatively, plot the SI as percentage of maximum SI.
28. Assign the fluorochromes into broad categories based on their SI, that is, bright, intermediate-high, intermediate-low, and dim.

Setting and maintaining target MFI Values

29. Set optimal PMT voltages as determined in **steps 1–23**.
30. Load a sample with reference beads (*see* **Note 3**) that provide an adequate positive signal in each channel being utilized on your flow cytometer and acquire 20,000 events.
31. Optional: Repeat acquisition of reference particles ten independent times to determine baseline MFI and standard deviation (SD) of signal.
32. Create histograms for all detectors and place an interval gate on the positive peak(s) to determine median fluorescent intensity (MFI).
33. Using an appropriate analysis program, export the MFI for all baseline runs and calculate average MFI and standard deviation.
34. After the daily instrument-specific start-up and QC procedures and prior to every experiment, acquire reference beads and adjust PMT voltage for each detector to set signals to match the target MFI ± 5% (or ± acceptable variability based off the optional standard deviation calculations).

3.3 Characterization of Spreading Error and Fluorochrome Brightness

Conventional fluorescent flow cytometry instruments use optical band pass filters placed in front of each PMT, balancing between catching the main peak of a given fluorescent signal and simultaneously avoiding signals coming from other fluorophores. Despite instrument manufacturers' best efforts, there will be spectral overlap between different fluorophores measured (Fig. 4a). One usually refers to the primary detector as the one intended to detect that fluorophore (e.g., G575 for PE, excited by green 532 nm laser) and the secondary detector as the one that collects spillover, that is, the remaining portion of the spectrum that is detected but does not represent the primary signal in that detector (e.g., G610, intended for PE-CF594 detection but also collecting signal from PE). It is important to note that spillover can not only occur on neighboring detectors off the same laser line but also on detectors off a different laser line if the fluorophore shows some excitation by that wavelength (e.g., U570, intended for BUV563 but will also collect signal from PE which is excited primarily by the 488/532 nm laser but to a certain extent by the ultraviolet 355 nm laser) (Fig. 4a, right panel).

Spectral overlap is corrected by a process termed compensation, a mathematical manipulation of the acquired raw data [29, 30]. The compensation calculation is based on single-stained controls, which are discussed in more detail in Subheading 3.6. After compensation, typically a percent value is reported that will describe the relative fluorescence detected in the secondary detector compared to the primary detector. During compensation, this

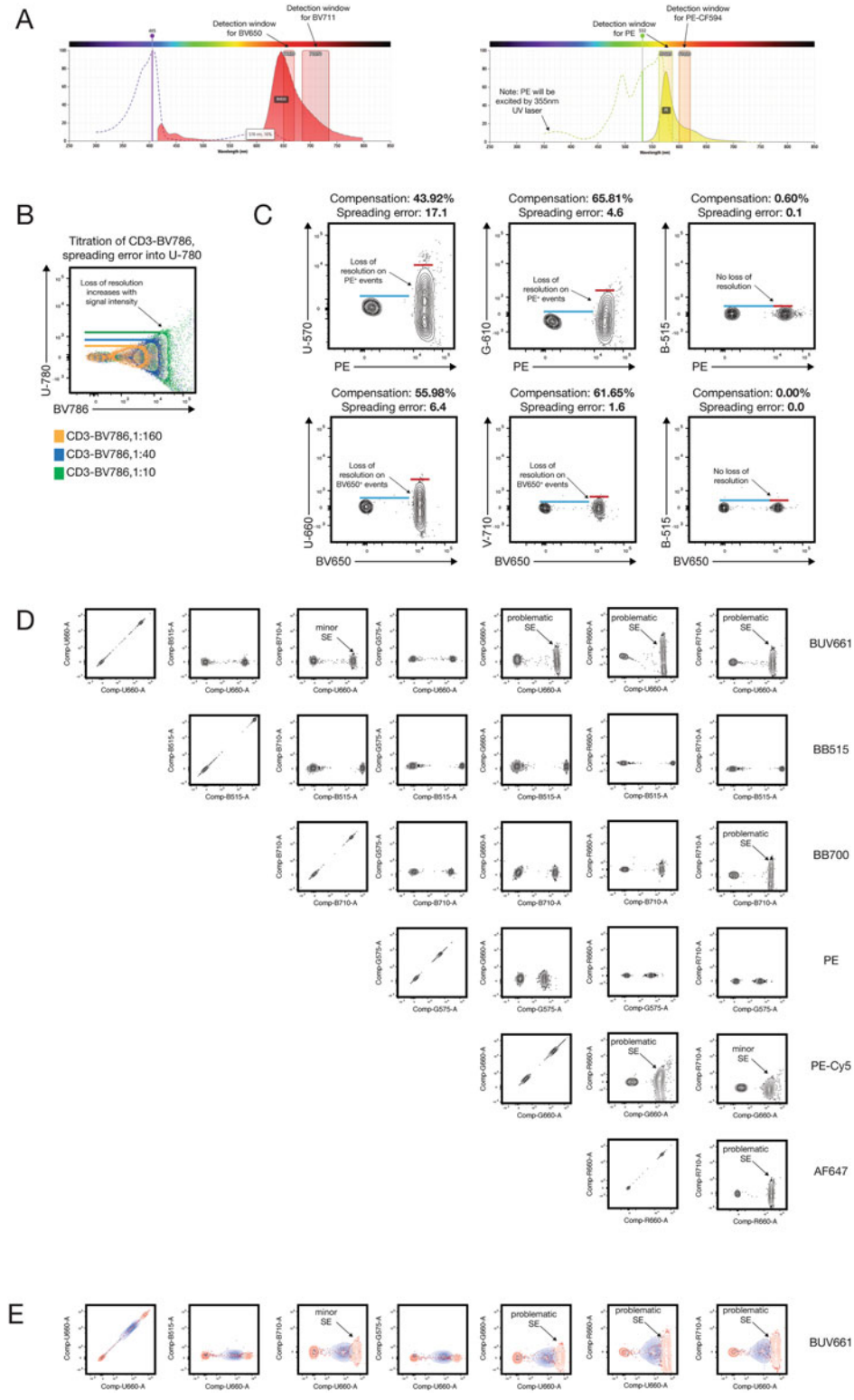

Fig. 4 Fluorochrome overlap, compensation, and spreading error (SE). (**a**) Fluorochrome spectra depicting excitation (dotted curve) and emission (solid filled curve) of Brilliant Violet 650 (BV650, left) and Phycoerythrin

signal portion is subtracted from the total signal detected in the secondary detector.

A very common historic misconception relates to which magnitude of a compensation value is considered "acceptable." Whenever the compensation wizard reports high values (especially approaching 100% or more), some users adjust PMT voltages to lower this value, assuming that this will improve their instrument setup. However, this is usually not the case and rather poses the danger of reducing the PMT voltage below the minimum value required to deliver optimal resolution (*see* Subheading 3.2). It is important to note that in high-dimensional fluorescent cytometry, some fluorochrome spectra will be so close, that at optimal detector settings compensation values can often approach or even exceed 100%, which does not necessarily negatively impact assay performance. An excellent description and sample data demonstrating this effect has been published by Ashhurst and colleagues [31].

The correct metric to use in this context is the so-called spreading error (SE), which is an extremely useful metric that was only recently developed by the Roederer lab at NIH [32]. In a nutshell, the SE describes the spreading of the negatives of any given compensated population in the secondary detector. Often this increase in the spread (measured by robust standard deviation, rSD) is attributed to compensation, which is incorrect—compensation does not generate the SE but merely makes it visible at the low end of a logarithmic/biexponential scale. The underlying reason for the SE is the fact that the actual measurement of the

Fig. 4 (continued) (PE, right curve) (adapted from http:/www.bdbiosciences.com/us/s/spectrumviewer). The shaded boxes indicate typical detection windows that are used to collect the emission peaks of these fluorophores. Spectral overlap will occur into the neighboring detectors, as well as the same detection window on different laser lines (indicated for PE, which is not only excited by the 488 nm and 532 nm laser, but also by the 355 nm UV laser). (**b**) Connection between signal intensity and spreading error (SE). Three different titers of single-stained anti-human CD3-BV786 PBMCs are shown as an overlay contour plot, with the U-780 detector (excitation laser: 355 nm, detection window: 785/62) on the *y*-axis. The limit of detection on $CD3^+$ events is indicating by a solid line, highlighting that the SE increases with signal intensity. (**c**) Examples showing the disconnect between the compensation value and SE. Compensation beads were stained with anti-human CD4-PE (upper panels) and CD4-BV650 (lower panels). PE will show significant spillover into the U-570 (excitation laser: 355 nm, detection window: 570/40) and G-610 (excitation laser: 532 nm, detection window: 610/20) detector, but no spillover into the B-515 detector (excitation laser: 488 nm, detection window: 515/20). BV650 will show significant spillover into the U-660 (excitation laser: 355 nm, detection window: 660/40) and V-710 (excitation laser: 405 nm, detection window: 710/40) detector. Note that the relative extent of SE does not correlate with the compensation value (numbers above plots). (**d**) $N \times N$ view of some selected compensated single-stained controls from a 28-color experiment. Fluorophore detector pairs with relevant SE have been marked with arrows. (**e**) The same view as in the first row of (**d**), but with single-stained samples derived from human whole blood. Using an anti CD4-antibody, this will deliver a bright population (T cells) as well as an intermediate population (monocytes, colored in blue) and allows to visually assess the extent of SE at different signal intensities

fluorescence signal is imprecise and will carry some variance due to the Poisson error in photon counting [32]. This measurement error will depend on many factors (such as the PMT model and the wavelength of the detected signal) and is an intrinsic property of the measurement process.

There are three key aspects of SE that will be relevant for panel design as discussed in Subheading 3.4. First, SE is proportional to signal intensity, that is, the brighter a signal in the primary detector, the more pronounced the SE in the secondary detector will be (Fig. 4b). Second, SE is reducing the resolution in the secondary detector, that is, the detector that is collecting spillover (Fig. 4c). Third, SE is additive, that is, if a detector collects SE from multiple different fluorophores, the overall increase of the rSD of the negatives (and thus the loss in sensitivity) will be larger.

As a result, an essential tool for modern panel design is the spillover spreading error matrix (SSM), which is different from the compensation matrix. The SSM will provide a comprehensive overview for a given instrument on (1) the relative contribution of any fluorophore to SE in secondary detectors and (2) the relative loss of resolution in any secondary detector due to SE collected from all other fluorophores. The mathematical formula for calculating the SSM is provided in the original publication [32] and has also been made available online by Ashhurst and colleagues, but the SSM can be calculated more easily in a common data analysis package, FlowJo (version 10.4 and higher).

It is important to note that the extent of SE cannot be predicted from the corresponding value in the compensation matrix, which is exemplified in the plots displayed in Fig. 4c. Below, we describe the steps for obtaining an SSM for a given flow cytometer using antibody capture beads (compensation beads). Alternatively, one can directly utilize the data that has been generated during PMT voltage baseline setup in Subheading 3.2. Of note, these data sets can not only be used to calculate the SSM, but can also serve as a visual reference to judge the extent of SE. After appropriate compensation, visualizing the single-stained control samples in the form of $N \times N$ plots (i.e., each fluorophore by each detector) will provide a quick visual reference of the amount of SE into all corresponding detectors to identify potential "watch-out" channels for coexpressed markers (Fig. 4d). If these plots are based on human whole-blood samples utilizing CD4 as an antigen, the added advantage is that the impact of signal intensity on SE can be visualized, as monocytes will show as CD4-intermediate cells, while T cells will show a CD4-bright population (Fig. 4e). This approach reiterates the fact that SE is proportional to signal intensity, and can provide a useful "generic" biological reference as you plan your fluorochrome assignments.

Overall, the concept of SE and the SSM is invaluable for panel design, and an example SSM and how to interpret it is shown in

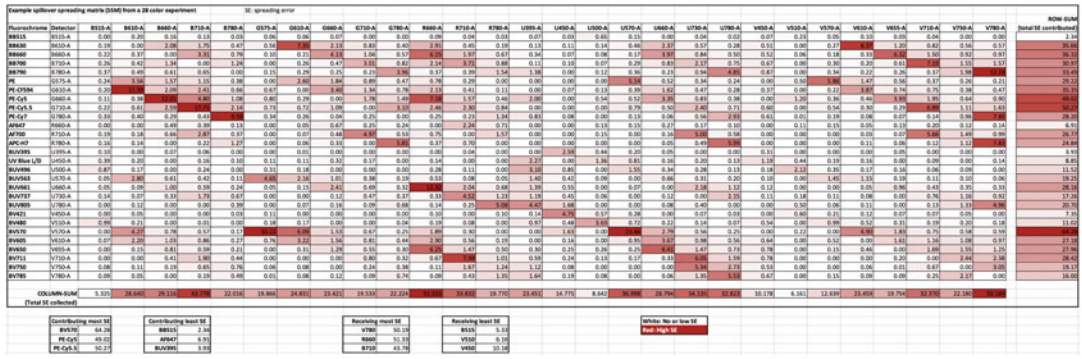

Fig. 5 The spillover spreading matrix (SSM). (**a**) A representative spillover spreading matrix (SSM) calculated from a 28-color experiment is shown. Color coding is from no SE (white) to high SE (red). Note that the SSM is instrument specific and does not provide generalizable conclusions about the extent of SE, and SE is a unitless, relative number. For this particular instrument, the three fluorophores contributing the least SE are BB515, AF647 and BUV395. Thus, these three dyes would be optimal to assign for lineage markers as they are contributing minimal SE to all the other detectors. In turn, the three detectors receiving the least SE are B-515, V-510 and V-450. Since BB515 as well as BV421 are typically two very bright fluorophores (*see* Fig. 3c), on this particular instrument these two detectors would be optimal for dimly expressed targets. Note that when assessing the row-sums, that is, the total SE contributed by a given fluorophore, the case can be that this sum is driven mostly by one particular value, for example BUV661, where SE into R-660 is the major contributor to the sum. In this case, BUV661 can still readily be used without major impact to all other detectors except R-660

Fig. 5. The use of the SSM is more extensively discussed in the next section.

1. Obtain antibodies carrying all fluorochromes that are to be included in the new panel (the antigen targets do not necessarily matter at this point in time, but should match the fluorochromes that will be used) (also *see* **Note 4**).
2. Aliquot antibody-capture beads (i.e., compensation particles) into as many wells as required into a 96-well plate.
3. Resuspend in 100 µl of staining buffer.
4. Add 1 µl of a single antibody into each well and resuspend using a 100 µl pipette. Incubate for a minimum of 15 min at RT in the dark.
5. Add 200 µl of stain buffer to all wells, centrifuge plate at 400 × *g* for 5 min, flick supernatant, and dry the top of the plate by carefully placing it on a paper towel. Repeat this wash once.
6. Optional: add 100 µl of fixative to each well, resuspend, and incubate for 20 min at RT in the dark.
7. Add 200 µl of stain buffer to all wells, centrifuge plate at 400 × *g* for 5 min, flick supernatant, and dry the top of the plate by carefully placing it on a paper towel.

8. Resuspend in 200 μl of stain buffer and store at 4 °C until acquisition.
9. Perform flow cytometer startup and daily QC as required per manufacturer's instructions.
10. Acquire the single-stained control tubes at the optimal voltages determined in Subheading 3.2.
11. Export data in FCS3.1 file format and load into a flow cytometry data analysis program capable of computing the SSM (FlowJo).
12. Open the compensation wizard, calculate the compensation matrix and then select "compute SSM" (*see* http://docs.flowjo.com/d2/experiment-based-platforms/plat-comp-overview/spillover-spreading-matrix/).
13. Export the SSM as a CSV-file and color-code it based on SE using a spreadsheet program (for example low SE no color, large SE red) (*see* Fig. 5).
14. Apply the calculated compensation matrix to the single-stained control samples.
15. Arrange the samples in the form of an $N \times N$ plot and visually assess the extent of SE.

3.4 In Silico Panel Design Using the Spillover Spreading Matrix (SSM)

The two key considerations for successful panel design are (1) fluorochrome brightness, and (2) dealing with spreading error (SE), whereby the latter is the more relevant issue. There are several ways that SE and the resulting loss of resolution can be controlled. The most straightforward way is to use fluorophore–detector pairs with high SE for mutually exclusive markers (e.g., CD3 and CD19) because in this case the SE will not interfere with detection of either signal. However, this tactic is only applicable to a limited extent as most researchers' experimental aim is to analyze as many target antigens as possible on one or two cell types.

Thus, if fluorophore–detector pairs with significant SE are to be used for molecules expressed on the same cell type, one has to assess the expression level of the target antigens. This can be done either by an internet search, or by preliminary experiments assessing antigen density on the target population. An excellent resource in this context are optimized multicolor immunophenotyping panels (OMIPs) [33, 34], which typically show raw data in their supplements and cover many of the commonly used targets in immunology [13, 35, 36]. If the target expression level is known, one can use a fluorophore generating a large SE for low-expression antigens, which will thus generate only weak signals, which in turn will reduce the amount of SE (as SE is proportional to the signal intensity). Alternatively, one can use a highly expressed antigen in the secondary detector, and assess whether the signal of the target

in the secondary detector is above the spread of the negatives generated by the SE.

Another useful approach for dealing with SE is to purposefully titer down signals of lineage markers, which typically show bimodal expression patterns and are only used to delineate positive from negative cells. Again, lowering the overall signal by lowering the used antibody titer will also decrease the SE generated (*see* example in Fig. 4b). Of note, working at nonsaturating staining conditions has important drawbacks that will be discussed in more detail in Subheading 3.5 below.

Finally, one has to consider relative fluorochrome brightness (*see* fluorochrome brightness chart, Fig. 3c) such that low-expression antigens are paired preferably with bright fluorochromes, while high-expression antigens can be paired with dim fluorochromes. However, it is important that this assignment takes SE into account. Below, we describe the steps that we typically perform for the in silico design of a new panel. The example SSM shown in Fig. 5 summarizes some of the key aspects that are listed below.

1. Print out the color-coded SSM as well as the fluorochrome (reagent) brightness chart generated in Subheading 3.4.
2. Identify the three highest values in the SSM and assign these to mutually exclusive antigen targets.
3. Identify the fluorophores in the SSM with the lowest row sums, that is, the ones that contribute the least amount of overall SE to all the remaining detectors.
4. Assign these fluorophores to your most relevant lineage markers (e.g. CD3 for a T cell-centric analysis).
5. Identify the detectors in the SSM which have the lowest column sums, that is, collect the least amount of SE and thus will not have any increased spreading of the negatives introduced.
6. Assign the most critical/dimly expressed antigen targets to the fluorophores used in these detectors. Make sure that the corresponding fluorophores fall into the "bright" or "intermediate" category of brightness.
7. Identify the detectors in the SSM which have the highest column sums, that is, those receiving the most SE. For these detectors it is essential that the assigned antigen–fluorophore pairs deliver a signal that is above the SE. This has to be assessed in preliminary experiments using fluorescent-minus-one (FMO) controls compared to a full staining panel (*see* Subheading 3.6).
8. For the remaining antigens and fluorochromes, pair the antigens with higher expression levels to the fluorochromes generating less SE, and the antigens with lower expression levels to the fluorochromes generating higher SE.

9. As there will be numerous possibilities in **step 7**, design up to three different panel combinations. Note that in most cases, there will not be one single optimal panel, but several possible combinations.
10. Order the necessary reagents in small test-size aliquots.

3.5 Titration of Reagents

After selection of reagents for the panel run, titration is an essential step that is often omitted. The primary reason for titrating antibodies is twofold: first, to minimize background from nonspecific binding and second, to ensure that staining is performed at saturating concentration (if necessary) [37]. For a typical titration experiment, it is recommended to do 8–12 twofold serial dilutions starting from a highest concentration of 2 μg per staining reaction. After titration, the stain index (SI) can be plotted relative to the dilution factor. This will reveal the saturating titer, which is the concentration at which further addition of antibody does not increase the signal intensity, that is, all target binding sites have been occupied. If any quantitative measurements (i.e., extracting MFI as a means of estimating target antigen density) are intended, antibodies have to be used at the saturating concentration. However, for some lineage markers (e.g., CD3 or CD4) it is sufficient to work at nonsaturating concentration. The benefits are that it saves reagent and also minimizes spreading error into other detectors. If one chooses to do so, it is important to note that signal intensity at nonsaturating concentration can change with the number of cells stained, incubation length, and temperature. Thus, one has to work with standardized cell numbers and staining conditions. For different examples of antibody titrations, we direct the reader to the supplementary materials of recent OMIPs [13, 35] and the information found in the "Guidelines" [23].

1. Obtain cells (typically cryopreserved PBMCs or murine splenocytes). *See* **Note 5** for targets that are not readily detected on steady-state cells.
2. Aliquot cells in as many wells as required into a separate 96-well plate.
3. Spin plate at 400 × *g* for 5 min. Flick supernatant and dry the top of the plate by carefully placing it on a paper towel and draining remaining liquid.
4. For a given titration experiment, add 96 μl of stain buffer to the empty well 1 of a 96-well plate, and 60 μl of stain buffer to wells 2–12.
5. Add 24 μl of antibody stock to well 1 and mix. If the specific antibody concentration is known, use 2 μg of antibody stock solution and add it to well 1 with additional stain buffer as required to achieve a final volume of 120 μl.

6. Transfer 60 µl of the solution in well 1 into well 2, mix, and then transfer 60 µl from well 2 into well 3 and so on. From the last well (well 12), remove 60 µl and discard.
7. Using a multichannel pipette, take up 50 µl from the prepared antibody dilutions and use this to resuspend the cells and incubate for 20 min at RT in the dark.
8. Add 200 µl of stain buffer to all wells, centrifuge plate at 400 × *g* for 5 min, flick supernatant, and dry the top of the plate by carefully placing it on a paper towel. Repeat this wash once.
9. Optional: add 100 µl of fixative to each well, resuspend, and incubate for 20 min at RT in the dark.
10. Add 200 µl of stain buffer to all wells, centrifuge plate at 400 × *g* for 5 min, flick supernatant, and dry the top of the plate by carefully placing it on a paper towel.
11. Resuspend in 200 µl of stain buffer and store at 4 °C until acquisition.
12. Start up the flow cytometer and perform daily QC as required per instrument-specific instructions.
13. Acquire all samples at the optimal voltages determined in Subheading 3.2.
14. Export data in FCS3.1 file format and load into a flow cytometry data analysis program.
15. For a given titration sample, draw gates delineating singlets, and subsequently the positive peak, and the negative peak.
16. Calculate the median fluorescence intensity (MFI) of both the positive and the negative peak (gates are typically assigned by the compensation wizard), as well as the rSD for the negative peak.
17. Calculate the staining index (SI) for each titration sample by using the following formula: $SI = MFI(pos) - MFI(neg)/(2 \times rSD)$.
18. Using a spreadsheet program, plot the SI relative to the dilution.
19. Determine the saturating titer by identifying the dilution at which the SI does not show any further increase (i.e., plateaus).
20. If there is no saturating concentration, identify the dilution at which the SI is highest, while maintaining a low background signal of the negative population.
21. Record the optimal as well as the saturating concentration together with the antibody clone, lot, and target in a spreadsheet.

3.6 Single-Stained Controls and Testing of Full Panel

Once all reagents have been titrated, the full panel can be tested. The most essential aspect of this validation experiment is not necessarily the full stain but rather the inclusion of the appropriate controls, specifically fluorescence-minus-one controls (FMOs) [38]. An FMO contains the full antibody mix except one antibody, and thus allows assessing problems that can potentially arise from unforeseen spillover or unexpected interaction between dyes [39]. While it is beneficial to prepare FMOs for every single reagent in a panel, this can be tedious and might not always be necessary. Instead, one should include FMO controls for every target where the expected signal is either not known or includes potentially problematic fluorochrome–detector pairs (also *see* **Note 6**).

The quintessential prerequisite (and most common source of error) for the success of any polychromatic experiment is the quality of single-stained controls [30]. This is an area where several misconceptions are commonly found as to what constitutes a "good" single-stained control, in particular whether one should use cells or antibody-capture beads (compensation particles). There are four generalizable rules that apply for single-stained controls, and as long as these are met one can use either cells or beads (*see* also **Note 7**): (1) Within one single-stained control, the positive and negative particles must be of the same kind, that is, have the same autofluorescence. This means that both populations must be beads (of the same batch and type), or both populations must be the same type of cells (and not one population comprising T cells and the other one monocytes, as would be the case if using human PBMCs and staining them with CD3). The reason for this is that during compensation, the compensation value is calculated such that MFIs of positive and negative populations are equal [30]. If the autofluorescence, that is, the background fluorescence of these two populations are different, this will introduce an error into the compensation matrix. (2) The positive signal of the single-stained control must be as bright as or brighter than the experimental sample, but remain within the linear range of a given detector. The reason for this rule is that the error that is associated with a measurement is proportional to the square root of the signal intensity, such that a dimmer signal will carry a proportionally larger measurement error. Therefore, it is better if the single-stained control is bright but does not exceed the linear range of the detector (instrument-dependent), which would again introduce errors into the compensation matrix. (3) The single-stained control must use the exact same fluorophore as the real experimental sample. Though this is an obvious rule, it is sometimes not followed because researchers use different antibodies for their single-stained control relative to their experiment. However, this can be detrimental in particular for tandem dyes because different product lots will show different conjugation efficiencies. Therefore, it is required that the antibody–fluorochrome molecule used to prepare the

single-stained control is the same type and lot as the one used to stain the actual sample. (4) The single-stained control should be treated exactly the same way as the real experimental sample, including fixation. The reason for this is that some fixation protocols can impact fluorophore characteristics, and similar to the previous point, the single-stained control should carry the exact same fluorophore as is present in the sample.

1. Prepare a spreadsheet showing antibody volumes to be pipetted in the planned experiment.
2. Obtain cells (typically cryopreserved PBMCs or murine splenocytes).
3. Resuspend cells in the appropriate volume and dispense into a 96-well plate.
4. Centrifuge plate at 400 × *g* for 5 min. Flick supernatant and dry the top of the plate by carefully placing it on a paper towel.
5. Add 100 μl of freshly prepared Fc-Block/Viability dye solution and incubate for 15 min at room temperature (RT) in the dark (*see* **Note 8**). In the meantime, start preparing the antibody master mix (and the required FMO mixes) using Brilliant Staining Buffer (*see* **Note 9**).
6. Wash by adding 200 μl of stain buffer, centrifuge plate at 400 × *g* for 5 min, flick supernatant, and dry the top of the plate by carefully placing it on a paper towel.
7. Add 50 μl of antibody staining mix containing the correct final dilutions of all antibodies, resuspend, and incubate for 20 min at RT in the dark.
8. In parallel, dilute 7–15 drops of compensation beads with stain buffer to a total volume of 1.5 ml, aliquot 50 μl into the wells of the 96-well plate, add 0.7 μl of a single antibody into each well. Incubate for a minimum of 15 min at RT in the dark.
9. Add 200 μl of stain buffer to all wells, centrifuge plate at 400 × *g* for 5 min, flick supernatant, and dry the top of the plate by carefully placing it on a paper towel. Repeat this wash once for a total of two washes.
10. Optional: add 100 μl of fixative to each well, resuspend, and incubate for 20 min at RT in the dark.
11. Add 200 μl of stain buffer to all wells, centrifuge plate at 400 × *g* for 5 min, flick supernatant, and dry the top of the plate by carefully placing it on a paper towel.
12. Resuspend the cells in 100–200 μl of stain buffer and store at 4 °C in the dark until acquisition.
13. Startup flow cytometer and perform daily QC as required per manufacturer's instructions.

14. Adjust the PMT voltages to match your MFI target values of your reference particles as described in Subheading 3.2, **steps 29–34**.
15. Record 10,000 events from each single-stained control, and perform compensation using instrument-specific acquisition software.
16. Record as many events as desired from the full stain as well as the FMO controls.
17. Export data in FCS3.1 file format and load into a flow cytometry data analysis program. Follow the analysis steps outlined in Subheading 3.7.

3.7 Analysis and Troubleshooting

Within this section we focus on manual analysis and quality control of the newly established panel. This can be performed using any software that is able to read and visualize FCS files. Among the commonly used stand-alone packages are FlowJo (BD Biosciences), and a powerful cloud-based solution is offered by Cytobank (www.cytobank.com), but there are several other options available on the market (e.g., FCS Express from De Novo Software).

For fluorescent data an often-undervalued aspect is the appropriate transformation and visualization of the data, which is essential for successful manual analysis. Historically, flow cytometric data has often been displayed using a purely logarithmic scale. This can introduce visual artifacts at the lower end of the scale, which is why the logicle/biexponential transformation was developed in the mid-2000s [40–42]. These so-called hybrid scales will be logarithmic at the high end of the scale and gradually approach a linear scale around 0, after which they would transition back to a logarithmic scale below 0. In the biexponential implementation, the "width" basis will determine how much space around 0 is displayed in a linear domain, thus either "compressing" or "widening" the appearance of the negative populations. Currently, there is no reliable automated solution available, and thus researchers need to adjust the width basis as well as the amount of negative decades manually. This should be done in a way that (1) all events are visible on a plot and (2) the negative population appears as a normally distributed (i.e., round) population. In particular for complex polychromatic data sets, it is essential to use logicle/biexponential (or any other appropriate version such as arcsin) transformation of the data in order to visualize spreading error (SE) accordingly.

After the first run of a newly established panel, validation and troubleshooting is critical, as even with the best possible planning, unexpected issues can arise from SE or improper compensation. To assess this, two different strategies should be employed: (1) Prepare an $N \times N$ view of all parameters against each other and visually screen for erroneous patterns. This include either "leaning," triangular populations or the so-called "super-negative" populations that appear below 0 (Fig. 6a). Of note, the negative population

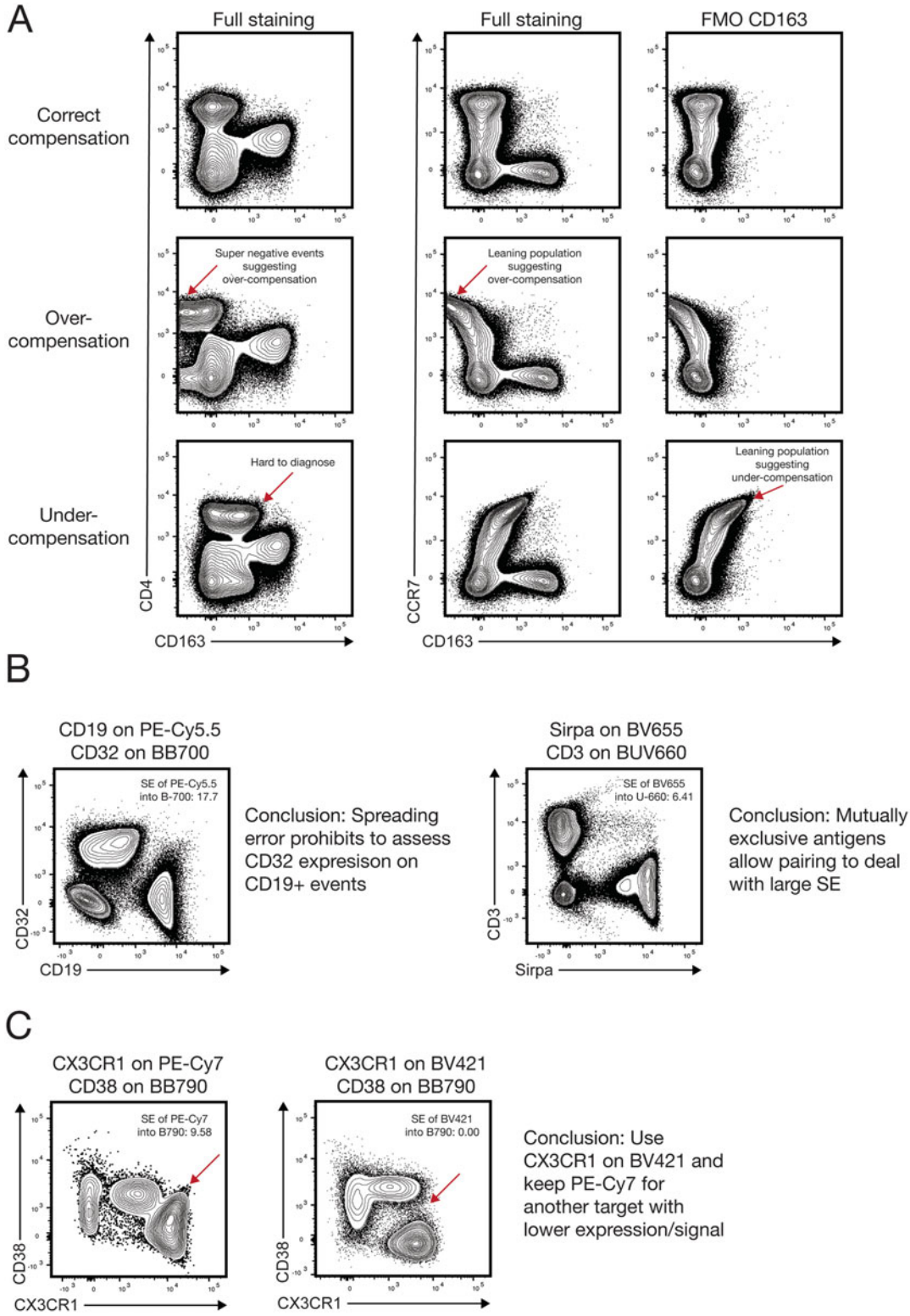

Fig. 6 Examples of erroneous staining patterns and (SE) spreading error. Example data are from the development of a 28-color immunophenotyping panel centered on myeloid cells (published in OMIP-044).

for a highly compensated fluorochrome pair can sometimes appear as slightly tilted oval, which is not an issue, but a result of the compensation calculation [43]. (2) Compare the fully stained panel with all FMO controls. If there is a positive signal present in the controlled detector of the FMO control, this usually can be traced back to either erroneous compensation, or SE that has not been anticipated. The source of this SE can usually be found by visualizing the detector in question against all other fluorophores present in the experiment.

An example where SE was dealt with by assigning fluorophores to mutually exclusive markers is shown in Fig. 6b. Figure 6c depicts an example where spreading error from one antigen (CX3CR1-PE-Cy7) was lowering the resolution in the B790 detector. The solution to this problem was to move the highly expressed CX3CR1 to another fluorochrome (BV421) that did not generate SE in the B790 detector, and instead assigning PE-Cy7 to a target generating less intense signal (and thus less SE).

1. Load all the data in your preferred flow cytometry data analysis program and make sure that compensation has been applied.
2. Assess signal/time for all parameters by either displaying a signal off each laser versus time, or use the automated function of your analysis program (in FlowJo: press cmd + I). Signal over time should be constant. Major, recurrent fluctuations in signal over time could indicate issues with the stability of instrument fluidics.
3. Do a cleanup gating based on FSC-A vs SSC-A to exclude debris.
4. Exclude doublets by gating on FSC-A vs FSC-H and FSC-A vs FSC-W.
5. Exclude dead cells by gating on live/dead stain negative events. This population should be used for all further analysis and validation.

Fig. 6 (continued) (**a**) Upper panel shows correctly compensated data. If overcompensated (middle panel), this would be visible either as a population of "super-negative" events (if displaying two unrelated detectors) or as a leaning, banana-shaped population pointing below 0 (if displaying the fluorophore–detector pair that is wrongly compensated). If the population is undercompensated, this would be very hard to diagnose solely from the full stain, but the FMO control clearly identifies the issue by showing a leaning, banana-shaped population pointing toward the upper right. (**b**) Two examples where spreading error (SE) was dealt with by assigning the corresponding fluorophores to mutually exclusive antigens. (**c**) Example for improving a suboptimal panel after initial testing. Left plot shows CX3CR1 on PE-Cy7, which will show significant SE into the B-780 detector (excitation laser: 488 nm, detection window: 780/60) and makes it hard to discern $CD38^{+}$ events in this detector. To improve this, CX3CR1 was moved to BV421, a fluorophore that does not show any SE into the B-780 detector. PE-Cy7 should instead be used for a target that delivers a dimmer signal, which will in turn diminish the SE generated

6. Use the fully stained sample and generate an $N \times N$ view of all parameters against each other.
7. Visually screen for "leaning" population patterns as displayed in Fig. 6a (lower panel).
8. Visually screen for "super-negative" populations as displayed in Fig. 6a (middle panel).
9. Prepare an overview gating delineating your populations of interest, and at least one plot of every marker in your panel.
10. Using this gating hierarchy, compare the fully stained sample to all FMO controls. Assess whether there is any spreading of the negatives in the respective channel of interest in the FMO control. Note that the FMO control can be used to set reliable gates during manual analysis.
11. Assess whether all the targets in the panel are resolved.
12. If this is not the case, usually this is due to spreading error (SE) from another fluorophore. Trace poorly resolving pairs and assess whether the signal is too dim or whether there is unexpected SE from another signal.
13. If the signal is too dim, assess whether a brighter fluorophore can be used instead.
14. If SE is the problem, assess the problematic pair and try to switch it to another fluorophore combination, keeping the aspects in mind that were discussed during panel design in Subheading 3.5 (*see* example in Fig. 6c).

4 Notes

1. Fixation is not a prerequisite for every experiment, but can be considered optional. If processing human samples, fixation might be required by shared resource laboratories prior to acquisition for biosafety reasons, while for murine samples this is not the case. In general, unfixed samples should be acquired within 6–12 h after processing. If samples have been fixed, they can be stored in the dark at 4 °C for several days. However, as we discuss in Subheading 3.6 it is imperative that all single-stained controls are treated the same way as the sample (i.e., fixation).
2. If in a voltration experiment maximal resolution (i.e., plateau of SI) has not been reached before the CD4 population has exceeded the linear range of the detector, an antibody with a lower antigen density can be chosen for that detector and the voltage titration repeated. Alternatively, a PMT voltage can be chosen that sets the median fluorescence intensity (MFI) of the CD4 positive peak no higher than 60,000. As a rule of thumb,

at this MFI most common higher density antigens will remain on scale for that detector while maintaining sufficient resolution of dim antigen targets. Finally, as discussed in more detail in the following ref. 28, one can use quantum simply cellular beads (QSC beads, available from Bangs laboratories) for the voltration experiment, which will provide multiple signal intensities within one sample.

3. The key requirements for fluorescent reference particles are (1) long-term stability, (2) an appropriately bright signal in all detectors and (3) an intrinsically small coefficient of variation (CV). Commonly used are single-peak reference beads such as Spherotech Supra Rainbow MidRange Fluorescent particles (#SRCP-35-2A) or MidRange Rainbow Fluorescent particles (#RFP-30-5A). However, since instrument configuration and detection optics vary widely, it might be required to use a combination of multiple fluorescent particles to obtain a sufficiently bright signal in all detectors. In this case, Spherotech Ultra Rainbow Particles (#URCP-38-2F) have been proven useful, containing six peaks of varying fluorescent intensity which are also suitable for detectors excited with a UV laser.
4. As discussed in Subheading 3.6, it is important that the positive signal of your compensation control is as bright or brighter than your experimental sample. The underlying reason for this is again the Poisson error during photon counting at the detector. This error is proportional to the square root of the signal intensity, that is, the error will be relatively larger for dimmer signals. As the error is propagated throughout the compensation calculation, the compensation matrix will only be accurate up to this signal intensity, and will be increasingly imprecise for signals that are brighter than the compensation control. In the author's experience, using 1 μl of antibody with standard compensation particles will deliver an appropriately bright signal, though it might be subsaturation stain of the binding sites on the compensation bead. If the signal is not sufficiently bright, one has to either use more antibody to saturate all binding sites or switch to the so-called Plus-compensation particles available by various vendors which are larger in size and deliver a brighter signal. In turn, if the signal from the compensations beads is off-scale (i.e., too bright), one should use appropriately diluted antibody solution to achieve a dimmer signal.
5. Depending on your biological targets, antigen expression evaluation and corresponding reagent titration may require either perturbation to your target cells (stimulation, etc.) or the addition of costains to identify small subsets of cells that express your antigen of interest. In the case of variable or inducible antigen expression, it is advisable to use a similar sample source to what will be used in your final assay as background staining

and receptor expression may vary and impact the choice of an appropriate reagent titer. Whenever possible, a primary cell that represents your target antigen expression level should be preferred over cell lines or other surrogates. In addition, your titration experiments should be performed on cells that are treated the same way as your experimental cells including any digestions, stimulations, permeabilization, or fixation steps.

If the target antigen is expressed on a small subset of your bulk cell population and you require costains, it is important to make sure that the primary antibody–fluorochrome needed to identify your targets do not show any SE into the detectors that are used for titrated antibodies. Appropriate fluorophores can be determined by utilizing the SSM as described in Subheading 3.3.

6. Commonly, FMOs are used to control only for a single fluorochrome. However, there are two situations where one could use FM2 or FM3 controls: First, if the detector to be controlled for is collecting SE from multiple fluorochromes, the FM2/3 can be used during panel optimization to assess the cumulative SE that is contributed from the two or three omitted fluorochromes relative to an FMO. Second, if FMO controls are to be included in repeated experiments, it can save pipetting effort (and sample) if FM2 controls are made in a way that the two omitted fluorochromes are explicitly tested to not have any cross talk between each other.
7. While compensation beads are the preferred way of preparing single-stained control samples, in the authors' experience, not all compensation particles work equally well with all fluorochromes, in particular some of the Brilliant-Violet and Brilliant Ultraviolet tandem dyes (e.g., BUV737, or BV786). For these dyes, the resulting compensation value might have variability compared to cells that is not appropriate for your specific assay requirements. Thus, users should always assess whether the chosen type of compensation particle delivers appropriate compensation values by utilizing appropriate FMO controls.
8. As shown by multiple labs, utilizing Fc-blocking reagents is critical to prevent possible artifacts arising from unspecific antibody binding to the Fc-receptors of various myeloid cells [44]. To save time, in the authors' experience it is possible to combine the Fc-blocking and live/dead staining step. This will result in a slight decrease in the intensity of the live/dead staining signal, as the amino-reactive dye will also react with the Fc-blocking reagent, but this does not negatively impact assay performance.
9. Polymer-based dyes of the Brilliant-Violet or Brilliant-Ultraviolet family are commonly used for high-dimensional

panels, and will likely become even more prevalent in the future [45]. Due to their chemistry, these polymer-based dyes can potentially interact with each other, causing artifacts in flow cytometry experiments that appear as inappropriately compensated data. To prevent this, it is advisable to use commercially available staining buffers preventing this interaction every time one is using more than two polymer-based dyes in an experiment. Since different vendors use different proprietary formulations for these buffers, users might need to test which buffer works best for their experimental setup.

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Chapter 2

Immunophenotyping by Mass Cytometry

Gregory K. Behbehani

Abstract

Mass cytometry is a novel technology similar to flow cytometry in which antibodies are tagged with heavy metal molecules rather than fluorophores and then detected with time-of-flight mass spectrometry. This enables measurement of up to 50 simultaneous parameters with no autofluorescent background and little or no spillover or required compensation. Mass cytometry has tremendous potential for the analysis of highly complex research or clinical samples and can measure 40–50 immunophenotypic markers at a time. This chapter describes most of the commonly used methods for performing basic immunophenotyping experiments by mass cytometry, and how this can be combined with measurement of cellular functional properties.

Key words Immunophenotyping, Mass cytometry, CyTOF, Flow cytometry, Minimal residual disease, Acute leukemia, Aberrant marker expression

1 Introduction and General Considerations

Flow cytometry is one of the most used technologies in the basic science and clinical research laboratory. It is now an essential tool in the study of malignancies, infectious diseases, and immune system function. Its utility stems from its ability to analyze single cells for their expression of virtually any antigen to which an antibody can be specifically bound. This enables the identification and quantification of immunophenotypically different cell types in complex biologic samples even if the cells of interest are extremely rare. Despite its utility, however, most current clinical flow cytometry technologies are limited to analysis of 4–15 simultaneous measurement parameters, primarily due to the dependence of the technology on fluorescent reporter molecules (with relatively broad absorption and emission spectra), which also makes flow cytometry susceptible to various artifacts due to the fluorescent properties of the cells being studied and interactions between the fluorescent reagents being used to study them. While the technology of fluorescent cytometry continues to advance rapidly with newer machines and

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newer reagents, the most transformative advance in cytometry has been the development of mass cytometry, which allows for immunophenotypic measurements without the problems created by fluorescent reporters and optic measurement [1].

1.1 Mass Cytometry Versus Fluorescent Flow Cytometry

Mass cytometry (MC) is a cytometry technique similar to fluorescent flow cytometry that detects cellular antigens through the use of mass spectrometry to detect binding of heavy-metal conjugated antibodies to the cells being studied. Mass cytometry experiments are typically performed using the same antigen-specific antibodies used in conventional flow cytometry, but the binding of these antibodies is measured by vaporizing and ionizing the cells (with the bound metal-conjugated antibodies) followed by the subsequent detection of the molecules of isotopically purified heavy metal atoms by time-of-flight mass spectrometry (ICP-MS). The primary advantage of this approach stems from the ability of ICP-MS to distinguish ions of different atomic weight with less than 0.5% signal spillover between adjacent masses (which are each conjugated to a particular antibody). The data generated from the analysis of each antigen is very similar to the data generated when the same antigen is analyzed by flow cytometry, and currently up to 40–50 parameters can be simultaneously recorded per cell with minimal or no signal compensation and no background due to autofluorescence. The greatest advantage of mass cytometry is the large increase in the number of simultaneous parameters that can be recorded. This is particularly useful in the analysis of highly complex or heterogeneous cell populations in which the additional parameters enable simultaneous identification of numerous cell types or functional states to be uniquely identified and characterized, even when some of these cell types are very rare. There are now numerous publications utilizing this technology and many excellent reviews [2–6]; this chapter will provide a basic protocol for immunophenotyping by mass cytometry, how this can be combined with measurement of intracellular antigens for functional studies, and focus on the unique practical considerations that are important in designing mass cytometry experiments.

Since mass cytometry experiments can typically be performed using the same exact antibody clones as fluorescent flow cytometry experiments, the immunophenotypes derived from the two techniques should be nearly identical. However, labeling of antibodies with chelated heavy metal and its detection by time-of-flight mass spectrometry creates several unique considerations that must be accounted for in the design of a mass cytometry experiment. These can be grouped into issues related to the antibody labeling polymer and the ion detector.

Antibody considerations: Mass cytometry antibodies are produced by conjugation of polymer of DTPA chelators (functionalized using a maleimide group) to available sulfhydryl groups of the

antibody of interest. The majority of antibodies are produced using a polymer called Maxpar® X8, which researchers can purchase in a kit from the manufacturer (Fluidigm) to custom-label their own antibodies (note that Fluidigm also uses a second polymer for a small subset of their preconjugated antibodies that has slightly different properties; *see* **Note 1**). The vast majority of commercially available antibodies can be labeled in this way (except for antibodies sensitive to reducing conditions, such as IgM clones), provided that the antibody can be obtained (from the manufacturer or by purification) without any other proteins in the antibody solution. Regardless of whether the antibodies are custom-conjugated or purchased preconjugated, most mass cytometry antibodies have a staining intensity similar to what would be achieved if that of the same antibody was conjugated to FITC and analyzed by flow cytometry. This can be advantageous in that most of the reagents will have similar measurement sensitivity, but this also means that antigens of very low abundance that typically show changes of only two- to threefold when measured by flow cytometry using antibodies conjugated to bright fluorochromes (e.g., APC or PE) can be difficult to detect with mass cytometry. Such low signals can potentially be increased by detecting the antigen of interest with multiple metal-labeled antibodies that are specific for the antigen (provided that the antibodies do not sterically interfere with one another). Additionally, the molecular structure of the metal chelating polymers appears to cause them to adhere nonspecifically to certain rare cell types such as eosinophils (likely due to interactions with intracellular cations), but this effect can generally be blocked using heparin, as described by Rahman et al. [7] Another advantage of mass-tagged antibodies is that they are generally stable to the majority of cellular processing and permeabilization methods, enabling staining of surface antigens followed by permeabilization or other additional processing steps without significant disruption of the bound antibodies or their attached metal. By using post-staining fixation, mass cytometry surface antigen staining has been combined with hybridization intracellular RNA [8], staining for nucleotide incorporation [9], and staining for hypoxia [10], along with detection of other routine intracellular antigens. Given this advantage, even in experiments that combine immunophenotypic assessment with measurement of intracellular functional markers, surface markers can be stained fresh or after only minimal cell manipulation (e.g., gentle fixation).

As with all antibodies, mass-tagged antibodies should always be titrated for optimal staining concentration. It is worth noting that most metal-labeled antibodies do exhibit very small amounts of nonspecific cell binding at high concentrations, meaning that (like fluorescent flow cytometry) saturation of the antigen of interest and optimal signal to noise ratio can occur at antibody concentrations below the maximal achievable staining intensity. It is thus

important to include good negative controls in all antibody titrations. Fortunately, the relative lack of spillover between mass channels allows numerous antibody reagents to be titrated simultaneously.

Detector considerations: The mass cytometer's detector is another obvious difference from a traditional fluorescent flow cytometer. Most notably, the mass cytometer only has a single ion detector. The ion detector is automatically tuned daily, greatly simplifying machine setup relative to fluorescent systems with multiple (often instrument-specific) filter sets and photomultipliers. This fact also creates a drawback, however, in that it is not possible to adjust detector sensitivity separately for each measurement channel. This makes it important to consider the mass channels used for antigens expected to be at high abundance, as discussed in Subheading 2.3. Additionally, unlike fluorescent flow cytometry detectors, the mass cytometer's detector has a finite lifetime and will typically need to be replaced approximately every 12–18 months depending on usage. Staining of samples with high levels of metal can greatly accelerate the wear of the detector and, since its sensitivity cannot be adjusted separately for each channel, it is important to ensure samples are not stained with excessively high amounts of metal.

1.2 General Method Design

In this chapter the most common approaches to mass cytometry cell processing will be described. However, if converting a previously established fluorescent flow cytometry protocol to a mass cytometry protocol, the same sample processing and antibody staining conditions can typically be used. The only absolutely required modifications needed to convert a flow cytometry immunophenotyping protocol to mass cytometry protocol are as follows: (1) use of metal-conjugated antibodies instead of the fluorochrome-conjugated ones, (2) the use of a fixative-containing metal intercalator solution after the completion of antibody staining (Subheading 3.6, **steps 1–3**), and (3) running the sample into the mass cytometer after dilution in either pure water or a specialized running buffer free from nonorganic salts (Subheading 3.6, **steps 4–9**). If using commercially prepared solutions for fixation and permeabilization, all solutions should be checked for heavy metal contamination on a regular basis (unless the solutions are specifically made for mass cytometry).

The minimum number of cells required for mass cytometry analysis varies based on the exact number of centrifugation steps, the type of centrifuge tubes employed, and the number of washes performed. For the protocol described here (utilizing standard 5 mL polystyrene FACS tubes), 500,000 cells is a good minimum number as the mass cytometer generates data from 30% to 50% of the cells that are injected (after staining and washing), necessitating a proportional increase in the number of starting cells (as compared

to fluorescent flow cytometry). As a general rule, we typically expect to get data events from 10% to 20% of the cells present at the beginning of the experiment, thus 500,000 cells will typically yield 50–100,000 final cell events in the final data. Utilizing smaller, polypropylene tubes, and reducing the number of staining and wash steps can allow for the analysis to be preformed with lower starting cell numbers (as low as 100,000). A good approach to determining the number of starting cells, is to estimate the expected frequency of the rarest cell population of interest and the absolute number of these events desired, the total number of starting cells necessary can then be calculated based on these numbers and the 10–20% rate at which starting cells result in data events. Cells fixed according to this protocol (Subheading 3.4) will be stable for at least several months at −80 °C, and we have successfully analyzed cells 2–3 years after fixation.

2 Materials

2.1 Precautions

To avoid background due to heavy metal contamination, all solutions for mass cytometry should be prepared using ultrapure water and all reagent solutions (when first prepared from new lots of stock reagents) should be tested for heavy metal contamination (*see* **Note 2**). Note that cisplatin, paraformaldehyde, and the Smart Tube buffer are all toxic and potentially mutagenic. They should be handled with appropriate protections (gloves, eye and respiratory protection as appropriate). It is also important to mention that standard laboratory dishwashers, autoclaves, and dishwashing detergents are commonly contaminated with heavy metals (particularly lead and barium) that can disrupt experiments or damage the mass cytometer. We use either disposable plastic containers or glassware that we wash by hand for producing and storing all mass cytometry solutions and reagents.

2.2 Fixatives, Buffers, and Tubes

1. Paraformaldehyde (PFA), 16% solution: This must be methanol-free (i.e., not formalin). We purchase this in 10 mL ampules (Electron Microscopy Sciences) and transfer it to foil-wrapped tubes, as it will lose activity over several weeks upon exposure to air and light. We will throw out any PFA that has been open for more than 1 month.
2. Smart Tube proteomic stabilizer (STPS; alternate fixative): can be purchased from Smart Tube incorporated (San Carlos, CA). This comes as a working solution that can be added directly to cell samples at a sample–buffer ratio of 1:1.4.
3. Pure methanol (optional): This should be kept cold (−20 °C to 4 °C) in a sealed bottle. This should not have any drying agents added, as these may contain heavy metals.

4. Cell staining media (CSM): Standard phosphate buffered saline (PBS), plus 0.5% bovine serum albumin (BSA), and 0.02% sodium azide, pH = 7.4. This is typically made this in 4 L batches by adding 20 g of BSA and 800 mg of sodium azide. Start with 3 L of sterile PBS and mix in the dry ingredients until dissolved. Then add additional PBS to a total volume of 4 L. Sterile-filter this using a 0.2 μM bottle-top filter into rinsed, 500 mL glass bottles. Rinse and hand-wash these bottles as necessary, but do not place them into standard laboratory dishwashers. Premade Maxpar® cell staining buffer can be substituted for CSM. Other premade PBS and blocking protein solutions could also likely be substituted for CSM; however, they should be checked for heavy metal contamination before use.
5. Intercalator solution is made by addition of the iridium-based intercalator solution (Fluidigm, 201192A or 201192B) to PBS at a final concentration of approximately 125 nM. To this add 1/10th volume of 16% PFA to achieve a final concentration of 1.5% PFA. This solution should be made fresh just before addition to the cells. The failure to use adequate amounts of fresh (i.e., active) fixative will cause cells to degrade while being analyzed (since analysis is typically performed in pure water).
6. Cisplatin: This can be purchased from a variety of manufacturers or can be purchased from Fluidigm as a natural isotopic mixture or in the form of its purified 194 or 198 isotopes. We typically prepare this ourselves as a concentrated 5 mM stock in DMSO and aliquot it into single-use tubes.
7. This protocol is written for use with standard 5 mL polystyrene FACS tubes, but a variety of other tubes can be used. We have successfully performed the protocol in 1.5 mL Eppendorf-style microcentrifuge tubes, and 1.1 mL polypropylene "cluster" microcentrifuge tubes, though additional washes will be required if the tube size is small relative to the residual volumes left after each wash step (*see* **Note 3**).
8. Cells: Essentially any cell that can be placed into a single-cell suspension (without disrupting the biology of interest) can be analyzed by mass cytometry; however, cells that normally grow or exist in a single-cell suspension tend to yield the best results. Note that each mass cytometry sample typically requires at least 500,000 cells per sample (we typically collect a minimum of two million cells to allow for one million cells to be stained and analyzed twice if necessary; *see* Subheading 3).

2.3 Antibodies and Antibody Panel Design Considerations

1. As described above in the introduction, antibodies can be purchased preconjugated from the manufacturer (Fluidigm) or can be conjugated with metal using a kit from Fluidigm in

accordance with the provided protocol. Additionally, other non-Fluidigm conjugation protocols are available [11].

2. This protocol is largely based on our experience using cells fixed using PFA or STPS fixation and methanol cell permeabilization, an approach used by a large fraction of other MC researchers. We have additionally performed similar experiments on fresh (unfixed) cells with good results (notably, most antigens exhibited similar staining regardless of fixation). We have not extensively tested staining under other permeabilization conditions (e.g., saponin), though other permeabilization methods would likely be compatible with this method as these same antigens have been successfully analyzed with other protocols and are supported by Fluidigm [12].
3. The relative lack of spillover in mass cytometry allows greater flexibility in panel design; however, many of the same considerations that are important in fluorescent panel design are still important for mass cytometry. Specifically, it is still important to pay close attention to situations in which high abundance antigens are on mass channels near very low abundance antigens. The mass cytometer itself has a spillover of only 0.3% into adjacent channels (technically termed abundance sensitivity); however, most of the metals used for metal conjugation are isotopically purified from natural populations of mixed isotopes (e.g., natural neodymium is purified into its seven isotopes, each of which is a separate mass channel) and this purification is usually not complete. Thus, most mass cytometry metals are contaminated with 0.5–2% of one or more other isotopes of that same metal (*see* **Note 4**). Additionally, all metals oxidize to some extent after ionization and before the TOF analysis; this effectively creates a 0.1–2.5% spillover into the channel 16 Da above (due to the mass of an oxygen atom; the exact percentage of oxide signal is dependent on the chemical properties of each element; *see* **Note 5**).
4. While generally not needed, compensation can be applied to mass cytometry experiments. We have performed manual compensation in rare cases where it is required, by either compensating for the known isotopic impurities of the relevant isotope, by using cells known to be negative for the antigen receiving the spillover, or by using a "minus one" control cell. In addition, Chevrier et al. [13] have recently developed an automated methodology for applying compensation to mass cytometry. While both approaches can effectively compensate spillovers (achieving a median spillover signal of 0 counts), signals of greater than several thousand counts can create spillovers that cannot be compensated with a linear matrix. Additionally, even lower intensity signals 1000–5000 counts can lead to widened population distributions (in channels receiving spillover) of

10–20 counts around the correctly compensated median intensity that can overwhelm changes in very low expression antigens being measured on the channel receiving spillover. Such spillovers issues should be avoided by designing the antibody panel so that high expression antigens are placed onto channels with minimal spillovers into channels measuring antigens with low expression levels. Alternatively, high abundance antigens can be placed onto mass channels with low sensitivity (e.g., 89Y), which can also facilitate measurement of more markers by saving the higher sensitivity channels for the low abundance markers that require the additional sensitivity (*see* **Note 6**).

5. Fluidigm does provide an online tool to assist with these panel design issues (available on their website). Alternatively, the use of premade commercial antibody cocktails, or antibody designs previously published by other researchers can alleviate many of the concerns around panel design.

3 Methods

3.1 Viability Staining (Optional)

If a significant fraction of the cells of interest could be dead at the time of collection, it is generally advisable to perform a viability (live–dead) stain prior to antibody staining or cell fixation. Dead or dying cells typically display different size and surface marker expression characteristics and almost universally will have altered levels of intracellular functional markers. It is thus critical to have a means of excluding dead cells (if expected to be present) as part of most experimental designs. This is particularly important for analysis of cryopreserved cells, in which the freezing and thawing process will reliably kill a fraction of cells by necrosis. The simplest method of live–dead staining is use of cisplatin (which nonspecifically and covalently binds to protein), based on the publication from Fienberg et al. [14], which will be described here. Alternative approaches include staining with bifunctional chelators loaded with heavy metal, such as barcoding reagents [15], or use of metal-containing DNA intercalators such as rhodium [16] (although in our experience the latter stain is generally not stable enough to be suitable if intracellular antigens will be measured). In all cases, the basic concept is to incubate cells in a reagent that does not rapidly cross the cell membrane of live cells but can rapidly enter the disrupted membrane of a dead cell where it will bind to intracellular DNA or protein, resulting in higher signal in the dead cells.

1. Fresh cells (from primary tissue or in vitro culture media, prior to any fixation) should first be washed one or two times with serum/protein-free PBS (*see* **Notes 7** and **8**).

2. The cells should then be resuspended in serum/protein free PBS at a concentration of one to ten million cells per mL and mixed to achieve an even single cell suspension.
3. Cisplatin should be added from the concentrated stock to the cell suspension to achieve a final concentration of 1–10 μM (the manufacturer recommends 5 μM; *see* **Note 9**).
4. Mix the cell suspension well and maintain at room temperature for 5 min. (The time of incubation can also be titrated if necessary.)
5. Following incubation with cisplatin, the reaction should be quenched with 5× volume of PBS with either BSA (0.5%) or serum (10–50%) added to it; alternatively pure human or animal serum or CSM could be used to quench the staining reaction (note that the sodium azide in CSM could interfere with subsequent functional studies). The protein in this solution will bind any remaining cisplatin and stop the staining reaction.
6. Wash cells once in either: PBS + BSA, serum, or CSM.
7. If properly titrated, this staining step will not cause significant short term (minutes to a few hours) toxicity to live cells [14], so subsequent stimulations or other brief functional assessments can be performed after cisplatin staining, if desired.
8. Platinum signal from natural platinum is typically recorded on the 195 Dalton channel; if using an isotopically purified cisplatin, measurement should be performed on the relevant channel (194Pt or 198Pt). When the Pt staining reaction has been properly titrated, there will be low levels of Pt staining on live cells and dead cells will demonstrate a 1–2 log increase in Pt incorporation.

3.2 Cell Stimulation (Optional)

While beyond the scope of this chapter, if the planned experiment calls for a cell stimulation prior to antibody staining (e.g., cytokine stimulation [17] or IdU incorporation for cell cycle analysis [18]) this should be performed after viability staining and before antibody surface staining. Typically, if a stimulation is performed, the cells will need to be fixed with PFA or STPS (or another fixative product) before antibody staining to preserve the intracellular response to the stimulation.

3.3 Fresh Sample Surface Staining

If the primary objective of an experiment is measurement of immunophenotype (rather than functional cell properties), cells can be stained live, provided that the relevant antibodies have been titrated on live cells using the staining time and temperatures that will be employed in the final experiment. It is worth noting that many of the original mass cytometry publications utilized cells that had been previously been fixed before staining and stained for longer

(45–60 min) incubations at room temperature, thus the published antibody concentrations from such publications may not perform the same using the protocol described below. Note that traditional barcoding methodologies [15, 19] will not perform well on live cells; however, antibody-based barcoding methods [20] can be used if barcoding is needed. (If this is desired, the relevant published protocol should be followed for antibody barcoding.) Live cell staining will provide the most accurate representation cell surface immunophenotype; however, the cell processing of the live cells is very likely to disrupt the functional state of the cells (intracellular signaling, cell cycle state, etc.) so if such measurements are important, gentle fixation prior to staining can be used to "freeze" the intracellular state of the cells at the expense of potentially decreasing staining of a subset of fixation-sensitive antigens.

1. The desired antibody cocktail should be prepared just prior to the start of cell processing (though we have prepared cocktails the day before with success). Each antibody should be diluted such that it will be at its empirically determined optimal staining concentration after the cocktail is added to the sample (*see* **Note 10**). For live cells, the cocktail can be made in CSM or alternatively (if the sodium azide will interfere with subsequently planned functional assessment) in PBS with added albumin or serum. We typically add 400 units per mL of sodium heparin to the antibody cocktail to prevent nonspecific antibody binding [7]. Typically, we will aspirate cells after each wash down to a final volume of 50 μL and make our antibody cocktail at a 2× concentration, so that we can add 50 μL of antibody cocktail to the residual 50 μL to achieve a final 1× antibody concentration in 100 μL.
2. Cells should be washed (*see* **Note 7**) in the desired staining buffer one or two times prior to the start of the staining reaction. It is desirable, but not absolutely required, that protein be present in this solution to block nonspecific antibody binding. Typically for live cell staining, serum from the relevant species is used directly or added to PBS. (CSM could also be used, but the sodium azide could create cellular toxicity and may need to be removed when staining live cells.) Cells can also be stained in whole blood or the primary in vivo body fluid the cells came from. We typically add sodium heparin to a final concentration of 400 Units per mL into the final wash of the cells. Typically, cells are cooled to 4 °C during this process (*see* **Note 11**). If blood is used, we typically use sodium heparin as the anticoagulant when the blood is initially collected.

3. *(Optional)* Add an Fc blocking reagent at the manufacturer's recommended concentration and incubate for 10 min (*see* **Note 12**).
4. Add the antibody cocktail at the previously determined optimal concentrations. Typically for staining of live cells, staining should be performed for 30 min at 4 °C or on ice. Cells should be gently mixed periodically or placed on a low speed rotator or shaker to keep the cells evenly suspended.
5. Wash twice in CSM or PBS.
6. If performing intracellular staining skip to Subheading 3.5 on intracellular staining; if only performing a surface stain skip to Subheading 3.6 on DNA intercalation.

3.4 Fixed Sample Surface Staining

1. Fixation: If the goal of the experiment is to study the intracellular properties of the cells of interest, it is typically advantageous to gently fix the cells prior to antibody staining. Commonly used fixation methods utilize PFA at 1.5–4% for 10–15 min at room temperature. For analysis of fresh primary human cell suspensions (typically, human peripheral blood or bone marrow aspirate), we will typically utilize Smart Tube proteomic stabilizer (STPS) solution in accordance with the manufacturer's instructions. A variety of other commercial fixative solutions are available and would likely be compatible with this protocol. This solution utilizes a proprietary fixative cocktail, but is sufficiently gentle to allow subsequent lysis of red blood cells as well as detection of antigens known to be disrupted by standard PFA fixation. An alternative fixation procedure developed by Chow et al. [21] utilizing PFA fixation followed by Triton X-100 can also work well for ex vivo sample fixation; however, this method tends to be slightly more disruptive to fixation-sensitive antigens in our experience. With either approach, cells can be frozen at −80 °C after fixation but before red cell lysis. If freezing other cell types after fixation with PFA, cells should be washed twice in CSM and then resuspended in CSM + 10% DMSO before snap-freezing at −80 °C (*see* **Note 13**). As previously noted, some surface antigens can be disrupted by fixation, so the effect of fixation should be empirically tested for each antigen–antibody combination of interest.
2. Red cell lysis (optional): If blood is present in the sample, it should be lysed before proceeding with antibody staining. If using STPS, we lyse the red cells in accordance with the manufacturer's instructions, if using the protocol of Chow et al. [21] lysis is performed with a detergent containing lysis buffer. After the completion of red cell lysis the cells should be washed twice in CSM.

3. Barcoding (optional): If performing cellular barcoding [15], this should be performed prior to antibody staining.
4. The desired antibody cocktail should be prepared just prior to the start of cell processing (though we have prepared cocktails the day before with success). Each antibody should be diluted such that it will be at its empirically determined optimal staining concentration after the cocktail is added to the sample (*see* **Note 10**). We typically add 400 units per mL of sodium heparin to the antibody cocktail to prevent nonspecific antibody binding [7]. Typically, we will aspirate cells after each wash down to a final volume of 50 μL and make our antibody cocktail at a 2× concentration, so that we can add 50 μL of antibody cocktail to the residual 50 μL to achieve a final 1× antibody concentration in 100 μL. (If using a saponin permeabilization to stain surface and intracellular antigens simultaneously, both should be added to this cocktail; *see* **Note 14**.)
5. Cells should be washed in CSM one or two times prior to the start of the staining reaction. We typically add sodium heparin to a final concentration of 400 Units per mL into the final wash of the cells. When using fixed cells, all steps can be performed at room temperature if desired, but antibody titration and staining should always be performed under the same conditions. (If using a saponin-based staining protocol, saponin should be present in this wash solution.)
6. *(Optional)* Add an Fc blocking reagent at the manufacturer's recommended concentration and incubate for 10 min (*see* **Note 12**).
7. Add the antibody cocktail at the previously determined optimal concentrations. Stain for 30–60 min at room temperature. Cells should be gently mixed periodically or placed on a low speed rotator or shaker to keep the cells evenly suspended.
8. Wash twice in CSM.
9. If performing intracellular staining, continue to Subheading 3.5 on intracellular staining; if only performing a surface stain (or using saponin to simultaneously stain surface and intracellular antigens), skip to Subheading 3.6 on DNA intercalation.

3.5 Intracellular Staining

1. Surface Antibody Fixation (optional): While not absolutely required, we typically fix surface antibodies in place after surface staining. After the last wash of the surface stain protocol (Subheading 3.3, **step 5** or Subheading 3.4, **step 8**), add 1 mL of PBS plus 100 μL of 16% of PFA (1.5% final PFA concentration) and incubate for 15 min at room temperature.
2. Pellet cells by centrifugation and aspirate supernate down to a minimal volume (typically ~50 μL; *see* **Note 15**). Vortex cell pellet to resuspend it to an even single cell suspension

(*see* **Note 16**). Rapidly add 1 mL of ice-cold methanol immediately after vortexing the cell pellet then place the cells onto ice. It may be helpful to vortex the cell suspension at low speed during methanol addition to ensure that an even suspension is maintained. Incubate cells on ice for 10 min. If desired, cells can be stored at −80 °C overnight (or for up to a week) in methanol.

3. Add 1 mL of PBS to the cells in methanol. Then fill the remainder of the tube with CSM (*see* **Notes 17** and **18**).
4. Centrifuge cells for 5 min at 600 × *g*. Aspirate supernate to a minimal volume. Vortex to resuspend the cell pellet.
5. Wash 2 times in CSM.
6. Add a staining cocktail containing with the desired intracellular antibodies. We typically stain one to two million cells in 100 μL staining reaction for 40–50 min at room temperature, but other staining volumes, incubation times, or incubation temperatures can be used provided the antibodies are titrated for optimal staining under the same conditions.
7. After the completion of staining, wash cells at least twice with CSM.

3.6 DNA Intercalation and Data Acquisition

1. After the final wash following the surface or intracellular staining reaction, resuspend the cell pellet by vortexing thoroughly.
2. Poststaining fixation (optional): If the cells of interest are particularly fragile, or if clogging of the mass cytometer has been experienced when running similar cell samples, it may be helpful to perform an additional fixation step, by resuspending the cell pellet in PBS plus 1.5% PFA and incubating for 10–15 min at room temperature. This additional fixation will reduce lysis of particularly fragile cells during sample acquisition, which can lead to clogging of the mass cytometer's fluidics. Ensure that that the PFA is fresh. After this fixation, pellet the cells by centrifugation and resuspend the cell pellet by vortexing thoroughly.
3. Add at least 100–200 μL of intercalator solution for each million cells in the cell pellet. This can also be titrated for the specific cell types being stained. Mix or vortex cells gently to resuspend them evenly in the intercalator solution. Place the cells at 4 °C for at least 20 min. The cells will be stable in the intercalator solution at 4 °C for at least a week.
4. After intercalation, cells should be washed twice in either ultrapure (heavy metal free) water or in a cell acquisition solution (*see* **Note 19**). Cells should then be resuspended at a density of approximately one million cells per mL in either water or cell acquisition solution (if used). Four element EQ beads should

be added to the solution at the manufacturer's recommended concentration (which should result in analysis of 4–10 beads per second).

5. Begin analysis of cells on CyTOF mass cytometer. Exact acquisition settings are dependent on which CyTOF version is being used and the cell types being studied. Startup procedures and cell injection protocol will depend on the exact CyTOF instrument version being used; follow the manufacturer's recommended procedures.
6. Once acquisition has begun, a preview of the data should be performed with the cytometer in TOF mode to ensure that there is not significant metal contamination (*see* **Note 20**).
7. Once acquisition is started, the frequency of cell events per second should be verified. An ideal rate is typically 200–400 cells per second (though the Helios machines may be able to run slightly faster). Rates higher than this will lead to larger numbers of cell doublet events and commonly leads to cell clogs in the cytometer fluidics. If the sample acquisition rate is too high, samples should be diluted with a mixture of water and EQ beads (or cell acquisition solution mixed with EQ beads) until the cell event rate reaches the desired level.
8. The number of that needs to be collected will be experiment-specific; however, it is advisable to predetermine the expected frequency of the most rare cell of interest and the minimum number of such cell events that will be needed for the experimental objectives, then back calculate the number of total events that will be required (*see* **Note 21**).
9. Water or cell acquisition solution should be run for several minutes between each sample. The data preview function should be used to ensure that no further cell events are visible when running water before starting the next cell sample. Should cell events persist for more than several minutes, the cytometer's fluidics should be cleaned with either wash solution (Fluidigm) or with 3% Nitric acid (trace metal free), followed by several minutes of water (*see* **Note 22**).

3.7 Initial Data Processing

There are now a large number of data analysis approaches for high dimensional mass cytometry data ranging from large scale analysis of hundreds of standard biaxial plots to complex high-dimensional analysis strategies. A thorough discussion of the pros and cons of each approach is beyond the scope of this chapter, but some guidance can be found in several recent publications [1, 3, 6]. Regardless of the final analysis approach, several steps should always be performed to ensure the data quality before more complex analysis is undertaken. It is always ideal to still view the data in the relevant key biaxial plots that have been traditionally used to define relevant cell

populations (either before or after more complex analyses), to ensure that the cells of interest would have been similarly gated by the majority of other researchers.

1. Normalization: Upon completion of cell acquisition, the resulting data should be normalized, using either the mass cytometers built-in software or the Matlab application developed by Finck et al. [22] This normalization step is critical for experiments in which total cell acquisition requires more than an hour, as a significant loss of measurement sensitivity can occur in as little as 1–2 h of sample analysis. Without use of EQ beads and postanalysis data normalization, samples run later in the experiment acquisition will appear to have lower signal across all channels as compared to samples run earlier during data acquisition. The normalization process will effectively correct this effect in final data files. Note that samples that have not been adequately fixed will frequently lose antibody signal during prolonged incubations in water (*see* **Note 23**).
2. Debarcoding (optional): If barcoding was performed, debarcoding should be performed after data normalization and before any subsequent data analysis.
3. Singlet gating: Once data has been uploaded into the flow cytometry analysis software of choice, a singlet gate should be applied to the remove cell doublets and debris. This gate is created in a biaxial plot of event length (i.e., the number of "pushes" of the TOF analysis that ions from each cell event are spread across) vs. DNA intercalator signal (measured on the 191 or 193 channel). In this plot, doublet events will appear as a tail of cells with high DNA signal and a sharply increased event length. The gate on singlets should exclude this tail of event length high cell events as well as any cells with low amounts of DNA intercalator signal, that are likely to represent debris. It is best to determine the exact borders of these gates empirically whenever possible. This can be done by identifying events that are clearly doublets (e.g., cell events positive for CD3 and CD15), then plot these doublet events on the event length vs. DNA biaxial plot. The same can be done for events that are clearly debris. When determined empirically and drawn properly, this singlet gate will remove 50–60% of cell doublets without removing a significant amount of real cell events. To more completely exclude doublets, cells need to be run very slowly (75–150 cells per second) or barcoded with a nondegenerate code. Under these conditions, more than 95% of doublet events can be excluded.
4. Viability gating (optional): After normalization, debarcoding, and singlet gating, a viability gate should then be applied to remove dead cells from experimental analysis (unless

measurement of cell viability is an objective of the experiment). This gate is created on a plot of DNA vs. platinum (195, 195, or 198 Pt). If a significant fraction of dead cells was present at the time of viability staining, those dead cells should form a population with an approximate 1–2 log increase in Pt signal, compared to the background staining in the remaining live cells.

5. After these steps, the data are ready for more complex analyses as appropriate for the given experimental objectives.

4 Notes

1. The several Fluidigm-manufactured, preconjugated antibodies utilize a different polymer for metal chelation and attachment to antibodies. This polymer enables conjugation of more metal atoms to each antibody but also has slightly higher nonspecific binding and can exhibit very high background on cells that have been permeabilized with certain saponin preparations. (This is lot specific, and saponin-containing buffers purchased directly from Fluidigm generally do not exhibit this issue.) As of December 2018, the specific antibodies labeled with this polymer are anti-human CD19/b4 (clone: hib19) on 142nd, 165ho, and 169tm; anti-human CD25 (clone: 2a3) on 149sm and 169tm; anti-human CD45ra (clone: hi100) on 143nd, 153eu, 155gd, 169tm, and 170er; anti-human CD127 (clone: a019d5) on 143nd, 149sm, 165ho, 168er, and 176yb; anti-human CD45 (clone: hi30) on 89y; anti-human CD196/ccr6 (clone: g034e3) on 141pr and 176yb; anti-human CD196/ccr6 (clone: 11a9) on 141pr; anti-human CD49d/α4 integrin (clone: 9f10) on 141pr and 174yb; anti-human tgfbeta/lap (clone: tw4–6h10) on 163dy; anti-human CD41/gpiib (clone: hip8) on 89y; anti-human CD16 (clone: 3g8) on 209bi; anti-human CD47 (clone: cc2c6) on 209bi; anti-human CD61 (clone: vi-pl2) on 209bi; anti-human CD11b/mac-1 (clone: icrf44) on 209bi; and anti-mouse CD45 (clone: 30-F1130-F11) on 89Y.
2. Reagents can be tested using solution mode on the mass cytometer (recording all metal channels) or by collecting data in the preview mode using the TOF setting that shows the entire mass range. Do not inject any concentrated solution that may contain significant amounts of heavy metal directly into the mass cytometer without first testing a 1/10th or 1/100th dilution of the solution. Note that during regular data acquisition, only the metal masses selected in the reagent panel for that experiment will be shown, as a result, if there is contamination from a metal not being used for an antibody reagent

(even if at very high levels) it will not be visible unless TOF mode is used.

3. After each antibody staining step, sufficient washes should be performed such that residual unbound antibody is diluted by at least 1000- to 5000-fold. Note that washing away unbound antibody is more important in mass cytometry than in flow cytometry as there is no sheath fluid for excess reagents to dissipate into and excess unbound reagents will increase wear on the mass cytometer's detector.
4. While almost all mass channels have less than 4% spillover into any channel, the mass channels with the highest spillovers (>1%) are In113; Nd(143 and 145); Sm(147 and 149); Gd (156, 157, and 158); Dy(161, 162, 163, and 164); Er (166, 167, and 168); and Yb(171, 172, and 173).
5. The mass cytometer's plasma temperature is tuned to ensure oxide formation is always less than 2.5%; however, individual metals have different propensities to form oxides. The metals with the highest levels of oxide formation (1–2.5%) are La, Ce, Pr, Nd, and Gd.
6. Current mass cytometers are significantly less sensitive to metals with masses below 139 Daltons or above 176 Daltons (approximately three- to tenfold less sensitive than the other metal channels). Across the remainder of the mass range, sensitivity generally only varies by threefold with the most sensitive channels in the range of 159Tb to 169Tm).
7. In this method, a "wash" refers to addition of CSM or PBS to resuspend the cell pellet in 50–100 volumes (~5 mL for standard FACS tubes), followed by a 5 min centrifugation, then aspiration of the supernate down to a minimal volume (typically about 50 μL for a standard FACS tube). The cell pellet should then be resuspended to an even single cell suspension in the residual volume (by tapping, or gentle vortexing). All centrifugation steps prior to methanol permeabilization can be performed at 300–600 × *g* (or whatever centrifugal force is known to be optimal for the cells of interest) for 5 min. After methanol permeabilization, centrifugation should be performed at a minimum of 600 × *g*. We recommend use of a refrigerated swinging bucket centrifuge (e.g., Sorvall Legend XTR [Thermo/Fisher], or similar) set to 4 °C or 20°C. If the volume of the tube being used is not large enough to allow for 50–100 volumes of CSM to be added, perform additional washes until the total dilution is greater that 1000-fold. Note that washing is much more important in mass cytometry than fluorescent flow cytometry, since residual metal reagents in solution can create significant background, measurement error, and may increase wear on the mass cytometer's detector.

8. We have successfully optimized this cisplatin staining protocol for staining in cells in protein-containing media; however, this requires much larger concentrations of cisplatin and addition of concentrated protein solutions to quench the reaction. The protocol can also be optimized for use with different incubation periods or temperatures, provided that these conditions will be very consistent during the final experiment.
9. The exact concentration that provides optimal live–dead discrimination will depend on the exact cell numbers, the amount of residual protein that is not washed away in the previous washes, the fraction of dead cells, and the length of incubation. This should be empirically titrated for the planned experimental protocol and cells of interest.
10. Titrations should be performed for all antibodies (with the possible exception of preoptimized antibody cocktails developed for a specific cell type and staining protocol). It is important that titrations be performed under the conditions that will be used to stain the cells, particularly similar staining volume, cell number, staining time, and staining temperature. Once the optimal conditions have been established, if staining a larger number of cells we will increase the total staining volume to achieve the same ratio of cells per volume of staining reaction. We typically titrate our antibodies for staining one to two million cells in a 100 μL staining reaction, and scale up or down the staining volume (without changing antibody concentration) to maintain a constant ratio of cells to volume of staining cocktail (e.g., 100 μL per two million cells).
11. Live cells are typically stained at 4 °C to prevent antigen internalization; however, it is not clear if antigen internalization would actually alter mass cytometry staining (since it would likely take many minutes for a cell to break down and excrete the chelated heavy metal).
12. We typically use a commercial Fc block from Biolegend, but a variety of other Fc blocking reagents are available. Care should be taken to use a blocking agent that does not disrupt measurement of Fc receptors (e.g., CD64, CD32, CD16) if these antigens will be measured. We have also successfully used total mouse IgG for blocking. For samples being stained in whole blood or whole serum, blocking may not be necessary due to the serum IgG already present.
13. At the desired time of sample analysis, the fixed cells should be thawed at 4 °C; this can be done rapidly in a circulating 4 °C water bath or by placing cells on ice.
14. If using a saponin-based permeabilization to allow for simultaneous staining of surface and intracellular antigens, cells should be fixed (using PFA, STPS, or a commercial fixative

preparation) and the protocol in Subheading 3.4 should be used. It is important to use the appropriate saponin-containing buffer for the washes before staining and include saponin at the appropriate concentration (typically 0.2%) in the staining reaction. After staining, skip to Subheading 3.6; the other steps of the protocol are typically unchanged. *See* also **Note 1** regarding interactions between certain Fluidigm reagents and some lots of saponin.

15. When aspirating the CSM (or PBS + PFA) from the cells, remove as much as possible (without aspirating the cell pellet), preferably to a pellet of 50 μL or less. (This will enable a final methanol concentration of 90–95% to be achieved.)
16. It is absolutely critical that cells be in an even single-cell suspension prior to the addition of methanol. If cells are not evenly resuspended, they will reliably form large clumps and aggregates.
17. The BSA in the CSM will precipitate if added to a solution with a high concentration of methanol, so it is important to reduce the methanol concentration with PBS before adding the CSM to the cell suspension.
18. If the volume of methanol added in **step 1** was more than 20% the volume of the tube being used, then additional CSM washes should be performed so that the final methanol concentration of the residual buffer and cell pellet (just before adding the antibody staining cocktail) is less than 0.5%. If using 1.1 mL polypropylene "cluster" microcentrifuge tubes, the cells will first have to be centrifuged in methanol, the methanol aspirated, then add 0.5 mL of PBS followed by 0.5 mL of CSM and centrifuge again. Then perform two additional CSM washes to remove residual methanol.
19. The purpose of using a running buffer rather than pure water for analysis of cells is to reduce the chance of cell breakdown or antibody disassociation while samples are being run or waiting to be run (after having been washed into pure water). This comes at the cost of increased mass cytometer sensitivity degradation due to solute buildup on the mass cytometer's cones. Typically, this step is unnecessary if cells are well fixed; however, we and others have observed this behavior in certain cell types (or with certain antibody–antigen pairs) in situations when fixation would have been expected to have been adequate. If a running buffer is used, inorganic metal salts (e.g., sodium chloride) should be avoided as inorganic (mineral) salts seem to cause the mass cytometer's signal to degrade much faster, requiring more frequent machine cleaning. Fluidigm sells a ready-made cell acquisition solution for this purpose.

20. Note that in regular data collection mode, only signal within the time-of-flight windows of the masses set in the panel will be recorded or displayed on the preview window of the computer. As a result, if the sample is contaminated with a metal not being measured in the experiment's antibody panel, this contamination (even if at relatively high levels) can be invisible to the user. A quick preview in TOF mode will show all of the ion species striking the detector, and if the preview shows cell events without significant amounts of metal contamination, the chance of problematic contamination is quite low.
21. When calculating the number of required cell events, it is important to remember that up to 10% of events will be from EQ beads and will not be present in the final data file. Additionally, if significant amounts of debris are present in the sample, these debris particles will often be counted as cell events. Thus, if debris is observable in preview screen of the cytometer during sample acquisition, additional events will need to be collected in proportion with the approximate frequency of debris relative to true cell events. It is thus not uncommon to need to collect 10–50% more events than desired final number of cell events.
22. If running wash buffer (which contains acid) or nitric acid between samples, it is important to ensure that all acid has been washed away before starting the next sample. Persistent acid will strip metal from the antibodies attached to the cells in subsequent samples until the acid is neutralized.
23. If samples have not been adequately fixed before being placed into pure water the antibodies can disassociate from their antigens and/or cells may lyse during prolonged cell acquisition. This effect is directly dependent on the time cells are in water and cannot be normalized by the EQ bead-based normalization algorithms. This problem can be minimized by ensuring that the PFA used in initial sample fixation and in the intercalator solution is sufficiently fresh, by minimizing the time cells are kept in water prior to being loaded in the cytometer, and by keeping cells cold between water washes and analyses. Additionally, use of the optional poststaining fixation steps (Subheading 3.5, **step 1**; Subheading 3.6, **step 2**), or use of a cell acquisition solution can minimize this problem.

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Chapter 3

Fluorescent Cell Barcoding for Immunophenotyping

Valentina Giudice, Giovanna Fantoni, and Angélique Biancotto

Abstract

Immunophenotyping using flow cytometry highly benefits from multiplexing samples for generation of more robust data, because of reduction of antibody consumption, batch effect and technical variations. One way to multiplex is via fluorescent cell barcoding (FCB) prior to staining procedure.

FCB is a high-throughput multiplexed assay using various concentrations of different fluorescent dyes. Individual samples are uniquely labeled, then mixed together, stained and analyzed as a single sample, decreasing technical variations and increasing throughput and speed of acquisition. In addition, FCB simplifies implementation of normalization using a bridge control sample.

In this chapter, we illustrate the protocol for FCB and recommendations for choosing barcoding dyes and concentrations among other technical considerations.

Key words Fluorescent cell barcoding, Immunophenotyping, Multiplexing

1 Introduction

Standardization of flow cytometric analysis process is critical in order to have reproducible high-quality data that are comparable at intralaboratory and interlaboratory levels over time [1–3]. Fluorescent cell barcoding (FCB) enables high-throughput multiparameter flow cytometry by mixing multiple samples in a single tube for further staining and analysis [4]. Using covalently bound fluorescent dyes at various concentrations for labeling, samples are "barcoded" and distinguished based on fluorescence emission wavelengths and intensities. Multiplexed barcoded samples can be mixed together and subsequently stained with antibodies in a single tube. Thus, FCB minimizes staining variability, antibody consumption and decreases total sample volume needed. However, to be implemented FCB requires optimization [5].

In this chapter we describe FCB procedure for immune cell phenotyping that can be performed on whole fresh blood, peripheral blood mononuclear cells (PBMCs), or cryopreserved PBMCs.

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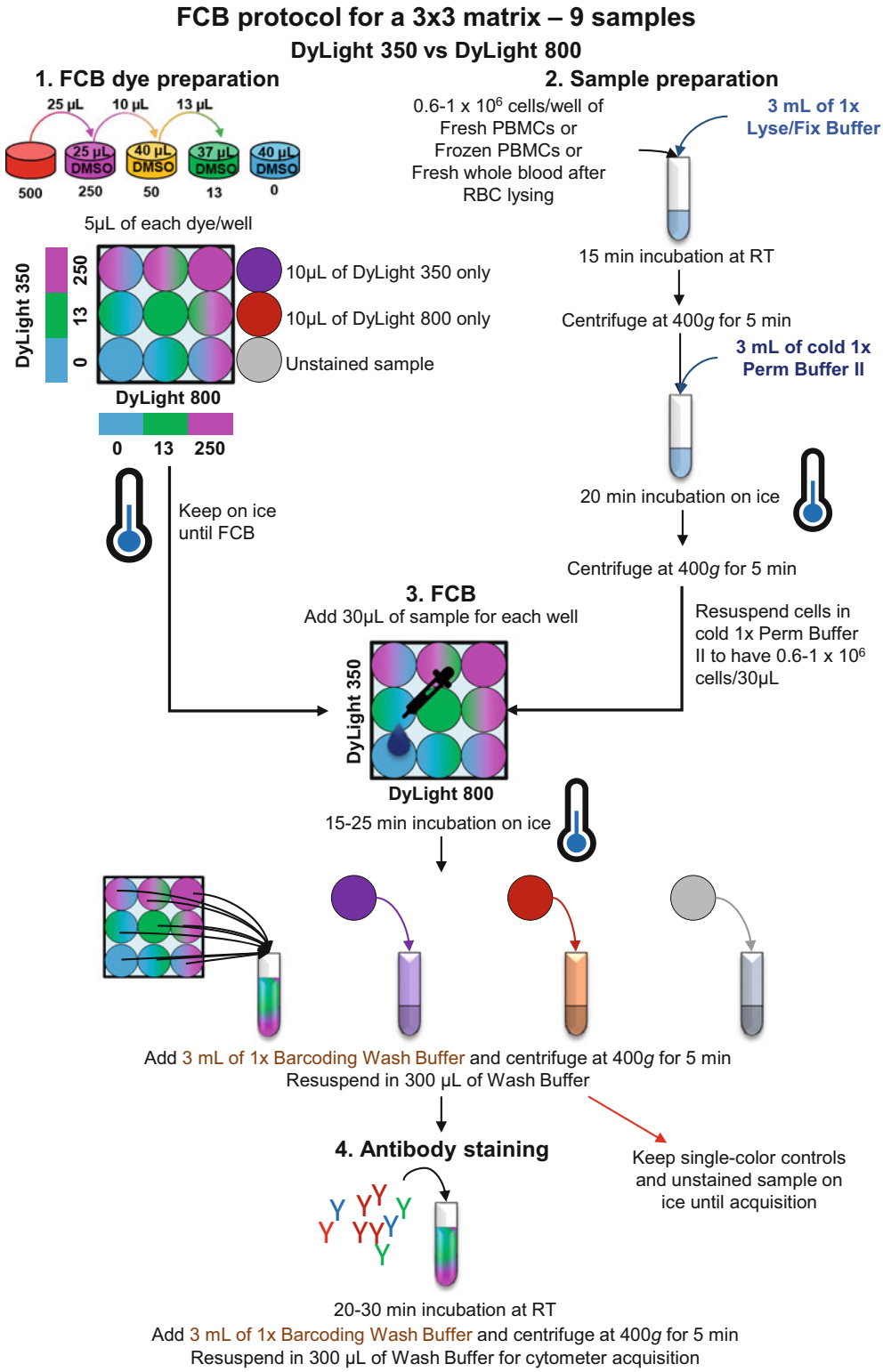

Fig. 1 Illustration of FCB protocol for a 3 × 3 matrix using DyLight 350 and DyLight 800. In (1) FCB dye preparation is shown how to perform dye dilution (Subheading 3.1); then, 5 µL of each dye at various

In this protocol, FCB is obtained using two amine-reactive fluorescent dyes with active esters (succinimidyl ester, NHS) (DyLight 350 and DyLight 800) because of their efficiency in tethering molecules [6].

In this chapter, we describe barcoding of nine or 12 samples combined with a five-color staining, by listing reagents needed and a detailed protocol procedure (Fig. 1). FCB is performed using 96 round-bottom well plate; however, 1.5 mL Eppendorf tubes can also be used. In addition, gating strategy to analyze samples is described for manual and semiautomated analyses by using available software. Finally, Notes describe technical recommendations based on immunophenotyping of interest (phospho-flow, fluorochromes and instrument used).

2 Materials

2.1 FCB Buffers and Dyes

Barcoding dyes from Thermo Fisher Scientific (Waltham, MA, USA): DyLight 350—NHS; DyLight 800—NHS; and Pacific Orange—NHS (*see* **Note 1**).

Barcoding buffers from BD Biosciences (San Jose, CA, USA): BD Perm Buffer II; 5× BD Phosflow Lyse/Fix Buffer; 4× BD Phosflow Barcoding Wash Buffer.

Other reagents: Ficoll-Paque gradient centrifugation (MP Biomedicals, LLC, Santa Ana, CA, USA), according to the manufacturer's instructions for PBMC isolation; Ack Lysing buffer (Quality Biological, Gaithersburg, MD, USA) for red blood cell lysis; Phosphate buffer saline (PBS) and dimethylsulfoxide (DMSO) Sigma-Aldrich (St. Louis, MO, USA).

Fig. 1 (continued) concentrations are pipetted in appropriate wells (according to colors). In two separate wells, 10 μL of DyLight 350 or DyLight 800 are added and will be used as single-color controls. In another well no dye is added and 40 μL of unstained sample will be pipetted, will be considered unstained control for cytometer setting. Once prepared, dyes can be kept on ice until use. In (2) Sample preparation, 0.6–1 × 10^6 cells/well per sample of fresh PBMCs or frozen PBMCs or fresh whole blood after RBC lysing are incubated for 15 min at RT with 3 mL of BD Lyse/Fix Buffer. After centrifugation, supernatants are removed and cell pellets loosen; then, 3 mL of cold 1× Perm Buffer II are added and samples incubated for 20 min on ice. After incubation, cells are washed with 2 mL of PBS and centrifuge at 400 × *g* for 5 min. Cells are subsequently resuspended in cold 1× Perm Buffer II to have 0.6–1 × 10^6 cells/30 μL. In (3) FCB, samples are added in appropriate wells prepared with FCB dyes and incubated on ice for 15–25 min. Samples from the 3 × 3 matrix are combined together, while single-color controls and unstained sample are transferred to separate tube. Then cells are washed with 3 mL of 1× Barcoding Wash Buffer and centrifuge at 400 × *g* for 5 min. In (4) Antibody staining, after centrifugation, cells are resuspended in 300 μL of Wash Buffer and antibodies are added according to the manufacturer's instructions. After 20–30 min incubation at RT, samples are washed with 3 mL of Barcoding Wash Buffer, centrifuged, and resuspended in 300 μL of the same buffer for cytometer acquisition

2.2 Buffer and Dye Preparation

All buffers and dyes are prepared using PBS and stored as indicated.

1. Prepare 1× BD Perm Buffer II by diluting 1:2. Add 10 mL of BD Perm Buffer II at room temperature (RT) and 10 mL of cold PBS to a 50 mL Falcon Conical tube (Fisher Scientific, Loughborough, UK), mix by inversion and store at +4 °C.
2. Prepare 1× BD Phosflow Lyse/Fix Buffer from the 5× stock solution. Add 10 mL of 5× BD Phosflow Lyse/Fix Buffer and 40 mL of PBS at RT to a 50 mL Falcon Conical tube, mix by inversion and store at RT.
3. Prepare 1× Barcoding Wash Buffer by diluting 1:4. Add 5 mL of cold 4× Barcoding Wash Buffer and 35 mL of PBS at RT to a 50 mL Falcon Conical tube, gently mix by inversion and store at RT (use the solution within 1–2 days).
4. Dyes used in this protocol are DyLight 350 and DyLight 800. Upon arrival dry powder dyes are reconstituted at 500 μg/mL concentration in DMSO in 15 mL Falcon Conical tubes (Fisher Scientific, Loughborough, UK). For 1 mg of dry powder, add 1 mL of DMSO to the tube containing the powder and transfer to a 15 mL Falcon Conical tube; then rinse with 1 mL of DMSO the tube and transfer to the 15 mL Falcon tube. Mix well by pipetting and aliquot in 1.5 mL Eppendorf tubes. Store at ≤−80 °C protected from light (*see* **Note 2**).

3 Methods

3.1 Sample Preparation

FCB for lymphocyte phenotyping can be performed on fresh samples, as whole blood or peripheral blood mononuclear cells (PBMCs) or cryopreserved PBMCs. If frozen PBMCs are used, thaw cells in a water bath at +37 °C for 1–2 min. Add 2 mL of PBS and centrifuge at 400 × *g* for 5 min. Discard supernatant and vortex to loosen cell pellets. If fresh PBMCs are used for barcoding, start from **step 5**.

If whole fresh blood is employed in the experiment:

1. Collect at least 0.2 mL of whole blood in the presence of anticoagulant (EDTA or sodium heparin).
2. Lyse with 1:10 Ack Lysing buffer. For 0.2 mL of fresh blood, add 2 mL of Ack Lysing buffer and incubate for 10 min on ice (*see* **Note 3**).
3. Centrifuge at 400 × *g* for 5 min and discard supernatant.
4. Repeat **steps 2** and **3**.
5. Add 3 mL of 1× Lyse/Fix Buffer, gently mix by pipetting and incubate at RT for 15 min.
6. Centrifuge at 400 × *g* for 5 min. Discard supernatant and vortex to loosen the cell pellets.

7. Add 3 mL of cold 1× Perm Buffer II. Incubate on ice for 20 min (*see* **Note 4**).
8. Add 2 mL of PBS and centrifuge at 400 × *g* for 5 min. Discard supernatant and vortex to loosen cell pellets.
9. Resuspend cells in cold 1× Perm Buffer II and keep cells on ice until use (*see* Subheading 3.4).

   Calculate the volume of cold 1× Perm Buffer II for resuspension in order to have 0.6–1 × 10^6 cells/30 μL. In addition, in order to have controls for cytometer set up, for one sample calculate: 30 μL for unstained sample; and 30 μL for each single-dye control (e.g., DyLight 800 and DyLight 350).

3.2 Nine-Sample Matrix Preparation (3 × 3)

Prepare dye working concentrations with DMSO from stock concentration immediately before starting the experiment, as DMSO tends to precipitate.

1. Thaw dye stock solution (500 μg/mL) at RT protected from light. After thawing, vortex vigorously.
2. Prepare dye working concentrations with DMSO in round bottom 96-well plate and label wells as "DyL350-0", "DyL350-13", "DyL350-250" and "DyL800-0", "DyL800-13", "DyL800-250" (*see* **Note 5**).

   For each dye, prepare the following dilutions:

   250-dilution = 25 μL of 500 μg/mL dilution + 25 μL of DMSO

   50-dilution = 10 μL of 250-dilution + 40 μL of DMSO

   13-dilution = 13 μL of 50-dilution + 37 μL of DMSO

   0-dilution = 30 μL of DMSO

   The 50-dilution is used only for preparing the 13-dilution and not for FCB matrix preparation (*see* **Note 6**).
3. Add 5 μL of diluted dye to appropriate well in a round bottom 96-well plate as shown in Fig. 1. For a final volume of 40 μL/well (10 μL of dyes +30 μL of sample), final concentrations will be 0, 3.25, and 62.5 μg/mL.
4. Seal or cover the plate and keep at RT protected from light.
5. Proceed to Subheading 3.4.

3.3 Twelve-Sample Matrix Preparation (4 × 3)

Prepare dye working concentrations from stock concentration right before starting the experiment, as DMSO tends to precipitate.

1. Thaw dye stock solution (500 μg/mL) at RT protected from light. After thawing, vortex vigorously. In a round bottom 96-well plates, prepare dye working concentrations with DMSO as follows (Fig. 2).
2. For DyLight 350, name tubes/wells as follows: "DyL350-0", "DyL350-30", "DyL350-150" and "DyL350-500". Prepare dilutions as follows:

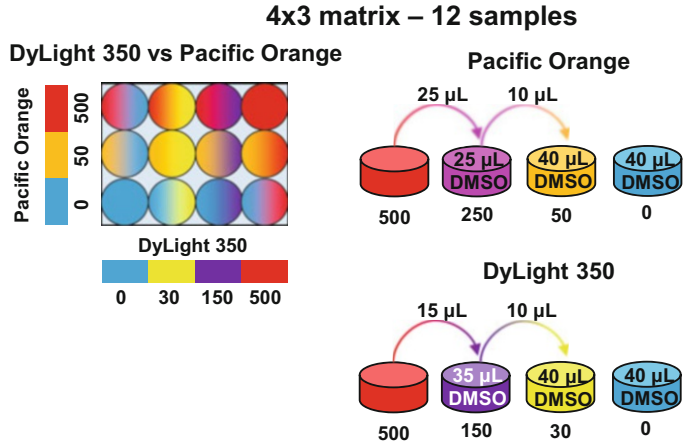

Fig. 2 Illustration of 4 × 3 matrix preparation. Using four concentrations of DyLight 350 and three of Pacific Orange, a 4 × 3 matrix is prepared for 12-sample FCB. Detailed information on how to prepare dye dilutions is described in Subheading 3.3

500-dilution = 50 µL of 500 µg/mL dilution

150-dilution = 15 µL of 500 µg/mL dilution +35 µL of DMSO

30-dilution = 10 µL of 150-dilution +40 µL of DMSO

0-dilution = 40 µL of DMSO

For a volume of 40 µL/well combining dye and samples, DyLight 350 final concentrations are 0, 3.8, 18.8, and 125 µg/mL.

3. For Pacific Orange, label wells as "PO-0", "PO-50", and "PO-500".

   Prepare dilutions as follows:

   500-dilution = 50 µL of 500 µg/mL dilution

   250-dilution = 10 µL of 500 µg/mL dilution + 10 µL of DMSO

   50-dilution = 10 µL of 150-dilution + 40 µL of DMSO

   0-dilution = 40 µL of DMSO

   The 250-dilution is made only for preparing the 50-dilution and not for barcoding. For a volume of 40 µL/well combining dye and samples in a 12-sample matrix with PO final concentrations are 0, 6.3, and 125 µg/mL.

4. Add 5 µL of diluted dye to appropriate well in a round bottom 96-well plate.
5. Seal or cover the plate and keep at RT protected from light.
6. Proceed to Subheading 3.4.

3.4 Fluorescent Cell Barcoding

Once matrix and samples are prepared, proceed to FCB as follows. Perform all steps at RT unless otherwise specified.

1. Add 30 μL of each sample into appropriate well, according to their matrix position. Mix well the solution as dyes tend to precipitate.
2. Add 30 μL of samples into one well with 10 μL of DyLight 350 and other 30 μL of sample into a different well with 10 μL of DyLight 800. These samples were prepared in **step 9** of Subheading 3.1 and will be used as single-color controls for cytometer setting.
3. Seal or cover the plate and incubate for 15–25 min on ice. Longer incubation time is preferable for whole blood barcoding (*see* **Note 7**).
4. Transfer all nine barcoded samples into one FACS tube named "Combo". Transfer single-color controls prepared in **step 2** in two separate FACS tubes labeled "DyL350" and "DyL800."
5. Wash with 3 mL of 1× Barcoding Wash Buffer (*see* **Note 8**). Centrifuge at 400 × *g* for 5 min and discard supernatant. Vortex to loosen cell pellets. Repeat **step 5** (*see* **Note 9**).
6. Resuspend "Combo", "DyL350", and "DyL800" in 300 μL of 1× Barcoding Wash Buffer. Keep "DyL350" and "DyL800" on ice until cytometer setting.
7. Add antibodies according to the manufacturer's instructions to the appropriate tube and incubate at RT in the dark for 20–30 min.
8. Wash with 3 mL of 1× Barcoding Wash Buffer. Centrifuge at 400 × *g* for 5 min, then discard supernatant and vortex to loosen cell pellet. Repeat washing step one more time.
9. Resuspend cells in 300–500 μL of 1× Barcoding Wash Buffer and keep on ice or +4 °C until acquisition.
10. Acquisition can be carried out using appropriate lasers (355, 407, or 630 nm) on cytometers. Preacquisition compensation can be calculated using bead standards for each fluorochrome (anti-mouse Ig, κ/Negative Control Compensation Particles Set, BD Biosciences) and prepared barcoded cells with the highest concentration of each dye. For each fluorochrome, cells as well as single-color controls can be used. A minimum of 50,000 lymphocytes needs to be recorded.

3.5 Control Samples for Protocol Optimization

During protocol optimization, a bridge sample within one matrix is used in order to measure intra-assay variability. Indeed, percent of positive cells and MFI values should not significantly differ across all 9/12 barcoded populations within the same matrix.

Control samples are (1) "No fix/perm" control, without fixation, permeabilization, and barcoding; and (2) "No FCB" control,

a fixed and permeabilized sample without barcoding. These two controls are used to compare percentages of positive cells before and after barcoding.

After optimization, it is preferable to include the same bridge control used for protocol optimization across all experiments in order to measure interassay variability.

3.5.1 "No fix/perm" Control Preparation

1. Resuspend cells from **step 4** of Subheading 3.1 in PBS.
2. Add antibodies according to the manufacturer's instructions and incubate at RT for 20–30 min in the dark.
3. Wash with 3 mL of PBS, centrifuge at 400 × *g* for 5 min and discard supernatant. Vortex to loosen cell pellets.
4. If intracellular staining is required, add 200 μL of CytoFix/CytoPerm BD buffer and incubate at +4 °C for 30 min. Otherwise skip to **step 7**.
5. Wash with 2 mL of 1× Perm Wash, centrifuge at 400 × *g* for 5 min, discard supernatant, and vortex to loosen cell pellets.
6. Resuspend cells in 175 μL of 1× Perm Wash and add intracellular markers antibodies according to the manufacturer's instructions. Incubate at +4 °C for 30–40 min in the dark.
7. Wash with 2 mL of 1× Perm Wash. Centrifuge at 400 × *g* for 5 min, discard supernatant and vortex to loosen cell pellets.
8. Resuspend cells in 300 μL of 1× Perm Wash for acquisition.

3.5.2 "No FCB" Control Preparation

1. Prepare cells as in Subheading 3.1 (**steps 1–4**).
2. After centrifugation, add 3 mL of 1× Lyse/Fix Buffer and incubate at RT for 15 min.
3. Centrifuge at 400 × *g* for 5 min. Discard supernatant and vortex to loosen the cell pellets.
4. Add 3 mL of cold 1× Perm Buffer II. Incubate on ice for 20 min.
5. Add 2 mL of PBS and centrifuge at 400 × *g* for 5 min. Discard supernatant and vortex to loosen cell pellets.
6. Resuspend cells in 200 μL of 1× Barcoding Wash Buffer.
7. Add antibodies according to the manufacturer's instructions to the appropriate tube and incubate at RT in the dark for 20–30 min.
8. Wash with 3 mL of 1× Barcoding Wash Buffer. Centrifuge at 400 × *g* for 5 min, then discard supernatant and vortex to loosen cell pellet. Repeat washing step one more time.
9. Resuspend cells in 300–500 μL of 1× Barcoding Wash Buffer and keep on ice or +4 °C until acquisition.

For measurement of inter-assay variability across matrixes and over time, we suggest including in each matrix one of the donors used for FCB optimization. This sample will be used as internal control and bridge through different experiments facilitating longitudinal studies. This bridge sample should always be barcoded with the same dye concentrations, thus the same position in the matrix. By comparing percentages of positive cells and/or MFI values from bridge internal controls across matrixes, inter-assay variability can be measured and outliers can identify experiments that should be repeated.

3.6 Post-Acquisition Analysis

Post-acquisition analysis can be carried out using FlowJo or RStudio software. Recommended analysis steps using both software are described below.

3.6.1 FlowJo

Flow cytometry analysis of barcoded samples, or deconvolution, can be performed in FlowJo software as follows (Fig. 3):

1. Make post-acquisition compensation matrix and apply it to barcoded samples. Export the matrix as .csv file.
2. Identify lymphocytes and/or monocytes based on forward scatter area (FSC-A) and side scatter area (SSC-A).
3. Exclude doublets based on FSC-A vs FSC-H.
4. On single cells, identify barcoded populations based on FCB dyes (e.g., DyLight 800 vs DyLight 350).
5. On each barcoded population, perform your gating strategy according to antibody staining.

3.6.2 RStudio

RStudio also can be used using the following packages (flowCore, flowClust, flowViz, flowWorkspace, ggcyto, and flowType) (Fig. 4a). In RStudio, antibodies are identified by cytometer channel used during acquisition (e.g., on LSR Fortessa cytometer, DyLight 350 was acquired using the channel BUV396-A and DyLight 800 with APC-H7-A channel).

1. Remove debris by filtering the data using *rectangleGate* function and FSC-A and SSC-A parameters. Events with FSC values lower than 20 k are removed by using the *Subset* method.
2. Compensate data using the matrix made in **step 1** of FlowJo analysis and *compensate* function of flowWorkspace.
3. Transform compensated data using *arcsinh* function.
4. Filter data for FCB dye channels (e.g., BUV.396.A and APC.Cy7.A) by *rectangleGate* function.
5. Identify barcoded populations using *flowClust* function. Clustering is performed by setting *varNames* parameter on FCB dye channels (e.g., BUV.396.A and APC.Cy7.A) and K is set based on the number of barcoded samples in the matrix (9 or 12).

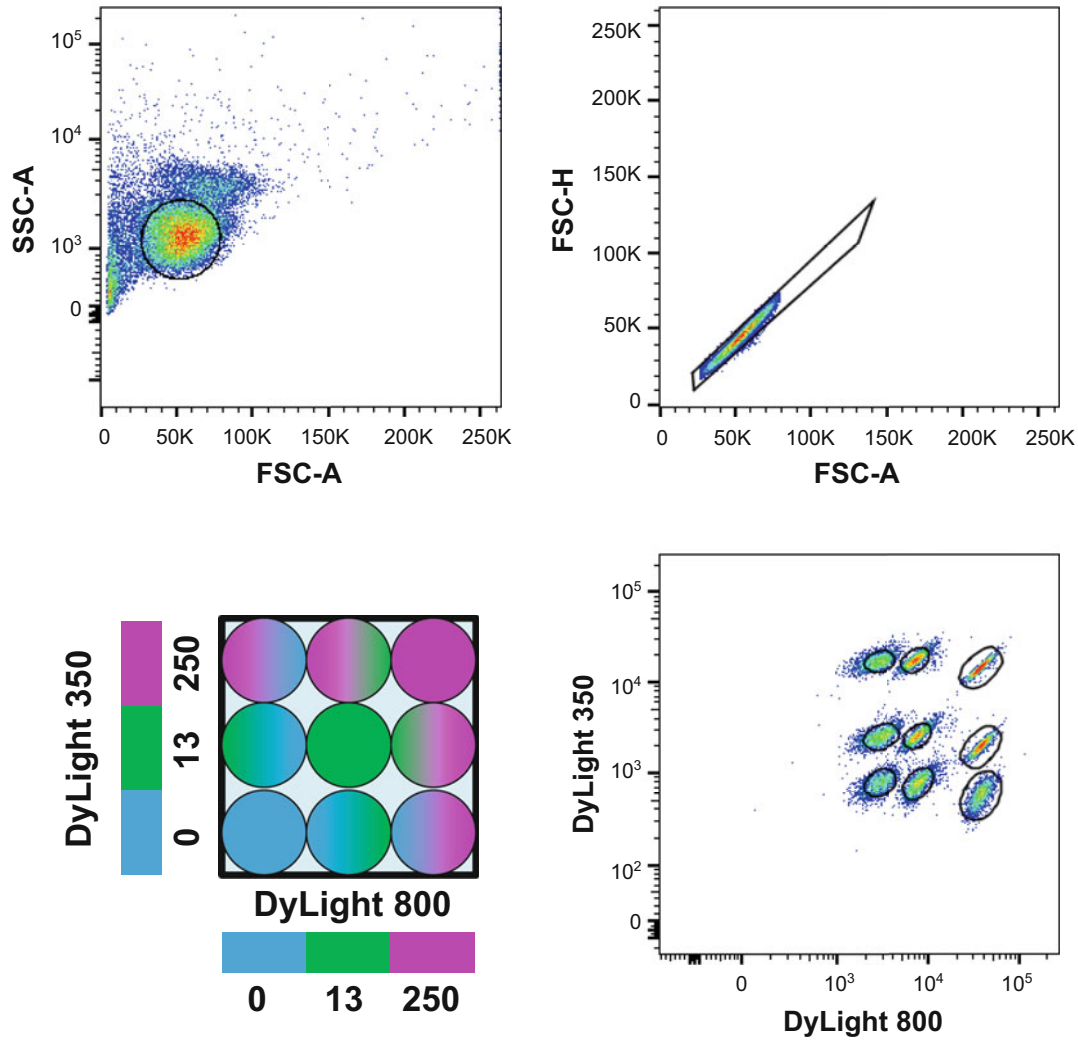

Fig. 3 Manual deconvolution using FlowJo. In order to gate the nine barcoded populations using FlowJo software, first lymphocytes are identified based on linear parameters (FSC-A vs SSC-A) and double cells excluded (FSC-H vs FSC-A). On single cells, barcoded populations are shown using FCB dye parameters (DyLight 350 vs DyLight 800) and samples are visualized on a dot plot according to dye concentrations (bottom left panel)

6. After clustering, data can be shown using ggcyto package.
7. Barcoded populations are identified individually and a flowset created for further analysis.
8. Gating strategy can be performed employing *rectangleGate* function using parameters of interest (e.g., CD3 vs SSC-A or CD4 vs CD8) and barcoded populations included in one flowset shown simultaneously using ggcyto.

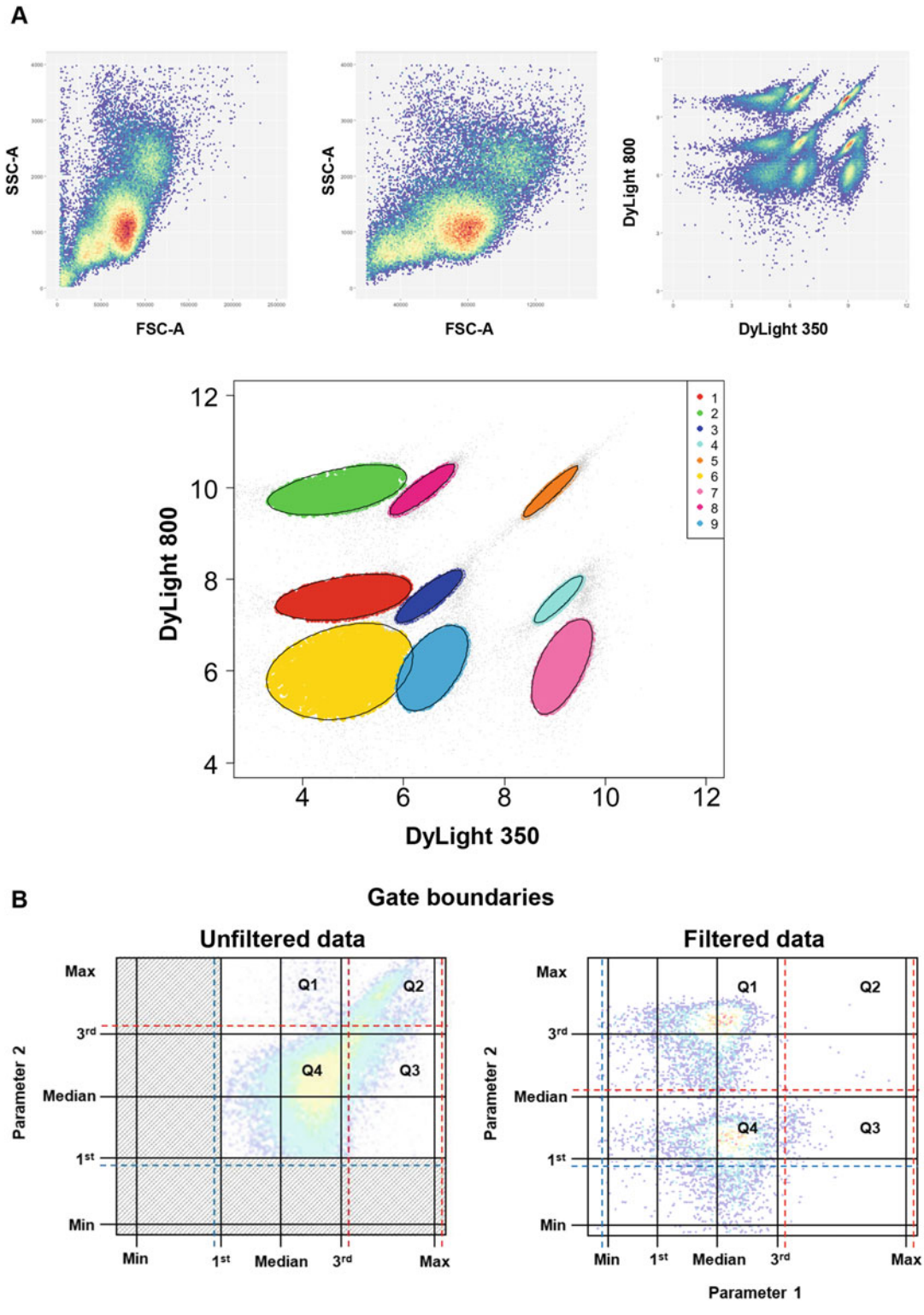

Fig. 4 Automated deconvolution using RStudio. (**a**) Using RStudio software, first debris (events $<$20 k in FSC) are removed by filtering for linear parameters (FSC-A vs SSC-A) with *rectangleGate* function. After compensation and arcsinh transformation, data are filtered for FCB dye parameters by *rectangleGate* and subsequently, nine populations are identified using *flowClust* function. Barcoded populations are identified individually and a *flowset* created for further analysis. (**b**) Gate boundaries for unfiltered and filtered data. A detailed description is provided in Subheading 3.6.2

For standardization of gate boundaries in RStudio, Minimum (Min), first quartile (1st), Median, third quartile (3rd), and maximum (Max) values of individual antibody can be extracted from each barcoded population and fluorochrome. Variations in fluorescence intensity among populations are considered, identifying the lowest and highest values for each parameter. On unfiltered data, below the lowest first values are removed from the analysis, because considered as outliers. Gates (Q1–Q4) are then defined as following: Q1, Parameter 1 (lowest 1st, highest 3rd) and Parameter 2 (highest 3rd, highest Max); Q2, Parameter 1 (highest 3rd, highest Max) and Parameter 2 (highest 3rd, highest Max); Q3, Parameter 1 (highest 3rd, highest Max) and Parameter 2 (lowest 1st, highest 3rd; and Q4, Parameter 1 (lowest 1st, highest 3rd) and Parameter 2 (lowest 1st, highest 3rd). For filtered data, gates were defined as follows. Gates (Q1–Q4): Q1, Parameter 1 (lowest min, highest 3rd) and Parameter 2 (highest Median, highest Max); Q2, Parameter 1 (highest 3rd, highest Max) and Parameter 2 (highest Median, highest Max); Q3, Parameter 1 (highest 3rd, highest Max) and Parameter 2 (lowest Min, highest Median; and Q4, Parameter 1 (lowest Min, highest 3rd) and Parameter 2 (lowest Min, highest 3rd) (Fig. 4b). Based on FCS data, the first quartile can be substituted with Min and Median with the Mean in order to adjust gate boundary. However, we suggest keeping the same gate boundary strategy during analysis of samples within the same experiment.

3.7 Recommended Assessment FCB Efficacy and Intra-assay/Interassay Variability

Purity of deconvolution is defined as the distance between MFIs and CVs to obtain 70% of the respective barcoded population with 95% purity. Others reported that MFIs should be separated by a three-fold increase for a good deconvolution (Fig. 5a).

3.7.1 MFI Fold Increase Calculation

1. Make post-acquisition compensation matrix in FlowJo software and apply it to barcoded samples.
2. Identify lymphocytes and/or monocytes based on forward scatter area (FSC-A) and side scatter area (SSC-A).
3. Exclude doublets based on FSC-A vs FSC-H.
4. On single cells, shown each FCB dye as histogram and identify each dye concentration used and name populations accordingly (e.g., “DyL350-0”, “DyL350-13”, “DyL350-250” and “DyL800-0”, “DyL800-13”, “DyL800-250” when a nine-sample matrix is made).
5. Calculate MFI and CV for each FCB dye dilution. Export data as .csv or .xls file.
6. In a spreadsheet, calculate MFI fold change as follows: $\text{MFI fold increase} = \left[\text{MFI}_{\text{peak}_2} - \text{CV}_{\text{peak}_2}\right] / \left[\text{MFI}_{\text{peak}_1} + \text{CV}_{\text{peak}_1}\right]$.

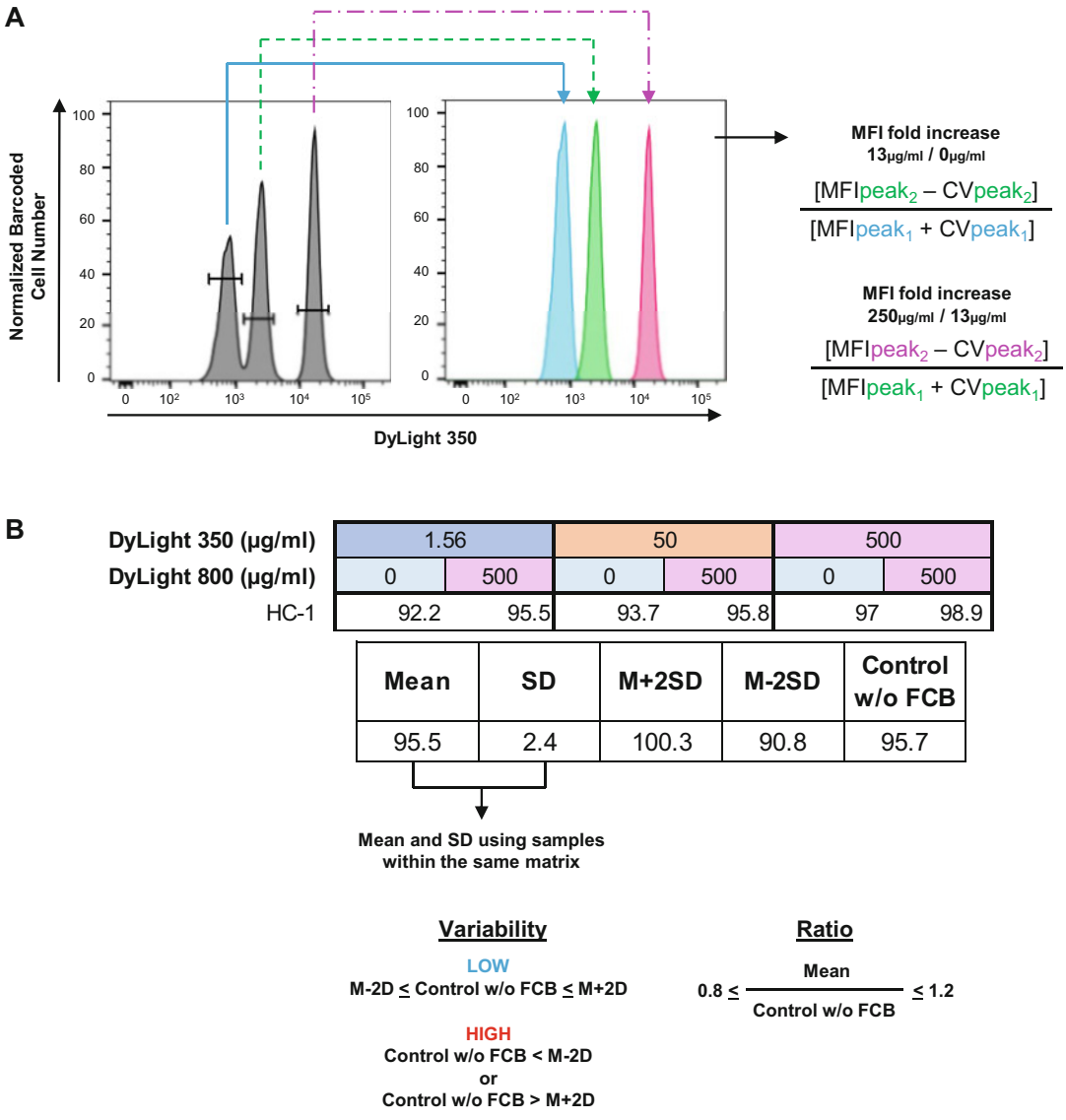

Fig. 5 FCB efficacy and inter-assay/intra-assay variability. (**a**) FCB efficacy is calculated using the MFI fold change. For each dye, MFI values are calculated for each of the three concentrations and MFI fold change is determined according to the following formula: MFI fold increase $= \left[\mathrm{MFI}_{\mathrm{peak}_2} - \mathrm{CV}_{\mathrm{peak}_2}\right] / \left[\mathrm{MFI}_{\mathrm{peak}_1} + \mathrm{CV}_{\mathrm{peak}_1}\right]$. (**b**) Inter-assay/intra-assay variability is assessed for each fluorochrome and percent of cell population (Subheading 3.7). For intra-assay variability, using a 3×3 matrix with the same donor in all nine positions and matched No-FCB controls, range of variability is calculated as $<$ mean in controls –2SD or $>$ mean in controls $\pm$2SD and percent of barcoded populations after deconvolution should be within this range. For inter-assay variability, the average of all barcoded samples across all experiments is calculated and compared to the average of all matched control samples used. Values should be within mean of controls $\pm$2SD. The ratio of variability is calculated as the ratio between the mean of barcoded samples and the mean of matched No-FCB controls. The ratio should be within 0.8–1.2

For example, MFI fold increase = $[MFI_{DyL350\text{-}13} - CV_{DyL350\text{-}13}]/[MFI_{DyL350\text{-}0} + CV_{DyL350\text{-}0}]$MFI fold increase = $[MFI_{DyL350\text{-}250} - CV_{DyL350\text{-}250}]/[MFI_{DyL350\text{-}13} + CV_{DyL350\text{-}13}]$.

3.7.2 Intra-assay/Inter-assay Variability of Percent of Positive Cells Calculation

1. Calculate percent of positive cells (e.g., CD3+ cells, CD4+ cells) in control samples using FlowJo software and export data as .csv or .xls file.
2. Calculate the range of variability as <mean in controls –2SD or >mean in controls +2SD (Fig. 5b).
3. Compare percent of barcoded populations after deconvolution to matched control samples. Values should be within ±2SD.
4. For intra-assay variability, for each cell population (e.g., CD3+ cells), calculate the average across all barcoded samples within one matrix and compare to the average of all matched control samples used in that matrix. Values should be within mean of controls ±2SD.
5. For inter-assay variability, of each cell population (e.g., CD3+ cells), calculate the average of all barcoded samples with the same FCB dye dilution (e.g., 0 μg/mL) across all matrix and compare to the average of all matched control samples used. Values should be within mean of controls ±2SD.

3.7.3 Intra-assay/Inter-assay Variability of MFI Values for Each Fluorochrome Calculation

1. Calculate MFI for each antibody used in barcoded samples using FlowJo software and export data as .csv or .xls file.
2. Calculate the range of variability as <mean in controls –2SD or >mean in controls +2SD.
3. Compare MFI values of barcoded populations after deconvolution to population barcoded with lowest concentration (e.g., 0 μg/mL) (*see* **Notes 10** and **11**). Values should be within ±2SD.
4. For intra-assay variability, for each cell population (e.g., CD3+ cells), calculate the average across all barcoded samples within one matrix and compare to the average of all matched control samples used in that matrix. Values should be within mean of controls ±2SD.
5. For inter-assay variability, for each cell population (e.g., CD3+ cells), calculate the average of all barcoded samples with the same FCB dye dilution (e.g., 0 μg/mL) across all matrix and compare to the average of all matched control samples used. Values should be within mean of controls ±2SD.

4 Notes

1. We describe combination of FCB dyes using DyLight 350 and DyLight 800 but the user can choose a different dye (e.g., Pacific Orange) depending on cytometer setting and

fluorochromes used. Indeed, if DyLight 350 and DyLight 800 are chosen, cytometer needs to be equipped with ultraviolet (355 nm) and red (633 nm) lasers. If ultraviolet laser is not available, we recommend the use of Pacific Orange and DyLight 800 for barcoding, as they are excited by violet (407 nm) and red (633 nm) lasers. However, Pacific Orange limits the use of other fluorochromes excited by violet laser because of the spillover in other violet channels.

2. Aliquots of 500 μg/mL stock solution can be prepared in order to avoid repeated thawing cycles. However, do not make small volume aliquots as solutions could not be as homogenous and stable as the ones in larger volumes.
3. Gently mix samples with Ack lysing buffer by pipetting and repeat occasionally during the incubation time.
4. If pSTATs are included in the antibody staining, for permeabilization, use 3 mL of cold Perm Buffer III instead of 1× Perm Buffer II. Avoid sample incubation below +4 °C, because DMSO can freeze.
5. If 0- and 13-dilutions do not achieve an MFI fold changes ≥3, instead of preparing a 13-dilutions, make a 15-dilution by mixing 15 μL of 50-dilution and 35 μL of DMSO.
6. Higher concentrations of FCB dyes might change MFI values of same fluorochromes because of the increase in autofluorescence of barcoded samples. For this reason, prefer higher dye concentrations or 12-sample matrix concentrations for surface marker staining when MFI values are not required (e.g., immunophenotyping or T cell subset characterization).
7. After incubation, it is preferable to combine samples in a tube already filled with 3 mL of 1× Barcoding Wash Buffer, because residual unbound dyes are quickly diluted in a larger volume of wash buffer upon transfer.
8. To get rid of residual unbound dyes that might react with cells by the time of acquisition repeating the wash step twice, especially if barcoded samples are not acquired in the same day.
9. Barcoded "Combo" cells can be used for up to 3 different antibody-staining by transferring 100 μL of resuspended cells in each of three separate tube. Label tubes accordingly as "Staining 1," "Staining 2," or "Staining 3." If only one staining and/or the acquisition of more events are required, cells can be resuspended in 200 μL of 1× Barcoding Wash Buffer and antibodies directly added to the sample.
10. Deconvolution can be carried out at any step of gating strategy; however, percent of positive cells may differ for two reasons:
    (a) If deconvolution is performed later in the analysis, not barcoded cells are included in all gates before identification of barcoded populations.

(b) Percent of positive cells is calculated using a given population as total (parent, 100%). For example, if we want to know the percent of CD4+/CD8+ cells:

- What if we do deconvolution first? Each barcoded population is our parent population on which all percentages will be calculated.
- What if we do deconvolution later? If we identify first CD4+ and CD8+ cells, these subsets become our parent populations. Then, if we do deconvolution of CD4 + cells for example, we will identify nine barcoded populations but percentages represent the proportion of each of them within the CD4+ subset.

11. During optimization of protocols for phosphoFlow in combination with FCB, in addition to percent of positive cells and MFIs, MFI fold change values for pSTATs (MFI of stimulated samples/MFI of unstimulated samples) need to be calculated for each barcoded population and compared to matched controls.

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Chapter 4

Immunophenotyping Using Dried and Lyophilized Reagents

Marc Langweiler

Abstract

Antibody reagents that are used for flow cytometry immunophenotyping have traditionally been prepared by combining individual liquid antibody conjugates into mixtures. These cocktails have limited shelf-life, and their preparation is time-consuming and prone to laboratory error. Manufacturers of these reagents, in collaboration with several clinical and research centers, have made advances in constructing dried antibody cocktails which have addressed many of the problems inherent in preparing the liquid cocktails on the lab bench. This chapter discusses methods for the use of dried reagents.

Key words Flow cytometry, Immunophenotyping, Harmonization, Dried antibody cocktail, Lyophilization

1 Introduction

From the outset, one of the fundamental uses for flow cytometry has been immunophenotyping of cell suspensions using antibody cocktails. Throughout the evolution of this methodology, the paradigm for the procedure has been and continues to consist of (1) preanalytical steps including processing of a cellular substrate to be stained and preparation of antibody cocktails, (2) analytical steps including cell staining and data acquisition using cytometers, and (3) postanalytical steps including off-line data analyses. There have been marked advances in each of these steps, including the number of markers contained in cocktails, the variety of fluorochromes available to be conjugated with these markers, the number of parameters available on cytometers that are manufactured with various technologies for creating flow streams, and the progression of the data analysis strategies.

Throughout this process, there have always been the goals of maximizing accuracy, precision, and reproducibility, while minimizing variability and cost. These goals are essential in performing good science, but they also have a financial impact on the operations in the research and clinical laboratory realm [1]. One of, if not

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the most difficult, stages of the process outlined above is the construction, preparation, and storage of liquid antibody cocktails. The challenges in preparing liquid cocktails are varied and include the need for repetitive pipetting of small volumes of reagent and degradation of cocktail constituents due to aging and exposure to light and temperature variation.

Antibody cocktails that have been prepared by drying them into lyophilized buttons or, more recently and effectively, by drying directly to the plastic of staining vessels have addressed these issues. Dried reagents have been utilized for what has been referred to as "multicenter harmonization" studies [2] in clinical centers investigating immunodeficiency syndromes and hematologic malignancies. For example, members of the *EuroFlow Consortium* have developed exhaustive strategies for standardization of immunophenotyping panels to study normal, reactive, and malignant human leukocytes [3]. Several of the panels are composed of dried formulations of antibody cocktails manufactured by BD Biosciences and Cytognos [4, 5].

Similar products manufactured by Beckman-Coulter have been used to develop a screening tube for lymphocyte subsets in peripheral blood, bone marrow aspirates and body fluids [6, 7]. More recently, the *Laboratory of Immunomonitoring in Oncology* has used dried cocktails and liquid "drop-in" reagents in an initiative to characterize the immune status of cancer patients [8].

Another multicenter harmonization project, organized by the *Human Immunophenotyping Consortium* (HIPC), under the auspices of the *Federation of Clinical Immunology Societies* (FOCIS) attempted to minimize several contributory variables in multicenter clinical trials [9]. The markers used for implementation of this study were manufactured in a lyophilized form by BD Biosciences [10] utilizing a set of standard markers for the phenotyping of T-cells, B-cells, NK-cells, monocytes, and dendritic cells to enumerate the basic subsets of the above cell types and their activation status [11]. The methodology described in this chapter developed following the culmination of this study, as a means to augment the functionality of the antibody cocktails and staining protocol reported in that study [12].

2 Materials

2.1 Assay Lyoplate, BD Biosciences Designate 175 (Fig. 1)

Each of the five rows contains 12 replicates of the five cocktails (*see* **Note 1**).

A: T-cell.

B: Treg.

C: B-cell.

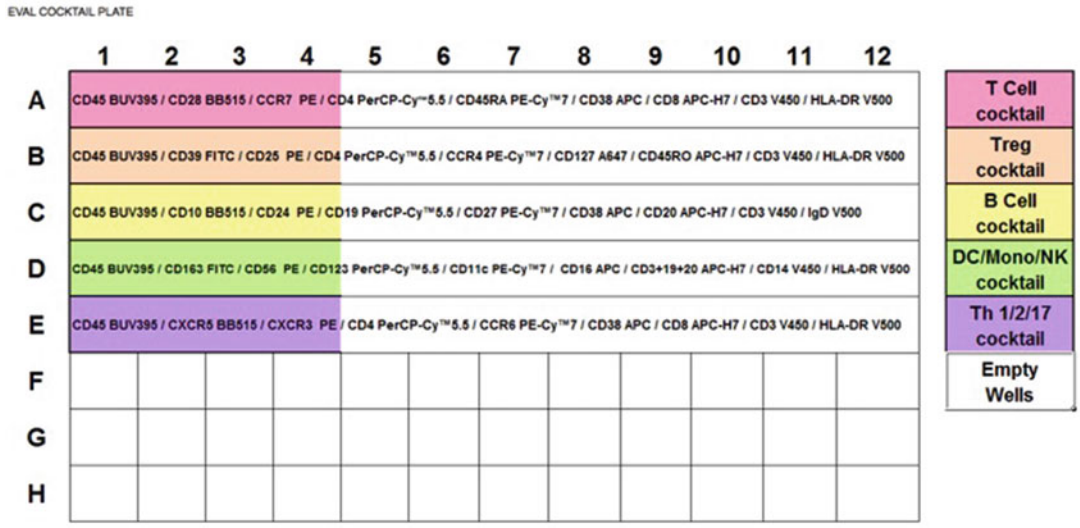

Fig. 1 Assay Lyoplate (number 175) from BD Biosciences is a 96-well plate with rows A through F and columns 1 through 12 containing lyophilized antibody cocktail buttons. Each row includes 12 replicates of the complete cocktail as follows: T-cell (**A**), Treg (**B**), B-cell (**C**), DC-Mono-NK (**D**), and T-helper (**E**)

D: DC-Mono-NK.

E: T-helper.

2.2 Compensation Lyoplate, BD Biosciences Designate 177 (Fig. 2)

Each row contains wells with individual antibodies that are constituents of one of the five cocktails, as well as negative and positive compensation bead particles. Beads are not used to incorporate viability dyes into the compensation matrix. Instead, cellular material is stained separately, in tubes with cells, and these are acquired on the cytometer at the same time as bead acquisition. Several strategies are available for calculating the compensation matrices for each cocktail (*see* **Note 2**).

2.3 Fluorescence-Minus-One (FMO) Lyoplate, BD Biosciences Designate 176 (Fig. 3)

Each row contains antibodies specific for each of the five cocktails in a similar fashion as the compensation Lyoplate. Columns A and B include all the cocktail antibodies. Cells that have been stained with viability dye are added to column A which represents the "complete" cocktail. Cells that have not been stained with viability dye are added to column B which represents the viability dye FMO. The remaining columns contain the antibody cocktail minus one of the antibody conjugate constituents (*see* **Note 3**).

2.4 Phosphate-Buffered Saline (PBS)

1×, pH 7.4 (Quality Biological, catalog number 114-058-101).

2.5 Supplemented PBS

Staining Buffer: PBS supplemented with 1% fetal calf serum, 1% normal mouse serum, 0.02% sodium azide (*see* **Note 4**).

COMP PLATE

	1	2	3	4	5	6	7	8	9	10	11	12	
A	[open]	CD45 BUV395	CD28 BB515	CD4 PCP-Cy™5.5	CD38 APC	CD8 APC-H7	CD3 V450	HLA-DR V500	CCR7 PE	CD45RA PE-Cy™7			T cell comps
B	[open]	CD45 BUV395	CD39 FITC	CD4 PCP-Cy™5.5	CD127 AF647	CD45RO APC-H7	CD3 V450	HLA-DR V500	CD25 PE	CCR4 PE-Cy™7			T reg comps
C	[open]	CD45 BUV395	CD10 BB515	CD19 PCP-Cy™5.5	CD38 APC	CD20 APC-H7	CD3 V450	IgD V500	CD24 PE	CD27 PE-Cy™7			B cell comps
D	[open]	CD45 BUV395	CD163 FITC	CD123 PCP-Cy™5.5	CD16 APC	CD3+19+20 APC-H7	CD14 V450	HLA-DR V500	CD56 PE	CD11c PE-Cy™7			DC/ mono/NK comps
E	[open]	CD45 BUV395	*CXCR5* BB515	CD4 PCP-Cy™5.5	CD38 APC	CD8 APC-H7	CD3 V450	HLA-DR V500	CXCR3 PE	CCR6 PE-Cy™7			Th1/2/17 comps
F													Empty Wells
G													
H													

Fig. 2 Compensation Lyoplate (number 177) from BD Biosciences is a 96-well plate with rows A through F and columns 2 through 10 containing lyophilized buttons of a single antibody from each cocktail per well along with both negative and positive beads that are included in BD Biosciences Anti-Mouse Ig Compensation Particles Kit (part number 552843). Each row contains the constituents of one of the five cocktails as follows: T-cell (**A**), Treg (**B**), B-cell (**C**), DC-Mono-NK (**D**), and T-helper (**E**). The antibody in well E3 is a rat monoclonal anti-CXCR5 conjugate; therefore, the compensation bead set for this well is derived from the BD Biosciences Anti-Rat Ig Compensation Particle Kit (catalog number 552844)

FMO Plate:

	1	2	3	4	5	6	7	8	9	10	11	12
A	T Cell cocktail	T Cell cocktail	FMO CD45 BUV395	FMO CD28 BB515	FMO CCR7 PE	FMO CD4 PCP-Cy5.5	FMO CD45RA PE-Cy7	FMO CD38 APC	FMO CD8 APC-H7	FMO CD3 V450	FMO HLA-DR V500	
B	Treg cocktail	Treg cocktail	FMO CD45 BUV395	FMO CD39 FITC	FMO CD25 PE	FMO CD4 PCP-Cy5.5	FMO CCR4 PE-Cy7	FMO CD127 A647	FMO CD45RO APC-H7	FMO CD3 V450	FMO HLA-DR V500	
C	B Cell cocktail	B Cell cocktail	FMO CD45 BUV395	FMO CD10 BB515	FMO CD24 PE	FMO CD19 PCP-Cy5.5	FMO CD27 PE-Cy7	FMO CD38 APC	FMO CD20 APC-H7	FMO CD3 V450	FMO IgD V500	
D	DC/Mono/ NK cocktail	DC/Mono/ NK cocktail	FMO CD45 BUV395	FMO CD163 BB515	FMO CD56 PE	FMO CD123 PCP-Cy5.5	FMO CD11c PE-Cy7	FMO CD16 APC	FMO CD3+19+20 APC-H7	FMO CD14 V450	FMO HLA-DR V500	
E	Th 1/2/17 cocktail	Th 1/2/17 cocktail	FMO CD45 BUV395	FMO CXCR5 BB515	FMO CXCR3 PE	FMO CD4 PCP-Cy5.5	FMO CCR6 PE-Cy7	FMO CD38 APC	FMO CD8 APC-H7	FMO CD3 V450	FMO HLA-DR V500	
F												
G												
H												

Fig. 3 FMO Lyoplate (number 176) from BD Biosciences is a 96-well plate with rows A through F and columns 1 through 11 containing lyophilized antibody buttons. Columns 1 and 2 are complete cocktails as follows: T-cell (**A**), Treg (**B**), B-cell (**C**), DC-Mono-NK (**D**), and T-helper (**E**). Columns 3 through 11 are "minus-one" of the constituents of the cocktail for that row

2.6 Fixation Medium

2% formalin solution in PBS, pH 7.4: Prepared fresh from a 10% methanol-free buffered formalin stock (Polysciences, catalog number 04018-1).

2.7 Viability Dye

Fixable Blue Live/Dead Cell Stain Kit (ThermoFisher Scientific, catalog number L23105): The kit includes four vials of frozen dye and one vial of dimethyl sulfoxide (DMSO) diluent. Stock solutions are prepared by addition of 50 μl of DMSO to each of the four vials of dye, which are pooled. Aliquots of 16 μl are placed in screw-capped freezing vials which are stored at −80 °C. One of these vials is sufficient to stain cells from five different samples.

2.8 Cell Samples

PBMC: peripheral blood mononuclear cells that had been prepared using density gradient centrifugation and preservation in liquid nitrogen for long-term storage (*see* **Note 5**).

3 Methods

The methods outlined below refer to the overall protocol used to perform staining of cells using three different lyoplates. These plates include—in the order of use—Compensation, FMO, and Assay. The compensation plate is handled in a slightly different manner than the latter two plates, as described below.

An adherent foil wrapping covers all three types of lyoplates, beneath the lid of the plate. The foil is removed from the plate at the time of use, and buttons within the wells are inspected, with any anomalies noted, in the event unexpected results are obtained that might be associated with any defects. The Compensation and FMO plates are used in their entirety once they are unpackaged. However, the Assay plates may have unused wells, if there are less than 12 samples to be assayed. In these cases, the foil wrapping is only partially removed so that it remains adhered to unused plate wells. If the plate is used again for a subsequent staining procedure, the wells are evaluated for evidence of hydration of the remaining lyophilized buttons. If this is noted in any well the plate is discarded.

Once the substrate is added to the lyophilized antibody cocktail button, the workflow for surface-marker immunophenotyping using dried reagents is not markedly different from that used with liquid reagents. Hence, this chapter does not proceed past the preparation of samples to be presented for data acquisition on a flow cytometer; these steps, as well as data analysis strategies, are particular to individual flow cytometry laboratories that utilize liquid antibody cocktails. Depending on the vendor of the dried cocktails, they may be manufactured in tubes or plates. Hence, the particulars for the procedure following admixture are contingent on the staining vessel. There are advantages and disadvantages

inherent in each type of containment, regarding staining versus cytometer data acquisition (*see* **Note 6**).

3.1 Processing the Compensation Lyoplate

1. PBS is added to each well in 100 µl volumes, starting in well A1, proceeding down the column then moving to the top of successive columns.
2. After addition of PBS to all wells, the plate is incubated for 30 min at the ambient temperature, protected from light. There is a 5-min period during which the added buffer dissolves the lyophilized button; the plate is placed on a rotary plate mixer at low speed for the remaining 25 min.
3. At the end of this interval, the plate is centrifuged (ambient temperature, 400 × *g*, for 5 min) and flicked to remove supernatants, followed by addition of 100 µl of Staining Buffer. The contents of each well are transferred into labeled polystyrene tubes and held at 4 °C until cytometer acquisition, which is completed within 30 min of staining. Beyond this interval, the beads will begin to deteriorate, which may compromise the validity of the compensation matrices.

3.2 Processing Cell Samples

1. After cells have been thawed and washed, 1 ml of PBS is added to the residual volume remaining in each tube.
2. Before the addition of 1 µl of the viability dye working solution, a 50 µl aliquot of cell suspension is removed and added to another labeled tube containing 100 µl of Staining Buffer, to be acquired on the cytometer as *unstained* samples.
3. The tubes with cells and viability dye are incubated, at the ambient temperature, protected from light for 5 min, followed by the remaining 25 min on a rotating plate mixer.
4. At the end of this interval, the tubes are centrifuged (ambient temperature, 400 × *g*, for 5 min), decanted one-by-one, and the pellets resuspended by gentle repipetting in their residual supernatant with addition of 450 µl of Staining Buffer, to ensure sufficient volume for staining the five wells for each sample (100 µl per well).

3.3 Processing the FMO and Assay Lyoplates

1. For either plate, once removed from its pouch, and peeling off of the foil cover, 100 µl of cells from Subheading 3.2 is added into rows A through E in the column assigned to each sample. Pipetting of sample starts in column 1 and proceeds through column 10, using a new pipette tip for each well.
2. Once all wells are filled, a 30-min incubation at the ambient temperature ensues, with the plate protected from light. After the first 5 min, the cells are resuspended, column-by-column, using an eight-channel multipipettor set at 80 µl. The suspensions are mixed by gentle repetitive pipetting. After addition to each column, the tips are discarded and replaced with new tips

before proceeding to the next column. Following the resuspension of cells, there is an additional 25-min incubation, protected from light, at the ambient temperature on the rotary plate mixer.

3. Washing steps (*see* **Note 7**).

   Wash 1: 100 μl of PBS is added to each well, mixed by gentle repetitive pipetting, then *centrifuged and flicked to remove supernatants.*

   Wash 2: 200 μl of PBS is added to each well, mixed by gentle repetitive pipetting, then *centrifuged and flicked to remove supernatants.*

   Wash 3: 200 μl of 2% formalin is added to each well, mixed by gentle repetitive pipetting. The plate is then incubated at the ambient temperature, protected from light, for 30 min, then *centrifuged and flicked to remove supernatants.*

   Wash 4: 200 μl of Staining Buffer is added to each well, mixed by gentle repetitive pipetting, then *centrifuged and flicked to remove supernatants.*

4. The final suspension volume of 200 μl of Staining Buffer is added, followed by gentle repetitive pipetting. The contents of each well are transferred to polystyrene tubes labeled with the row/column identifier, already containing 250 μl of Staining Buffer. Samples are collected on the cytometer immediately following the staining procedure. In the interval prior to acquisition on the cytometer sample tubes are protected from light and held at 4 °C until ready for acquisition, either refrigerated or on wet ice.

4 Notes

1. The original iteration of the Lyoplate product for the HIPC project [11] did not include the utilization of a 355 nm laser since not all instruments from participating centers were equipped with one. With our desire to use this laser, a CD45∗BUV conjugate (BD Biosciences, catalog number 563792) was included in each lyophilized cocktail. The original iteration directed usage of a viability dye excited by the 488 nm laser; this was modified to use the 355 nm laser-excited viability dye discussed above in Subheading 2.6. This made the 488 nm laser-excited conjugates available for incorporation of an additional conjugate - either FITC (rows B and D) or BB515 (rows A, C and E) - in each cocktail:

   Row A (T-cell) <viability dye/CD45∗BUV/CD3∗V450/HLA-DR∗V500/CD28∗BB515/CD4∗PerCP-Cy5.5/

CCR7∗PE/CD45RA∗PE-Cy7/CD38∗APC/CD8∗APC-H7>

Row B (Treg) <viability dye/CD45∗BUV/CD3∗V450/HLA-DR∗V500/CD39∗FITC/CD4∗PerCP-Cy5.5/CD25∗PE/CCR4∗PE-Cy7/CD127∗AF647/CD45RO∗APC-H7>

Row C (B-cell) <viability dye/CD45∗BUV/CD3∗V450/IgD∗V500/CD10∗BB515/CD19∗PerCP-Cy5.5/CD24∗PE/CD27∗PE-Cy7/CD38∗APC/CD20∗APC-H7>

Row D (DC-Mono-NK) <viability dye/CD45∗BUV/CD14∗V450/HLA-DR∗V500/CD163∗FITC/CD123∗PerCP-Cy5.5/CD56∗PE/CD11c∗PE-Cy7/CD16∗APC/CD3 + CD19 + CD20∗APC-H7>

Row E (T-helper) <viability dye/CD45∗BUV/CD3∗V450/HLA-DR∗V500/CXCR5∗BB515/CD4∗PerCP-Cy5.5/CXCR3∗PE/CCR6∗PE-Cy7/CD38∗APC/CD8∗APC-H7>

2. The original iteration of the Lyoplate product for the HIPC project [11] used a single plate that contained the compensation beads and the assay cocktails. Combining the assay and compensation wells in one plate results in the compensation beads being washed more aggressively and incubating longer than necessary, leading to inconsistencies when calculating matrices. An alternative strategy is to use separate plates for compensation and assay. Using a separate compensation plate—with more wells available—allows the use of the full complement of the conjugates for each cocktail. This offers the opportunity for two strategies to construct the matrices. In the first scenario, separate matrices are constructed for each cocktail, using the full complement of wells in each row. In the second scenario, only one well of conjugates with replicates present in more than one cocktail is acquired. Following data acquisition, the experiment in the cytometer software is repeatedly duplicated resulting in five copies, one for each cocktail. The data files that are not relevant for a given cocktail are then deleted before the matrix is constructed. With regards to the frequency of preparation/data acquisition/data analysis of the Compensation Lyoplate, the expectation at the outset, when the compensation wells and antibody conjugate wells were included on a single plate, was that new matrices would be constructed for every plate. In the protocol described herein, matrices were constructed with much less frequency—only when there were significant changes to the cytometer instrument optics/fluidics/electronics during servicing. The relatively low number of parameters associated with the panels

described above makes it possible to construct $N \times N$ plots of all fluorochrome combinations within the cytometer software experiment template before exporting files for off-line analysis. With the use of the experiment template copied from the previous acquisition session, changes to matrix values are easily accomplished to address minor changes inherent in day-to-day variability in cytometer performance.

3. In the original iteration of the BD Lyoplate used in the HIPC study [10–12], there was no provision for performing FMO analysis. Given its importance of in immunophenotyping [13], the use of dried reagents for this procedure is a significant addition to the protocol. Therefore, a limited number of FMO lyoplates were prepared during the production run. The use of dried FMO cocktails in plates is a significant time and cost-saving step when compared with using liquid cocktails in tubes. That said, once the initial quadrant gate placements are derived from the initial FMO files analyses, subsequent FMO plates are only run following significant alterations following servicing of the cytometer. The strategy for confirming proper quadrant gate placement, after initial placement from analysis of FMO data files, involves the use of Boolean gating strategies to derive "NOT" gates in order generate internal "negative" cell populations in the absence of markers that would definitively identify contextual leukocyte subpopulations.
4. Several stages of the procedure are carried out using PBS without any additives, while others utilized this buffer supplemented with 1% fetal calf serum, 1% normal mouse serum and 0.02% sodium azide (Staining Buffer). PBS, instead of Staining Buffer, is used during cell processing before addition of viability dye to prevent diminution of viability dye uptake in the presence of protein. PBS is used during processing of the Compensation lyoplate because the presence of normal mouse serum in the Staining Buffer would block binding of murine antibodies to the compensation particles. PBS is used in the first two wash steps following staining, before the formalin fixation step. All other steps utilize Staining Buffer.
5. Though several of the cited references report on the use of dried antibody cocktails to study bone marrow aspirates and body fluids, the primary substrate used is peripheral blood, which is prepared using lysis techniques (before or after addition to antibodies), or density gradient separation. In most cases, the latter procedure is an antecedent to long-term storage in liquid nitrogen. The samples used in this procedure were not processed in the same laboratory that performed the staining; hence, the methodology is not described. There are recommendations for using certain types of cell processing,

depending on the nature of the study being conducted. In studies that involve single-time point determinations at a single testing site, the use of lysed blood would be preferable to minimize cell loss and possible phenotypic modification that might occur with cell separation methods and long-term storage. In studies that involve temporal sampling and/or testing at multiple sites, as described herein, the use of frozen peripheral blood mononuclear cell preparations is a necessity.

6. The choice of whether to use dried antibodies prepared in microtiter plates or 12 × 75 mm polystyrene tubes has depended on the format chosen by the vendor manufacturing the product. For example, the original Lyoplate product from BD Biosciences was only available in plates, and the original Duraclone product from Beckman-Coulter was only available in tubes. It is an open question as to whether there will be the opportunity to request one or the other format. That being the case, regarding cell preparation, the use of microtiter plates is an advantage compared to tubes concerning processing time and nonantibody reagent costs. The same applies to data acquisition on instruments with high-throughput acquisition modules. The techniques described herein used staining in plates, with transfer to tubes before cytometer acquisition. The reason for transferring to tubes was due to a preponderance of paucicellular samples, and a desire to collect at low flow rates. Since the plates take several hours to run to completion, and there is no mechanism to cool the plate during collection, it is necessary to keep the samples chilled until the time that they would be acquired, to prevent cell and conjugate degradation.
7. Lyoplate washing procedures, including centrifugations (400 × *g* for 5 min), are carried out at the ambient temperature. Supernatants are removed by flicking the plates into a sink. The sink is disinfected with a 10% bleach solution following each wash step. Centrifugations in tubes, carried out during the preparation of cellular material before staining, are performed at the ambient temperature at 400 × *g* for 5 min, with supernatant removal by decanting.

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Chapter 5

Guidelines for Gating Flow Cytometry Data for Immunological Assays

Janet Staats, Anagha Divekar, J. Philip McCoy, Jr, and Holden T. Maecker

Abstract

"Gating" refers to the selection of successive subpopulations of cells for analysis in flow cytometry. It is usually performed manually, based on expert knowledge of cell characteristics. However, there can be considerable disagreement in how gates should be applied, even between individuals experienced in the field. While clinical software often automates gating, and some guidelines do exist (especially for clinical assays), there are no comprehensive guidelines across the various types of immunological assays performed using flow cytometry. Here we attempt to provide such guidelines, focused on the most general and pervasive types of gates, why they are important, and what recommendations can be made regarding their use. We do so through the display of example data, collected by academic, government, and industry representatives. These guidelines should be of value to both novice and experienced flow cytometrists analyzing a wide variety of immunological assays.

Key words Flow cytometry, Gating, Analysis

1 Introduction

The analysis of flow or mass cytometry data uses a process commonly called "gating," whereby populations of cells are virtually separated from one another. This is accomplished through the use of specialized flow cytometry software programs to identify subsets within a sample of particles or cells, and to assess the expression levels of specific markers of interest on those selected subsets, without confounding data from other cellular subsets. In this context, some authors may refer to an "analysis region" rather than a "gate," which formally refers to physical sorting of cells; but we will not distinguish the two here. As widely practiced today, gates are set by human input based on visual examination of the flow cytometry data.

Subsets of interest can range in magnitude from very abundant to exceptionally rare. The relative abundance of a subset, as well as its separation from other populations, affects the ease with which

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that subset can be accurately and reproducibly gated, as well as the purity of the gate.

Most software programs used for flow cytometry analysis allow for gates to be drawn in one or two dimensions (i.e., on histograms or on two-parameter plots). Two-dimensional gates may be rectangular, elliptical, or irregular polygons; and the plots on which they are drawn may depict contours, density, or individual events (dot plots). In theory, gates could be drawn in more than two dimensions at once, but this is practically not feasible for humans. Hence, subsets that are distinguished by a series of markers are usually identified by sequential one- or two-dimensional gates (known as Boolean hierarchical gating).

An important aspect of hierarchical gating is knowledge of the expression profile of a population of interest across many different markers. Hierarchical gates can include both positive and negative markers; and the sequential order of the hierarchical gates can influence the ability to clearly resolve the final population. Therefore, accurate and robust gating should be based on a strategy that involves an optimal sequence of marker-based gates, rather than just a single terminal gate based on one or two markers.

Gating is subjective; substantial variations in gating can be observed between the gate set by different analysts. In addition to operator expertise, this is in part dependent on the quality of staining. Many factors influence the observed fluorescence intensity of the samples, as summarized in Table 1. While most of these factors have been reviewed previously, it is generally assumed that the operator will make appropriate adjustments for these factors in terms of gating, although, ideally, these factors should be first

Table 1
Factors affecting population resolution in flow cytometry

Root level	Factor	References
Biological	Donor variability in expression levels, background	[1]
	Sample integrity (shipping, cryopreservation/thawing, viability, etc.)	[2–4]
	Aggregates	[5]
Instrument	Optical configuration (lasers, laser power, filters)	[6]
	Daily performance, including laser delays, voltage calibration	[7]
	Hydrodynamics (air bubbles, clogs)	[8, 9]
Reagents	Antibody clones, titers, and lot-to-lot variability	[10, 11]
	Panel design (fluorochrome brightness and spillover)	[12]
	Reagent degradation, especially tandem dyes	[12]
	Staining protocol, including activation, fixation, permeabilization, intracellular vs. cell-surface staining, washing	[11]
Data transformation	Accuracy of compensation for fluorescence spillover	[13, 14]
	Scaling (logarithmic vs. biexponential, valley artifact)	[15, 16]

minimized through technical means (e.g., experimental design and careful adherence to protocol and to best practices).

Because of the vast numbers of different assays that can be performed, flow cytometry has developed into a widely used technology in a number of scientific endeavors. These applications can be roughly divided into four major areas: clinical/diagnostic, clinical/translational research, biopharma/drug discovery, and basic research. While these four areas use a common technology, each has its nuances that relate to the specific applications. Thus, when guidelines for flow cytometry are established, some elements might be common to all applications while others might apply to one or a few of the applications. Perhaps for this reason, there are few general guidelines on how to place gates that are applicable to the vast majority of flow cytometry analyses.

As one might expect, the clinical environment has led the way in efforts to establish practice guidelines for flow cytometry, in large part due to the implementation of CLIA-88 (Clinical Laboratory Improvement Act of 1988). These laws were enacted to assure reliable, high quality, accurate, and safe testing in all clinical laboratories. The laws encouraged a standard of practice, as well as proficiency testing and inspections. CLIA, in turn, led directly to the issuance of guidelines for the performance of flow cytometry in the clinical environment by the College of American Pathologists (CAP) and the Clinical Laboratory Standards Institute (CLSI) (formerly NCCLS). These guidelines dealt with a variety of issues ranging from lot testing to instrument set up to establishing standard operating procedures (SOPs) for nearly every aspect of the laboratory.

In the clinical and translational research environments, there have been a number of assay-specific guidelines that have been established. Perhaps the best known are the guidelines for CD4 T cell counting in the context of HIV disease. One of the first of these were the guidelines published by the US Centers for Disease Control and Prevention (CDC) in 1992, with supplements and revisions to these guidelines issued in 1994, 1997, and 2003 [17–20]. Similar guidelines for enumeration of CD4 T lymphocytes have been put forward by the World Health Organization [21].

Guidelines for additional clinical tests have also been published and gained wide acceptance. Among these are guidelines for enumeration of CD34+ hematopoietic stem cells (proposed by the International Society of Hematotherapy and Graft Engineering [22]), and guidelines for detecting paroxysmal nocturnal hemoglobinuria (PNH) [23]. These included specific directions for how the gating is to be performed for these assays.

While the clinical arena is heavily regulated with enforceable guidelines, the remaining areas in which flow cytometry is used are far less regulated. Except for some network clinical trials work, for which GCLP compliance is required, most often adherence to

guidelines in research environments is voluntary rather than mandated. For biopharmaceutical and translational research cytometry, there have been guidelines established to promote high-quality practices, although these pertain more to general laboratory practice rather than being specific for flow cytometry. In the realm of translational research, several guidelines have been proposed which would also be applicable to basic research. Examples of these include guidelines to intracellular cytokine staining, proposed by the Cancer Immunotherapy Consortium [24] and guidelines for HLA-peptide multimer assays proposed by the Cancer Vaccine Consortium [25]. Of these, only the former included details concerning how the gating should be performed.

Basic research using flow cytometry is the area in which the least has been done to standardize or harmonize how assays are performed. This is due to a number of factors including the vast array of assays that are performed by flow cytometry in basic research, the fiercely independent nature of many researchers, and the lack of the ability to enforce compliance with guidelines in this area. Nomura and colleagues demonstrated that gating of flow cytometric data was a significant factor in interlaboratory variations [26], a finding that was later confirmed by Finak et al. [27]. The latter study also explored advantages offered by an automated gating strategy.

A number of automated gating algorithms have been introduced to reduce the subjectivity of manual gating, decrease the time and labor of manual analysis, and/or discover populations not anticipated by manual gating (reviewed in [28, 29]). These range from tools that help to define the edges of a selected cell population, to supervised approaches for analyzing a complete data set, and even fully unsupervised clustering algorithms. In general, with increasing complexity and decreasing supervision comes the prospect that the automated method may produce spurious or undesirable results; or at least, will not match the manual gating results of recognized experts. As such, automated gating tends to still require some manual confirmation. It is not our purpose here to debate the relative merits of automated approaches; but rather, recognizing that the vast majority of gating is still done manually, to propose guidelines that could improve the consistency and accuracy of such manual gating. Certainly, some of these guidelines might be useful to designers of automated algorithms as well.

2 Guidelines

2.1 Acquisition Threshold or Trigger Gate

Definition: When acquiring samples using a flow cytometer, the cells of interest are usually in a suspension that contains many other cells or particles. Each cell or particle is measured by the cytometer as an event in the form of a pulse; some events represent signals of

interest and some are noise. An operator may set a limit for the type of pulse that is acceptable to be included in the resulting data file; this is called an acquisition (detection) threshold or trigger gate. Trigger gates are set prior to acquisition as a means of excluding events of noninterest or noise. The most common type of trigger gate is used to exclude debris based on size, measured using forward scatter. Using forward scatter, the trigger gate is set to exclude events that are too small to be considered cells. Another type of commonly used trigger gate is based on CD45 expression, where CD45 is a panleukocyte marker (*see* Fig. 1). Setting the trigger gate on CD45+ cells will essentially exclude all nonleukocyte events.

Note that on some cytometers, a *storage gate* can be set in addition to an acquisition threshold or trigger. Care should be taken that the storage gate as well as the acquisition gate includes all populations of potential interest for later analysis. For example, a storage gate set on lymphocytes (using forward vs. side scatter) would preclude downstream analysis of monocytes.

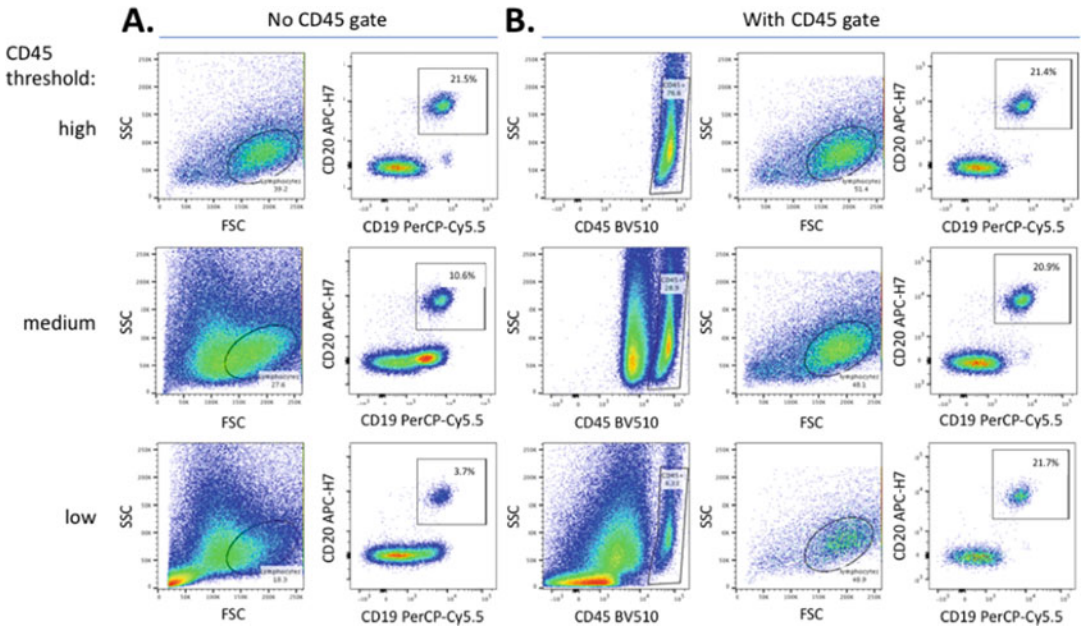

Fig. 1 A CD45 trigger gate (threshold) is used to exclude nonleukocytes. In a sample heavily contaminated with erythrocytes, a CD45 threshold can help to exclude these cells and "clean up" the downstream gates. In this example, the same erythrocyte-contaminated sample was run with a high, medium, or low threshold on CD45. Left panels: With no further gating on CD45, the initial FSC vs. SSC gate for lymphocytes (first column) is uncontaminated with a high CD45 threshold (top row), but progressively more contaminated as the CD45 threshold is reduced. This results in an artificially low observed percentage of B cells (second column), due to dilution of lymphocytes by contaminating erythrocytes. Right panels: The same data can be effectively rescued by applying a postacquisition gate on CD45high events (first column), prior to lymphocyte gating (second column). This results in similar observed percentages of B cells (third column), albeit at the expense of greatly diminished cell numbers when there was a low CD45 threshold on acquisition

How data is affected: Trigger gates have a "Goldilocks" zone. A low or no trigger gate leads to excess noise and reduces overall signal-to-noise ratio. But when trigger gates are set too high, they partially or completely exclude the target population of interest and decrease the overall signal. When applied properly, trigger gates effectively remove unwanted events or cells, thus decreasing noise and improving the overall signal-to-noise ratio.

Conclusion: Threshold gating is a very effective tool for reducing noise. To ensure the removal of noise and not signal, setting trigger gates properly requires both prior knowledge of the sample and the target population of interest.

2.2 Time Gate

Definition: Time was first introduced as a flow cytometry parameter to measure kinetic responses [30]. Subsequently, in 1987, James Watson first described the concept of using time as a quality control (QC) parameter [8]. The principle of flow cytometry is based on regulating the differential pressure between sample and sheath fluid to establish a laminar flow, enabling a single cell to pass in front of the laser for interrogation. The steps involved with acquiring a sample influence the fluid dynamics throughout the course of acquisition. When a sample is first introduced into the cytometer, a burst of pressure may cause a high density of events to pass in front of the laser during the first few seconds. After the initial sample introduction, the flow rate stabilizes and becomes laminar. Nearing the end of acquisition, the sample volume is greatly reduced, which may influence the fluid dynamics again. During acquisition, fluidic changes may be observed as either an increase or decrease in the event rate. The most frequent culprit is air bubbles or clogs. For these reasons, the time parameter may be used during analysis to exclude those events that were acquired during periods of nonlaminar flow. This is called a time gate. For manual analysis, a time gate is created by visualizing time versus scatter or fluorescence. Alternatively, there are automated software options, such as FlowClean [9], that may be used to effectively remove anomalies, such as those caused by changes in the event rate, from an FCS file. Of note, the time parameter may be displayed differently based on the software being used for acquisition or analysis.

How data is affected: Anomalies in the flow rate result in wider CVs and/or shifts in scatter and fluorescence parameters (*see* Fig. 2). The effect of nonlaminar flow on the resulting data may be modest or severe, depending upon the degree of fluidic instability and the integrity of any subsequent quality control gates, such as singlet and fluorescence gates. In the most severe case, an entire data file may be unacceptable for analysis due to unstable fluidics that occurred throughout the time of acquisition.

Conclusion: The time parameter is an effective QC tool for identifying temporal changes in fluid dynamics that occurred

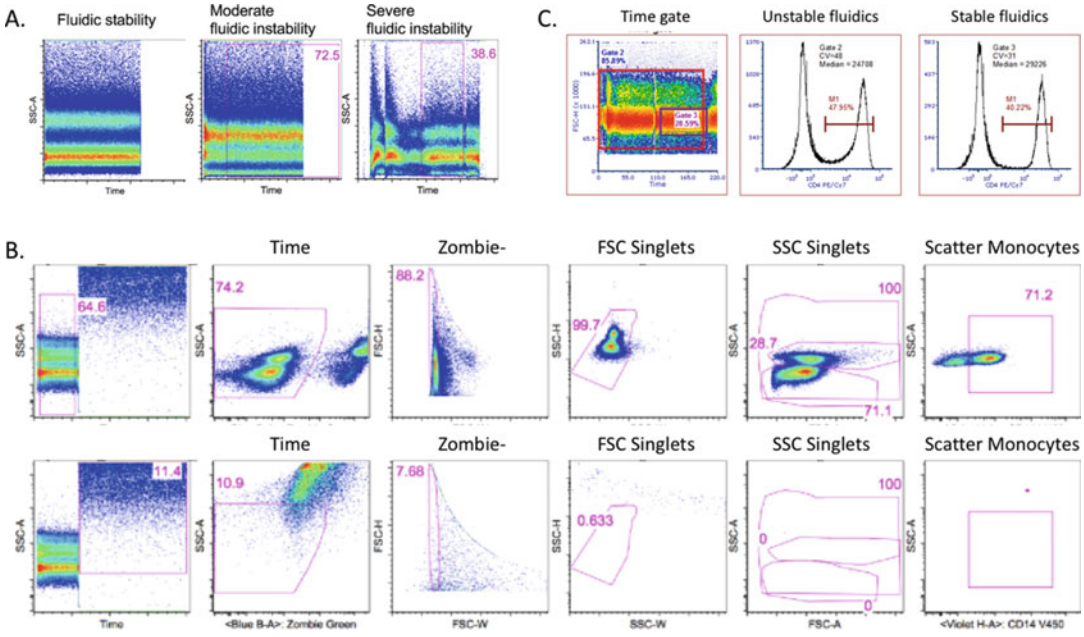

Fig. 2 Use of time gates. (**a**) Time as a QC parameter. PBMCs were thawed, permeabilized, stained, and acquired. Time versus SSC-A is used to visualize the temporal changes in fluid dynamics that occur throughout the sample acquisition. *Left plot* illustrates an ideal time parameter plot. The burst of sample at the beginning, observed as the red or higher density lower scatter population, has an initially wider SSC CV that rapidly resolves into a tight SSC CV. The SSC CV remains the same throughout the remainder of acquisition and slightly decreases near the end of acquisition, as the sample rate decreases (red turns to orange). There are no skips or jumps in the number of events relative to time and no shifting of SSC over time. *Middle plot*: moderate nonlaminar flow. A more noticeable sample burst at the beginning of acquisition with wide SSC-A CV. SSC-A CV changes throughout the acquisition in an oscillating pattern, caused by swirling fluidics. The time gate, indicated in pink, is set to include the most stable portion of the events acquired. *Right plot*: illustrates nonstable fluidics that occurred throughout the entire sample acquisition. Clogs are indicated by skips or jumps in time, where the event rate is greatly reduced followed by bursts of events when the clog breaks. Changes in SSC-A CV and shifting of SSC-A are prominent throughout the acquisition. The time gate, in pink, is placed around the most stable area, although no area in this example is ideal. While a significant portion of events are excluded due to unstable fluidics, the data included in the time gate represents those events with the highest integrity. (**b**) Time gate used to exclude shifting scatter created by nonlaminar flow. LyoCell PBMC control product was stained with CD14. The gating strategy shown was used to identify CD14+ monocytes by first parsing through time, then viability, singlets (FSC and SSC), and scatter gates. Both rows represent analyses using the same FCS file. *Top row*: the time gate is set around the region with best laminar flow, identifying approximately 71% of monocytes as CD14+. *Bottom row*: using a time gate set on the region with nonlaminar flow there are no CD14+ monocytes. (**c**) Time gating affects CV of downstream gates. Human PBMC were stained with CD4 PE-Cy7. The leftmost plot is ungated, while the middle and the right histograms are gated on FSC/SSC Singlets. If placement of time gate includes sections of the plot where nonlaminar flow exists then the CV as well as median of the CD4+ cell peak and the frequency of CD4+ cells is affected. Inclusion of events from the unstable fluidics results in a wider CV and higher percent positive cells and exclusion of these events results in a tighter peak with lower CV and higher fluorescence intensity

throughout sample acquisition. Gating on "good" acquisition interval(s) can improve data quality.

2.3 Singlet Gates

Definition: Aggregates consist of one or more cells or particles; they form for many reasons, including cells clumping together due to dying/death, adherent properties, antigen presentation, and also high sample concentration, high flow rate, and even dye–dye aggregation. When running clumped samples with many aggregates, it is important to filter the samples prior to acquisition to remove as many aggregates as possible. When filtering is insufficient to remove all aggregates, they may be further removed using singlet gating. To understand how singlet gates work, it is important to understand how signals are detected by a flow cytometer. When a particle passes in front of the laser, the cytometer measures the resulting photons of light in the form of a pulse. Digital cytometers are capable of collecting three values that define a pulse: *Width* is a measurement that represents the time a cell passes in front of the laser. Width is proportional to the size of the cell or particle and is unaffected by PMT voltage settings [31]. *Height* is the measurement of the intensity of the signal (maximum fluorescence, size, or scatter) and is affected by PMT voltages. *Area* is not measured; it is derived using both width and height measurements, and is only slightly affected by PMT voltage, owing to its relationship to both width and height. Area scaling is used in some digital instruments to equalize height and area. Pulses can be distorted by coincidence, when two events (a doublet) pass in front of the laser simultaneously. This distortion results in a twofold stretching of the area and width, while the height remains unchanged. During analysis, singlet gating uses the distortion aggregates impose on pulse width and area to distinguish singlets from aggregates. Singlet gating is performed using either forward scatter (FSC) or side scatter (SSC) parameters. It is important to remember that FSC is collected using a diode, unless you have an instrument specially equipped with FSC PMT, and SSC is collected using a PMT. Since PMTs are more sensitive than diodes, a better resolution between singlets and aggregates may be visualized using the SSC over FSC parameter. As a best practice, ideally both FSC and SSC singlet gates would be used sequentially to remove coincidental events occurring in different orientations. There are two common approaches to singlet gating; one is dependent upon area scaling and one is not: area versus height and width versus height. For area versus height singlet gating, coincidence is identified by the events for which there is an increase in area without a corresponding increase in height. Thus, cells along the diagonal are singlets and should be included in the singlet gate. Use of area versus height singlet gating requires the area scaling to be properly adjusted on your cytometer using cells; otherwise, there is a risk for gating errors. Maximum resolution between singlets and aggregates is observed using width versus

height, as both independently measured values. Another advantage to using width versus height singlet gating is that neither value is affected by area scaling [32]. For width versus height singlet gating, coincidence is identified by an increase in width, while height remains unchanged. Irrespective of the pulse value used to establish singlet gates, debris may be readily distinguished from cells in the lower corner and may be excluded using the same gate as the aggregates. Examples of singlet gates are shown in Fig. 3.

How data is affected: It is important to remove aggregates during data analysis of immunophenotpying data, as these can give false double positive events. As an example, if a cluster of cells includes B (CD19+ CD3−) and T (CD19− CD3+) cells and if these are not removed from the data analysis, then the operator may report the presence of a cell population that is CD3+ CD19+ (a T-B cell), which does not exist. Applying doublet discrimination helps remove these oddities from data.

Conclusion: In the context of immunophenotyping, singlet gates are necessary for accurate identification of cells that are coexpressing two markers or double positives. Once singlet gating is applied, in some instances, this will "clean up" data and thus result in loss of a certain cell population, whereas in other cases it may result in enrichment of a cell type due to loss of nonspecific populations. There are, however, circumstances under which one might want to evaluate aggregates, and for those assays the use of singlet gating would not be applicable [33].

2.4 Exclusion Gates

Definition: In the most general sense, an exclusion gate is any gate that removes unwanted cell populations, before positive gating on markers of interest. Practically, this is often useful to prevent the interference of these excluded populations on the downstream analysis. Besides time and singlet gates already discussed above, two common variations of such exclusion gates are viability gates and lineage marker cocktails (often called a "dump channel"). Viability is most frequently assessed by the fact that live cells have intact membranes and thus exclude dyes that would otherwise enter the cell and bind, for example, DNA. Propidium iodide and 7-AAD are examples of such dyes. Alternately, agents that react with free amine [4] or thiol groups can be used, taking advantage of the fact that relatively few such reactive groups are present on the cell surface, but many more are available in the interior of cells. Many dyes are available in this category, with different fluorescent properties, such that they can be combined with almost any combination of fluorochrome-labeled antibodies. A major advantage of these dyes is that they form a covalent bond with cellular proteins, and thus do not diffuse away if cells are fixed, permeabilized, or otherwise processed after application of the viability stain. For this reason, they are often referred to as "fixable" viability dyes.

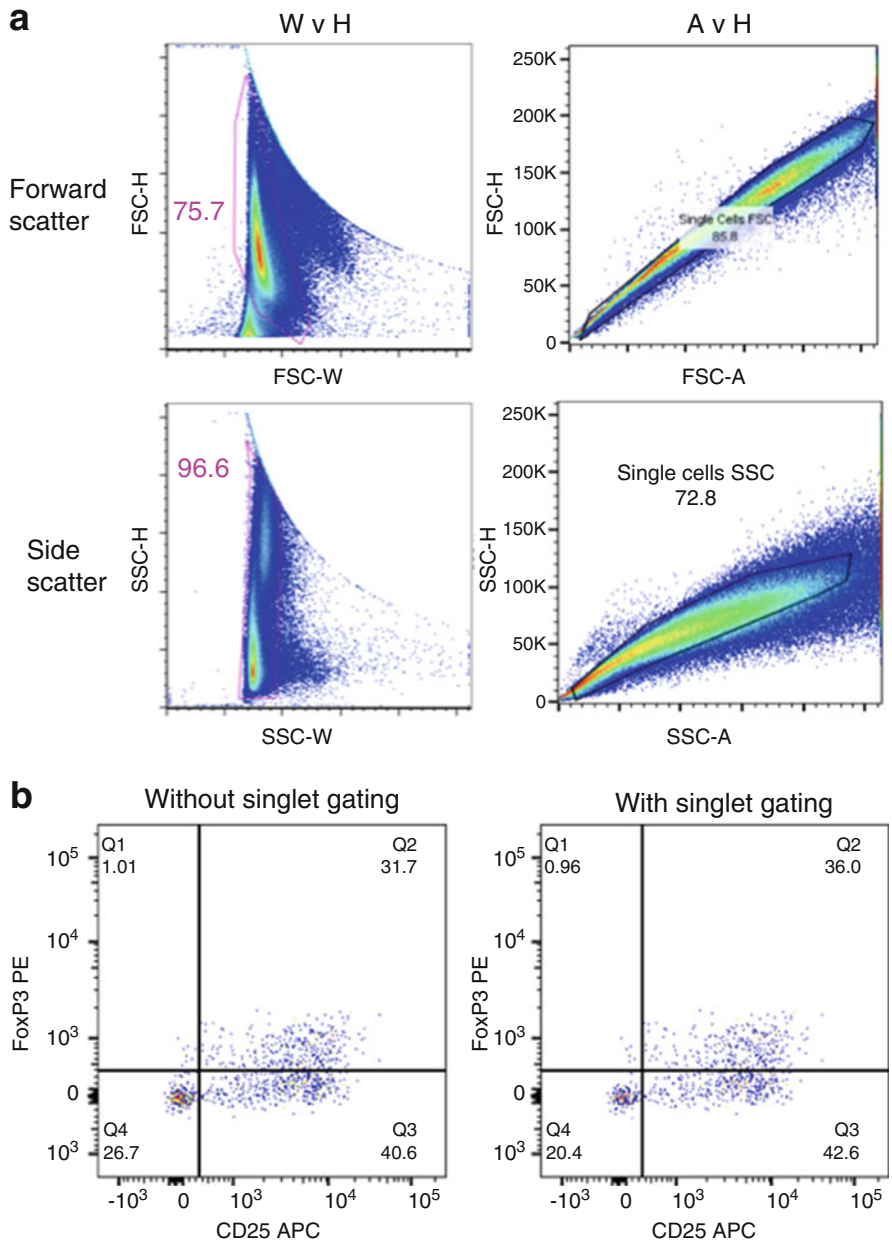

Fig. 3 Singlet gating. (**a**) Width vs height and area versus height singlet gates using FSC and SSC. Whole blood was subjected to erythrocyte lysis, stained, and acquired by flow cytometry. *Top row*: Forward scatter parameter is used to create singlet gates. *Bottom row*: side scatter is used to create singlet gates. *Left column*: width (-W) versus height (-H) plots are used for singlet gating and to exclude debris in the lower corner. Aggregates are those events for which there is an increase in -W relative to -H. *Right column*: area (-A) versus -H is used for gating singlets; aggregates are those events for which -A increases disproportionately to -H. (**b**) Exclusion of doublets can alter the frequency of gated populations. Whole blood was stained with a cocktail containing CD3, CD4, CD127, CD25, and Foxp3. Left image depicts frequency of Treg cells without doublet exclusion and right panel shows frequency of Treg cells with singlet gating or doublet exclusion. Note increase in frequency of CD25+ Foxp3+ cells following gating on singlets. Singlet gating was performed using SSC-A vs SSC-H followed by FSC-A vs FSC-H

How data is affected: Dead cells tend to bind antibodies and/or fluorochromes nonspecifically and as such interfere with the quantitation of cells identified with positive markers alone. This is particularly important when quantifying rare populations like antigen-responsive T cells (Fig. 4). In such assays, dead cells may add to the "background" or unstimulated response, as well as potentially obscuring the distinction between positive and negative populations.

A lineage cocktail uses markers for one or more cell lineages that are not of interest for downstream analysis. For example, B, T, NK, and monocyte markers are commonly combined in a lineage cocktail for the downstream gating of dendritic cells (DCs), which bear none of these markers. This exclusion improves the ability to perform positive gating on DCs (commonly gated as lineage-HLA-DR+). But other applications are also possible. Identification of antigen-specific T cells with certain reagents may be improved by first gating out monocytes and B cells, which express Fc receptors. Samples that have erythrocyte contamination may benefit from exclusion gating on CD235a, an erythrocyte-specific marker. And use of a platelet-specific antibody like CD62P can be used to remove platelet-bound lymphocytes and thereby clean up intracellular cytokine staining of antigen-responsive T cells [34].

To some extent, lineage cocktails also help to remove dead cells via their nonspecific binding; but they are not a perfect surrogate for a viability dye and can serve other functions as noted above. Of course, a viability dye and a lineage cocktail can also be combined in a single channel.

Conclusion: Exclusion gates reduce noise, thus enhancing the overall signal-to-noise ratio. Rare populations are more accurately quantitated with exclusion of dead cells and/or irrelevant cell lineages.

2.5 Scatter Gates

Definition: Fluorescence flow cytometry typically measures forward and right-angle (side) scatter of the laser light used to excite fluorochromes on the particles of interest. These two parameters contain information on cell size and granularity, respectively. As such, they are frequently used as initial gates on immune cell populations, such as lymphocytes, monocytes, and granulocytes.

How data is affected: While a population such as lymphocytes may appear to have a relatively homogeneous scatter profile, there are in fact subtle differences in the scatter profile of different lymphocyte subsets. Most dramatic is the increased forward scatter of proliferating lymphocytes (blasts); a tight lymphocyte gate thus misses most of the proliferating cells. But even populations such as B cells or CD8+ T cells can have subtle shifts in their scatter relative to the rest of the lymphocytes. The result is that the placement of the lymphocyte gate can have small but potentially significant

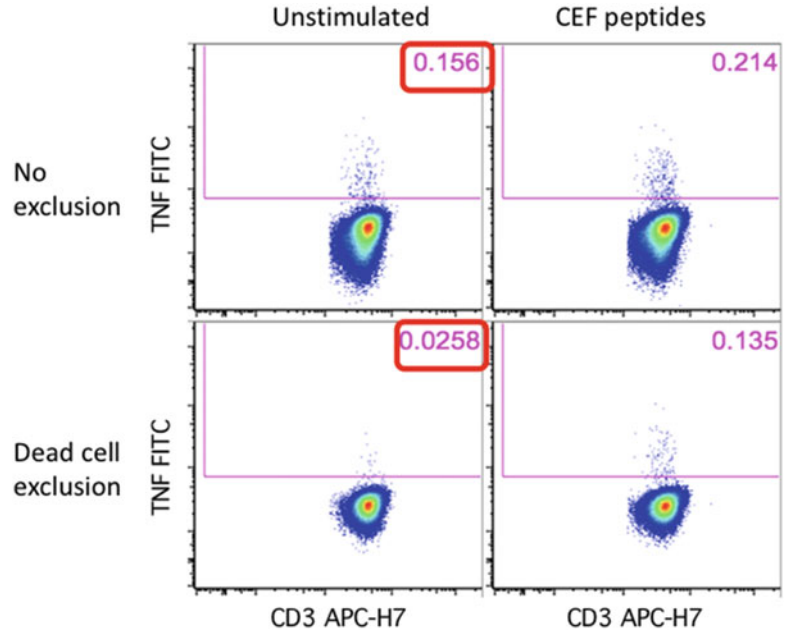

Fig. 4 Excluding dying/dead cells reduces noise. PBMCs were thawed, stimulated with either no specific antigen or CEF peptide mix (optimal 8–9mers for CMV, EBV, and influenza) in the presence of protein transport inhibitors, permeabilized, stained, and acquired. For all plots, CD3 versus IFN-γ + IL-2 are shown after gating on CD8+ T cells. For this ICS assay, a positive response is defined as a response that is at least 2× background and greater than 0.10% and values are reported after background subtraction. *Left column*: unstimulated. *Right column*: CEF stimulated. *Top row*: The background subtracted CEF response in the absence of a dead cell exclusion gate is 0.058% or no response. *Bottom row*: When the same FCS file as was used in the top row is analyzed using a dead cell exclusion gate, the background is reduced, and the net response is 0.109% and thus positive

effects on the relative percentages of different subsets reported within that gate (Fig. 5).

Conclusion: Scatter gates should be used with caution and checked by backgating to avoid unintended exclusion of relevant cells (recovery) and inclusion of irrelevant cells (purity). Backgating refers to the visualization of a final gated population in all prior gating steps (generally with those prior gates removed). This allows one to see the effect of each parent gate in the hierarchy, and can quickly identify parental gates that create undesirable effects (e.g., a too-tight scatter gate that cuts through a specific downstream subpopulation of interest).

2.6 Specific Population Gates

Definition: A specific population gate defines a cell population such as CD4+ T cells, B cells, or monocytes, focusing further subset identification within those populations. The size, placement, and

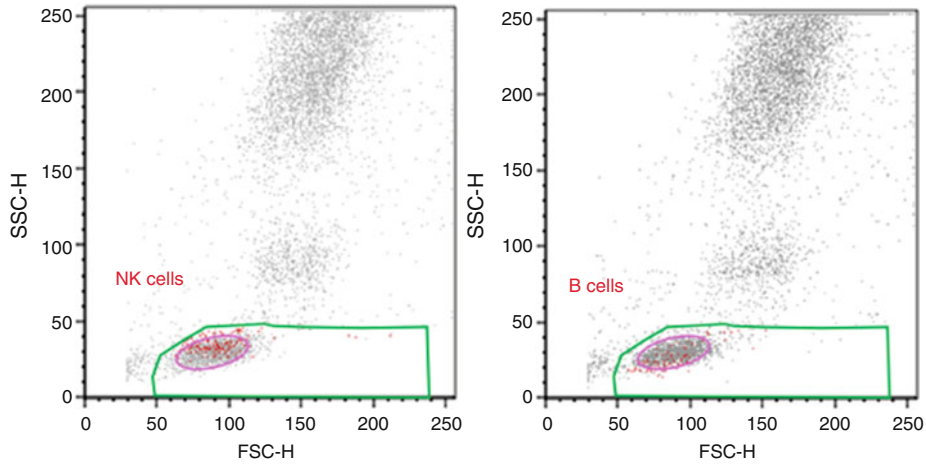

Fig. 5 Differential scatter profiles of specific lymphocyte subsets. NK cells (left panel) and B cells (right panel) were gated separately using specific markers from the green, liberal scatter gate for lymphocytes and then projected back into the scatter dimensions as red dots. Note that the pink (tight) scatter gate for lymphocytes would exclude a subset of both NK and B cells because of their biased and/or diffuse scatter profile relative to most lymphocytes

order of such gates can influence the reported results in ways that may not be obvious to an inexperienced operator.

How data is affected: Knowledge of the expression properties of specific subsets of interest can be useful for setting a parental population gate. For example, CD3 may be used as a parental gate prior to analysis of T cell subsets such as γδC5; T cells. By examining a dot plot of CD3 vs. SSC, one can identify the γδC5; T cells as CD3high relative to the rest of the CD3+ population (Fig. 6). Knowing this, care should be taken not to exclude CD3high cells when enumerating γδC5; T cells as a subset thereof.

Another example in T cell gating concerns the simple discrimination of CD4+ and CD8+ populations. Since these are largely mutually exclusive populations, it may seem irrelevant whether one gates on CD3+ CD4+ and CD3+ CD8+ populations, or rather sequentially gates on CD3+ and then CD4 vs. CD8. However, the presence of a small but variable population of CD4+ CD8+ T cells means that these two strategies will not give identical results. In fact, the former strategy will double-count these CD4+ CD8+ events as belonging to both populations, while the latter method allows their true discrimination.

Still another example with regard to T cell gating is the down-modulation of CD3, CD4, and CD8 on activated T cells. When performing stimulation assays, the gates for these markers need to be sufficiently "loose" to avoid cutting off potentially small sub-populations of activated (and thus responding) T cells that have partially down-modulated these markers.

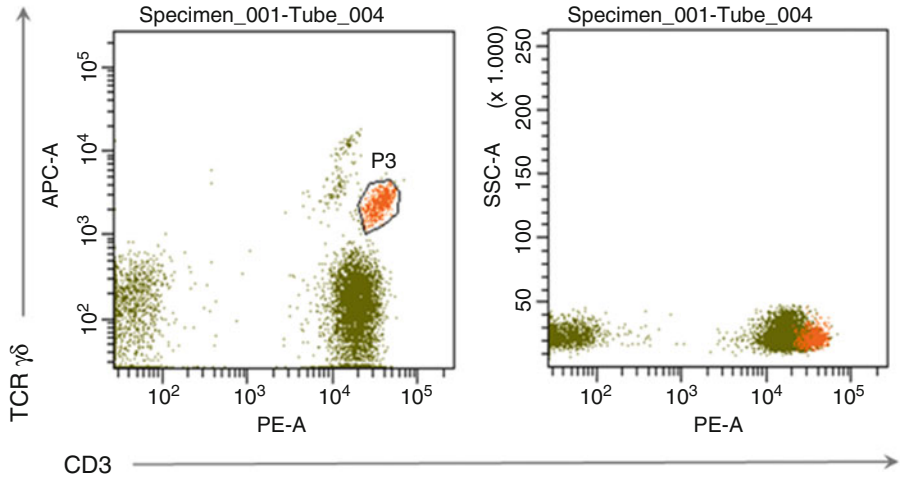

Fig. 6 γδC5;T cells are CD3high. Human lysed wash blood was stained with CD3 and TCRγδC5; antibodies. Orange population indicates TCRγδC5; cells on a plot of CD3 vs TCRγδC5; (left) and CD3 bright staining on the TCRγδC5; population depicted in CD3 vs SSc plot (right image)

Rare event gates are another source of potential variability. For example, gates on cytokine+ cells responding to specific antigen, or MHC-tetramer+ T cells, require consistent placement relative to the negative population. If the gate is drawn too close to the negative population, there will be increased and variable "background" created, from cells that are on the edge of the negative population but counted as positive. Conversely, if the gate is drawn too far away from the negative population, dim positives will be missed. In this regard, a gating control (e.g., unstimulated cells for cytokine assays or an FMO for MHC-tetramer staining) may be useful for setting the boundary of the positive gate.

Conclusion: Knowledge of the staining properties of subsets within an initial population gate is important for accurate and consistent downstream enumeration. Backgating is a useful technique for checking these initial population gates to be sure they are not unwittingly excluding populations of interest. A gating control can also be useful, particularly for rare and/or dim populations.

2.7 Data Display

2.7.1 Compensation

Definition: The premise of flow cytometry is that one marker is tagged with one fluorescent dye and the photons generated by the dye are measured in a single detector. Spectral overlap occurs when the fluorescence emission from one dye emits photons into the fluorescence emission spectrum of a neighboring detector. In a multicolor panel, spectral overlap from multiple dyes may be observed in a single detector. Electronic subtraction is applied to correct the interference caused by the spectral overlap in each channel; this is called compensation. A compensation matrix is generated to correct the spectral overlap for each dye versus all of

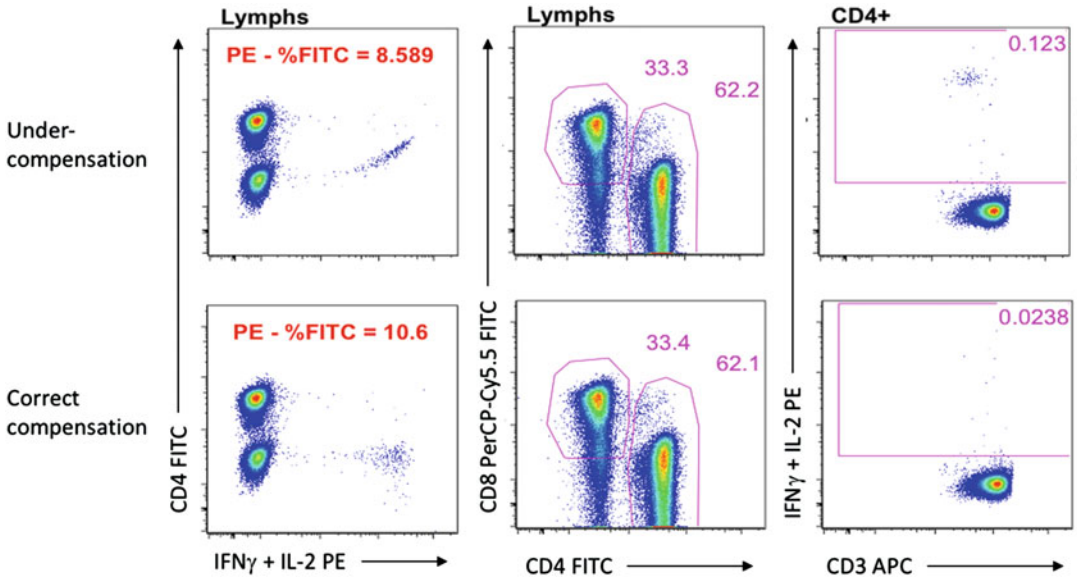

Fig. 7 Undercompensation leads to a false positive response in an ICS assay. Undercompensation between PE (IFNγ + IL-2) and FITC (CD4) created the appearance of CD4+ IFNγ + IL-2+ cells, when in fact with correct compensation these cells were CD4−. In this case, the operator used software to create a compensation matrix but neglected to verify the accuracy of the compensation matrix by visual inspection the data (e.g., using an $N \times N$ plot matrix as described in the text)

the other dyes in the panel, respectively. The goal of compensation is to specifically detect fluorescence from each dye in its on respective detector. Compensation does not change the raw data, but it merely changes how data are displayed. By reducing the apparent interference from neighboring fluorophores using compensation, the markers tagged with those fluorophores are displayed with more separation, enhancing the appearance of distinct populations.

How data is affected: During analysis, where visualizing a clear separation of populations is essential, an error in compensation may lead to an erroneous classification of cells, including false positives or false negatives (Fig. 7). It is therefore important to assess the accuracy of compensation by examining biaxial plots of all combinations of fluorescence channels, before the application of gating on fluorescence channels. Typically, an $N \times N$ set of plots is generated, with appropriate transformation (*see* Subheading 2.7.2), where N is the number of fluorescence channels in the experiment. Some flow cytometry software allows the automatic creation of such a plot layout. The user can then judge the accuracy of compensation by the degree to which populations that should be orthogonal to each other for particular markers do in fact align orthogonally. Undercompensation or overcompensation can be easily identified as an artificial curved edge to such populations.

Conclusion: It is critical to appropriately compensate cytometry data prior to analysis. To ensure proper compensation, operators should manually verify compensation for accuracy, looking for "hooks" created by overcompensation or undercompensation, as well as subnegative populations resulting from overcompensation with a neighboring detector. An easy way to review compensation is by visualizing a compensation layout containing $N \times N$ dot plots.

2.7.2 Transformation

Definition: The range of signals in flow cytometry can span very negative values (particularly after compensation) to very high values (10^4 or greater). Given the approximately log-normal distribution of many populations in flow data, a linear scale will severely distort populations and make inefficient use of the display space. However, a simple logarithmic scale is unable to display zero or negative values. Therefore, transformations have emerged that preserve the ability to display negative and zero values while approximating a logarithmic scale in the positive space. These have variably been referred to as logicle, biexponential, or VLog scales [15, 35, 36]. Some version of these is available in all modern flow cytometry software, often with automated application in a data-dependent fashion.

How data is affected: Choosing an optimal transformation is important for a number of reasons. First, visualizing the effects of compensation requires a scale that smoothly transitions through zero and displays negative data. Undercompensation or overcompensation can then be accurately judged based on how orthogonally the edges of populations align in the negative to low positive range. Second, the choice of transformation is important to efficient use of the graphical display space. If a majority of the axis length is devoted to high values (e.g., $>10^2$), and if the data consists entirely of dim events, then the transformation is not appropriate. Conversely, if a majority of the axis length is devoted to negative values, and high positive populations are compressed and difficult to distinguish, the transformation is not appropriate. Finally, an inappropriate transformation can create artifacts such as "splitting" of a negative population into what appears to be two populations ("valley artifact"). This particular artifact is a result of displaying too many decades of logarithmic scaling at the low end of the log scale (usually below 10 or 1). Since judgement of positive and negative expression is often a goal of flow cytometry, such artifacts can clearly be an impediment to interpretation.

Conclusion: Choosing the right transformation is critical for accurate visualization of compensation, for efficient use of display space, and for complete visualization of negative populations without introducing the artificial appearance of breaks in the data.

2.8 Analytical Controls

2.8.1 Unstained Cells

An unstained cell sample is often used for calculation of compensation, relative to single positive samples for each fluorochrome. However, this is not strictly required for performing compensation, if negative populations are included in each single-color control. An unstained control is, however, still useful for judging the PMT voltage gain in each sample, since it represents the position of the most negative possible population. It is generally not useful for determining gate placement on stained samples, since it does not account for either spillover from other fluorescent channels, or nonspecific binding in the channel of interest.

2.8.2 Isotype Controls

Isotype controls are sometimes useful when deciding gate placement in flow cytometry. These controls are made of the same isotype as the antibody of interest and have the same fluorescent dye attached to them. In specific instances, such as when monocytes are being stained with an antibody tagged with tandem dyes such as PE/Cy7, APC/Cy7, APC/Fire 750, APC-H7, or PE-CF594, the use of an isotype control antibody is extremely helpful. Monocytes tend to nonspecifically bind to dyes such as Cy7, Fire 750, and H7. Figure 8 shows the effect on tandem dye binding to isotype controls. Some manufacturers formulate their antibodies with reagents to reduce this background and others may sell buffers to overcome these issues (e.g., Monocyte Blocker from BioLegend).

2.8.3 Fluorescence Minus One Controls (FMOs)

In the absence of situations like those shown in Fig. 8, the major background signals in high-dimensional flow cytometry tend to come from spillover rather than nonspecific binding. As such, an FMO control, which leaves out a single antibody conjugate but maintains all other stains in the panel, is often more useful for setting gates than an isotype control in the absence of other stains.

2.8.4 Combined Isotype and FMO Controls

A control which leaves out one antibody conjugate, but replaces it with an isotype control antibody, can be considered a "combined" control. With such a control, one attempts to combine the benefits of FMO (accounting for spillover) and isotype controls (accounting for nonspecific binding). While this in theory may be the "best of both worlds," it is worth mentioning that there are still drawbacks to the use of isotype controls that are not alleviated by combining it with FMO. Specifically, the particular isotype control antibody may or may not have similar nonspecific binding properties as the test antibody [37]; and it will likely require titration of the isotype control to at least approximate those properties.

2.8.5 Internal Controls (e.g., Unstimulated Cells)

For functional readouts like cytokines in intracellular cytokine staining (ICS), the best gating control may be an internal control (e.g., unstimulated cells from the same donor). Even so, it can be difficult to decide exactly where to place the cytokine or functional

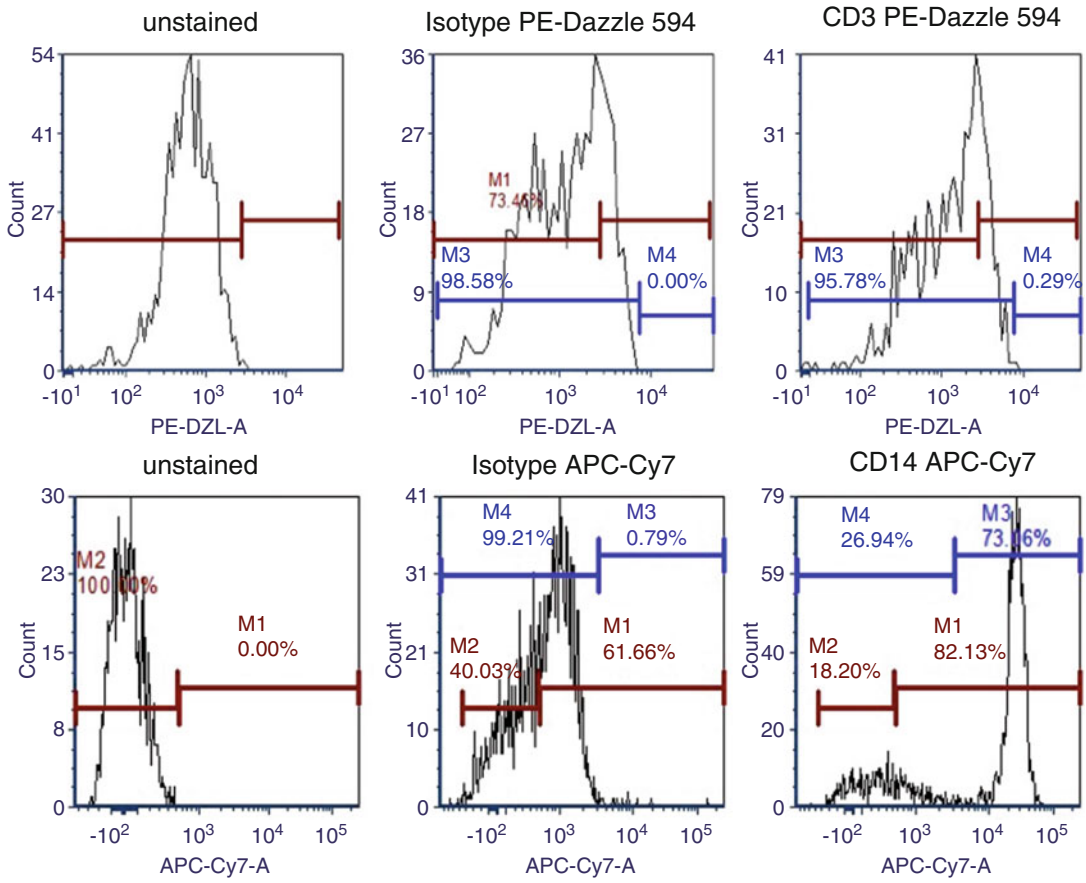

Fig. 8 Tandem dyes nonspecifically stain monocytes and thus isotype should be used to set marker gate (s) appropriately. *Top row*: Human lysed washed blood was not stained or stained with isotype control PE/Dazzle 594 or CD3 PE/Dazzle 594. Histograms are gated on FSC and SSC Singlets followed by monocyte gate (scatter based). Red marker gates (M1 and M2) indicate gate placement using unstained cells (leftmost panel) and blue marker gates (M3 and M4) depict frequency of CD3 positive cells based on gate placement with isotype control (middle panel). If care is not taken interpreting data based on unstained cells, then users may report monocytes to be CD3+. *Bottom row*: A similar case using APC/Cy7. Here we show that gates based on the isotype control are more appropriate for the positive monocyte stain, CD14

marker gate relative to the negative population. Placing the gate too close to the negative population increases the background of false positive events; but placing it too far away can reduce the readout of true positives. This becomes especially critical when dealing with rare events such as antigen-specific responses. Guidelines for this have been published, both for manual gating [24] and for gate selection based on a statistical measure [38]. For manual gating, it is generally advised to set the gate above the "halo" of cells at the edge of the negative population, since increased background, which may be sample-specific, will be the most detrimental to detection of low-level responses.

2.9 Flow Cytometry Units

It is worth noting that the readout of a gated population can be expressed in different units. Most gating software by default reports percent-of-parent statistics, that is, the percentage of cells in the parent population that are contained in the gate of interest. However, this may not always be the best readout to report.

Consider the case of lymphocytes, which have major subpopulations of B, T, and NK cells. If an individual's T cell count increases, the percentage of lymphocytes that are B and NK cells will necessarily decrease, even if their absolute frequency is unchanged. In order to avoid the effect of such reciprocal relationships, one can report absolute cell counts (per microliter of blood). However, this requires a volumetric measurement or use of counting beads. It can be done most accurately by single-platform methods (where the counting beads or volumetric measurement is done in the same flow cytometry assay as the cell population staining). Alternately, a dual platform method can be used, relating the results of a counting assay to the flow cytometry staining. For example, total lymphocyte count can be measured by a complete blood count with differential (CBCD). The total lymphocyte count can then be multiplied by the percentage of lymphocytes gated as B, T, and NK cells, to derive absolute counts of these three populations. Because of variations in the way lymphocytes are defined in the CBCD versus flow cytometry platform, however, this will necessarily be less accurate than single platform counting. Still, both methods can be made relatively reproducible [39].

Short of reporting absolute counts, one can also report population percentages but using a higher-level ancestor gate. For example, each gated population in a PBMC sample could be reported as a percent of all live cells (after exclusion of aggregates). While this is not the same as absolute counting, it does diminish the effect of changes in reciprocal populations. It is also easily done using the reported percent-of-parent statistics, by simply multiplying percentages of each ancestor gate up to the top level gate being reported. Some software programs can provide such reporting automatically by choosing the desired ancestral gate from which to report percentages.

Finally, it is possible to report not the frequency or percentage of cells in a gate, but rather their mean or median fluorescence intensity (MFI). This is particularly useful when reporting data on markers that may not have distinct positive and negative populations, but whose distribution represents a continuum ("smear") or for which shifts in expression level are the biologically important readout. Examples include CD80 and CD86 expression on dendritic cells, whose intensity relates to cell maturation/activation. Rather than measuring the percent of "positive" cells using a potentially arbitrary cutoff, it makes more sense to report MFI of the entire population. However, when comparing MFI across

experiments, day-to-day variations in staining and instrument setup will confound such results. Calibration of MFI to measured equivalent soluble fluorochrome (MESF), using beads of standardized intensity, is recommended in such cases [40].

2.10 Special Considerations

Flow cytometry experiments and therefore associated results can be affected by artifacts in the assay, which are not considered or known prior to performance of a particular assay. These artifacts can include samples with too few events or difficult-to-discern rare populations; or shifts in a cell population from one sample to another. Visual inspection of gates on all samples is therefore critical before finalizing the gating. In determining whether to move a gate for a specific sample, one should attempt to maintain a fair and unbiased approach, that is, keeping gates as similar as possible except where clear shifts in a population warrant changing the gate. Samples to be directly compared, such as stimulated and unstimulated conditions from the same donor, need to have the exact same gating applied.

When gating a large study, or when multiple operators and/or sites are involved, it becomes necessary to create a gating template. This is a document with default gates provided, that is the starting point for analysis of each batch of samples. It represents not only the hierarchy of all gates to be applied but also a "best guess" of where those gates should lie. Movement of gates from the template position should only be undertaken when necessary, and with the caveats described above in mind. In general, central analysis by a single operator (or at least verified by a single operator) is more consistent than separate analysis by different individuals or sites [27, 41].

Some types of artifacts can be anticipated and possibly avoided, including many of the factors listed in Table 1. Some variables that should be controlled to avoid excessive population shifts include: reagent lot control, consistent washing between wells or tubes, and avoiding degradation of problematic tandem dyes that can be idiosyncratic between samples. It is of course always preferable to avoid artifacts that would complicate gating in the first place, but this may not always be perfect. An example of a "preventable" population shift is that caused by brilliant dye interaction, as shown in Fig. 9.

Finally, a piece of idiosyncratic advice regarding gate placement relative to X and Y axes. Even with appropriate transformation and data display, there are almost always events that are below the axis for negative populations. As such, gates on the negative population need to be placed *over* the relevant axis to include events that are below that axis. Depending on the plot size and visual resolution, this difference may be insidious; two gates may look essentially identical, but one includes events below the axis and one does not. Experienced cytometrists therefore typically draw a gate and then drag it well beyond the axis in order to avoid this issue.

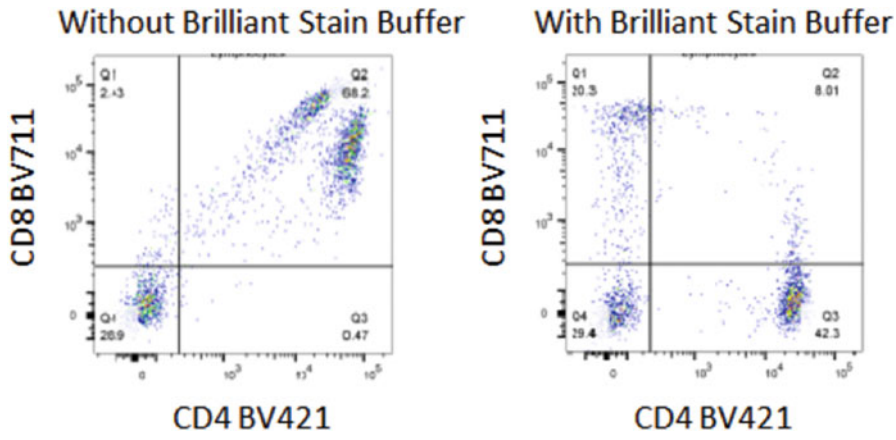

Fig. 9 Interaction between fluorophores may affect populations. Human lysed whole blood was stained with CD4 Brilliant Violet 421™ and CD8 Brilliant Violet 711™ in the presence (left) or absence (right) of Brilliant Stain Buffer™. Note loss of mutually exclusive expression of CD4 and CD8 in the left plot

3 Discussion/Conclusions

In research and clinical trial settings, the application of gates to flow cytometry data has been done largely in the absence of wide-ranging consensus or written guidelines. This is surprising given the pervasiveness of flow cytometry in immunology research and immune monitoring settings. The guidelines set forth here were initially drafted by a small group of experienced flow cytometrists from academic, industrial, and government labs. They were further vetted by presentation to the Federation of Clinical Immunology Societies (FOCIS) 2017 and 2018 annual meetings. The result is an attempt at a consensus document on the highest-level principles for gating flow cytometry data in immunological assays.

The guidelines presented here mainly focus on types of gates that are (or should be) common to most immunological assays. These include acquisition thresholds (or triggers), time gates, singlet gates, exclusion gates, and scatter gates. Examples of how the definition of these gates can affect downstream data highlight their general usefulness. We also discuss specific population gates, which tend to be more assay-centric, for example, T cell and rare event gates in the setting of in vitro-stimulated and/or antigen-specific T cell assays. Finally, we discuss the quality control of gating. This can include the use of "back-gating" to identify the effect of each gate in a gating hierarchy, allowing for optimization of the recovery of a desired population. It can also include the review of compensation for artifacts that cause downstream gates to report inaccurate results. Finally, the application of gates to multiple conditions and donors is discussed, including the need to visually inspect all files,

creating gates that can be applied across samples while still allowing for donor-specific corrections if needed.

Much of the advice presented here can be broadly summarized as follows.

1. *All relevant parameters in the data file should be used to most purely and completely define populations of interest.* These parameters include time, which can give clues to artifacts such as clogs or bubbles in the data acquisition. They also include the height, area, and/or width of the light scatter parameters, which can be used in combination to exclude cell aggregates. Expression of one or more parameters may be used to exclude irrelevant events (exclusion gating), in addition to parameters used to include relevant events. And backgating can be useful to judge the adequacy of individual gates in a hierarchy, including light scatter gates, with regard to their unbiased selection of the population of interest. Importantly, the fact that a parameter is not used in the gating scheme does not mean that it may not affect the gated results; so visual inspection of all parameters in the data is recommended.
2. *Once defined for a single sample, gates should be checked to ensure fair application across all samples.* This may require adjusting gates between donors, but it should generally not entail changes in the gate among samples or conditions to be compared within the same donor. Attempts should be made to avoid gates that slice arbitrarily through an obvious cluster of cells, including cases where part of a cell cluster lies below the axis of a histogram or dot plot. In some instances, an otherwise homogeneous cell population will display a continuous distribution of intensity for a given parameter. It may then be more sensible to report the mean (or median) intensity of the population rather than arbitrarily gating a positive and negative subset for that parameter and reporting percent positive or negative.

The eventual goal of these guidelines is not only accuracy in defining populations of interest but also reproducibility between experiments, between operators, and between donors and cohorts. This is particularly important in longitudinal and/or multisite studies. In these cases, it is best to have all gating verified by the same personnel, even if initially performed by different personnel. Further, it is advisable to continually refer to historically gated data, to ensure reproducible treatment relative to contemporary samples, for studies that are analyzed over a period of time.

In this document, we have tried to illustrate the most overarching principles that apply to gating flow cytometry data from immunological assays. There may be exceptions to these principles, but they are likely to be few. There are also undoubtedly situations where one can avoid some of these recommendations without

peril. But it is also likely that following most or all of the recommendations increases the confidence that artifacts and false conclusions will be avoided. Furthermore, there are certainly additional and more detailed recommendations that could be added to these guidelines. But with increasingly detailed recommendations comes the likelihood that they will not apply as broadly across different situations and assays. So we have mostly avoided too much specificity in these guidelines, in favor of exposing the high-level concepts behind the gating strategies discussed. We hope that wide dispersion of these concepts leads to an overall higher level of accuracy and reproducibility of flow cytometry gating.

Acknowledgments

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Chapter 6

Strategies and Techniques for NK Cell Phenotyping

Chen Ziqing, Andreas Lundqvist, and Kristina Witt

Abstract

Therapies based on activating the immune system, that is, immunotherapy, are now widely implemented in clinical praxis in patients with advanced cancer. Although cancer immunotherapy can result in long-lasting clinical responses, the majority of patients do not respond or develop resistance. Furthermore, cancer immunotherapy is being increasingly combined with other forms of immunotherapy or conventional cancer therapies. It is therefore much needed to identify biomarkers that can precisely classify what patients will benefit from the treatment without any major adverse events and to further develop the efficacy of cancer immunotherapy. While much attention has been focused on monitoring T cell responses in cancer immunotherapy, recent reports have shown that NK cells also play a major role in the response to cancer immunotherapy. The gold standard for immunoprofiling of NK cells is flow cytometry, but other technologies have emerged and include mass cytometry, multiplex immunohistochemistry, and single-cell RNA--sequencing. In this chapter we provide a detailed protocol to profile NK cells using flow cytometry, and a brief introduction to other techniques.

Key words NK cells, Flow cytometry, Immunoprofiling

1 Introduction

NK cells are innate lymphocytes that can kill virus infected and malignant cells without any prior sensitization of antigens. They play a major role in cancer immunosurveillance, and high frequency and activity of NK cells is correlated with improved prognosis in several different solid tumors and hematological malignancies. In recent years, immunotherapy based on activating NK cells has been explored in several clinical trials including bispecific antibodies or adoptive transfer of unmodified or genetically modified NK cells [1, 2]. Cancer immunotherapy and in particular treatment with immunomodulatory antibodies has now established itself as one of the pillars in cancer care. While this treatment can result in long-lasting clinical responses in patients with advanced cancers, many patients do not respond or develop resistance to the treatment. Recently, reports have demonstrated that NK cells may play an important role in the response to immunomodulatory antibodies

J. Philip McCoy, Jr (ed.), *Immunophenotyping: Methods and Protocols*, Methods in Molecular Biology, vol. 2032,
https://doi.org/10.1007/978-1-4939-9650-6_6,

[3]. Thus, it is increasingly important to perform immunoprofiling of NK cells to identify immunological predictive, pharmacodynamic, and prognostic biomarkers. The gold standard for a comprehensive analysis of immune cell subsets including NK cells in blood and tissues has for long been flow cytometry and the growing list of fluorescently labeled antibodies available for intracellular and cell surface markers make flow cytometry even more applicable. In parallel, new technologies have been developed to profile NK cells. In this chapter we describe sample processing and preparation for NK cell phenotyping using flow cytometry. We also briefly discuss novel technologies including mass cytometry, multiplex immunohistochemistry, RNA sequencing, and spatial transcriptomics that have evolved in recent years and can be used to perform immunoprofiling of NK cells.

2 Materials

2.1 Flow Cytometry and Fluorescence-Activated Cell Scanning (FACS)

1. Ice.
2. FACS tubes.
3. 1× DPBS.
4. Tabletop centrifuge.
5. FACS buffer.
   (a) 1× DPBS.
   (b) 2% FBS.
6. FcBlock.
7. Fixation solution (*see* **Note 18**).
8. Permeabilization buffer (*see* **Note 19**).
9. Fluorochrome-labeled antibodies (*see* **Note 6**).
10. DCM (*see* **Notes 12** and **16**).

3 Methods

3.1 Flow Cytometry

1. NK cell phenotyping using flow cytometric analysis requires single cell suspensions of viable cells. Possible starting materials are whole blood (*see* **Note 1**), fresh polymorphonuclear cells (PBMC) (*see* **Note 2**), frozen PBMC (*see* **Note 3**), fine needle aspiration biopsy (*see* **Note 4**), and fresh organ or tumor material (*see* **Note 5**).
2. The panel to study NK cells in the blood or tissue should be designed according to the study interest and the available flow cytometer. To simply identify human NK cells in blood or tissue the following markers are widely used and accepted: CD45, CD3, CD56, and CD16. Additional makers that are

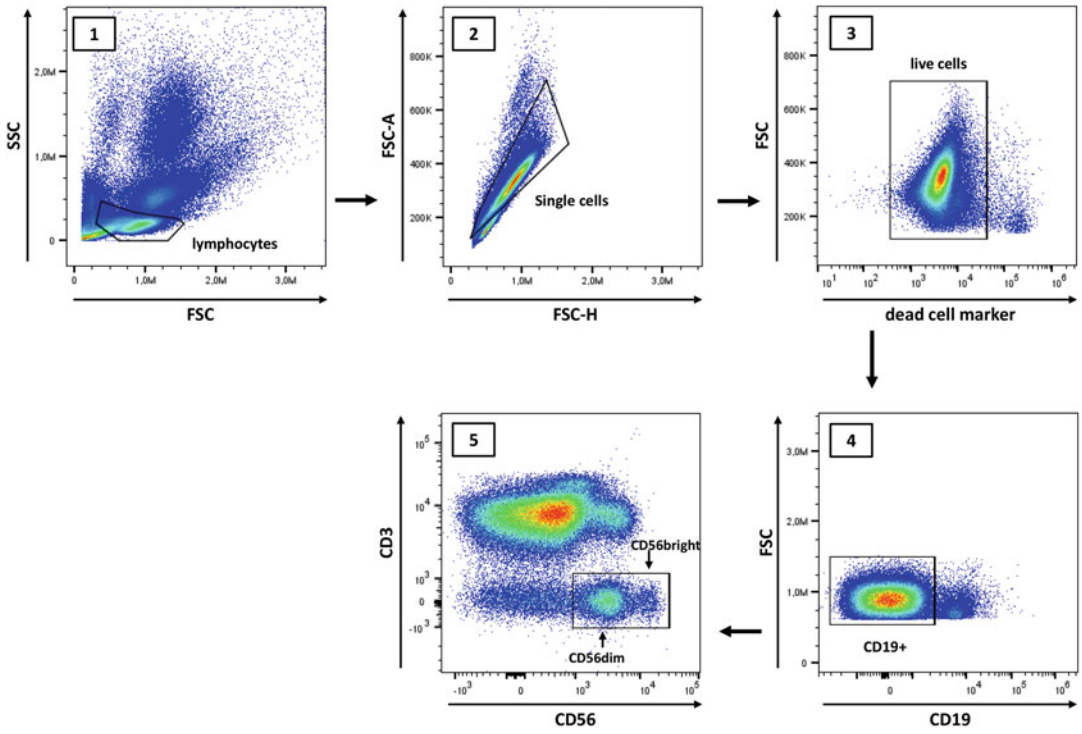

Fig. 1 Identification of NK cells in human PBMC. Human PBMC are stained and acquired on flow cytometry. NK cells are identified with the following gating strategy. (1) Gate on lymphocyte population in FSC–SSC plot. (2) Next gate on linear population in FSC-A–FSC-H plot. (3) Gate on live cells in dead cell marker–FSC plot. (4) Within the live cells gate on CD19neg cell population in CD19–FSC plot. (5) From the CD19neg population gate on CD56+CD3− cells in CD56–CD3 plot. In this plot the $CD56_{dim}$ and $CD56_{bright}$ cells appear as two separate populations

studied on NK cells are described in **Note 6**. The gating strategy to identify NK cells in human PBMCs is shown in Fig. 1.

3. The panel should be designed for the flow cytometer used in the study. Details are described in **Note 7**.
4. Samples can be stained directly in FACS tubes. Prepare FACS tubes according to the number of samples and panels (*see* **Note 8**).
5. Resuspend all samples in DPBS and count all samples.
6. Transfer 2×10^5 cells into each prepared FACS tubes (*see* **Note 9**).

   If nonfixable dead cell marker is used, continue with ***step 14****.*
7. Fill up to 1 ml with DPBS and centrifuge tubes at $700 \times g$ for 2 min.
8. Discard supernatant by inverting tube and blotting on tissue (*see* **Note 10**).

9. Vortex each sample for 3 s to loosen cell pellet (*see* **Note 11**).
10. Repeat **steps 7** and **8**.
11. Vortex each sample for 3 s to loosen cell pellet and resuspend cells in leftover DPBS.
12. Add fixable dead cell marker to samples according to the manufacturer's protocol (*see* **Notes 12** and **13**).
13. Incubate according to the manufacturer's protocol.
14. Add 1 ml of FACS buffer and centrifuge tubes at 700 × *g* for 2 min.
15. Discard supernatant by inverting tube and blotting on tissue (*see* **Note 10**).
16. Vortex each sample for 3 s to loosen cell pellet (*see* **Note 11**).
17. Repeat **steps 14–16** two times.
18. Resuspend cells to single cell suspension in leftover FACS buffer.
19. Add FcBlock to each sample at a concentration of 2.5 μg for 1×10^6 cells.
20. Prepare master mix containing surface staining antibodies. Mixes should be filled up with FACS buffer to a volume of 10–20 μl added to each sample (*see* **Note 14**).
21. Incubate samples for 20 min at 4 °C in the dark (*see* **Note 15**).
22. Repeat **steps 14–16** two times.

    For intracellular or intranuclear staining continue with ***step 26****.*
23. Vortex each sample for 3 s to loosen cell pellet and resuspend cells in 200 μl DPBS.
24. If no fixable dead cell marker was used, add nonfixable dead cell marker to all cells and incubate for 5 min at 4 °C in the dark (*see* **Note 16**).
25. Keep samples on 4 °C until acquisition on flow cytometer (*see* **Note 17**).
26. Resuspend cells in fixation solution (*see* **Note 18**).
27. Incubate samples for 30 min at 4 °C in fixation solution or according to the manufacturer's protocol.
28. Prepare permeabilization buffer (*see* **Note 19**).
29. Add 1 ml of permeabilization buffer (Perm buffer) to each sample centrifuge tubes at 700 × *g* for 2 min.
30. Discard supernatant by inverting tube and blotting on tissue (*see* **Note 10**).
31. Vortex each sample for 3 s to loosen cell pellet (*see* **Note 11**).
32. Repeat **steps 29–31**.

33. Repeat **steps 29** and **30**.
34. Vortex each sample for 3 s to loosen cell pellet and resuspend cells to single cell suspension in leftover Perm buffer.
35. Prepare master mix containing surface staining antibodies. Mixes should be filled up with Perm buffer to a volume of 10–20 μl added to each sample (*see* **Note 14**).
36. Incubate samples for 40–60 min on ice (*see* **Note 20**).
37. Add 1 ml of permeabilization buffer (Perm buffer) to each sample centrifuge tubes at 700 × *g* for 2 min.
38. Discard supernatant by inverting tube and blotting on tissue (*see* **Note 10**).
39. Vortex each sample for 3 s to loosen cell pellet (*see* **Note 11**).
40. Repeat **steps 37–39**.
41. Repeat **steps 37** and **38**.
42. Vortex each sample for 3 s to loosen cell pellet and resuspend samples in 200 μl DPBS.
43. Keep samples on 4 °C until acquisition on flow cytometer (*see* **Note 21**).

3.2 Mass Cytometry

Mass cytometry, also called cytometry by time of flight (CyTOF) is a novel platform to perform even deeper analysis of immune cell subsets compared with flow cytometry [4]. Immunophenotyping by mass spectrometry provides the ability to measure >40 proteins at a rate of 1000 cells/s, compared with 18 proteins per cell, at >10,000 cells/s for fluorescence-based flow cytometry. Theoretically, mass cytometry is capable to measure up to 100 different stable isotope tags, but the high-purity requirement of these tags limits the usage down to around 40 rare earth metal tags [5]. The sample preparation is similar to flow cytometry. In contrast to the acquisition on a flow cytometer, the single cells are analyzed in a mass spectrometer. The antibodies are not coupled to fluorochromes but instead to heavy metals. Each heavy metal conjugated to an antibody binding an NK cell will give a unique peak at an exact molecular weight. The advantage is that there is no overlap between different heavy metals; thus, no compensation needs to be performed. More than 100 heavy metals are available for mass cytometry, giving the opportunity to analyze in depth NK cell phenotypes and subsets. Due to the high degree of customization, the library of preconjugated commercially available antibodies is limited. This requires self-conjugation of antibodies but at the same time allows for the design of large individualized and specific panels. Details on the design and optimization of an NK cell-specific mass spectrometry panel have been described by Kay et al. [6].

3.3 Multiplex Immunohistochemistry

Flow cytometry and CyTOF can identify the presence of rare immune cell subsets in single-cell suspensions. While these technologies are powerful, no information regarding spatial distribution can be obtained. The localization of immune cells and their local environment indeed differ between different areas in the tumor (stromal, intratumoral, and tumor margin). The understanding of cell localization can be of great prognostic value [7]. Multiplex immunohistochemistry has the ability to provide this missing information. Sequential staining of a single section allows for the understanding of spatial cell type distribution within a tumor and to draw conclusions on cell-specific protein expression. The sequential stainings are individually acquired on a fluorescent microscope and are overlapped upon image analysis. Since the majority of antibodies are produced in mice, rats, and rabbits, antibody stripping is required after individual rounds of staining. Stripping of antibodies always have the risk to remove or damage antigens. The order in which the antigens are stained requires therefore a lot of optimization. The technology has further been developed to allow for standardization and clinical application. For example, the whole-slide eight-plex mIHC platform is set up to analyze six antigens and cell nuclei in parallel in whole FFPE sections [8]. The staining procedure is standardized, and while the secondary antibodies are not changeable, the primary antibodies can be freely selected.

There are certainly developments to perform deeper analysis using multiplex immunohistochemistry. Imaging mass cytometry is one such development, combining mass cytometry with tissue localization [9]. In this application, fresh-frozen paraffin-embedded tissue or frozen sections are stained with mass spectrometry antibodies following standard immunofluorescence protocols. With a precisely directed laser beam, tissue pieces of 1 μm^2 are removed one after the other from the section and acquired in the mass spectrometer. This allows to analyze the protein expression on the tissue section to a resolution of 1 μm^2. Another development is the CO-Detection by indEXing (CODEX) technology to enable multiplexing of antibody-tagged target epitopes. In CODEX, antibody binding events are rendered iteratively using DNA barcodes, fluorescent dNTP analogs, and an in situ polymerization-based indexing procedure [10].

3.4 Genome-Wide Analysis

Omics technologies are typically considered to be based on genomics and presents a panoramic view of the unbiased molecular determinants of NK cells. To analyze NK cells using different levels of omics approaches, different technologies can be applied, including microarrays, RNA-sequencing, single-cell RNA sequencing, and microRNA sequencing. In microarrays, thousands of biologic reactions at DNA, RNA, or protein levels can be measured or even quantified in a single experiment. With decreasing costs, RNA-seq

is increasingly used for transcriptome analysis. Unlike microarray technology which relies on fluorescent labeling, RNA-seq transforms RNA into a cDNA library, which is followed by sequencing. RNA-seq is applied to analyze the differential elements of gene expression of the whole transcriptome in a more accurate, reproducible, wider, and more reliable manner than that of other methods. The data obtained from microarray and RNA-seq technologies represent average values of cell populations. With advances in separation of single cells and establishment of cDNA libraries, single cell RNA-seq technology has emerged into a technology that facilitates the analysis of molecular profiles of a single cell from cell populations.

4 Notes

1. Whole blood without further processing can be used for KN cell phenotyping. Staining antibodies are directly added to whole blood. After incubation, all samples are washed with FACS buffer followed by red blood cell lysis. The samples are then washed again and acquired using a flow cytometer. Starting with whole blood is in particular of interest when working with patient material. The processing is minimal and reduces the risk of losing or damaging surface proteins. Especially when investigating chemokines, it has been observed that expression of some chemokine receptors (i.e., CXCR2) is lost after Ficoll density centrifugation.
2. Prior to NK cell phenotyping in fresh blood samples, PBMC should be isolated using Ficoll. PBMC contain between 5% and 15% NK cells in healthy donors. Having fresh PBMC as a starting material allows not only to perform phenotyping on the material but also allows for functional studies. However, it has been shown that the isolation of PBMC can alter surface proteins, in particular chemokine receptor [11].
3. Frozen PBMC can be used for NK cell phenotyping as well. Vital freezing of PBMC is essential in order to use frozen PBMC as starting material. It is also recommended to test beforehand, if the staining gives a similar result on frozen PBMC as observed on fresh PBMC. The freeze–thaw process has been shown to alter and damage surface proteins and should be avoided if possible [12].
4. Single cells can be obtained from fine needle aspiration biopsies using trypsin. Following sample digestion, cells should be passed through a 70 μm cell strainer to remove tissue clumps. If free DNA is present in the sample, an additional treatment with DNase is recommended.

5. Fresh tumor or organ material requires mechanic dissociation. Samples should be first cut into small pieces with scalpels in PBS, supplemented with 5% FBS, 200 U/ml penicillin, and 200 U/ml streptomycin. Tissue pieces can then be passed through a metal sieve with 80 μm mesh size. Enzymatic digestion with collagenase and dispase can be additionally performed at this moment. If free DNA is present, DNase can be added to the enzyme mix. In the final step samples should be passed through a 40 μm cell strainer to remove remaining tissue clumps.
6. NK cells are defined as $CD45^{+}$, $CD3^{-}$, $CD56^{+}$ cells. NK cells are further divided into $CD56^{bright}$ $CD16^{low}$ cytokine-producing NK cells and $CD56^{dim}$ $CD16^{+}$ cytotoxic NK cells. The NK cell activation status can be evaluated using the following markers resembling protein associated to activation or inhibition of NK cells: DNAM-1, NKG2D, NKp30, NKp44, NKp46, NKG2A. KIR receptors (inhibitory: KIR3DL2, KIR3DL1, KIR2DL1, KIR2DL2/3; activating: KIR 3DS1, KIR2DS1–5) binding different MHC class I molecules are essential for the activity and function of NK cells but are not often analyzed in NK cell phenotyping. The following costimulatory proteins are expressed of NK cells and can be of interest in the NK cell phenotyping: CD137, OX40, and CD27). A number of immune checkpoint proteins (PD-1, TIGIT, TIM-3, and VISTA) can be expressed on activated NK cells and can be included in NK cell phenotyping panel.
7. The intensity of different fluorochromes varies, and low-expression proteins should be detected with antibodies conjugated to brighter fluorochromes. Further, spectral overlaps and machine settings should be considered. Every flow cytometer is unique despite having the same lasers and detectors. Proteins for which gradual expression or changes are to be evaluated should be detected with fluorochromes that have low spectral overlap with other fluorochromes to reduce compensation errors.
8. Prepare one tube for each staining panel and one unstained tube for each sample.
9. If intracellular staining is included, it is recommended to start with a higher cell number (300,000–500,000 cells). Fixation and permeabilization can decrease cell number due to an increased number of washing steps.
10. After centrifugation a small cell pellet should be visible at the bottom of the tube. After discarding the supernatant blot tubes directly on tissue without turning the tube back up. Cell pellet loosens and resuspends in remaining liquid as soon as tubes are turned back up.

11. Alternatively, cells can also be resuspended using a hand pipette. Vortexing between washes saves time.
12. Fixable dead cell markers are amine reactive dyes. They are membrane impermeable and react with amine groups of any protein. It is therefore important to stain cells with these dyes in the absence of free protein to ensure optimal staining of dead cells.
13. Add dead cell marker to unstained tube if you intend to analyze protein expression on live NK cells.
14. Preparation of antibody master mixes for each panel is recommended to ensure comparability between samples and to reduce pipetting related errors, especially when working with volumes in the range of 0.2–1 μl. Preparation of master mixes further reduces the total handling time of the samples. Always prepare the master mix for $n + 1$ samples.
15. Samples are best stored on ice in a closed container. If samples are stored in the fridge, samples should be covered with tin foil to prevent light exposure.
16. Commonly used nonfixable dead cell markers are 7-AAD and propidium iodide (PI).
17. Nonfixed sample should be acquired within 1 h.
18. Fixation method for your samples and application should be optimized. It is common to use PFA solutions for cell fixation or commercially available kits for intracellular and intranuclear staining. Resuspend samples to homogeneous single cell suspension. Doublets cannot be separated any longer after fixation.
19. Methanol and acetone are common detergents to permeabilize fixed cells. The choice of detergent is dependent on the application (intracellular vs intranuclear staining) and protein stability. Commercially available kits include permeabilization buffers and are available for both intracellular and intranuclear staining, including the staining for phosphorylated proteins.
20. Intracellular staining requires longer incubation time than cell surface staining.
21. Fixed samples can be stored for a few hours at 4 °C prior to acquisition. Stability of fluorochromes, especially conjugated fluorochromes, is the time-limiting factor for storage of fixated samples.

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Chapter 7

Immunophenotyping of Human B Lymphocytes in Blood and in Adipose Tissue

Alain Diaz, Maria Romero, Daniela Frasca, and Bonnie B. Blomberg

Abstract

The human obese subcutaneous adipose tissue (SAT) contributes to systemic and B cell intrinsic inflammation, reduced B cell responses, and increased secretion of autoimmune antibodies. Immune cells are recruited to the SAT by chemokines released by both adipocytes and infiltrating immune cells. We describe here the characterization of B lymphocytes from the SAT and blood (control) of obese females undergoing weight reduction surgeries (breast reduction or panniculectomy). We show how to isolate the immune cells from the blood and SAT, how to characterize B cells and their subsets, and how to measure markers of activation and/or transcription factors in SAT-derived B cells and B cell subsets. We also show how to evaluate other immune cell types infiltrating the SAT, including T cells, NK cells, monocyte/macrophages, in order to measure relative proportions of these cell types as compared to the blood.

Key words Adipose tissue (AT), Body mass index (BMI), Cardiovascular (CV), Dulbecco's modified Eagle's medium (DMEM), Fetal calf serum (FCS), Free fatty acids (FFAs), Germinal center (GC), Hanks' balanced salt solution (HBSS), Insulin resistance (IR), Insulin sensitivity (IS), Peripheral blood mononuclear cells (PBMC), Red blood cells (RBC), Reactive oxygen species (ROS), Room temperature (RT), Subcutaneous adipose tissue (SAT), Stromal vascular fraction (SVF), Type-2 diabetes (T2D), Toll-like receptor (TLR)

1 Introduction

Obesity is an inflammatory condition associated with chronic activation of cells of the innate immune system and consequent local and systemic inflammation, responsible for several chronic pathologic conditions including cardiovascular (CV) disease [1], type-2 diabetes (T2D) [2–4], cancer [5], psoriasis [6], atherosclerosis [7], and inflammatory bowel disease [8]. Increased body mass index (BMI) has also been significantly associated with insulin resistance (IR), which indicates the lack of appropriate response to circulating insulin in several tissues, including pancreas, liver, muscle, and adipose tissue (AT). Recent studies have shown increased prevalence of obesity over the past 25 years in 68 million individuals living in 195 countries [9], this global obesity pandemic

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affecting all age groups. The decreased proportion of obese patients responding to vaccination [10–12] or achieving remission in response to therapy [13–15] is an emerging world health problem. The combined effect of impaired immunity and altered responses to interventions may further affect the outcome of infection, with obvious health and monetary consequences at the individual and population level.

Inflammaging, the condition of increased chronic inflammation in the elderly, is an important link between obesity, IR, aging, and age-associated diseases. Chronic low-grade (sterile) inflammation causes IR and consequent transition from metabolically normal obesity to metabolic syndrome. This occurs through both systemic inflammation and metaflammation [16], a process whereby excess nutrients promote chronic low-grade inflammation, whose metabolic hallmarks are high levels of lipids, free fatty acids (FFAs), glucose, and reactive oxygen species (ROS).

The increase in AT size is characteristic of obesity. It is now well known that the AT is not only a storage for excess nutrients but it is an active endocrine tissue. Conversion of the AT from an insulin sensitive (IS) to a IR state during obesity involves expansion of adipocyte volume and remodeling of extracellular matrix components (collagens, elastins, and the associated blood vasculature) which parallels increases in the secretion of adipokines, proinflammatory cytokines and chemokines, which are involved in the recruitment of immune cells to the AT. Failure to undergo appropriate remodeling in response to overnutrition is detrimental to body metabolic homeostasis, as excess nutrients promote metaflammation [16, 17].

There is evidence that altered innate and adaptive immune responses occur in the "calorie-stressed" AT [18]. Our results recently published in humans [19] have shown that immune cells are recruited to the *obese* subcutaneous AT (SAT) and increased RNA expression of chemokines by the adipocytes (responsible for the recruitment of immune cells to the AT) as well as RNA expression of the corresponding chemokine receptors by the immune cells [19]. Moreover, we have shown that the SAT generates autoimmune antibodies. We have identified several mechanisms responsible for the release of "self" antigens in the human *obese* SAT, induction of class switch, and production of autoimmune antibodies. We have shown that reduced oxygen availability in the *obese* SAT (leading to hypoxia and reduced mitochondrial respiration), NK cell cytotoxicity, and DNA damage induce cell death and lead to further release of proinflammatory cytokines, "self" protein antigens, cell-free DNA, and lipids. All these stimulate class switch and the production of autoimmune IgG antibodies which have been described to be pathogenic [20]. Autoimmune antibody production has been shown to be dependent on the expression of the

transcription factor T-bet by several groups [21–23], and we have also found T-bet expression in the human *obese* SAT [19].

This paper describes the characterization of B lymphocytes from the SAT and blood (control) from obese individuals undergoing weight reduction surgeries at the University of Miami Miller School of Medicine. We show here how to isolate the immune cells from blood and SAT, how to characterize B cells and their subsets and how to measure markers of activation/transcription factors in AT-derived B cells and B cell subsets. We also show how to evaluate other immune cell types infiltrating SAT, including T cells, NK cells, monocyte/macrophages, in order to measure relative proportions of these cell types as compared to blood.

2 Materials

2.1 Isolation of Lymphocytes

1. Fresh SAT specimen (100–200 g of tissue) and blood as control are from females undergoing breast reduction surgery or panniculectomy surgery (removal of lower abdominal fat) at the Division of Plastic and Reconstructive Surgery at the University of Miami Hospital.
2. Supplemented HBSS: 1× Hanks' balanced salt solution supplemented with 200 nM adenosine and 1× penicillin–streptomycin.
3. Supplemented DMEM (s-DMEM): 1× High Glucose Dulbecco's modified Eagle's medium supplemented with 15 mM HEPES, 1 mM sodium pyruvate, 1× penicillin–streptomycin, 1% BSA, and 200 nM adenosine.
4. Complete RPMI: RPMI 1640, supplemented with 10% FCS, 10 μg/mL gentamicin, 2×10^{-5} M 2-mercaptoethanol, and 2 mM L-glutamine.
5. Collagenase Type V (Sigma).
6. Sharp scissors and blender (for mincing adipose tissue).
7. Forceps.
8. Siliconized glass beakers.
9. Stainless steel 316 syringe needle, blunt tip point (gauge 18, L 6 in).
10. 300 μm Mesh (Spectra/Mesh Nylon, 30 × 30 cm square).
11. 50 mL polypropylene centrifuge tubes (BD Falcon).
12. 20 cc syringe.
13. Sigmacote (Sigma).

2.2 Staining of Blood and SVF

1. BD FACS™ lysing solution (BD).

2. ACK lysing solution (Dissolve 8.29 g NH_4Cl, 1 g $KHCO_3$, and 37.2 mg Na_2EDTA in 1 L deionized H_2O).
3. Staining buffer (Dissolve 9.8 g Hanks' balanced salts, 0.35 g $NaHCO_3$, 1 g BSA, 0.2 g sodium azide in 1 L deionized H_2O).
4. BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit containing Fixation and Permeabilization Solution and BD Perm/Wash™ buffer.
5. Fluorochrome-conjugated antibodies for staining of major lymphocyte subsets:
   (a) LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (ThermoFisher).
   (b) Pacific Blue™ anti-CD45 (clone 2D1, Biolegend).
   (c) APC anti-CD19 (clone HIB19, BD).
   (d) FITC anti-CD3 (clone HIT3a, BD).
   (e) PerCP anti-CD4 (clone L200, BD).
   (f) APC-H7 anti-CD8 (clone SK1, BD).
   (g) APC anti-TCR α/β (clone IP26, BD).
   (h) PE/Cy7 anti-TCR γ/δ (clone B1, Biolegend).
   (i) APC/Cy7 anti-CD16 (clone 3G8, Biolegend).
   (j) PE anti-CD56 (clone B159, BD).
   (k) APC anti-CD14 (clone M5E2, BD).
6. Fluorochrome-conjugated antibodies for staining of γ/δ T cell subsets:
   (a) FITC anti-TCR Vδ1 (clone TS8.2, InVitrogen).
   (b) PE anti-TCR Vδ2 (clone B6, Biolegend).
   (c) Alexa Fluor® 700 anti-CD3 (clone OKT3, Biolegend).
7. Fluorochrome-conjugated antibodies for staining of B cell subsets and germinal center (GC) B cells:
   (a) APC anti-CD19, PE anti-CD27 (clone M-T271, BD).
   (b) FITC anti-IgD (clone IA6–2, BD).
   (c) Brilliant Violet 711™-anti-CD10 (clone HI10a, Biolegend).
   (d) PE/Cy7 anti-human/mouse Bcl-6 (clone 7D1, Biolegend).
8. Fluorochrome-conjugated antibodies for staining of GC T cells:
   (a) Anti-CD3.
   (b) Anti-CD4.
   (c) APC anti-CD279 (PD-1) (clone EH12.2H7, Biolegend).
   (d) APC/Cy7 anti-CD185 (CXCR5) (clone J252D4, Biolegend).

9. Additional fluorochrome-conjugated antibodies for single color control staining: Brilliant Violet 510™ anti-human/mouse CD45R/B220 (clone RA3-6B2, Biolegend).

2.3 Detection of Single Cell mRNA

1. Invitrogen™ PrimeFlow™ RNA Assay Kit (ThermoFisher).
2. Type 1 target probe for detection of T-bet mRNA (Affymetrix).
3. TLR7 agonist CL097 (InVivogen).

3 Methods

3.1 Isolation of SAT-Derived Blood and SVF

1. Weigh the AT. The sample should be collected in HBSS or similar buffer.
2. Remove connective tissue with scissors. Proceed to cut the tissue in smaller pieces and place them in 50 mL tubes until approximately half-filled. Remove connective tissue with scissors.
3. Wash tissue three times by filling the tubes with supplemented HBSS and carefully removing the buffer below the floating tissue by aspiration with syringe and blunt needle. Use at least 2 new 50 mL tubes to collect the first wash from this step that contains most of the blood surrounding the tissue. This will be used for blood cell staining subsequently.
4. After the last wash, add one and a half volumes of supplemented DMEM and transfer to a food blender.
5. Blend the washed tissue in the blender, using very short strokes to avoid damaging the cells.
6. Transfer tissue to new 50 mL tubes and centrifuge for 3 min at 1200 × *g*.
7. Weigh 1 mg/g of tissue of collagenase type V and dissolve in s-DMEM at 1 mg/mL.
8. After centrifugation (**step 6**), remove the precipitated debris and most of the liquid with the syringe and transfer the tissue to a siliconized beaker containing about an equal volume of s-DMEM containing 1 mg/mL of collagenase.
9. Incubate for 1 h in a 37 °C water bath, gently mixing the reaction mix every 10 min to help the digestion of the tissue.
10. After incubation, filter the reaction mix through the 300 μm Mesh into 50 mL tubes to eliminate undigested larger tissue pieces. This step will require several pieces of mesh since they get clogged rapidly, as well as squeezing the mesh by gloved hand in order to recover most of the digested sample.

11. Centrifuge the tubes containing the filtrate for 5 min at 300 × *g*. Use the syringe and blunt needle to collect the pellet, which comprises the SVF, into a new 50 mL tube.
12. Wash the SVF with s-DMEM by centrifugation for 5 min at 300 × *g*. Discard the supernatant.
13. Lyse the RBC in the SVF by resuspending the cell pellet in 3 mL of the ACK lysing solution, incubating for 5 min at room temperature (RT).
14. Add s-DMEM up to at least ten times the volume of ACK, and centrifuge for 5 min at 300 × *g*.
15. Discard the supernatant and resuspend the pellet in 10 mL of s-DMEM. Filter through a 40 μm cell strainer to remove any remaining clumps.
16. Proceed to do a viability count of the SVF with Trypan Blue.
17. The protocol of isolation of adipocytes and SVF from the human SAT is adapted from [24].

3.2 Staining of the SVF for Flow Cytometry Analyses

1. Distribute 500,000 to 10^6 SVF cells in five 5 mL FACS tubes for antibody staining. Add 1 mL of FACS buffer and centrifuge for 5 min at 500 × *g*.
2. Discard the supernatant and briefly vortex to resuspend the pellet.
3. Add the adequate amount of the following combination of antibodies for membrane staining in a volume of at least 50 μL of staining buffer:

   Tube 1 (B cell phenotyping and GC B cells): LIVE/DEAD™ Fixable Aqua Dead Cell Stain, Pacific Blue-CD45, FITC-IgD, PE-CD27, APC-CD19 and Brilliant Violet (BV) 711-CD10.

   Tube 2 (T/NK-T cells phenotyping): LIVE/DEAD™ Fixable Aqua Dead Cell Stain, Pacific Blue-CD45, FITC-CD3, PE-CD56, PE/Cy7-TCRγ/δ, PerCP-CD4, APC-TCRα/β, and APC/H7-CD8.

   Tube 3 (Tγδ subsets): LIVE/DEAD™ Fixable Aqua Dead Cell Stain, Pacific Blue-CD45, Alexa Fluor 700-CD3, FITC-TCRγδ1, PE-TCRγδ2, PerCP-CD4, APC-TCRαβ, and APC/H7-CD8.

   Tube 4 (GC T cells): LIVE/DEAD™ Fixable Aqua Dead Cell Stain, Pacific Blue-CD45, Alexa Fluor 700-CD3, PerCP-CD4, APC-PD1, APC/Cy7-CXCR5.

   Tube 5 (Monocytes/NK cells): LIVE/DEAD™ Fixable Aqua Dead Cell Stain, Pacific Blue-CD45, FITC-CD3, PE-CD56, PE/Cy7-CD19, APC-CD14, APC/Cy7-CD16.

4. Incubate for 20 min at RT in the dark.
5. After incubation, add 2 mL of staining buffer and centrifuge for 5 min at 500 × *g*. Discard the supernatant and repeat the centrifugation step.
6. Discard the supernatant and add 300 μL of BD fixation buffer to all tubes except tubes 1 and 4. Briefly vortex to resuspend cells.
7. Tubes 1 and 4 require additional steps for intracellular staining. Add 250 μL of BD Fixation and Permeabilization Solution and incubate for 20 min at 4 °C in the dark.
8. Add 2 mL of BD Perm/Wash 1× buffer to the tubes and centrifuge for 5 min at 500 × *g*. Discard the supernatant and vortex briefly to detach the cell pellet.
9. Add adequate amounts of PE/Cy7 anti-Bcl-6 antibody for intracellular staining in a volume of 100 μL of BD Wash/Perm buffer to each tube. Incubate for 20 min at RT in the dark.
10. After incubation, repeat **steps 5** and **6** for these tubes.

3.3 Staining of SAT-Derived Blood for Flow Cytometry Analyses

1. Use the blood collected in Subheading 3.1, **step 3** ("Isolation of the blood and the SVF from the AT"). Centrifuge the 50 mL tubes containing the first wash of the AT sample for 5 min at 500 × *g*. Carefully remove the supernatant by slowly inverting the tube without disturbing the more viscous bloody fluid and pellet that remain at the bottom of the tube. A volume of approximately 500 μL of blood fluid and pellet should remain in the tube.
2. Vortex briefly to resuspend the pellet and transfer 100 μL of blood to four 5 mL FACS tubes for antibody staining.
3. Add the same volume and combination of antibodies for membrane staining used in **step 3** in Subheading 3.2.
4. Incubate for 20 min at RT in the dark.
5. Add 2 mL of BD FACS™ lysing solution to each tube, vortex to mix and incubate for 15 min at RT in the dark.
6. Centrifuge the tubes for 5 min at 500 × *g*. Discard the supernatant and briefly vortex.
7. Add 2 mL of staining buffer to each tube and centrifuge for 5 min at 500 × *g*.
8. Perform **steps 6–10** from Subheading 3.2 for intracellular staining of blood.

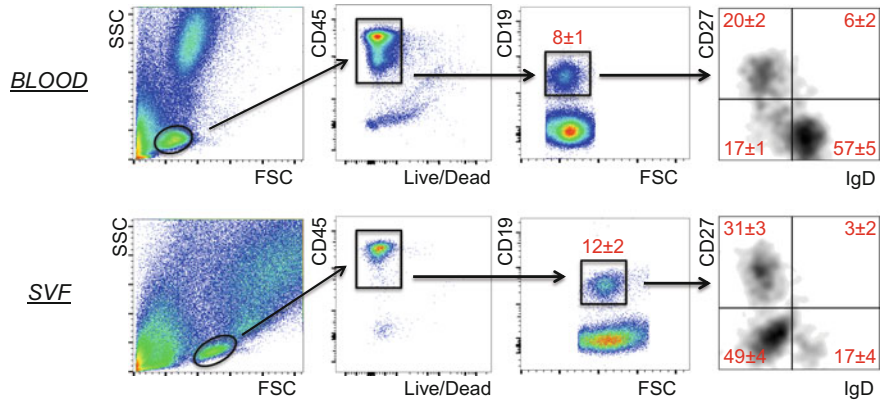

Fig. 1 Gating strategies and frequencies of B cells in the obese SAT versus blood. The blood and the SVF from the SAT of the same individual were stained to evaluate the frequencies of B cells. Gating strategies and a representative dot plot from one individual are shown. Means ± SE from 20 individuals are shown in red. The figure is adapted from Frasca D et al., PLOS One, 2018

3.4 Acquisition and Analysis of Lymphocyte Populations in SAT-Derived Blood and SVF (see Note 1)

1. Single color controls should be prepared for each experiment to assist with compensation. We routinely use cryopreserved normal PBMC from healthy donors for single color staining.
2. Follow your instrument guidelines to perform automatic compensation using the single color controls as well as the unstained cells.
3. Acquire at least 50,000 (or as many as possible) events in the Live/CD45+ gate.
4. After acquisition we export the files and perform the analyses at a later time with the FlowJo 10.0.6 software. Figure 1 shows our gating strategy and analysis of B cell subsets in blood versus SVF from the same obese individual.

3.5 Detection of Single Cell T-Bet mRNA by Amplified Fluorescence In Situ Hybridization Using PrimeFlow mRNA Assay (see Note 2)

1. Dispense the cells in 12-well culture plates at a concentration of 10^6 cells/mL in c-RPMI (2 to 5 × 10^6 cells/well).
2. Two culture conditions are set up. Cells are left unstimulated or stimulated with the TLR7 agonist CL097 at 5 μg/mL.
3. Place plate inside a 5% CO_2 incubator at 37 °C for 24 h.
4. Collect the cells with a serological pipette and count them with Trypan Blue. Up to 5 × 10^6 cells can be used per reaction.
5. Dispense the cells in FACS tubes for membrane staining. There should be a no probe control tube per condition, that is, unstimulated and TLR7-stimulated cells.
6. Perform membrane staining as described above with the following antibodies: LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (ThermoFisher L34966), Pacific Blue™ anti-CD45 (clone 2D1), APC Anti-CD19 (clone HIB19).

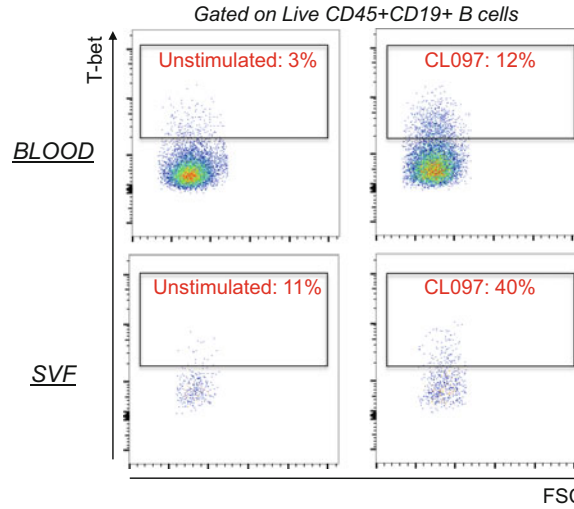

Fig. 2 Frequencies of T-bet-positive B cells in the obese SAT versus blood as evaluated by PrimeFlow RNA assay. SVF and PBMC from age-, gender-, and BMI-matched obese healthy donors were stained to evaluate the frequencies of T-bet-positive B cells. Briefly, cells were labeled with the live/dead detection kit and then with anti-CD45 and anti-CD19 antibodies to detect B cells. For T-bet mRNA detection, target probe hybridization was performed using type 1 (Alexa Fluor 647) probe for T-bet. Cells were unstimulated or stimulated with the TLR7 agonist CL097. Cells were incubated for 2 h with the probe in a precisely calibrated incubator set to 40 °C. All samples were then incubated with the PreAmplification (PreAmp) reagent for 2 h and the Amplification [9] reagent for an additional 2 h at 40 °C. After signal amplification, cells were incubated with the labeled probe at 40 °C for 1 h. Cells were washed and suspended in staining buffer prior to acquisition. The figure is adapted from Frasca D et al., PLOS One, 2018

7. After staining, proceed to the PrimeFlow mRNA Assay protocol as per manufacturer's instructions. We follow exactly the protocol provided by the manufacturer (ThermoFisher), and it will be briefly described in the following steps. For a better managed workflow we perform the assay in 2 days as recommended.

   Day 1: Fixation, permeabilization and target probe hybridization.

8. Wash once with Flow Cytometry Staining Buffer. Spin cells at 500 × *g* for 5 min at 2–8 °C.
9. Fix cells in PrimeFlow RNA Fixation Buffer 1 for 30 min at 2–8 °C.
10. Wash twice with 1× PrimeFlow RNA Permeabilization Buffer with RNase Inhibitors. Spin cells at 800 × *g* for 5 min at 2–8 °C.

11. Fix cells in 1× PrimeFlow RNA Fixation Buffer 2 for 60 min at RT.
12. Wash twice with PrimeFlow RNA Wash Buffer. Spin cells at 800 × *g* for 5 min at RT (room temperature).
13. Perform Target (T-bet) Probe hybridization for 2 h at 40 °C. Invert to mix after 1 h.
14. Wash once with PrimeFlow RNA Wash Buffer. Spin cells at 800 × *g* for 5 min at RT.
15. Wash once with PrimeFlow RNA Wash Buffer with RNase Inhibitors. Spin cells at 800 × *g* for 5 min at RT.
16. Store samples overnight.
    Day 2: Signal amplification.
17. Perform PreAmp hybridization for 1.5 h at 40 °C.
18. Wash three times with PrimeFlow RNA Wash Buffer. Spin cells at 800 × *g* for 5 min at RT.
19. Perform Amp hybridization for 1.5 h at 40 °C.
20. Wash twice with PrimeFlow RNA Wash Buffer. Spin cells at 800 × *g* for 5 min at RT.
21. Perform Label (fluorescent) Probe hybridization for 1 h at 40 °C.
22. Wash twice with PrimeFlow RNA Wash Buffer. Spin cells at 800 × *g* for 5 min at RT.
23. Wash once with PrimeFlow RNA Storage Buffer or Flow Cytometry Staining buffer. Spin cells at 800 × *g* for 5 min at RT.
24. Analyze samples on the BD LSR Fortessa flow cytometer.
25. Single-color controls consisting of stained PBMC are used for compensation in each experiment. We do not perform the beads based compensation suggested in the manufacturer's instructions.
26. Acquire at least 50,000 (or as many as possible) events in the Live/CD45+ gate.
27. After acquisition we export the files and perform the analyses at a later time with the FlowJo 10.0.6 software. Figure 2 shows the frequencies of T-bet-positive B cells in the obese SAT versus blood from age-, gender-, and BMI-matched obese healthy donors.

4 Notes

1. All FACS tubes containing stained blood and SVF should be filtered to eliminate clumps and prevent clogging of the flow cytometer. We recommend using Falcon tubes with a cell

strainer cap (BD Falcon) and Pasteur pipettes to perform the filtering. For the combination of colors that we use, the instrument used for acquisition should be equipped with at least the Violet (405 nm), Blue (488 nm), and Red (640 nm) lasers. Our instrument is a BD LSR Fortessa HTS analyzer running the DiVa-8 software, equipped with five lasers and capable of 19-color analysis.

2. To perform this assay we use the isolated SVF as well as PBMC from age-, gender- and BMI-matched obese healthy donors. The purpose is to detect the mRNA of the transcription factor T-bet in B cells using this novel technique in combination with flow cytometry. The transcription factor T-bet is encoded by the *tbx21* gene, and it is known to be associated with the secretion of IgG2a/c antibodies in mice [21–23] (IgG1 in humans), which include pathogenic (autoimmune) antibodies [20].

Acknowledgments

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Dandona L, Dandona R, Dharmaratne SD, Duncan BB, Eshrati B, Esteghamati A, Feigin VL, Fernandes JC, Furst T, Gebrehiwot TT, Gold A, Gona PN, Goto A, Habtewold TD, Hadush KT, Hafezi-Nejad N, Hay SI, Horino M, Islami F, Kamal R, Kasaeian A, Katikireddi SV, Kengne AP, Kesavachandran CN, Khader YS, Khang YH, Khubchandani J, Kim D, Kim YJ, Kinfu Y, Kosen S, Ku T, Defo BK, Kumar GA, Larson HJ, Leinsalu M, Liang X, Lim SS, Liu P, Lopez AD, Lozano R, Majeed A, Malekzadeh R, Malta DC, Mazidi M, McAlinden C, McGarvey ST, Mengistu DT, Mensah GA, Mensink GBM, Mezgebe HB, Mirrakhimov EM, Mueller UO, Noubiap JJ, Obermeyer CM, Ogbo FA, Owolabi MO, Patton GC, Pourmalek F, Qorbani M, Rafay A, Rai RK, Ranabhat CL, Reinig N, Safiri S, Salomon JA, Sanabria JR, Santos IS, Sartorius B, Sawhney M, Schmidhuber J, Schutte AE, Schmidt MI, Sepanlou SG, Shamsizadeh M, Sheikhbahaei S, Shin MJ, Shiri R, Shiue I, Roba HS, Silva DAS, Silverberg JI, Singh JA, Stranges S, Swaminathan S, Tabares-Seisdedos-R, Tadese F, Tedla BA, Tegegne BS, Terkawi AS, Thakur JS, Tonelli M, Topor-Madry R, Tyrovolas S, Ukwaja KN, Uthman OA, Vaezghasemi M, Vasankari T, Vlassov VV, Vollset SE, Weiderpass E, Werdecker A, Wesana J, Westerman R, Yano Y, Yonemoto N, Yonga G, Zaidi Z, Zenebe ZM, Zipkin B, Murray CJL (2017) Health effects of overweight and obesity in 195 countries over 25 years. N Engl J Med 377(1):13–27. https://doi.org/10.1056/NEJMoa1614362

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Chapter 8

Multiparametric Flow Cytometry Analysis of Naïve, Memory, and Effector T Cells

Ankit Saxena, Pradeep K. Dagur, and Angélique Biancotto

Abstract

Polychromatic flow cytometry enables the detection and characterization of markers which are helpful in defining phenotype of various cell subsets. Here we describe flow cytometry-based method to characterize phenotype of naïve, memory, and effector T cells. Being able to differentiate these cells is crucial in understanding immune response, and immune profiling. Naïve T cells enable the body to fight off new, unrecognized infections and diseases, and memory T cells are enriched for response to recall antigens. Furthermore, the antigen-experienced T cell populations can be broadly divided into effector and memory cell compartments, both of which are needed for sustaining a responsive immune system. Simplistically, the effector T cells require active antigenic stimulation to eliminate pathogens. On the other hand, memory T cells are described as cells which remain present in the absence of antigenic stimulation and have the capacity to expand rapidly upon secondary challenges. Recently, with the identification of central and effector memory T cell subsets, tremendous efforts have been devoted to characterize markers on the surfaces of these cells. Though, various markers have been used to identify the subsets, no single marker that segregates one subset from the other has been described. Thus, multiple markers are needed to subset the cells in order to characterize them. Here we report the verification of a nine-color panel (CD3, CD4, CD8, CD45RO, CD28, CD95, CCR7, Live/Dead Aqua, dump channel-CD19, CD14, CD56, CD16) that can successfully identify six distinct CD4 and CD8 T cell populations within the naïve and effector cell subsets from human donors.

Key words Immunophenotyping, Multicolor flow cytometry, Whole blood, T cells, Memory T cells, Naïve T cells

1 Introduction

T cell phenotype has long been used as means of functionally classifying T cell subsets [1, 2]. The development of techniques for proteins detection at the single-cell level allowed detailed correlations between the functional properties of T-cells and their phenotype [3]. Following positive and negative selection in thymus CD4+ and CD8+ T cells are released in the periphery as mature naïve T cells. Mature CD4 and CD8 T cells in the extrathymic environment are long-lived cells which can remain in interphase

J. Philip McCoy, Jr (ed.), *Immunophenotyping: Methods and Protocols*, Methods in Molecular Biology, vol. 2032, https://doi.org/10.1007/978-1-4939-9650-6_8,

for many weeks or months [4, 5]. In response to antigenic challenge naïve T (TN) cells with specificity for a certain antigenic epitope proliferate and differentiate and later die >95%, after antigenic clearance leaving behind a small pool of long-lived memory cells [6, 7]. The process of conversion of a naïve T cell to memory is a sequential process where less differentiated cells give rise to more differentiated progenies in response to antigenic stimulation or homeostatic signaling. These subsets are often associated with signature molecules and display phenotypic and gene specific properties which are useful to define them as cluster of cells. Though the progression of the naïve and effector memory cell subsets can be broadly staged as different subsets such as naïve T (TN), T-stem cell memory (TSCM), T-central memory (TCM), T-terminal memory (TTM), T-effector memory (TEM), and T-terminal effector (TTE) [7]. While naïve T cells are fairly homogeneous, diversity becomes extreme in the antigen-experienced memory compartment. The precise identification of these subsets is pivotal for both basic as well as clinical immune monitoring of patients undergoing immunotherapy for T cell-based therapeutic approaches [8].

Since multiparameter flow cytometry approaches are becoming more popular, if not routine, in immunology laboratories, it would be logical that human T cell differentiation should be delineated using a minimum set of canonical markers, that is, CD45RO (or CD45RA), CCR7, CD28, and CD95. The differential expression of these markers allows the identification of six subsets in the peripheral blood of healthy humans: T_N, T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells (Fig. 1).

Multicolor flow cytometric analysis of T cells has revealed that a single marker or a combination of two markers does not allow the identification of pure TN cells [2]. Ideally, the more markers are included to define TN cells in a polychromatic panel, the better. We describe a nine-color panel to identify the abovementioned subsets (Table 1).

2 Materials

Prepare all solutions using distilled water (dH_2O) and analytical grade reagents. Prepare and store all reagents at room temperature (RT) (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials.

1. Sample material: human peripheral blood, bone marrow (in anticoagulant—heparin, EDTA etc.), lymphoid tissue.
2. RBC lysis buffer—ammonium chloride lyse (10× concentration) NH_4Cl (ammonium chloride) 8.02 gm; $NaHCO_3$ (sodium bicarbonate) 0.84 gm; EDTA (disodium) 0.37 gm; dissolve in 100 mL with dH_2O. Store at 4 °C for 6 months.

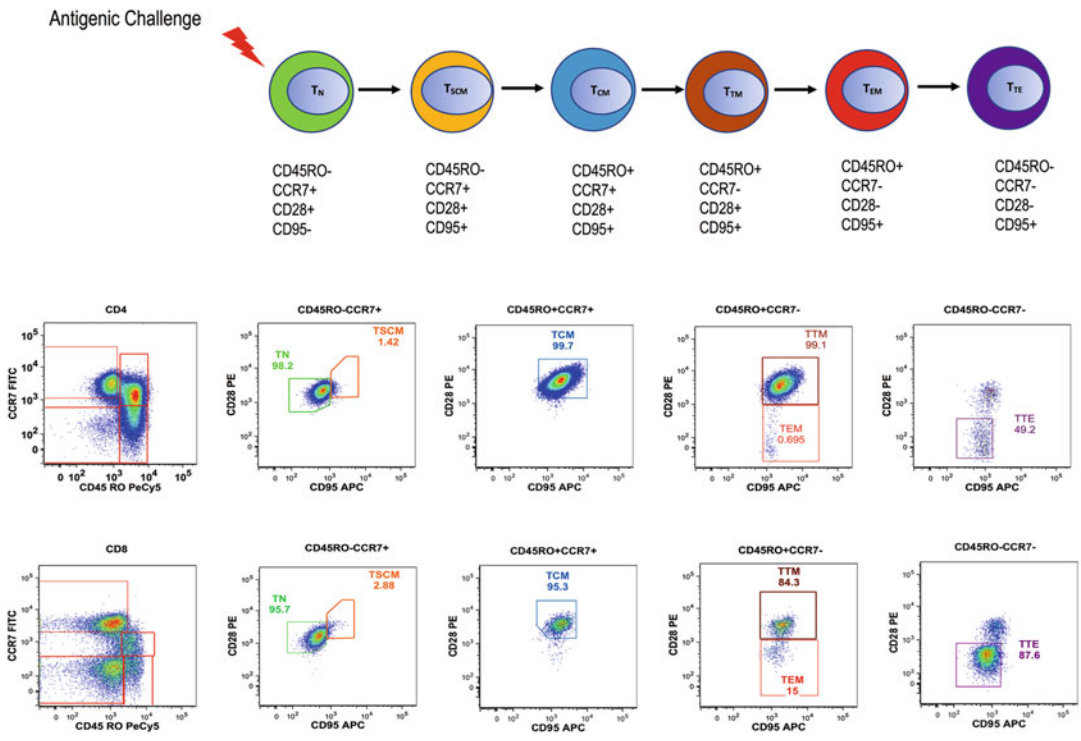

Fig. 1 T cell Heterogeneity among naïve and memory subsets. CD45RO, CCR7, CD28, and CD95 helps in identifying six major subsets of T cells. While differentiating (through activation) from T_{SCM} to T_{CM}, T_{TM}, T_{EM} and culminating in T_{TE} cells, memory T cells progressively lose or acquire specific surface protein markers. Following encounter with antigen, these naive T cells develop into effectors, for which phenotype is highly dynamic and unpredictable. When the antigen is cleared, surviving effector T cells that survive return to memory state. Bottom-Multiparametric flow cytometry identification of multiple T cell subsets among naïve and memory compartment in the peripheral blood of healthy individuals according to expression of CD45RO, CCR7, CD28, and CD95

Working solution dilute 10 mL 10× concentrate with 90 mL dH_2O. Refrigerate until use.

3. Staining buffer—1× phosphate buffered saline (PBS): dissolve 8 gm NaCl, 0.2 gm KCl, 1.15 g Na_2HPO_4, and 0.2 gm KH_2PO_4 in 800 mL distilled water (dH_2O), 10% FCS or 1% bovine serum albumin (BSA). Adjust the pH to 7.4 and the final volume to 1 liter (*see* **Note 1**).
4. Fluorochrome labeled antibody diluted to the appropriate concentration as determined by titration and viability dye (Table 2) (*see* **Note 2**).
5. 12 × 5-mm round-bottom polystyrene test tubes or 96-well round-bottom, V-bottom or flat bottom microtiter plates for high-throughput screening (HTS) (*see* **Note 3**).
6. 40–100-μm nylon mesh for isolating cells from lymphoid tissues.

Table 1
Instrument configuration. The panel was optimized for an LSR II with the listed optical elements

				Optical filters			
Laser wavelength (nm)	Laser power (mW)	Laser type	Spectral range for detector (nm)	Dichroic #1 (nm)	Dichroic #2 (nm)	Band pass (nm)	Fluorochrome
633	200	DPSS	750–810	–	740	780/60	APC-Cy7
			650–670	685	–	660/20	APC
532	150	DPSS	760–800	–	740	780/40	PE-Cy7
			640–680	690	640	660/40	PE-Cy5
			562–587	600	–	575/25	PE
488	100	DPSS	505–525	685	505	515/20	FITC
405	100	DPSS					
			590–620	630	595	605/40	BV605
			564–606	595	630	660/40	BV650
			540–580	557	535	560/40	AquaBlue
			425–475	535	–	450/50	BV421

In our set-up the dichroic filters act as long pass filters (Dichroic #2) for one detector, sending longer wavelengths through the respective band pass filters, and reflecting shorter wavelengths down the optical path to the next dichroic filter (Dichroic #1). *DPSS* Diode pumped solid state

Table 2
Antibody panel details: antigen specific mAb used for various cell surface antigens

Specificity	Fluorochrome	Ab clone	Vendor	Catalog number	Volume
CD3	BV450	UCHT1	BD Biosciences	560365	3.5 µL
CD4	APC-Cy7	RPA-T4	BD Biosciences	557871	3.5 µL
CD8	BV605	SK19	BD Biosciences	56416	3.5 µL
CD28	PE	CD28.2	BD Biosciences	555730	15.0 µL
Dead cells	AquaBlue	–	Life Technologies	L34957	100 µL[a]
CCR7	FITC	150503	R&D	FAB197F	10 µL
CD45RO	PE-Cy5	UCHL-1	BD Biosciences	555494	15 µL
CD95	APC	DX2	BD Biosciences	558814	15 µL
CD20	PE-Cy7	L27	BD Biosciences	335793	15 µL
CD56	PE-Cy7	NCAM16.2	BD Biosciences	335809	15 µL
CD14	PE-Cy7	M5E2	BD Biosciences	557742	3.5 µL
CD16	PE-Cy7	3G8	BD Biosciences	557742	3.5 µL

APC allophycocyanin, *APC-Cy7* allophycocyanin–cyanine H7 tandem, *FITC* fluorescein isothiocyanate, *PE* phycoerythrin, *PE-Cy7* phycoerythrin–cyanine 7 tandem, *Pe-Cy5* peridinin–cyanine 5 tandem, *BV450* brilliant violet 450, *BV605* brilliant violet 605 violet
aAquaBlue was prediluted 3.5 µL of Aqua blue stock in 1 mL of FACS buffer

7. Centrifuge with adaptors for 5 mL, 12 × 5-mm round-bottom test tubes, 50- and 15 mL falcon tubes or 96-well round-bottom microtiter plates.
8. Fixation buffer: 2% paraformaldehyde in 1× PBS.
9. Compensation beads (BD Biosciences).
10. Flow cytometer.
11. FlowJo analysis software (Tree Star Inc.).

3 Methods

3.1 Blood Sample Collection and Cell Preparation

1. Whole Blood Lysis: collect blood in sodium heparin, sodium citrate, or EDTA tube and keep it at RT on a nutator. Transfer 5–10 mL blood with a disposable Pasteur pipette into a 50 mL falcon tube and add 40–45 mL of RBC lysis buffer. Place the 50 mL falcon tube on a nutator for 2 min. Centrifuge tube at 300 × *g* for 5 min at 25 °C. Carefully remove the lysis buffer and break the cell pellet by slow vortex or gentle tapping. Repeat addition of 45–50 mL of RBC lysis buffer and keep cells on nutator for 1 min. Centrifuge tube at 300 × *g* for 5 min at 25 °C followed by lysis buffer removal and pellet breaking. Now add 10 mL of staining buffer (RT) to the cells and give a final wash to replace remnant RBC lysis buffer with the staining buffer. Pellet the cells (5 min, 300 g) and aspirate the supernatant. Centrifuge tube at 300 × *g* for 5 min at 25 °C to make a final cell concentration of 1–2 × 10^6cells/mL (*see* **Note 4**).
2. Add fresh staining buffer and adjust cell suspension to a concentration of 1–2 × 10^6 cells/mL in ice-cold PBS, 10% FCS. If large clumps seen the sample may be passed through the 40–100 μm mesh to get rid of clumps and obtain single cell suspension. Cells are usually stained in polystyrene round-bottom 12 × 75 mm^2 Falcon tubes. However, they can be stained in any container for which you have an appropriate centrifuge (e.g., test tubes, microcentrifuge tubes, and 96-well round-bottomed microtiter plates) (*see* **Note 5**).
3. Add titrated amount of fluorochrome-conjugated antibodies and live/dead aqua stain along with FcR blocker or in the presence of 2 μL of normal mouse serum to reduce background staining.
4. Incubate cells at 4 °C for 15 min (*see* **Note 6**).
5. Add 2 mL of staining buffer and wash the cells (5 min, 300 g). Resuspend the pellet in 0.4–0.5 mL of staining buffer.
6. If sample acquisition is to be done after 24 h, then fix cells using fixation buffer. Add 0.5 mL of fixation buffer to the cells and incubate at RT for 15–20 min in dark (*see* **Note 7**).

7. Pellet the cells (5 min, 500 g) and decant the fixation buffer-containing supernatant (paraformaldehyde is toxic: handle with care; dispose of according to material safety regulations) (*see* **Note 8**).
8. Resuspend cells with 0.5 mL of staining buffer keep it in dark and cold till samples are acquired on cytometer. (It is advised to run processed samples as soon as possible or within 72 h of staining.)

3.2 Data Collection on Flow Cytometer

Specific methods depend on the available make and model of flow cytometer. It is important to appropriately establish forward and side scatter gates to exclude debris and cellular aggregates from analysis. If cells are stained with multiple fluorescent labels, attention must be paid to balance the signal amplification among photomultiplier tubes (PMTs) in a way that particular fluorophore is brighter in its respective PMT in order to minimize potential spillover between fluorochromes and detectors.

1. Single-color controls for each fluorochrome is prepared and unstained cells are kept as negative control. Single-color controls can be prepared both by using cells or compensation beads as long as bright signal is observed in each respective detector PMT (*see* **Note 9**).
2. Here a BD LSR-II was used to acquire stained samples (Table 3) using BD FACS DIVA v8.0 (BD) acquisition and analysis software.

Table 3
Rationale for panel development. The table highlights important points to be considered in order to successfully design and execute a multicolor panel. Points to be considered are ordered under priority rating followed by rationale and reagents concerned

Priority rating	Category	Rationale for priority level	Reagents concerned
1	Dump channel	Detector with least number of good conjugates available	Monoclonal antibodies against CD16, CD14, CD56, and CD20 human antigen
2	Live dead	To gate out dead cells	AquaBlue live/dead dye
3	T cell subset markers	Identify CD4+ and CD8+ T cell compartments	Monoclonal antibodies against CD3, CD4, CD8
4	Differentiation markers	Reason for development of panel	CCR7 and CD45RO human antigen
5	Differentiation markers: additional	Identification of T_{TE} and T_{SCM} subsets within naïve-like cells	Monoclonal antibodies against CD95 and CD28 human antigen

3. Open DIVA software and connect to the instrument. Look for the green light appearing on the right bottom corner of the software to ensure proper connection. Once the instrument is connected, establish a new experiment in browser window and add appropriate number of tubes to acquire.
4. When the workstation is connected to the cytometer and an experiment is open, a gray pointer icon is displayed next to tubes in the browser. To activate acquisition controls, click the icon next to the tube you want to acquire to set as current tube pointer. The icon turns green and the tube becomes the active tube in the acquisition dashboard.
5. Under tab view select, Browser, Inspector, Cytometer, Acquisition, dashboard, Worksheet panels.
6. Click the "Parameters" tab in the "Cytometer" window and delete all the parameters that you do not need. To delete parameters, click the selection button next to each parameter that you want to delete. Hold down the Ctrl key to select more than one. After you are finished selecting, click the "delete" button. if needed, parameters can be added or modified by clicking the "Add" button.
7. Use the experiment layout to create labels, enter values for keywords, and enter acquisition criteria for each tube in an experiment.
8. Add or change labels. Select the field(s) listing the fluorochromes to be labeled, and type to enter a label.
9. Once you have created your experiment, you are ready to create compensation controls. Select experiment > compensation setup > create compensation controls. Alternatively, right-click the experiment icon and select compensation setup > create compensation controls. A dialog appears where you can add or delete controls, define label-specific controls, or change the order of the compensation controls.
10. Leave the "Include separate unstained control tube/well" checkbox selected when you are running unstained sample as one of your compensation controls.
11. Record data for the unstained and single-color controls approximately 5000 events for beads or cells, using appropriate signal amplification by PMTs to ensure that bright signal is observed in each respective detector PMT (*see* **Note 10**; compensation controls can only be acquired on normal worksheet, not on global worksheet).
12. At the end, select Experiment > Compensation setup > Calculate compensation.

13. Enter a compensation setup name, click Link and Save, and click OK. To apply compensation values to that experiment's cytometer settings, click "Apply Only."
14. Toggle back to global work sheet (in case gating strategy changes in different tube then use "sheet" instead of "Global work sheet").
15. Use menu commands or plot buttons to create dot plots, contour plots on worksheet.
16. To create a plot using a plot button, click the appropriate button on the worksheet toolbar, and click once on the worksheet to draw a plot of default size.
17. Acquire cells at an event rate of 3000–5000 events per second and record data.
18. Use a series of plots and establish a gating hierarchy to ensure recording of debris and doublet free live T cells (at least 250,000 single live T cells to ensure sufficient number of rare subsets to analyze in recorded data file, if needed).
19. Postacquisition, the fcs files should be transferred as follows: Right-click on the Experiment and select Export fcs Files. Save files in the desired folders. This will transfer all your compensation files as well as sample fcs files.

3.3 Data Analysis

1. On Flow-jo software open a new workspace and add compensation fcs files and samples fcs files in separate group folders.
2. If required, compensation can be recalculated using compensation fcs files and newly calculated compensation can be applied to the sample fcs files for further data analysis (*see* **Note 11**).
3. Open the fcs files and start creating gates as follows.
4. Define the gated populations as shown in (Fig. 2a, b).
5. Lymphocyte gate: Create bivariant polychromatic plot and gate click FSC-A on X-axis and SSC-A on Υ axis. Make a polygonal gate around lymphocyte population as shown. Please ensure in active gate check box gate events inside is checked on the flow-jo plot.
6. Singlet gating: select gate and right-click, select drill down bivariant polychromatic plot with FSC-A on X axis and FSC-H on Υ axis for preselected lymphocytes. Make a gate around singlets. If the area scaling for FSC detector and Blue LASER is properly adjusted while data recording, then singlets will be visible on a diagonal line when FSC-H and FSC-A parameters are plotted as X and Υ axis of bivariate plot. Doublets will fall toward FSC-A but not FSC-H and can be gated out to identify single cells.
7. Live cell gating: Gate Aqua LD negative subsets as live cells.

8. Gate dump negative cells excluding out B cells, NK cells, and Monocytes.
9. Draw a histogram and select CD3 cells.
10. On gated CD3 cells Select CD4 and CD8 populations.
11. Select CCR7 on *Y* axis and CD45RO on *X* axis for both CD4 and CD8 subsets and analyze as follows.
12. Divide this population into four subsets based on the presence or absence of CD45RO and CCR7 as CD45RO+CCR7+, CD45RO+CCR7–, CD45RO–CCR7+, CD45RO–CCR7– on CD4 and CD8 T-cell subsets.
13. Right-click on the CD45RO–CCR7+ gate and select option "Drill down" from drop down menu, make subset gate on bivariate plot for CD95 and CD28. Define CD28 bright CD95+ cells as T_{SCM} and CD95–CD28+ as T_N.

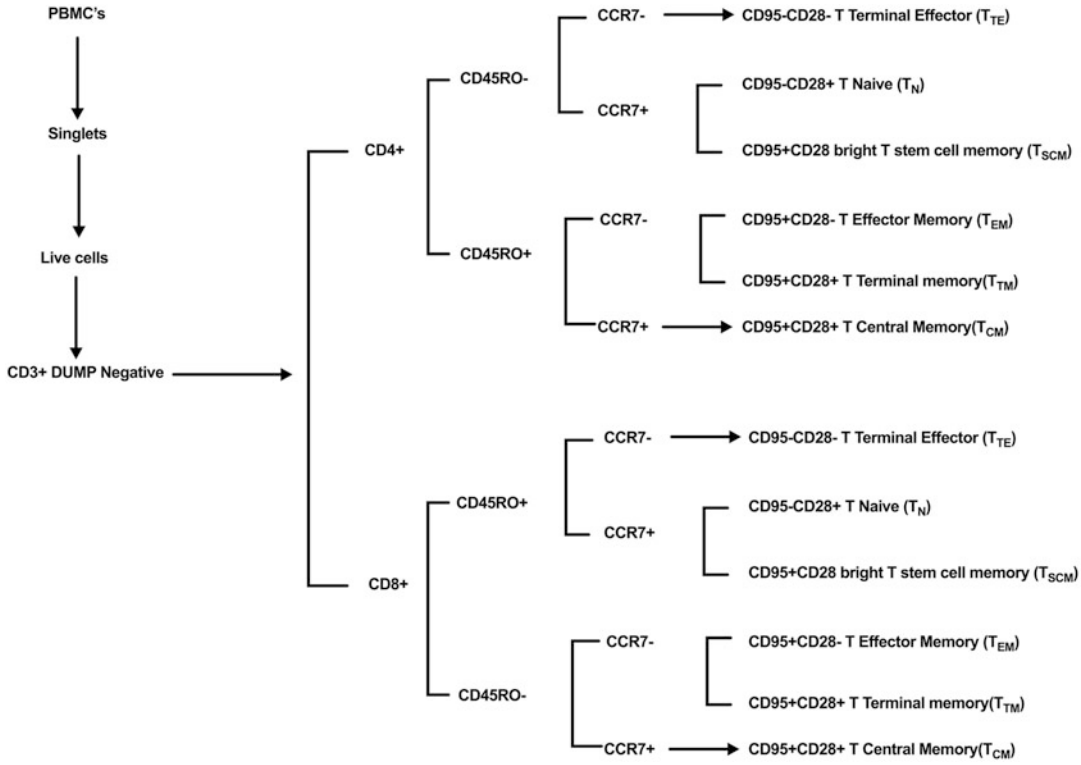

Fig. 2 Gating strategy for identifying naïve and memory T cell subsets. (**a**)The sequential gating strategy defines gated total lymphocytes from whole blood lysed sample. (**b**) Lymphocytes were gated for singlets, live and dump channel which excluded B, T, NK, and Monocytes. CD3 gated cells were further gated for CD4, CD8 followed by CD45RO and CCR7. On the basis of CD45RO and CCR7 the memory and naïve T cells were defined as follows. From CD45RO–CCR7+ subset gate on CD95 and CD28. T_{SCM}– CD45RO–CCR7+ CD28 ^{bright} CD95+, T_N– CD45RO–CCR7+ CD95–CD28+, T_{CM}– CD45RO+CCR7– CD95+CD28+, T_{TM}– CD45RO+CCR7– CD95 +CD28+, T_{EM}– CD45RO+CCR7– CD95+CD28–, T_{TE}– CD45RO–CCR7–CD28– and CD95–

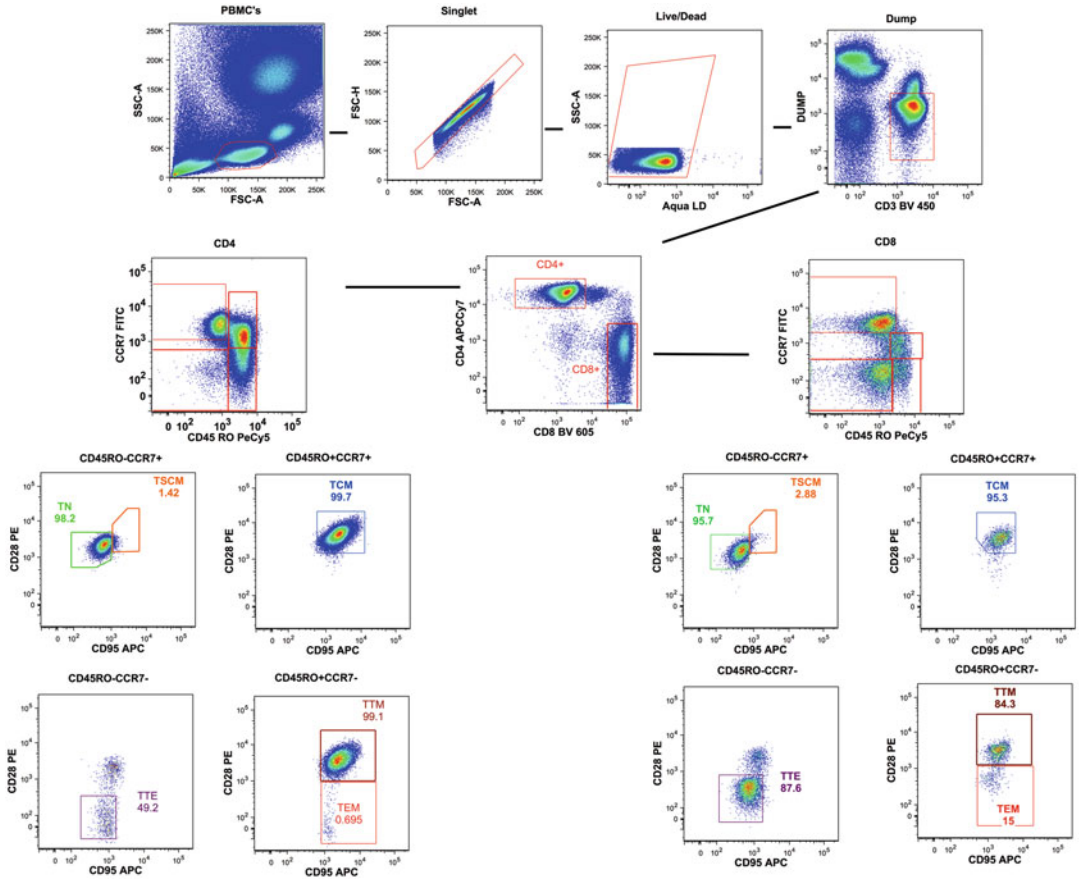

Fig. 2 (continued)

14. Drill down CD45RO+CCR7+ subsets as defined above and identify CD95+CD28+ expressing cells as T_{CM}.
15. Drill down CD45RO+CCR7– subset and identify CD95+CD28+ expressing cells as T_{TM} whereas CD95+CD28– as T_{EM} subset.
16. Drill down CD45RO–CCR7– subsets and identify CD28– and CD95– as T_{TE} cells.
17. Drag the gated population from work space to layout editor to prepare gating strategy schemes and to display data.

4 Notes

1. Use of azide in FACS buffer should be avoided especially if cells are to be sorted and used later for functional studies. Azide inhibits various metabolic functioning of cells, which could hamper the downstream functional studies of sorted cells.

Also, unfixed cells kept long in azide buffer may show more death than normal.

2. To avoid nonspecific binding of antibodies/reagents each fluorochrome-conjugated antibody/reagent should be optimally titrated before use.
3. High-throughput screen (HTS) should be set according to the manufacturer's instructions; please refer to the manual. It is Important to note that there is a dead volume of 30 μL for plates of all kind. The samples should preferably be run at a low flow rate between 1 and 2 μL/s for up to 10^6 cells.
4. Avoid prolonged (>5 min) incubation with ACK lysis buffer as it will significantly affect the lymphocyte viability.
5. Supplementing the medium with 20 μg/mL DNase helps to dissociate cell aggregates that result from DNA released by dead cells. This is especially helpful if working with cryopreserved cells as freeze–thaw may cause cell lysis.
6. Cell staining at 4 °C in the dark is a preferred method as it avoids antibody capping as well as photobleaching.
7. If there are any questions about the pathogenicity of the cells involved, then fixation of cells after staining and before running them through a flow cytometer is highly recommended for immunophenotyping purposes, not for live cell sorting.
8. Fixative solution should be removed after incubation by washing cells with FACS buffer. Prolonged fixation may alter fluorochrome properties and may lead to spillover in other channels.
9. Compensation beads should not be washed—as it may lead to loss of beads.
10. Optimize PMT voltages using unstained and single colors in order to obtain minimal spectral overlap.
11. Compensation matrix may still require to be adjusted during analysis. After adjusting the compensation electronically update biexponential transformation. On FlowJo—Select Platform<Custom transformation<Compensation matrix. Select appropriate compensation matrix. And appropriately adjust Biexponential scales in order to visualize the population clusters on scales.

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Chapter 9

Immunophenotyping of Human Regulatory T Cells

Janet Staats

Abstract

Regulatory T cells, also known as Tregs, play a pivotal role in maintaining homeostasis of the immune system and self-tolerance. Tregs express CD3, CD4, CD25, and FOXP3 but lack CD127. CD4 and CD3 identify helper T lymphocytes, of which Tregs are a subset. CD25 is IL-2Rα, an essential activation marker that is expressed in high levels on Tregs. FOXP3 is the canonical transcription factor, important in the development, maintenance, and identification of Tregs. CD127 is IL-7 receptor, expressed inversely with suppression, and is therefore downregulated on Tregs. Flow cytometry is a powerful tool that is capable of simultaneously measuring Tregs along with several markers associated with subpopulations of Tregs, activation, maturation, proliferation, and surrogates of functional suppression. This chapter describes a multicolor flow cytometry-based approach to measure human Tregs, including details for surface staining, fixation/permeabilization, intracellular/intranuclear staining, acquisition of samples on a flow cytometer, plus analysis and interpretation of resulting FCS files.

Key words Tregs, nTregs, iTregs, Effector tregs, Immunophenotyping, Flow cytometry, FoxP3, CD127, Suppressor T cells

1 Introduction

Regulatory T cells (Tregs) orchestrate immune responses, playing an essential role in immune tolerance, against self and nonself antigens, and inflammation [1–3]. Their ability to suppress inflammation is directly related to the production of cytokines IL-10 and TGF-β [1, 3]. Given their critical role in regulating the immune system, Tregs have been implicated in allergy, transplantation, infectious diseases, neoplastic disease, pregnancy, diabetes, autoimmune disease, colitis, atherosclerosis, and even sleep disorders [4–28]. A deeper understanding of Tregs, as well as their various subsets and states of being, may improve the existing standard of care for these diseases and disorders.

Because tregs are continually sensing and responding to their microenvironment, they are composed of diverse and dynamic subpopulations and exist in many different states of being [29]. Treg subpopulations include natural Tregs (nTregs), induced

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regulatory T cells (iTregs), Tr1, Th3, and Tfh regulatory T cells [30–32]. In the face of inflammation, these subpopulations respond to their environment by changing their phenotype. Therefore, Tregs exist in various states of being, including naïve (nvTreg), resting (rTreg), memory (mTreg), effector (eTreg), activated (aTreg), and proliferating Tregs [1, 3, 7, 33–39]. These phenotypically distinct subpopulations, and their states of being, are identified using cellular markers, where a marker is a molecule, usually a protein, that is located on or within a cell. Many different markers have been used to identify Tregs. The most commonly used Treg markers are CD3, CD4, CD25, FOXP3, CD127, CD45RA, CD45RO, CD194, HLA-DR, and Ki67 [7, 35]. CD3 is the T-cell receptor, used in conjunction with CD4 to identify helper T cells, of which Tregs are a subset. CD25 is interleukin-2 receptor alpha or IL-2Rα, a crucial activation marker expressed in high levels on Tregs, including nvTreg, mTreg, and eTregs [1, 3, 36, 40, 41]. It is important to note, however, that nonregulatory subsets of T cells, including effector T cells, also express high levels of CD25. FOXP3 and CD127 are the two quintessential markers of suppressor function, essential for identifying Tregs. FOXP3 is the canonical Treg transcription factor, required for Treg development, maintenance, and function [1–3]. Similar to CD25, FOXP3 is also expressed in subsets of activated T cells that lack suppressive function [3, 42, 43]. In humans, unlike mice, FOXP3 exists in different isoforms, resulting from posttranscriptional modifications [19, 44, 45]. FOXP3 is intranuclear, requiring fixation and permeabilization for flow cytometry assays. Fixed cells are not suitable for viable cell sorting; therefore, CD127 was identified as a surrogate for FOXP3 [41, 46]. Expression of CD127, interleukin-7 receptor alpha or IL-7Rα, is inversely correlated to suppressor function, meaning the lack of CD127 expression is a useful means of identifying Tregs [3, 7, 35, 40, 41]. One key intracellular marker for Tregs is Ki67, a proliferation marker [7]. rTregs proliferate in response to stimulation with specific antigen, becoming aTregs. CD45RA and CD45RO are CD45 isoforms that are mutually expressed on lymphocytes based on their maturational status. They may be used interchangeably or together to identify Treg subsets that exist in various maturational states. rTregs are CD45RA+ and CD45RO−, while mTreg, eTreg, and aTreg are CD45RO+ and CD45RA− [1, 3, 7, 35]. Chemokine receptors are involved in cell trafficking and play an important role in the migration of Tregs to inflammatory tissue and interactions with antigen presenting cells. Chemokine receptor expression is dependent upon the maturational state of Tregs. CD194, chemokine receptor 4 (CCR4), is expressed on terminally differentiated eTregs, the most suppressive phenotype [33, 35, 37, 38]. Finally, MHC-Class II or HLA-DR is upregulated on activated lymphocytes, including aTregs [1, 3, 34, 35]. However, nvTregs lack HLA-DR. While there is no single marker that

can be used to identify Tregs, a combination of markers, called a panel, must be employed to identify, qualify, and quantify Tregs.

Flow cytometry is the ideal tool for simultaneously measuring the expression of multiple markers on or within a single cell, and can be used to measure Tregs, their subsets, and their states of being [47–50]. This chapter describes how to measure human Tregs using flow cytometry for the purposes of immunophenotyping. Flow cytometry requires samples to be in a single cell suspension in order to establish a laminar sample flow through the point of acquisition. Human peripheral blood leukocytes are derived from a liquid sample and therefore require no manipulation to achieve a single cell suspension. Samples are prepared or stained, using a procedure that tags each of the markers of interest with a fluorophore that can be measured by the cytometer. When several fluorescently labeled reagents are used together in one assay, they are referred to as a cocktail. The cocktail of reagents and their corresponding combination of markers or panel are identified a priori in advance of staining cells [51–54]. Optimized, standardized, and harmonized Treg panels have been previously established [7, 35, 55–59]. The method for staining cells is determined by the location of the markers. Markers on the surface of the cell are readily accessible; however, intracellular or intranuclear markers often require fixation, to preserve the integrity of the markers, followed by permeabilization, to gain access to the intracellular or intranuclear compartments.

A flow cytometer has three main components: fluidics, optics, and electronics [47, 60]. The fluidic system injects the sample into a stream and introduces the sample, one cell at a time, for interrogation by the LASER(s). The optical system includes one or more LASERs, to excite the fluorophores from the various reagents on/in the cells, and collection optics. The collection optics capture the light emitted from the fluorophores plus light scattered by the cells based on their size and complexity. The electronic system converts the photons of light collected by the optics into a pulse, a digital signal that is interpreted by the software [61]. The digitized signals, collected from all of the various cells in a sample, are saved into a single data file using a standard format called flow cytometry standard or FCS file [62–64]. Flow cytometry software reads the FCS files and enables analysis of the various markers that are expressed in specific patterns by the cell types present in the sample.

The optical systems of a cytometer are not foolproof; as a result some fluorophores are excited by more than one laser, albeit not to the same degree, and the optical filters specific for one fluorophore will collect light from neighboring fluorophore(s). This is called spectral overlap [65, 66]. To deconvolute data collected from the various fluorophores, a compensation matrix is created by the flow cytometry software and used identify the fluorescence for one

specific marker from all of the other fluorescent signals present on the same cell [67, 68]. An operator then verifies the compensation matrix and analyzes the data by creating a series of sequential two-dimensional plots, where each plot displays fluorescence intensity of two different markers, X and Y. Some cells express a high amount of fluorescence, and are said to be positive, and some cells express low or no fluorescence and are said to be dim or negative, respectively. The combination of positive and negative populations creates patterns that are unique for a given population of interest. Electronic gate(s) or analysis region(s) are placed on the population (s) of interest based on these expression patterns. The act of placing gates and regions is called "gating" or analysis. The process of gating is highly subjective and; therefore, introduces a significant source of variability for flow cytometry-based assays (*see* Chapter 5, [7, 35, 69]. Regions are placed on plots for the purpose of generating statistical measures from a given population, such as frequency of parent (%) or median fluorescence intensity (MFI). The statistical data may be exported for subsequent bioinformatics or summary statistics.

Today, most cytometers are capable of detecting at least four colors simultaneously. Therefore, the premise of this Treg assay is to focus on a small set of four essential markers—the base panel. The required base panel includes CD3, CD4, CD25, and CD127 or FOXP3, where CD3 and CD4 are anchor markers, to gate on helper T cells, and CD25 is the key activation marker that is used with one marker of suppressor function, either CD127 or FOXP3, to identify Tregs. CD127 is preferred over FOXP3 when a surface staining assay is more desirable, either to conserve money or time.

While the use of FOXP3 to identify Tregs is well established, there is precedence in the literature that some clones of anti-FOXP3 antibodies may not work as well as others, causing Treg measurements to vary by choice of clone, and the choice of permeabilizing agent also introduces variance in the measured results [28, 70–72]. It is important to note that many of the clonal studies predated the discovery that humans have different isoforms of FOXP3; thus, the variation observed between clones may in part be related to different epitopes expressed by the FOXP3 isoforms. When using FOXP3 to enumerate Tregs by flow cytometry a nuclear permeabilizing reagent is required, along with a fixative to preserve the surface membranes. There are essentially two types of permeabilizing agents. A gentler solution, used to access intracellular antigens, and a harsher solution, required to access intranuclear antigens. Different permeabilizing agents use different mechanisms to create holes in the cellular membranes. Some reagents are detergent based, permeabilizing cells only when the detergent is present, and others create permanent holes in the membranes. Both the clone and permeabilizing agent should be considered potential sources of variance in Treg assays.

If the cytometer is not limited to four colors and a modestly expanded Treg panel is more desirable, then the base panel may be expanded by including a handful of optional Treg consensus markers; these include a fixable vital dye, CD45RA, CD45RO, CD194, HLA-DR, and Ki67. Expanding the Treg panel will exponentially increase the complexity of the assay and may not be necessary. To help inform the decision to expand the panel, the next few paragraphs provide guidance for when an expanded Treg panel may strengthen the overall results obtained from this Treg assay. The Subheading 3 will include provisions for all of the commonly used Treg markers, including the essential base panel and optional consensus markers.

There are two approaches to expanding the Treg panel that may be used together or separately. The first approach is to focus on the overall accuracy of the Treg measurement, and the second focuses on the Treg state of being. Accuracy of the Treg measurement can be improved by combining two different suppressor markers together in the same panel. The combination of FOXP3 and CD127 utilizes one marker that correlates positively with suppression and a second marker that is inversely correlated with suppressive activity. These two markers appear to identify slightly different Treg populations; albeit, with a high degree of overlap between the populations identified using CD127 and FOXP3 [73]. Two different clones of FOXP3 addresses the issue that not all clones of FOXP3 perform the same and FOXP3 exists in different isoforms [74]. The use of two Treg markers is recommended only when it is critical to hone the accuracy of the true FOXP3+ Treg measurement.

If you have access to an instrument that is capable of measuring up to 8-colors simultaneously and the scientific goal is to characterize the Treg state of being, then including the optional Treg consensus markers (a vital dye, CD45RA, CD45RO, CD194, HLA-DR, and Ki67) will add value to your results. The vital dye is useful when working with less pristine samples, such as overnight-shipped blood, cryopreserved/thawed PBMC, or samples collected from patients with conditions or medications that severely affect lymphocytes (some chemotherapies and immunotherapies). The remaining optional markers represent the combined markers from two unique and highly standardized panels; select one of these two panels based on your specific needs.

Both of the highly standardized panels were meticulously developed, optimized, and standardized by teams of expert immunologists who gathered specifically to create a standardized Treg assay for the purposes of immune monitoring and comparing data across laboratories and across human clinical trials. The Human Immunology Project Consortium (HIPC) standardized not only the panel of markers used to identify Tregs, but also standardized the assay and approach to data analysis [35, 75]. The HIPC Treg

assay uses a surface staining protocol with lyophilized plates containing a pretitered antibody cocktail that consists of the following eight markers: a vital dye, CD3, CD4, CD25, CD127, CD45RO, CD194, and HLA-DR. The HIPC assay is used to measure Tregs in general, as well as eTregs, aTregs, and mTregs. Of note, the HIPC assay was designed to measure multiple leukocyte subsets, including Tregs; this chapter focuses only on the Treg portion of the HIPC panels. In a separate and independent harmonization effort, the CIMT Immunoguiding Program, CIP, systematically composed a ranking of Treg markers, tested the markers to identify differences and overlap across the most commonly used Treg definitions, then evaluated the resulting panels for their ability to measure Tregs in various human tissues, primarily in the context of neoplastic disease [7]. Like HIPC, CIP also provided a robust gating strategy to guide the data analysis. The CIP harmonized panel includes the following seven markers: CD3, CD4, CD25, CD127, FOXP3, Ki67, and CD45RA. It is used to measure Tregs in general, as well as eTregs, nvTregs, aTregs, and mTregs. Both standardized panels include an essential base panel to identify Tregs. However, the two panels differ in the staining method, surface versus intracellular/intranuclear, and markers used to identify the Treg states of being. Note, different markers are used by the two standardized panels to identify similar states of being. For this reason, it is critical to use the same definition of a population, both in terms of markers and gating strategy, throughout the course of a given study. The essential marker list included in this chapter encompasses all of the markers in both the HIPC and CIP standardized Treg panels, enabling the use of either panel in the Treg assay described below.

This chapter describes how to measure human Tregs using analytical flow cytometry. The Treg assay preparation method includes a surface stain plus fixation and permeabilization. This permeabilization method resembles the one used by CIP and has been reported to be the most reliable intranuclear procedure [7]. To complete the Treg assay, the preparation method must be used in conjunction with a panel of markers to identify Tregs. Given the complexity involved in developing flow cytometry panels, a list of the most common Treg markers is provided. These include CD3, CD4, CD25, FOXP3, CD127, a vital dye, CD45RA, CD45RO, CD194, HLA-DR, and Ki67. The common markers may be used to in three different combinations or panels. To complete the assay, select the panel best suited to address the scientific question. The base panel is required to identify Tregs, and can be configured as a four-color assay. If the based panel is used with CD127, and not FOXP3, it is a surface only procedure. If used with FOXP3, and not CD127, fixation and permeabilization is required. To measure Treg states of being such as aTreg, mTreg, and the highly suppressive eTreg, there are two highly standardized panel options, HIPC and

CIP panels. Both standardized panels include the required base panel to identify Tregs. The HIPC Treg panel utilizes a simple surface staining procedure, while the CIP panel requires permeabilization and is more challenging technically. For all three panels, there is the option to incorporate a vital dye, second clone of FOXP3, and/or both suppressive markers, CD127 and FOXP3. Note, incorporating these additional options into one of the seven to eight-color standardized panel options may require a higher dimensional flow cytometer, with up to 12-color capabilities. The use of nonhuman or solid tissues, cell sorting, and more advanced Treg assays, designed measure the plethora of subpopulations of Tregs, their respective states of being, or function, are beyond the scope of this chapter.

2 Materials

1. Personal protective equipment, based on institutional guidelines.
2. Cytometer, capable of measuring at least four colors simultaneously, or up to 12 colors for the HIPC or CIP Treg panels.
3. Flow cytometry analysis software.
4. Centrifuge, refrigerated, with buckets to hold staining tubes or plates, depending on which is used in the assay.
5. Microfuge.
6. FACSWash buffer: PBS containing 0.5% hiFBS. Use a serological pipette to remove 2.5 mL PBS from a newly opened bottle and add 2.5 mL hiFBS to the PBS. Cap the bottle and invert 5 times to mix. Store at 4 °C for up to 1 month after preparation (*see* **Note 1**).
7. Blocking agent: commercially available, store according to manufacturer recommendations until expiration (*see* **Note 2**).
8. Red blood cell lysing agent: commercially available, usually as a concentrate, prepare and store according to the manufacturer's instructions (*see* **Note 3**).
9. Fixable vital dye stain: commercially available in several colors, prepare and store according to the manufacturer's instructions (*see* **Note 4**).
10. Fluorescent conjugated monoclonal Abs (mAb) for surface and intracellular/intranuclear staining: Essential mAb reagents include CD3, CD4, and CD25, plus CD127 and/or FOXP3. Additional antibody reagents include HLA-DR, CD194, Ki67, plus CD45RA and/or CD45RO. They are commercially available; store at 4 °C until expired (*see* **Note 5**).

(a) Basic panel with CD127 includes CD3, CD4, CD25, and CD127. Requires surface stain only.

(b) Basic panel with FOXP3 includes CD3, CD4, CD25, and FOXP3. Requires surface and intranuclear stain. CD4, CD25, CD127, FOXP3, Ki67, and CD45RA.

(c) HIPC panel CD3, CD4, CD25, CD127, CD194, CD45RO, and HLA-DR. Requires surface stain only.

(d) CIP panel includes CD3, CD4, CD25, CD127, FOXP3, CD45RA, and Ki67. Requires surface stain and intracellular/intranuclear stain.

11. Nuclear fixation/permeabilizing solution (Fix/Perm): commercially available, usually as a concentrate, prepare and store according to the manufacturer's instructions (*see* **Note 6**).
12. Permeabilization buffer (Perm buffer): commercially available, usually comes with the Fix/Perm as a concentrate, prepare and store according to the manufacturer's instructions.
13. Compensation beads (*see* **Note 7**).
14. 1% Fixative: PBS with 1% formalin. Add 5 mL 10% formalin to a 45 mL PBS in a 50 mL conical tube, cap and mix by inverting several times, store at ambient temperature for up to 1 month after preparation (*see* **Note 8**).
15. Vacuum manifold, if staining in plates (*see* **Note 9**).

3 Methods

1. Read all steps and Notes prior to performing the assay, as much advanced preparation is required to perform this assay.
2. Perform all steps on ice or at 4 °C unless otherwise noted, including centrifugation.
3. Store buffers on ice until use unless otherwise noted.
4. *Vital dye stain*.

(a) Add 100 μL whole blood or viable PBMC viable cells to the staining tubes or wells (*see* **Note 10**).

Note, for the remainder of the assay, through sample acquisition, the samples must be protected from light and kept on ice, unless otherwise noted (*see* **Note 11**).

(b) Wash cells by adding FACSWash buffer, 2 mL for tubes or 100 μL for wells.

(c) Centrifuge tubes for 5 min or plates for 3 min at 350 × *g* and 4 °C (*see* **Note 12**).

(d) Decant tubes or aspirate wells to remove supernatant (*see* **Note 13**).

(e) Add 100 μL vital dye stain at a final concentration based on pretitration (*see* **Notes 14** and **15**).

(f) Mix cells with the vital dye by vortexing tubes for 3 s at a moderate setting of 3 or pipetting the entire volume in wells up and down 10 times (*see* **Note 16**).

(g) Incubate cells with the vital dye for 30 min at ambient temperature.

(h) During the vital dye incubation prepare the surface mAb stain mix, a cocktail of all conjugated mAbs at the optimal concentrations in FACSWash buffer (*see* **Note 17**).

(i) Wash cells by adding FACSWash buffer, 2 mL for tubes or 200 μL for wells.

(j) Centrifuge tubes for 5 min or plates for 3 min at 350 × *g* and 4 °C.

(k) Decant tubes or aspirate wells to remove supernatant.

5. *Surface stain.*

(a) If using liquid mAbs, microfuge mAb reagent vials for 5 min immediately prior to use (*see* **Note 18**).

(b) Add fluorescently labeled monoclonal antibodies (mAb) to cells at pretitered concentrations in a final volume of 100 μL (*see* **Notes 15** and **19**).

(c) Mix cells with surface mAb mix by vortexing tubes for 3 s at a moderate setting of 3 or pipetting the entire volume in wells up and down 10 times.

(d) Incubate cells with surface mAb mix for 30 min.

(e) Wash with FACSWash buffer by adding 2 mL in tubes or 100 μL in wells.

(f) Centrifuge tubes for 5 min or plates for 3 min at 350 × *g* and 4 °C.

(g) Decant tubes or aspirate wells to remove supernatant.

6. *RBC lyse.*

(a) Add ambient temperature 1× lysing solution to cells, 1 mL in tubes or 100 μL in wells (*see* **Note 20**).

(b) Mix cells with lyse by vortexing tubes for 3 s at a moderate setting of 3 or pipetting the entire volume in wells up and down 10 times.

(c) Incubate cells with lyse for 10 min at ambient temperature, or according to the manufacturer's recommendations.

(d) Wash with FACSWash buffer by adding 2 mL to tubes or 100 μL to wells.

(e) Centrifuge tubes for 5 min or plates for 3 min at 350 × *g* and 4 °C.

(f) Decant tubes or aspirate wells to remove supernatant.

(g) If performing surface stain only assay skip to Fixation step; otherwise, continue with permeabilization and intracellular/intranuclear staining.

7. *Permeabilization.*

(a) Permeabilize cells by adding fixation/permeabilization reagent, 1 mL for tubes or 100 μL for wells (*see* **Notes 6** and **21**).

(b) Mix cells with Fix/Perm reagent by vortexing tubes for 3 s at a moderate setting of 3 or pipetting the entire volume in wells up and down 10 times.

(c) Incubate cells with Fix/Perm for 45 min at ambient temperature or according to the manufacturer's recommendations.

(d) Wash cells with Perm buffer by adding 2 mL to tubes or 100 μL to wells.

(e) Centrifuge tubes for 8 min or plates for 5 min at 500 × *g* and 4 °C (*see* **Note 22**).

(f) Decant tubes or aspirate wells to remove supernatant.

8. *Block.*

(a) Add 5–15 μL blocking agent, depending upon the manufacturer's recommendations.

(b) Mix cells with block by vortexing tubes for 3 s at a moderate setting of 3 or pipetting the entire volume in wells up and down 10 times.

(c) Incubate cells with block for 15 min.

(d) During the block incubation prepare the intracellular/intranuclear mAb stain mix (*see* **Notes 15**, **17**, and **23**).

9. *Intracellular/Intranuclear stain.*

(a) Microfuge mAbs.

(b) Add fluorescently labeled monoclonal antibodies (mAb) to cells in a final volume of 100 μL.

(c) Mix cells with intracellular/intranuclear mAb mix by vortexing tubes for 3 s at a moderate setting of 3 or pipetting the entire volume in wells up and down 10 times.

(d) Incubate cells with intracellular/intranuclear mAb mix for 30 min.

(e) Wash with Perm buffer by adding 2 mL to tubes or 100 μL to wells.

(f) Centrifuge tubes for 8 min or plates for 5 min at 500 × *g* and 4 °C.

(g) Decant tubes or aspirate wells to remove supernatant.

(h) Repeat wash two more times for a total of three washes, using 2 mL for tubes and 200 μL for wells.

10. *Fix.*

(a) After removing supernatant add 200 μL 1% Fixative to cells.

(b) Mix cells with Fixative by vortexing tubes for 3 s at a moderate setting of 3 or pipetting volume in wells up and down 10 times (*see* **Notes 8** and **24**).

(c) Store at 4 °C in the dark until acquisition.

11. *Stain compensation bead controls.*

(a) Mix compensation beads by vortexing at a setting of 8 for 5 s.

(b) Add 50 μL or one drop of Negative compensation beads to a staining tube or well (*see* **Note 25**).

(c) Add 50 μL or one drop of Positive compensation beads to separate staining tubes or wells, one for each fluorescent marker used in the panel (*see* **Note 25**).

(d) Add conjugated mAb to each respective compensation bead tube, matching the Ab concentration used to stain cells (*see* **Note 26**).

(e) Mix beads with mAbs by vortexing tubes for 3 s at a moderate setting of 3 or pipetting the entire volume in wells up and down 10 times.

(f) Incubate beads with mAbs for 20 min.

(g) Add 2 mL FACSWash buffer to each tube or well.

(h) Centrifuge tubes or plates at 350 × *g* for 5 min and 4 °C.

(i) Decant tubes or aspirate wells to remove supernatant.

(j) Add 200 μL 1% Fixative to each tube or well (*see* **Notes 7**, **8**, and **24**).

(k) Mix beads with 1% Fixative by vortexing tubes for 3 s at a moderate setting of 3 or pipetting the entire volume in wells up and down 10 times.

12. *Instrument setup and acquisition.*

(a) Verify the optical configuration of the cytometer will accommodate the fluorophores used for Treg assay (*see* **Note 27**).

(b) Empty waste tank (*see* **Note 28**).

(c) Fill sheath tank (*see* **Note 28**).

(d) Verify cytometer performance is acceptable for use (*see* **Note 29**).

(e) Enter annotation for samples and compensation beads in cytometer, including samples, conditions, markers, and fluorophores.

(f) Calibrate cytometer to set fluorescence voltage, scatter gains, and detection threshold (*see* **Note 30**).

(g) Use unstained cells to set collection and storage gates (*see* **Note 31**).

(h) Acquire compensation tubes.

(i) Calculate compensation matrix and apply compensation (*see* **Note 32**).

(j) Acquire cells (*see* **Note 33**).

(k) Clean the cytometer (*see* **Note 34**).

(l) Transfer data from cytometer to network or analysis workstation immediately following acquisition (*see* **Note 35**).

13. *Compensation.*

(a) Create a compensation matrix using the compensation beads (*see* **Note 36**).

(b) Apply the compensation matrix to all of the files (*see* **Note 37**).

(c) Using a file that contains all of the markers, verify compensation values are correct by visualizing each X axis versus all of the respective Y axes (*see* **Note 38** and Fig. 1).

(d) Manually adjust compensation as necessary (*see* **Note 39**).

(e) Save the adjusted compensation matrix.

14. *Gating.*

(a) Follow the gating scheme provided in Fig. 2 (*see* **Note 40**).

(b) Use sequential two-dimensional gating, to create gates for Time, Singlets (FSC and SSC), and Lymphocytes (*see* Chapter 5).

(c) From the Lymphocyte gate, display CD3 versus CD4 and create a CD3+CD4+ gate to identify Helper T lymphocytes.

(d) From the Helper T cell gate, display CD25 versus either CD127 or FOXP3, depending upon the panel used. Create the Treg gate as CD25+CD127low of CD25 +FOXP3+ (*see* Figs. 2 and 3).

(e) To gate on markers included in the HIPC panel, follow the gating scheme provided in Fig. 4. Gate on Tregs, using either CD25 versus CD127 or FOXP3, then gate on CD194+ to identify effector or eTregs.

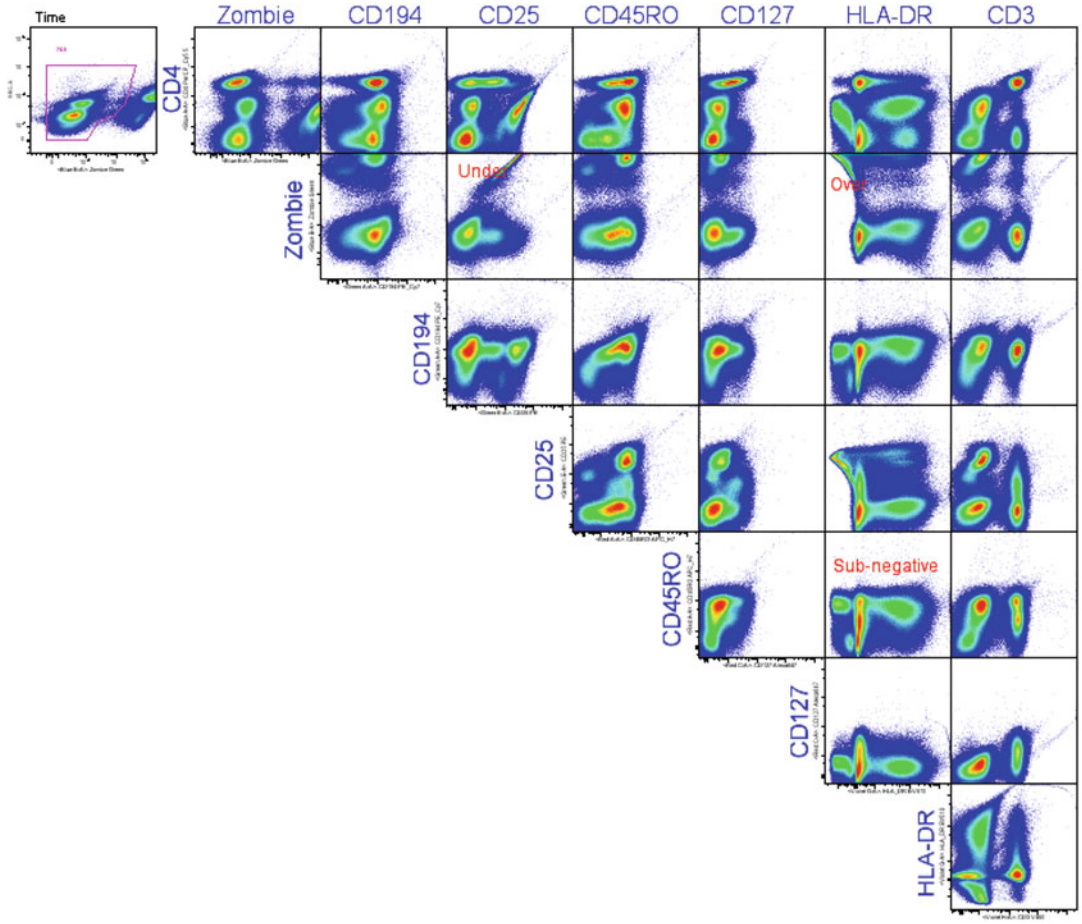

Fig. 1 $N \times N$ compensation plot. Dot plots in Fig. 1 represent an $N \times N$ plot for using to visualize compensation errors. Overcompensation and undercompensation errors are noted. The over compensation of Zombie from the HLA-DR creates a subnegative HLA-DR population that is visible in the HLA-DR versus CD45RO dot plot, as well as other *Y* axes markers paired with HLA-DR. Subnegative values are usually caused by overcompensation

(f) From the CD194+ gate, create CD45RO+ and CD45RO− regions to identify memory or mTregs and naïve or nTregs, respectively.

(g) Also from the CD194+ gate, create HLA-DR+CD45RO + regions to identify activated memory Tregs.

(h) To gate on markers included in the CIP panel, follow the gating scheme provided by Santegoets et al. [7].

15. *Controls.*

(a) Many types of controls are used in flow cytometry [76]. The essential controls for multicolor flow cytometry are compensation controls, single stained controls used to

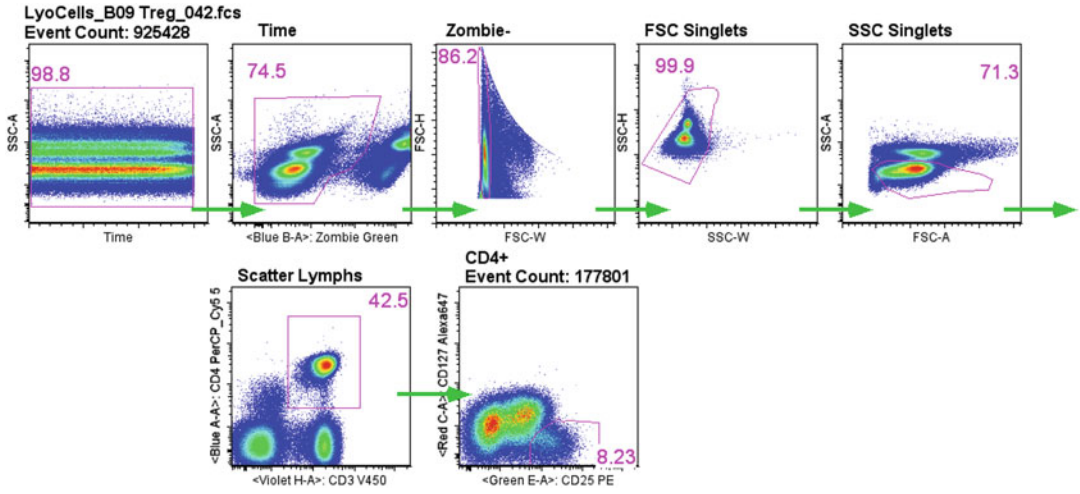

Fig. 2 Treg gating scheme. Cells obtained from a normal donor were surface stained with the HIPC panel using lyoplates and acquired on a BD LSRFortessa. CD25 versus CD127 is used to identify Tregs. The gates are drawn as described for Time, Zombie-, Singlets (FSC and SSC), Lymphocytes, CD3+CD4+ Helper T cells, and CD25+CD127low Tregs. The proportion of Tregs is 8.23% of Helper T cells, in the normal range of approximately 5–9%. The gates are listed above each respective dot plot and green arrows indicate the gating hierarchy from one gate to the next. The number of events are shown for the whole FCS file (925428) and CD3+CD4+ (labeled CD4+) (177801); 8.23% of 177801 is 14626 Treg events collected from a total of 2×10^6 viable PBMCs added to the staining well. This is a sufficient number of events to continue with analyzing Treg states of being, if desired

create compensation matrices to distinguish one fluorescence from another.

(b) Hard-dyed microbeads are commonly used to assess cytometer performance [77, 78]. Hard-dyed beads contain fluorescence molecules inside the bead itself, shielding it from many harmful elements found in the microenvironment. The choice for what bead to use and how to use it is generally up to flow core managers. Instrument manufacturers design quality control measures into software packages to facilitate routine instrument performance assessments. These are also based on hard-dyed beads.

(c) Controls used to calibrate cytometers include a number of options ranging from hard-dyed beads to stained beads to stained cells. These assay-specific settings are up to flow core managers and sometimes left to the end user's discretion. The most commonly used control to calibrate cytometers is hard dyed beads.

(d) Fluorescence minus one (FMO) controls contain all of the fluorescence markers in a panel except for one (*see* Figs. 3 and 5) [79]. For example, a FOXP3 control for a basic Treg panel would contain a vital dye stain, CD3, CD4,

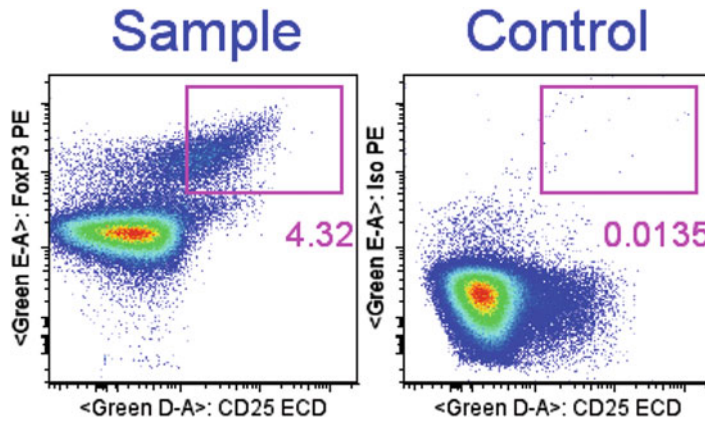

Fig. 3 CD25 versus FOXP3 Treg analysis region. Cells obtained from an HIV+ patient were stained for surface CD25 and intranuclear FOXP3 (PCH101 clone) using the optimized panel in OMIP 006 and acquired on an LSRII [56]. The sample in the left plot is stained with FOXP3 (*Y* axis). The frequency of Tregs in the HIV+ sample are low, but close to the lower limit of the normal range. The control on the right plot is a gating control. It contains all markers except FOXP3, like an FMO control. However, unlike an FMO control, the gating control includes an isotype control for PE, used at the same concentration of the FOXP3 mAb. In this instance the gating control was not helpful due to a shift in the PE negative. The shift in the PE negative population is an artifact with two possible causes. Negative drag-up created by improper washing that leaves unbound conjugated mAbs in solution that is excited by the LASER. Staining fixed/permeabilized cells with intracellular and intranuclear markers. This example is likely a combination of the two artifacts. In this example, the PE gate was placed between the visually discrete populations of FOXP3− and FOXP3+ populations in the sample, called an internal negative control. If a dimmer fluorophore or different clone for FOXP3 had been used, the resulting FOXP3 staining might not have been so clearly separated, making the analysis much more challenging. Proper washing and blocking the intracellular or intranuclear mAb helps to reduce these artifacts

and CD25 but not FOXP3. FMO controls are used to identify the boundary between positive fluorescence and negative fluorescence, taking into account any increase in background from neighboring detectors. They are helpful controls to use when deciding where to place boundaries of fluorescence positive and negatives when creating gates and analysis regions. The downside is that they are costly to incorporate in terms of both cells and reagent, and my not always work, as when there is negative drag-up or sometimes with intracellular/intranuclear markers. At an absolute minimum, FMO controls should be used during panel development to inform decisions regarding degree of interference from spillover.

(e) Internal negative controls are populations of cells that do not express the marker of interest but are present in a tube

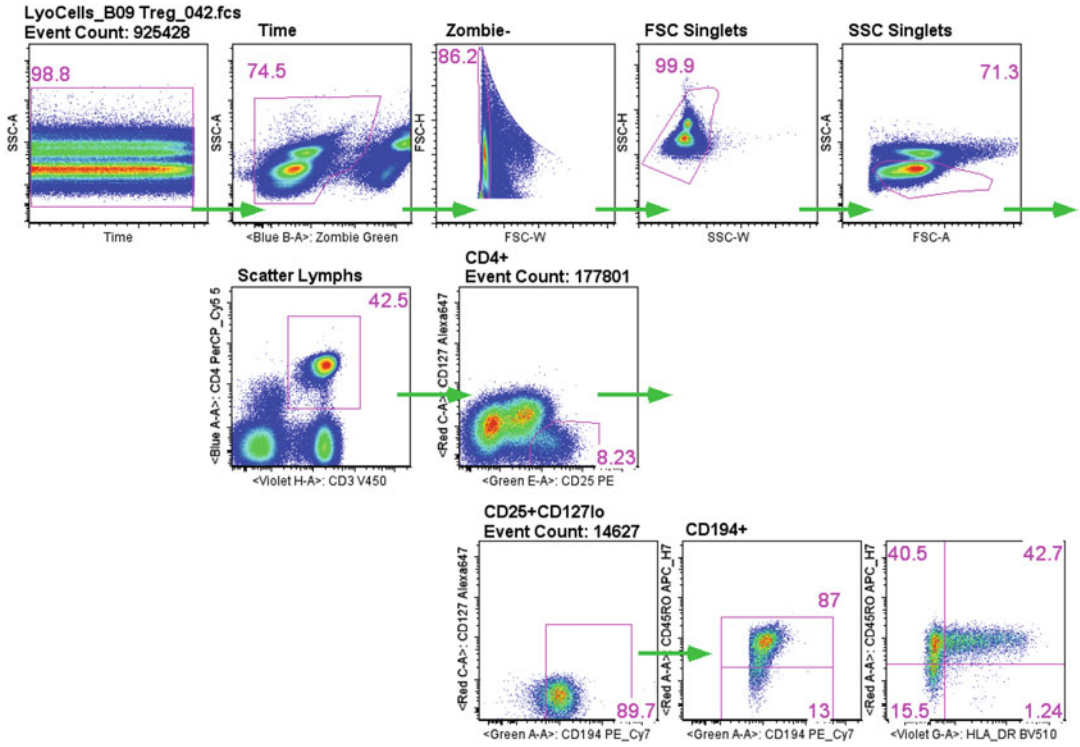

Fig. 4 HIPC panel gating scheme. Cells obtained from a normal donor were surface stained with the HIPC panel using lyoplates and acquired on a BD LSRFortessa. CD25 versus CD127 is used to identify Tregs. The gates are drawn as described for Time, Zombie-, Singlets (FSC and SSC), Lymphocytes, CD3+CD4+ Helper T cells, and CD25+CD127low Tregs. In addition to the Treg markers, the HIPC panel includes markers to measure the Treg states of being, namely maturation and activation. From the Treg gate, eTregs are identified as CD194+ and from the eTregs memory (CD45RO+) and activated memory (HLA-DR+CD45RO+) Tregs are also identified. CD25, CD127, CD194, CD45RO, and HLA-DR gates and regions were set using FMO controls. Figures 2 and 4 are created from the same data file

that is stained with all of the fluorescence markers in the panel [80]. In Fig. 3, an internal control was used to set the FOXP3 analysis region when the FMO control failed. In this example, we are using the biological knowledge about the sample, that not all Helper T cells express FOXP3. Internal negative controls only work when using a mixed population of cells, where some cells express the antigen of interest and some do not express the antigen of interest.

(f) Biological controls consist of normal donor cells that are either fresh, individual samples or in bulk form from leukopaks or buffy coats, or prepared as a bulk commercial product. Figures 2 and 4 were obtained using a commercial product derived from stabilized buffy coat leukocytes. Biological controls provide helpful information regarding

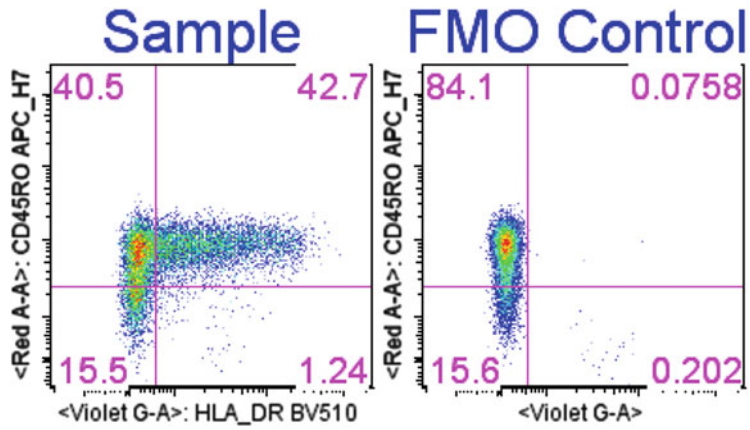

Fig. 5 FMO control for HLA-DR. Cells obtained from a normal donor were surface stained with the HIPC panel using lyoplates and acquired on a BD LSRFortessa. CD25 versus CD127 is used to identify Tregs. Markers for maturation and activation, namely, CD194, CD45RO, and HLA-DR, were included in the panel. The plot on the left is a sample stained with all markers in the panel (a vital dye, CD3, CD4, CD25, CD127, CD194, CD45RO, and HLA-DR). The plot on the left is an FMO control for HLA-DR, stained with all markers in the panel except for HLA-DR. The X quadrant was placed using the HLA-DR FMO control

the overall quality of the assay performed and can be used to assess staining, permeabilization, and analysis, depending upon how the control is designed. When the same biological control is used across multiple assays, the results can be used as a measure for overall variability between assays.

16. *Reporting flow cytometry results.*

(a) Flow cytometry results are reported using three units: frequency, absolute counts, and fluorescence intensity.

(b) Frequency is based on the number of cells that express a particular marker, or combination of markers relative to gate. Most commonly, frequency is reported as the percentage of the parent, meaning a proportion of the parent gate in the gating hierarchy. For example, Tregs are reported as a frequency of Helper T cells, CD4+CD3+ lymphocytes. It is possible to report Tregs as a proportion of lymphocytes; this would be frequency of grandparent. Most software packages enable users to report frequencies for a given population as a proportion of any gate in the gating hierarchy. Frequency is a qualitative measure.

(c) Absolute counts represent a means for converting a frequency to a quantitative measure. Specific counting controls are required to report values in absolute counts or cells/μL [81–84]. Counting controls are added to the

original sample during the staining process. This is called a single-platform method, meaning both the counting and the frequency measures are obtained using one instrument, the flow cytometer. Reporting Tregs as cells/μL requires both the use of whole blood samples and counting controls. A second, more variable dual-platform method, may be used generate absolute counts using the flow cytometry-derived frequencies and the hematology-derived absolute blood count and differential. This method requires a means of relating the flow cytometry analysis to the whole blood used in the hematology assay, usually the percentage of lymphocytes.

(d) Fluorescence intensity is directly proportional to the density of the antigen expressed on or inside a cell. The units of fluorescence intensity, median or mean (median/mean fluorescence intensity or MFI), are qualitative. With the appropriate controls, MFI values can be converted to quantitative units of antibody binding, such as antibody binding capacity (ABC) or molecules of equivalent fluorescence intensity (MESF) [85–88].

4 Notes

1. Flow cytometry wash buffers: at a minimum include must be buffered to neutrality and isotonic, such as PBS (calcium and magnesium free). Common ingredients included in flow cytometry wash buffers are FBS, EDTA, sodium azide, and DNAse/RNase. Protein, usually in the form of FBS, is added to protect cells from apoptosis, prevent heterophilic antibody interference, and block nonspecific binding of antibodies [89]. FBS must be heat-inactivated prior to use. Heat inactivation removes complement proteins that might otherwise cause unwanted cell lysis. Heat-inactivated FBS is commercially available or may be prepared in the lab by placing FBS in a 56 °C water bath for 1 h. Heat-inactivated FBS is aliquoted and frozen for up to 1 year or until expiration. Each aliquot can be subjected to a total of three freeze–thaw cycles, then discard. Use FBS at a final concentration from 0.5% to 10%, depending upon how much blocking is needed. EDTA prevents cell clumping and is most useful when specimens are clumpy, such as derived from solid tissue or subjected to cryopreservation and thawing. If EDTA is needed, use at a final concentration from 0.5 mM to 5 mM. Sodium azide is a preservative. It also inhibits capping, antigen internalization and shedding of surface antigens. If sodium azide is needed, use at a final concentration of 0.1–1%. If the sample contains dead cells, usually

seen in disaggregated tissue or thawed cells, the sample will also contain a fair amount of extracellular DNA and RNA derived from the dead cells. DNA and RNA are sticky and lead to cell clumping. To remove extracellular DNA and RNA, add DNAse and RNase to the wash buffer at a final concentration of 25–50 μg/mL.

2. Blocking agents contain immunoglobulin and are used to bind Fc receptors present on cells. They may be comprised of simply serum or a mixture of antibodies specific to Fc receptors. During flow cytometry assays, that use antibodies as a tool to stain cells, the Fc portion of the staining antibody (Ab) may also bind to Fc receptors on the surface of cells expressing FcR, causing increased background or nonspecific staining. When fluorescently tagged Abs bind via the FcR and not binding their specific antigen on the cell, the resulting expression pattern is a diagonal streak across the dot plot, running from roughly the double negative region of the plot toward the double positive region of the plot. The nonspecific binding or higher background interferes with the ability to accurately classify cells as positive or negative during analysis. For this reason, Fc receptors are often blocked during the staining process. Some cell types express more FcR than others and some patient cells are more prone to nonspecific binding. Blocking is especially important for intracellular/intranuclear staining, such as FOXP3.
3. Red blood cells or RBCs, when present in samples, obfuscate white cells during analysis and increase the overall variability of the measurements. Obvious red cell contamination, and even observing a small number of red cells in the cell pellet, warrants consideration to remove RBCs. There are two approaches to removing RBCs. The first method is to lyse RBCs and the second is to use CD45 gating, either with or without CD235a. There are many commercially available lysing solutions and most work by increasing osmotic pressure inside the cell through a net influx of NH4Cl, causing the red cells to burst. Most commercially available lysing agents are sold as concentrates and may or may not include a fixative. Because lysing agents work by changing osmolarity, it is important to read the manufacturer's instructions carefully and use the appropriate diluent, usually either water or PBS. Using the inappropriate diluent or not diluting properly, may cause an overlysis or underlysis. Overlysis may also lyse some leukocytes and under lysing results in red cells remaining after treatment with the lysing agent. Both over and under lysing can change the observed scatter properties on a forward versus side scatter plot. Lysing agents do not work well with nucleated RBCs. Some samples contain nucleated RBCs, such as those obtained

from pediatric patients, patients receiving chemotherapeutic agents or radiation, or patients who might be reticulating for other reasons. Nucleated RBCs do not lyse well. When nucleated red cells are present or when there is a need to avoid lyse in general, one can use specific markers to identify white cells and red cells through gating. CD45 is a pan-leukocyte marker and can be used to effectively to identify lymphocytes when combined with either SSC. CD235a is a red blood cell marker, can be used to remove red cells making it easier to identify the CD45+ white blood cells. The addition of CD45 is preferred when using samples that are derived from tissue that contains scarce leukocytes or when accuracy of the resulting measurement is paramount.

4. Use a fixable vital dye to exclude dead cells [90, 91]. Small-molecule dyes such as PI, AO, and 7AAD, will not work with the fixative used in Fix/Perms or the 1% Fix. Small molecule dyes are also notorious for binding the tubing inside of the cytometer tubing, causing a significant increase in background and decrease in signal-to-noise ratio.
5. A list of the most commonly used Treg markers is provided, including consensus markers used to identify Treg states of being. The reagent used to identify each respective marker is a fluorescent conjugated mAb. For a given study, optimal results are obtained when using the same clones, fluorophores, and lots for each respective reagent.
   (a) Most of the commercially available mAb clones are acceptable for use; however, the choice of FOXP3 clone matters [28, 69–71]. To know which anti-FOXP3 clone works best for your particular application, it is highly recommended that you compare anti-FOXP3 clones using your own samples.
   (b) Not all fluorophores are equal and it is important to understand how the fluorophores work and how to obtain the best performance for each dye [92–96]. The process of determining which fluorophore is conjugated to each respective mAb is called panel design and requires knowledge about the fluorophores, expression pattern of each marker (how it relates to the other markers of interest, whether it is coexpressed on the same cell or on a different lineage), plus knowledge about the optical configuration of the cytometer to be used [51, 53, 97]. The general rule for panel development is that markers with a high antigen density, such as CD3 and CD4, are conjugated to fluorophores with dim or moderate fluorescence intensity, while markers with low antigen density, such as FOXP3 and CD127 low on Tregs, are conjugated to fluorophores

with bright intensity. Additionally, markers that are coexpressed on a single cell, such as CD4 and CD3, should be conjugated with fluorophores that are on opposing ends of the fluorescence spectrum and on different LASERs. Overlapping fluorescence emission from markers expressed on the same subset leads to spreading error and masks the low or dim fluorescence positive cells. Because there are many fluorophores with emission in the 600 nm range, spreading error is most likely to happen when using fluorophores within the 600 nm range. A proper panel design minimizes the effect spreading error has on the ability to classify cells as positive or negative appropriately [51, 56, 97]. Investigators must work with their flow core manager to identify fluorescent conjugates that work on the cytometers available and optimize panel design.

(c) For the HIPC panel, the combination of fluorophores has been carefully designed to work well for most cytometers [35, 75]. The HIPC panel is available commercially in a lyoplate format that contains prealiquoted cocktail mix of all fluorescently conjugated mAbs in a lyophilized pellet. The prealiquoted kit eliminates the need for titering mAbs and reduces overall pipetting time and error.

(d) Should there be a need or desire to develop a panel that is not listed in this chapter, some pointers for selecting markers to include in a panel are provided: Begin by stratifying markers into four levels of priority. The first priority is the end points of the assay; these markers represent the values that are reported in the final results. When simply measuring the presence of Tregs, the end-point markers are CD25 with either FOXP3 or CD127 (or both). However, there are instances in the literature that suggest that it is not the presence of Tregs but their state of being or functional activity that is clinically relevant. In this instance, markers for Tregs and markers for their respective state of being would be considered the first tier of markers, including CD194, HLA-DR, Ki67, and CD45RO and/or CD45RA. The second priority tier is gating. Since Tregs are a subset of helper T cells, CD4 together with the T cell receptor, CD3, is used as anchor gates to identify the subset of lymphocytes that contain Tregs. Arguably, if the Treg state of being is the scientific end point for a given study, then one might consider the Treg markers CD25, FOXP3, and/or CD127 as the second priority tier. However, since they are essential to scientific end point for Treg state of being, there are included in the first tier. The first and second priority

tiers always make it into the final panel; therefore, our minimal recommended panel includes CD3, CD4, CD25, and FOXP3 and/or CD127. The third priority tier includes cleanup markers—dead cell markers to exclude nonviable cells that are present in less pristine samples, such as whole blood that has been shipped overnight, cryopreserved cells, disaggregated tissue or samples from patients with conditions or medications that severely affect lymphocytes, such as chemotherapy or immunotherapies. The third priority tier is sometimes included in the panel but not always; this is determined by instrument configuration, available reagents/conjugates, time to develop the panel, and overall costs. The final priority tier contains subcategories that are based on findings in the literature, preliminary data, and finally hunches. The fourth priority tier is rarely included in the panel; this is determined by instrument configuration, available reagents/conjugates, time to develop the panel, and overall costs. No fourth priority Treg markers are included in this chapter.

(e) Knowing what how to treat each type of fluorophore is critical to a successful flow cytometry assay [90, 92–96]. In general, fluorescence conjugated reagents should be kept in the dark, on ice, and in neutral pH solutions, irrespective of whether the fluorescence is inside the mAb vial or in a staining tube. Tandem dyes, such as PE-Cy7, degrade easily and must be treated with great care, including during transport. A degraded tandem dye might work well when stained by itself. However, the only way to know if a tandem dye has degraded is to stain cells or beads with the dye and observe how much, if any, fluorescence is present in the fluorescence donor; this is PE in the PE-Cy tandem. Also, many fluorophores are sensitive to fixatives. When using fixatives, test the fluorophores with the fixative to know how it might affect the fluorescent results.

6. The choice of staining protocol may lead to very different results; for this reason, it is highly recommended that you compare nuclear permeabilization reagents using your own samples and your FOXP3 clone of choice to select the reagents that work best for your assay. Permeabilizing agents are designed for different purposes. There are permeabilization reagents for accessing intracellular antigens and intranuclear antigens. Permeabilization reagents for intracellular antigens will not work well for intranuclear markers, while permeabilization reagents for intra-nuclear markers work well for intracellular markers. Both types of permeabilization reagents may affect

expression of surface antigens. FOXP3 is a transcription factor and an intranuclear permeabilization reagent is required to measure FOXP3 expression. Ki67 is present in the cytoplasm and an intracellular permeabilization reagent is required to measure Ki67 expression. Intranuclear permeabilization reagents also work for measuring Ki67. Nuclear permeabilization buffers have been compared in the literature; the eBioscience FOXP3 permeabilization buffer is currently the most widely used for Treg assays [28, 70, 72]. Fixation is used together with the permeabilization reagent to maintain integrity of surface membrane and markers. Read the manufacturer's recommended instructions carefully when using permeabilization reagents and buffers. If the wrong diluent is used to create the Fix/Perm or Perm Wash, or if the wrong buffer is used to wash, then the permeabilization result may be suboptimal and yield poor FOXP3 or Ki67 staining. Prepare the Fix/-Perm and Perm buffer fresh and discard unused portion after using.

7. To conserve cells and for optimal signal, compensation beads are used in place of cells as single-stained controls for compensation [66–68, 79, 92, 98]. Compensation beads bind the same fluorescent conjugated mAbs used for staining cells via the Fc portion of the mAb. There are positive and negative compensation beads. Positive compensation beads bind mAb and are positive for the respective Ab used to stain the beads. Negative compensation beads do not bind mAb and therefore have no fluorescent tag; they are used as a negative control. Positive and negative compensation beads can be included in the same staining tube or separately. When positive and negative compensation beads are stained in the same tube, then a positive and negative gate must be created for the given fluorescence in each tube. When positive and negative compensation beads are stained in separate tubes, then the negative can be used as a universal negative during compensation. A universal negative is the negative for all fluorophores used in the given panel and saves gating time during analysis. Binding mAb to beads is not the same as binding mAb to cells; include some protein in the compensation bead wash buffer, and a fixative, such as 2–5% FBS, to help stabilize the mAb binding to beads. Otherwise, the fluorescence from the positive compensation beads might appear as a smear instead of a single bright peak. Compensation beads are smaller and less complex than cells; for this reason, the FSC and SSC detector settings will likely be different between compensation beads and cells. However, the proper use of the compensation beads requires that the fluorescent detector settings for the beads be identical to the settings used for cells acquired in the same experiment. The fixable vital dye

is not an mAb but rather binds to free amines inside or on the cell. There are special compensation beads available to use with the fixable vital dyes, called arc beads. Arc beads are a separate product from standard compensation beads. They are also smaller than the regular compensation beads, but can usually be seen using the same scatter channel settings as the regular compensation beads when the settings are adjusted properly.

8. Fixation is not a necessary part of the assay, but can be very helpful when used as the buffer cells are stored in after staining and prior to acquisition. Fixatives kill cell-associated infectious agents, when cells are stored in fix for at least 24 h, and are required by some facilities based on the type of sample being used and the relative biological safety risk. Fixatives also stabilize binding of fluorescent conjugated mAbs to cells. If acquisition is delayed beyond 6 h, fixation will preserve the fluorescence intensity and integrity of staining if present during the delay. Fixatives can increase the level of autofluorescence observed in otherwise unstained cells and some fluorescent dyes are sensitive to some fixatives. For this reason, testing the combination of fluorescent dyes and fixatives is recommended prior to using the combination with test samples.
9. A vacuum manifold is used to aspirate supernatant from wells. Flicking plates to remove supernatant leads contamination between wells, higher backgrounds, and overall increased assay variability from inconsistent washing. To ameliorate problems caused by flicking plates, use a vacuum manifold. Vacuum manifolds standardize supernatant aspiration when using plates and have been shown to reduce background noise and improve overall assay variability [99, 100]. Using vacuum manifolds properly requires practice. Setting the vacuum pressure inappropriately is the most common problem; pressure set too high aspirates cells along with the supernatant and pressure set too low does not remove all of the supernatant which leads to increased backgrounds or drag-up of the negative. Check the total number of events in each resulting FCS file to know whether there is any unexpected cell loss. If the assay has been performed properly, and the manifold used correctly, the files will contain a consistent number of events for a given sample. If the pressure is too low, then supernatant remains after aspiration, leading to high backgrounds and increased assay variability. Not holding the manifold perpendicular to the plate creates uneven pressure when aspirating the supernatant, such that some wells do not receive enough vacuum pressure; this also leads to increased variability. To set the vacuum properly, add water to a tray, place the manifold tip just under the surface of the water, not resting on the bottom of the tray. While holding the manifold in position, very slowly turn on the vacuum

pressure. Apply as little pressure as possible, then very slowly increase pressure until the water just begins to aspirate. The pressure should be set so that there is just enough pressure to pull the water gently from the tray. Avoid pressures high enough to aspirate the most of the liquid at once. After the vacuum pressure is set, and before using the manifold to aspirate actual samples, practice using the manifold with a plate. To practice with a plate, fill the wells in a plate with water and use the manifold to aspirate water from the wells in the plate. Observe or measure the residual volume remaining in wells after aspirating the supernatant. If the volumes are too high the pressure is too low, if the volumes are too low the pressure is too high, if the volumes are inconsistent, then the manifold is not properly positioned relative to the plate during aspiration. Continue practicing with the manifold until the residual volumes for all wells are as expected. Each manifold has a known residual volume to expect; consult the manufacturer for more details. Remove water from the manifold tip before using to aspirate supernatant from cells by wiping the tips with clean gauze. Residual water may lyse cells. If not used properly, vacuum manifolds may pose a safety risk. To minimize the safety hazard, do not apply more vacuum pressure than what is needed to aspirate the supernatant and implement a holding chamber, to house the aspirated supernatant waste between the vacuum line and source of sample. Consulting with institutional safety is recommended before installing or using a vacuum manifold.

10. Cell staining may be performed in either tubes or plates. The number of cells to add per tube or per well ranges from 0.5×10^6 to 4×10^6 viable leukocytes, depending upon the number, type, and location of the markers. The number of cells recommended is based on the fact that the normal range for Tregs is approximately 5–9% of CD4 lymphocytes, meaning the gating hierarchy or the denominator influences the number of cells to be added, smaller denominators require more cells. For an assay that measures Tregs (CD3, CD4, CD25, and CD127 or FOXP3), $0.5–1.0 \times 10^6$ viable cells is the minimum number of cells to add per tube or well. In normal blood the number of white cells range from approximately $1–2 \times 10^6$/mL, meaning that 250–500 μL of whole blood will be needed to achieve 0.5×10^6 cells. For Treg assays that measure surface expression of CD127 and do not require permeabilization, 0.5×10^6 cells is sufficient. Permeabilization causes the cells to be more buoyant and increases cell loss; therefore, 1×10^6 cells is the minimum for an assay that utilizes the intranuclear marker FOXP3 to identify Tregs. To measure Treg states of being, the end value to report is a proportion of Tregs, a

smaller subset of CD4 lymphocytes. To accurately report Treg states of being a minimum of 2–4 $\times$ 10^6 viable leukocytes is needed. Use two million cells for the HIPC surface assay and four million for the CIP intracellular/intranuclear assay. To recover a sufficient number of cells for analysis, add at least the recommended minimum number of cells (more when available), wash when necessary but avoid unnecessary washing, and permeabilize only when necessary.

11. Keep fluorescent reagents and samples stained with fluorescent reagents protected from light at all times when not directly using them. Fluorophores are sensitive to light and if exposed will result in diminished fluorescence intensity.
12. Do not leave the centrifuge unattended while cells are in the centrifuge. Do not leave cells in the centrifuge after spinning. Cells left in a pellet become hypoxic rapidly, inducing cell death. Leaving cells on a pellet longer than necessary is a leading cause of poor-quality flow cytometry assay results. Dead and dying cells may still stain fluorescent markers; however, their expression may be variable in intensity, and/or falsely negative or positive. Avoid unnecessary cell death after centrifugation by working as quickly as possible to remove cells from the centrifuge, remove the supernatant, and resuspend cells. Use the centrifugation and incubation times to prepare for the next step in advance so that cells do not have to sit on a pellet waiting for the next step. Advanced preparation enables one to resuspend cells in the next solution quickly and efficiently, improving viability and flow cytometry results. To help with troubleshooting, position tube labels opposite from the rotor when putting tubes into the bucket and the bucket into the centrifuge (*see* **Note 13**). After centrifugation, the cell pellet will be positioned toward the side of the tube that is furthest away from the rotor. If tubes are positioned as noted, the pellet will be located on the side of the tube with the label. Follow these recommendations for all centrifugation steps.
13. Do not disturb the cell pellet when removing the tubes or plate from the centrifuge or when removing the supernatant. After centrifugation, visually inspect each tube or well for a visible pellet. If no pellet is observed in any tube or well, do not decant the supernatant and consider repeating the centrifugation step. Check the centrifuge settings. When repeating a centrifugation step, first resuspend cells by vortexing or pipetting; this will minimize the time cells are left on a pellet. If using tubes, note the position of the cell pellet prior to decanting the supernatant. If there are too few cells to see a pellet, the pellet can still be identified by marking the tube relative to the rotor (*see* **Note 12**). To minimize cell loss, decant the supernatant

from the tubes with the pellet positioned on the top of the tube. Follow these recommendations for all decanting steps.

14. Practice using pipettes prior to using them to pipette small volumes of vital dye or antibodies. When pipetting smaller volumes check the pipette tips to verify that the volume is accurate. Quality tips are graduated and have lines for verify the volumes. After dispensing the liquid verify that the liquid is no longer in the tip. If pipettes are not used properly the appropriate amount of liquid is not added stain the cells, resulting in variable results, usually missing or lower staining than expected. Refer to manufacturer instructions for more details. Calibrate pipettes every 6 months to ensure accuracy. Follow these recommendations for all steps that involve pipetting small volumes.
15. Fluorescent reagents, including vital dyes and mAbs, should be pretitered, under the conditions of the assay, to determine the optimal concentration for each reagent [101, 102]. Optimal staining of vital dyes and fluorescent conjugated mAbs is achieved by using saturating amounts. Saturation is determined through titrations based on mass, usually performed using serial twofold dilutions. Reagents to be used for surface stain must be titered as a surface stain using the same temperatures, wash buffer and fixative as will be used in the final assay. Reagents to be used for intracellular or intranuclear stain must be titered as intracellular or intranuclear stain using the same temperatures, Fix/Perm, Perm buffer, and fixative as will be used in the final assay.
16. Mix cells thoroughly after adding each reagent added. When mixing cells avoid creating air bubbles as these increase surface tension, leading to cell death. After mixing cells, visually inspect the tubes or wells to see if a pellet remains. If a pellet is observed, cells were not mixed properly and should be remixed until no pellet remains. If cells are not mixed properly, the stain results will be poor quality. In addition, cells that are not resuspended properly are left on a pellet, causing increased cell death and assay variability. Avoid overmixing cells, they only need to be mixed enough to resuspend the cell pellet in the added solution. Overmixing might also result in poor quality results.
17. Create mAb mixes immediately prior to use. Do not allow them to set longer than 30 min prior to use. Use the appropriate buffer to create mAb mixes, FACSWash buffer for surface stain mix and Perm buffer for intracellular/intranuclear stain mix. If more than one brilliant dye is used to stain cells, then a commercially available stabilization buffer that is specific for brilliant dyes should be used to prepare the mAb mix. The mAb

mix should be prepared such that a final volume of 100 μL will include all of the mAbs at their optimal concentration when added to the cells in their residual volume. Residual volume is usually around 35 μL but should be measured for each operator to ensure accuracy. If using preconfigured lyoplates, such as for the HIPC panel, follow the manufacturer's instructions for staining.

18. Microfuge the mAbs to pellet aggregates of the antibody and/or fluorophore. When pipetting mAb from the reagent vial, avoid disturbing the pellet and pipette liquid from the top of the vial, avoid touching the pipette tip to the bottom of the vial. Fluorophore aggregates appear in a freckling pattern higher than the cell staining and increase assay variability.
19. Surface staining mAb reagents include CD3, CD4, CD25, CD127, CD45RO, CD45RA, HLA-DR, and CD194, depending upon the preferred Treg panel. CD194 is CXCR4. Chemokines recirculate in the membrane when incubated at 37 °C; for this reason, optimal staining for chemokines is observed when prestaining at 37 °C for 10 min prior to continuing with the remainder of the surface stain [101]. If using prealiquoted lyoplates, follow the manufacturer's instructions for staining.
20. Lysis is required when using whole blood but optional for PBMCs. Lysis, when used properly, improves results by reducing noise (*see* **Note 3**).
21. Intranuclear permeabilization is required for FOXP3 detection. Intracellular permeabilization is required for Ki-67.
22. Permeabilized cells are more buoyant than cells that have not been permeabilized. Thus, increased centrifugal force and time are needed to pellet permeabilized cells. More harsh Fix/Perm reagents may necessitate additional centrifugal force and time. If you recover overall fewer cells than expected in the final data files, first verify that the operator is acquiring all of the sample, then verify that the postperm centrifugation is set to a higher force and longer time.
23. Intracellular/intranuclear mAb reagents include FOXP3 and Ki76. CD3 and CD4 may also be added during the intracellular/intranuclear staining step, if using clones that are made against fixed antigens, such as the SK7 and SK3, respectively, and the fluorescent conjugates are titered for intracellular/intranuclear usage. The buffer used to create the intracellular/intranuclear mAb mix must be the Perm buffer and not FACSWash buffer. If more than one brilliant dye is used, then the commercially available brilliant dye stabilization buffer must be added, comprising 10–20% of the final mix volume.

24. Resuspending cells in a fixative protects samples in case longer storage time is needed and inactivates infectious agents. If not resuspending cells in fixative, reconstitute cells in FACSWash buffer for surface stained cells and Perm buffer for intranuclear/intracellular stained cells.
25. Compensation beads by be stained and acquired in either tubes or plates. If using lyoplates, reconstitute beads according to the manufacturer recommendations.
26. Optimal bead staining may require more volume of mAb than what is used to stain cells. The amount of mAb needed to stain compensation beads should be predetermined during panel development. The fluorescence intensity of beads should be the same as or higher than the fluorescence intensity of cells when stained with the same mAb.
27. Compare LASER (excitation) and filter (emission) optics against the suggested excitation and emission spectra for each fluorescence dye used in the panel. Flow core operators and manufacturers can provide you with the optical configuration of their equipment. In addition, most companies that sell fluorescent conjugated mAbs offer technical support to facilitate panel design as well as on-line charts and tools to help identify which optics are compatible with their specific fluorescent products. Ideally, the process of comparing collection optics on the cytometer with fluorescent reagents occurs both prior to panel develop to guide the process and during the assay, to confirm that each respective fluorophore is being excited by the appropriate LASER and collected in the appropriate detector.
28. Begin each acquisition with full sheath tank and empty waste tank. Depending upon the instrument used, when a tank is half full or less the sheath pressure changes, causing fluorescence to shift and the event rate to change. These changes affect the resulting data by increasing variability.
29. Verify instrument performance by confirming that the performance criteria are within acceptable limits based on established ranges. Instrument performance criteria and their respective acceptable ranges may vary from lab to lab. If there are no established performance criteria for a given instrument, Perfetto et al. describes a very useful method for qualifying instrument performance [77].
30. Set fluorescence PMT voltages by running a fluorescent standard control and setting the median fluorescence to a predetermined value within the acceptable range. Instrument calibration is derived from the instrument verification used by manufacturers or as described by Perfetto et al. [77, 78]. Detector settings for scatter (FSC, SSC), as well as the detection

threshold, is set by running unstained cells and placing the lymphocytes on scale. The entire lymphocyte population should be visible, separate and distinct from debris, monocytes, and dead cells. Do not exclude lymphocytes by setting the detection threshold too high. Calibrating the instrument before each experiment (and prequalifying your reagents) ensures positive fluorescence and scatter settings are standardized. Standardized data enable centralized gating, applying the same gates to all files across all experiments, and improve assay reproducibility.

31. Set collection gate to acquire 25,000–50,000 CD3+ lymphocytes for Treg assays or 50,000–100,000 CD3+ lymphocytes for Treg state of being assays. This may mean that the entire volume of the tube is aspirated. The increased number of cells acquired is necessary for HIPC and CIP panels to evaluate states of being with some degree of statistical confidence. Do not hard-gate data, meaning collect all of the events and not just lymphocytes, as it may be necessary to adjust the lymphocyte gate during analysis.
32. Compensation can be set either during or after acquisition. It is not necessary to set compensation during acquisition; however, compensating data during acquisition facilitates collecting a predetermined number of cells, such as CD3+ lymphocytes.
33. Clogs and air bubbles are the most common problem encountered when acquiring samples on a cytometer. They can render the data in a file uninterpretable. While they are not entirely preventable, their frequency and effect on resulting data can be minimized. To avoid clogs, inspect all samples after staining and before acquisition. All samples with any visible clumps should be filtered prior to acquisition. To avoid bubbles, fill the sheath tank and empty the waste tank prior to each acquisition. Also, be vigilant at keeping track of the event rate during the acquisition of each tube or well. If event rate changes dramatically (higher or lower), or declines steadily over three consecutive refreshes, then there is a clog or air bubble. When this happens, stop the acquisition and troubleshoot to remove clog or air bubble before continuing. Observing the event rate is the only opportunity to identify and correct acquisition problems before they are permanently recorded into data files.
34. After each acquisition, stringently follow either the manufacturer or lab-specific cleaning protocol. Flow cytometers are fluidic instruments containing salt solutions. For the fluidics to work properly they must be cleaned regularly. After cleaning, place a tube filled half-full with deionized water on the sample port. Do not allow the sample port to dry. When they are not cleaned regularly or properly, the number of clogs from cells,

parts of cells, or salt solutions increase significantly and are more severe when they occur, often requiring service engineers to correct.

35. The biggest risk of data loss occurs while the data are located only on the cytometer workstation. To minimize the risk of data loss, transfer data files from the cytometer either during acquisition or while cleaning the cytometer. Verify all files are successfully transferred immediately after transferring them from the cytometer workstation. All data files are removed from the cytometer workstation during regular maintenance procedures, usually each month.
36. Ensure that there is a stained compensation bead for each fluorophore used in the panel [67, 68, 79, 92, 98]. In order for the compensation matrix to be calculated properly, confirm that the parameter selected in the file matches the appropriate channel for the marker that was stained. For example, when PE is collected in the Green E detector on the cytometer, then the data file for PE compensation bead should use the Green E parameter. If Green D parameter is selected for the PE compensation bead, then there is mismatch and the resulting compensation will be inaccurate. Some software have autocompensation calculations that can make these types of mismatch errors when the compensation bead is brighter in the secondary detector (Green D in this example) than it is in the primary detector (Green E in this example). Staining that is brighter in a secondary channel than in the primary channel happens when the instrument is not calibrated properly and necessitates significantly more compensation, if the primary signal is to be resolved (*see* **Notes 29** and **30**)
37. Check that the appropriate compensation matrix is applied to all files. Some software packages are notorious for changing compensation matrix, especially reverting back to an acquisition matrix. It is helpful to show the compensation in the workspace window throughout the entire analysis process, to ensure that the proper compensation matrix has been applied to each file.
38. To verify compensation is set properly, use $N \times N$ plots, meaning to view all X parameters versus all Y parameters in a given file simultaneously. Some software packages generate $N \times N$ plots automatically, if not then the plots need to be created manually. When compensation is set properly the populations appear symmetrical in a two-dimensional dot plot. Compensation errors are visible as a "hook" or parabola, where positive events are turning either toward or away from the respective axis. If the hook is away from the axis (toward the double positive quadrant), then the compensation error is said

to be undercompensation. If the hook is toward the axis, then the compensation error is said to be overcompensation. If a sample appears to be both over and undercompensated simultaneously, and the error remains even after adjusting compensation, this is spreading error. Spreading error cannot be corrected by adjusting compensation; it can only be prevented by using quality panel design [51–53, 97].

39. Correct undercompensation by increasing the compensation value. Correct overcompensation by decreasing the compensation value. Adjust compensation manually by using the matching means method. In a two-dimensional dot plot, with X fluorescence versus Y fluorescence, view the cell population along the X axis first. There are cells that are positive for X and negative for X. The Y mean of the cells that are positive for X should match the Y mean for the cells that are negative for X. For mutually exclusive populations, like CD4 or CD8 T cells, the populations should appear symmetrical when compensation is set accurately. Repeat this process for each combination of X versus Y for all parameters in the file. Because compensation from one parameter affects other parameters in the file, it may be necessary to repeat the entire process of checking all combinations of X versus Y for two to three passes. Biexponential transformation helps to visualize the negative [103–105]. When biexponential transformation is calculated in the presence of a severe overcompensation error, then the resulting biexponential display can create a valley artifact, where the negative population becomes merged with the positive population [106]. Correct the compensation error then recalculate the biexponential transformation to correct the valley artifact. The more markers used in a panel, the more complex and time-consuming compensation will be. A well-designed panel [together with appropriate instrument settings (*see* **Note 29** and **30**)] requires much less manual tweaking of the compensation matrix. If help is required for compensation, core operators or technical applications specialists can provide assistance.

40. Data analysis, including compensation, as well as the placement of gates and analysis regions, is the single largest source of variability in flow-based assays [69, 99, 107–109]. To ameliorate overall assay variability, great care has been taken to standardize panels, such as the HIPC and CIP panels, reagents such as the HIPC lyoplates, and instruments through calibration. After standardizing panels, reagents, and instruments, the cell populations should fall in the same relative positions for each dot plot displayed during analysis. The conundrum is that the actual placement of gates and regions, to separate negative and positive populations, is highly subjective and relies upon

the expertise of the operator performing the analysis. To reduce the variability associated with the placement of gates, a gating template or uniform gates may be employed. Gating templates are template file formats, created in the analysis software and applied across multiple files and/or multiple experiments without moving the gates. Uniform gating is achieved by applying the same gates across all samples from a given donor. In either case, there are exceptions to the rule. Time gates are changed with each file to include all of the events that are acceptable in the file. Fluorescence from fixable vital dyes seems to shift more than conjugated mAbs, causing the live cell gate to be moved even within a single donor. The same is true for a scatter lymphocyte gate. Light scatter properties are less stable than fluorescence, owing to changes within the microenvironment of each tube during the staining process. As a result, scatter gates sometimes need to be moved, even within a single donor. When following the methods described in this chapter, it is rare that gates or regions drawn on markers from fluorescent conjugated mAbs need to be moved within a donor. It is feasible that fluorescent conjugated mAb gates and regions might need to be adjusted slightly between donors, to accommodate different backgrounds; however, shifts in the positive populations are usually not observed. When shifts in the positive population occur, they are usually due to staining, acquisition, or instrument errors. Careful note taking during the staining and acquisition steps, documenting any errors with each tube, can be helpful in tracking these types of errors and deciding which data may be excluded based on errors.

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Chapter 10

Immunophenotyping of Human Innate Lymphoid Cells

Sara Trabanelli, Alejandra Gomez-Cadena, and Camilla Jandus

Abstract

In the last years, the family of innate lymphocytes has been growing following the discovery of innate lymphoid cells (ILCs). ILCs are lymphocytes able to rapidly produce a wide range of soluble mediators in an antigen-independent fashion. So far, three main subsets of ILCs have been discovered, ILC1, ILC2, and ILC3, expressing respectively the transcription factors T-bet, GATA3, and Rorγt and secreting distinct types of cytokines. After their discovery, several studies showed that different pathologies, such as allergic airway diseases and inflammatory disorders, are sustained by dysfunctional ILCs before adaptive immune sets in. In this regard, considerable efforts are currently performed to harmonize the identification and monitoring of ILCs in healthy and pathologic conditions to streamline a uniform immunophenotyping. Standardized ILC monitoring techniques will accelerate our understanding of these effector innate immune cells and ultimately facilitate their targeting in the context of infection, cancer, autoimmune disease, and transplantation.

Key words Human, ILCs, Flow cytometry, Staining, Isolation, Cytokines, Transcription factors, Expansion

1 Introduction

Innate lymphoid cells (ILCs) are the most recently identified family of lymphocytes that belong to the innate immune system [1]. Indeed, they lack rearranged antigen-specific receptors, such as the T-cell receptor (TCR, specifically expressed by T lymphocytes) or the B-cell receptor (BCR, specifically expressed by B lymphocytes), on their cell surface. As a consequence, ILCs do not recognize, bind, and respond to specific antigens but to soluble factors or to molecules expressed on other immune and nonimmune cell types. Therefore, they rapidly respond to signals coming from the extracellular environment and constitute one of the first-line defense of our body. For this reason, ILCs are considered "early sentinels" enriched at mucosal and barrier surfaces. They are also present in primary and secondary lymphoid organs, and they can be found circulating in the peripheral blood.

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ILCs differentiate from the common lymphoid precursor in the fetal liver or in the bone marrow [2]. Their development is similar to the one of natural killer (NK) cells, since it requires the expression of the common γ chain of the interleukin-2 (IL-2) receptor and the transcriptional repressor Id2. Different from NK cells, though, ILC differentiation is dependent on GATA3 and IL-7Rα expression. Further sequential engagement of transcription factors, cytokines and microbial signals is critical for the development of three distinct groups of mature ILCs. ILC1 express T-bet, are responsive to IL-12, and produce IFN-γ. ILC2 highly express GATA3, are responsive to the alarmins IL-25, IL-33 and TSLP, and produce IL-4, IL-5, IL-9, IL-13, and amphiregulin (Areg). ILC3 express RORγt, are responsive to IL-1β and IL-23, and produce IL-17 and/or IL-22. Notably, because of their transcriptional and cytokine profiles, mature ILCs are considered the innate counterpart of CD4 T helper cells. Indeed, ILC1 mirror the Th1, ILC2 the Th2, and ILC3 the Th17/22 T helper subsets. However, similar to CD4 T helper cells, this classification is not totally fixed, since heterogeneity and plasticity are prominent features of ILC behavior across all subsets and even beyond ILC themselves. Indeed, interconversion between NK cells and ILCs has also been reported [3].

The different ILC subsets actively participate to the initial phases of immune responses against different pathogens or during physiological and nonphysiological situations that alter homeostasis. In particular, ILC1 have been described as important players in fighting intracellular bacteria and protozoa, as well as in sustaining chronic inflammation. ILC2 are involved in the resolution of helminth infections and in allergic diseases and asthma. ILC3 are engaged during immune responses against extracellular bacteria and fungi; they are involved in intestinal homeostasis and participate in lymphoid tissue development.

To date, only few protocols are available for the identification and functional characterization of human ILCs. Since this recently described population of innate lymphocytes has become the focus of extensive investigations that aim at understanding their physiological and pathological roles in humans, uniform and robust methodologies for their careful identification and functional evaluation are essential [4].

This chapter represents a detailed description of materials, methods and notes of laboratory techniques, with the aim to promote common procedures leading to standardization of assays, ultimately allowing for wide application and improved understanding of the role of the different ILC subsets in physiological and nonphysiological processes [5].

2 Materials

2.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Human Peripheral Blood

1. Heparinized human blood.
2. 50 mL tubes.
3. Lymphoprep™ (Axis-Shield PoC AS), store at 4 °C in the dark.
4. PBS, store at room temperature.
5. RPMI 1640, GlutaMAX™-I medium (Gibco). Store at 4 °C.
6. 10 mL and 25 mL sterile pipettes.

2.2 Isolation of Human Mononuclear Cells (MNCs) from Tonsils

1. Pair of sterile scissors.
2. Petri dishes.
3. 70 μm and 100 μm cell strainers.
4. 50 mL tubes.
5. Plunger of a plastic syringe.
6. 10 mL Sterile pipette.
7. Lymphoprep™ (Axis-Shield PoC AS), store at 4 °C in the dark.
8. PBS, store at room temperature.
9. RPMI 1640, GlutaMAX™-I medium (Gibco). Store at 4 °C.

2.3 Extracellular Staining of Ex Vivo Human ILCs

1. FACS buffer: PBS supplemented with 5 mM EDTA, 0.2% BSA, and 0.2% NaN_3. Store at 4 °C.
2. LIVE/DEAD™ fixable green dead cell stain kit for 488 nm excitation (Invitrogen) (*see* **Note 1**).
3. Monoclonal antibodies:
   (a) Lineage (Lin): CD4 (clone RPA-T4); CD8 (clone MEM-31) (ImmunoTools). CD3 (clone UCTH1); CD14 (clone HCD14); CD15 (clone HI98); CD19 (clone H1B19); CD20 (clone 2H7); CD33 (clone HIM3-4); CD34 (clone 561); CD203c (clone NP406); FcεRI (clone AER-37) (Biolegend). CD16 (clone RMO52) (Beckman Coulter), all FITC conjugated.
   (b) CD127 BV421 (clone AO19D5); CD56 Alexa 700 (clone HCD56); CD117 (c-Kit) APC-Fire (clone 104D2); CRTH2 PE (clone BM16); CD161 (clone HP-3G10) PE-Cy7; PerCPCy5.5 NKp44 (clone P44-8) or NKp46 (clone 9E2) (Biolegend) (*see* **Notes 2** and **3**).
4. 5 mL V-bottom tubes or 96-well plates.
5. 5 mL U-bottom tubes.

2.4 Fluorescence-Activated Cell Sorting (FACS) to Isolate Human ILC Subsets

1. Anti-human CD3, anti-human CD14 MicroBeads (Miltenyi Biotec). Store at 4 °C.
2. LD Columns (Miltenyi Biotec).
3. MACS® Manual Separators—MidiMACS™ (Miltenyi Biotec).
4. Sorting buffer: PBS supplemented with 5 mM EDTA and 0.2% BSA. Store at 4 °C.
5. Same antibodies described for the extracellular staining (*see* Subheading 2.3) (*see* **Note 3**).
6. 1.5 mL Eppendorf tubes.
7. Sorting collection buffer: 100% fetal calf serum (FSC) (Gibco).

2.5 Transcription Factor (TF) Analysis on Human ILCs (Fig. 2)

1. Monoclonal antibodies: T-bet PE-CF 594 (clone O4-46); RORγt PE (clone Q21-559) (BD).
   GATA-3 APC (clone 16E10A) (eBioscience™) (*see* **Notes 2–4**).
2. Foxp3/Transcription Factor Staining Buffer Set (eBioscience™).
3. FACS buffer: PBS supplemented with 5 mM EDTA, 0.2%. BSA and 0.2% NaN_3. Store at 4 °C.
4. 5 mL V-bottom tubes or 96-well plates.
5. 5 mL U-bottom tubes.

2.6 Functional Analysis of Human ILCs

2.6.1 Intracellular Staining for Evaluation of Cytokine Production by Human ILCs

1. 96-well plate.
2. ILC culture medium: RPMI 1640, GlutaMAX™-I supplemented with 8% human serum (HS) (recommended is the use of pooled A+ healthy donor's serum), 1% PSG Mix (penicillin and streptomycin) (Gibco), 1.5 mM L-glutamine (Gibco), 0.24 mM L-asparagine (Sigma), 0.55 mM L-arginine (Sigma), 1% HEPES buffer (Animed), 1% from the 100 mM sodium pyruvate (Gibco), kanamycin 100× (Gibco), 0.1% 2-β-mercaptoethanol (Gibco). Store at 4 °C.
3. Recombinant human (rh) cytokines: 100 IU/mL IL-2 (Roche); 5 ng/mL rhIL-7; 50 ng/mL rhIL-12; rhIL-15; rhIL-25; rhIL-1β; rhIL-23; rhTSLP; rhIL-33 (PeproTech). rhIL-18 (R&D). Store stock dilutions according to the manufacturer's recommendations or at −80 °C, respectively.
4. GolgiPlug and GolgiStop (BD Biosciences) or Brefeldin A (Sigma). Store stock dilutions according to the manufacturer's recommendations or at −20 °C, respectively.
5. 50 ng/mL PMA (Phorbol 12-myristate 13-acetate) (Sigma) and 500 μg/mL ionomycin (Sigma). Store stock dilutions at −80 °C.

6. Fixation buffer: PBS supplemented with 1% formaldehyde, 2% D-glucose and 5 mM NaN_3. Store at 4 °C.
7. 0.1% saponin (Sigma). Store stock dilutions and aliquots at −20 °C.
8. Monoclonal antibodies: IFN-γ (clone 4S.B3); TNF-α (clone Mab11); IL-4 (clone MP4-25D2); IL-5 (clone TRFK5); IL-9 (clone MH9A4); IL-13 (clone JES10-5A2); IL-17A (clone eBio64DEC17); IL-22 (clone 2G12A41); LT-α (clone 359-81-11).

2.6.2 Quantification of Cytokine Secretion in Human ILC Culture Supernatants

1. 96-well plate.
2. ILC culture medium (*see* Subheading 2.6.1).
3. Recombinant human (rh) cytokines: 100 UI/mL IL-2 (Roche); 5 ng/mL rhIL-7; 50 ng/mL rhIL-12; rhIL-15; rhIL-25; rhIL-1β; rhIL-23; rhTSLP; rhIL-33 (PeproTech). rhIL-18 (R&D). Store stocks dilutions according to the manufacturer's recommendations or at −80 °C, respectively.
4. Multiplex immunoassay for cytokine detection kit (i.e., MSD Th1/Th2 cytokines, LEGENDplex™ Human Th Cytokine Panel, BD CBA Human Inflammatory Cytokines Kit, Luminex® Multiplex Kit R&D or single staining cytokine array).

2.7 In Vitro Expansion and Cryopreservation of Human ILCs

1. 96-well plates.
2. ILC culture medium (*see* Subheading 2.6.1).
3. Recombinant human (rh) cytokines: 100 U/mL rhIL-2 (Roche); 5 ng/mL rhIL-7 (PeproTech).
4. FCS sterile (Gibco).
5. Dimethyl sulfoxide (DMSO), sterile.
6. RPMI 1640, GlutaMAX™-I medium (Gibco). Store at 4 °C.
7. Freezing solution: pure FCS supplemented with 10% DMSO. Make fresh as required.
8. Round-bottom 1.8 mL cryogenic vials.

3 Methods

3.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Human Peripheral Blood

1. Transfer freshly collected heparinized human blood in 50 mL tubes (*see* **Note 5**).
2. Dilute the blood at 1:1 ratio with sterile PBS.
3. Prepare 50 mL tubes with the Lymphoprep™ solution. The ratio of Lymphoprep™ to diluted blood is 1:1.5.
4. Carefully and slowly, overlay the diluted blood on the Lymphoprep™ layer, creating a sharp Lymphoprep™–blood

interface (*see* **Note 6**). Cap the tube, avoiding accidental contamination.

5. Centrifuge the tube at 528 × *g* for 20 min with slow acceleration and without brake, in a swinging bucket rotor, at room temperature (*see* **Note 7**). This step allows for the formation of a density gradient that, by centrifugation, stratifies on different layers peripheral blood mononuclear cells and other cells such as erythrocytes and polymorphonuclear leukocytes. It creates an opalescent ring at the Lymphoprep™–medium surface where you will find the PBMCs.
6. Carefully harvest the PBMC layer, using a 5 or 10 mL sterile pipette. Transfer the cells into a new 50 mL tube. Fill up the tube with PBS or RPMI 1640, GlutaMAX™-I, mix well, and centrifuge for 5 min, at 528 × *g* with break, at room temperature.
7. Remove the supernatant, add PBS or RPMI 1640, GlutaMAX™-I, then centrifuge the PBMCs at 234 × *g* for 10 min without brake at room temperature (*see* **Note 8**).
8. Wash the cells in PBS or RPMI 1640, GlutaMAX™-I (5 min at 528 g).
9. Resuspend the pellet in ILC culture medium and count the cells before using the cell suspension (*see* **Note 9**).

3.2 Isolation of Human Mononuclear Cells (MNCs) from Tonsils

1. Put 3 mL of RPMI 1640, GlutaMAX™-I in a petri dish with tonsils (*see* **Note 5**).
2. Cut tonsils into small pieces in the petri dish using sterile scissors (*see* **Notes 10** and **11**).
3. Take the pieces and ground through a 100 μm sterile strainer using a plunger of a plastic syringe above a 50 mL tube. Rinse the filter two or three times with additional 5 mL RPMI 1640, GlutaMAX™-I.
4. Filter again the obtained cell suspension, this time through a 70 μm sterile strainer and rinse the strainer at least 2 times with 5 mL RPMI 1640, GlutaMAX™-I. Complete up to 25 mL with the same medium (*see* **Note 12**).
5. Carefully overlay this cell suspension on 15 mL of Lymphoprep™.
6. Centrifuge at 760 × *g* for 20 min with slow acceleration and without brake at room temperature.
7. Collect the mononuclear cell layer into a 15 mL tube, wash with PBS or RPMI 1640, GlutaMAX™-I, 10% FCS and centrifuge for 5 min at 528 × *g*.
8. Remove supernatant, resuspend very well the cell pellet with ILC culture medium and count the cells.

3.3 Extracellular Staining of Ex Vivo Human ILCs

1. Transfer the cells into V-bottom tubes or V- or U-bottom 96 well plates.
2. Wash with PBS and centrifuge for 5 min at 528 × *g*. At the same time, prepare the LIVE/DEAD solution referred before according to the manufacturer's recommendations in plain PBS (*see* Subheading 2.3) (*see* **Note 13**).
3. Discard the supernatant.
4. Add 100 μL of LIVE/DEAD solution on the pelleted cells. Incubate for at least 20 min and up to 1 h at 4 °C in the dark.
5. Prepare extracellular antibody mix using FACS buffer (*see* **Note 14**).
6. Wash with 250 μL (V bottom tubes) or 150 μL (96 well plates) of FACS buffer and centrifuge for 5 min at 528 × *g*.
7. Resuspend the pellet with 50 μL of extracellular antibody mix. Incubate for 30 min at 4 °C (*see* **Note 15**).
8. Wash as in **step 6**.
9. Resuspend in 200 μL of FACS buffer if there is no further intracellular staining. Transfer the stained cells in U-bottom tubes compatible with your flow cytometry analysis instrument.

3.4 Fluorescence-Activated Cell Sorting (FACS) to Isolate Human ILC Subsets

1. Wash cell suspension with sterile sorting buffer.
2. Deplete $CD3^+$ and $CD14^+$ cells using magnetic cell sorting according to the manufacturer's recommendations.
3. Centrifuge and resuspend the pellet with 50 μL of the antibody cocktail (*see* **Note 14**).
4. Incubate for 30 min at 4 °C (*see* **Note 15**).
5. Wash with sorting buffer: add 2 mL of sorting buffer and centrifuge for 5 min at 528 × *g*.
6. Resuspend the cells in sorting buffer at 10×10^6 cells/mL (*see* **Note 16**).
7. To isolate ILC1, ILC2, and ILC3 classical subsets the gating strategy presented in Fig. 1 can be used (*see* **Note 17**).
8. Recover your cells either in sterile 1.5 mL Eppendorf tubes or 5 mL tubes with 200 μL of sorting collection buffer.
9. Centrifuge the collected cells 5 min at 528 × *g* and then aspirate carefully the supernatant.
10. Resuspend in ILC culture medium according to your needs.

3.5 Transcription Factor Analysis on Human ILCs

1. After the extracellular staining described in Subheading 3.3, samples are washed and resuspended in 100 μL of fixation/permeabilization buffer, prepared following the manufacturer's recommendations. Incubate for 20 min at room temperature.

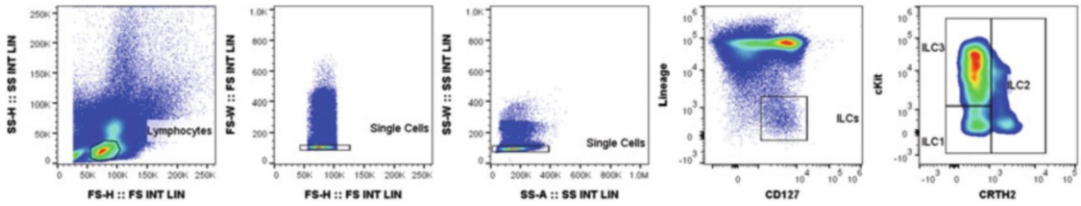

Fig. 1 Representative example of human ILC subset identification by multiparametric flow cytometry. ILCs were identified within the peripheral blood lymphocyte region on the basis of their forward (FSC) and side scatter (SSC) profiles (FSC low and SSC low) and by excluding from the analysis doublets (FSC H/FSC W dot plot, followed by SSC A/SSC W dot plot). Total ILCs were gated as $Lin^{-}CD127^{+}$ cells. Then, the different ILC subsets were determined according to the expression of CRTH2 vs cKit: ILC1 $CRTH2^{-}cKit^{-}$, ILC2 $CRTH2^{+}cKit^{+/-}$, and ILC3 $CRTH2^{-}cKit^{+}$. The dot plots shown are the result of a minimum of 10^6 MNCs acquired on a flow cytometer. Data were analyzed using FlowJo™ software (TreeStar)

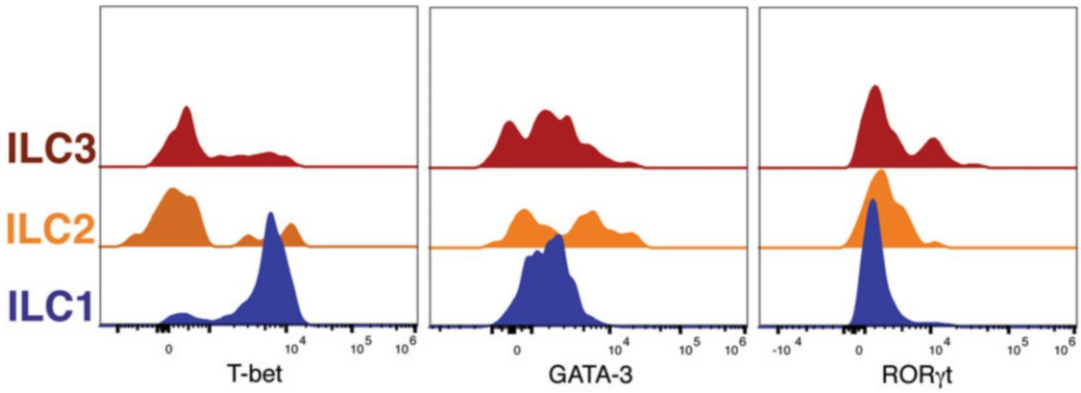

Fig. 2 Representative example of specific transcription factor expression by human ILCs. Total ILCs and ILC subsets were stained as in Fig. 1, then the cells were fixed and permeabilized and stained for T-bet, GATA3, and RORγt expression

2. Wash with permeabilization buffer 1× solution: add 100 μL if using 96-well plates or 200 μL if using V-bottom tubes and centrifuge for 5 min at 528 × *g*.
3. Prepare the transcription factor (TF) antibody mix using the permeabilization buffer.
4. Resuspend the pelleted cells in 50 μL of TF antibody mix. Incubate for 30 min minimum at room temperature (*see* **Note 18**).
5. Add 100 or 200 μL of Permeabilization buffer solution and centrifuge for 5 min at 528 × *g*.
6. Resuspend the cells in 200 μL of FACS buffer and transfer the cell suspension into U-bottom tubes compatible with your flow cytometry equipment.

3.6 Functional Analysis of Human ILCs

3.6.1 Intracellular Cytokine Staining for Evaluation of Cytokine Production by Human ILCs

1. After obtaining the total ILC suspension or isolating ILC subsets, ILCs can be stimulated to assess their intracellular cytokine production. To this purpose, different ILC subsets must be stimulated with their specific cytokine mix. ILC1 mix: hrIL-2 (100 U/mL); hrIL-7 (5 ng/mL); hrIL-12; hrIL-18; hrIL-15 (50 ng/mL). ILC2 mix: hrIL-2 (100 U/mL); hrIL-7 (5 ng/mL); hrIL-33; hrIL-25 and TSLP (50 ng/mL). ILC3 mix: hrIL-2 (100 U/mL); hrIL-7 (5 ng/mL); hrIL-1β; hrIL-23 (50 ng/mL) (*see* **Note 19**).
2. Incubate at 37 °C with 5% of CO_2 overnight with 2 μg/mL of Brefeldin A (*see* **Note 20**).
3. 1 h before starting the staining, stimulate with 50 ng/mL PMA and 500 ng/mL ionomycin.
4. At the end of the stimulation, harvest the cells and transfer them to V-bottom tubes or 96-well plates and proceed with the extracellular staining (*see* Subheading 3.3).
5. Wash with FACS buffer and centrifuge for 5 min at 528 × *g*.
6. Resuspend the pellet in 100 μL of fixation buffer (*see* Subheading 2.6.1). Incubate for 30 min at room temperature.
7. Add 100 μL, for the 96-well plates, or 200 μL, for the V-bottom tubes, of a 0.1% saponin solution (*see* Subheading 2.6.1) and centrifuge for 5 min at 528 × *g*.
8. Prepare your cytokine antibody mix in the 0.1% saponin solution (*see* **Note 21**).
9. Incubate for 30 min at 4 °C.
10. Finally wash the cells with the 0.1% saponin solution, centrifuge for 5 min at 528 × *g* and resuspend your cells in 200 μL of FACS buffer. Transfer the stained cells in U-bottom tubes compatible with your flow cytometer (Fig. 3).

3.6.2 Quantification of Cytokine Secretion in Human ILC Culture Supernatants

1. Collect the supernatant after 24 and/or 48 h stimulation of isolated ILC subsets (*see* Subheading 3.6.1) (*see* **Note 22**).
2. Supernatants must be stored at −20 °C for short periods of time or at −80 °C for longer periods (*see* **Note 23**).
3. Proceed with any multiplex quantitative protocol to assess soluble cytokine, according to the manufacturer's recommendations.

3.7 In Vitro Expansion and Cryopreservation of Human ILCs

1. Culture the sorted ILC subsets in 96-well plates, with ILC culture medium supplemented with rhIL-2 (100 U/mL) and rhIL-7 (5 ng/mL) for all the subsets.
2. Place the plates in an incubator at 37 °C, with 5% CO_2.
3. Refresh the medium every 48 or 72 h, and split the cells if necessary (*see* **Note 24**).

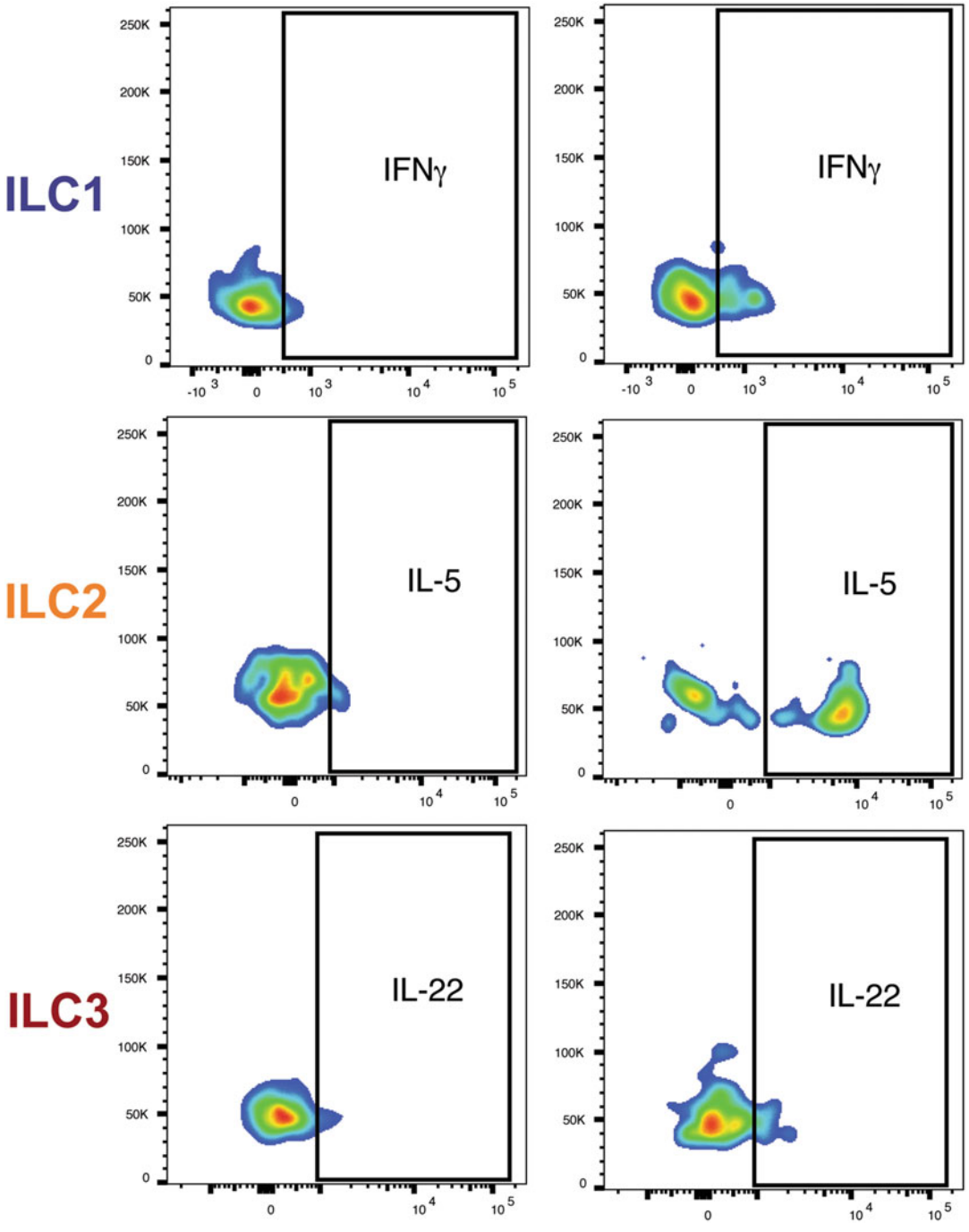

Fig. 3 Representative example of cytokine production evaluated by intracellular staining of human ILCs. ILCs were cultured with different cytokine cocktails overnight in the presence of brefeldin A. As negative control, PBMCs were cultured in absence of stimulating cytokines for the same period of time. After the culture, total ILCs and ILC subsets were stained as in Fig. 1, then the cells were fixed and permeabilized. Intracellular expression of IFN-γ, IL-5, and IL-22 was evaluated in each subset, respectively

4. For cryopreservation of expanded ILCs, prepare the freezing solution and put it on ice.
5. Label the cryogenic vials and put them on ice.
6. Count cells, wash them with RPMI 1640, GlutaMAX™-I, and resuspend in 1 mL of ice-cold freezing medium. Transfer to the cold cryogenic vials (*see* **Note 25**).
7. Close the tubes and mix gently. Immediately transfer the vials at −80°, or into dedicated freezing containers.
8. Cryogenic vials can be transferred to liquid nitrogen 24 h after freezing at earliest.

4 Notes

1. According to the configuration of your flow cytometer, other LIVE/DEAD Fixable dyes can be used to exclude dead cells.
2. This fluorochrome combination is our standard format for successful ex vivo monitoring of human ILCs. However, this format can be modified according to the configuration of your flow cytometer.
3. Antibodies should be titrated prior use in flow cytometry to determine the optimal dilution to be used for staining. In addition, always keep the antibody mix on ice or at 4 °C, in the dark, to avoid fluorochrome dissociation during the preparation of the mix, and rapidly store antibodies back after use. Also, staining should be performed avoiding the exposure to direct light.
4. In order to combine transcription factors and ILC surface marker expression for discrimination of subsets find the best fluorochrome combination. One option is to remove the NKp46 marker and include CRTH2 conjugated with the dye PerCPCy5.5.
5. Always wear gloves when handling human blood or tissue and perform PBMCs isolation in sterile conditions, under a clean laminar flow hood.
6. High-quality separation of PBMCs after density gradient centrifugation depends on a sharp interface between lymphocytes and the separation solution after the layering step. This step can be achieved by a slow overlay of the diluted peripheral blood onto the Lymphoprep™ solution using a 10 mL pipette. The second method involves inclining the two tubes (one containing the Lymphoprep solution and the other the diluted blood) in an upside down "V" position with the rims firmly touching each other, such that the blood can be carefully and continuously poured onto the solution along the side of the tube.

Avoid any shaking of the tubes to avoid mixing of the two solutions and progressively straighten the receiving tube as it becomes filled.

7. If the blood is stored for more than 2 h, increase the centrifugation time to 30 min.
8. This step allows to eliminate platelets by lower speed centrifugation and without brake. For small amounts of blood, like patient samples, this step can be removed.
9. After this step, a red blood cell lysis step can be performed if erythrocytes are contaminating your isolated PBMC. If you progress to fluorescence-activated cell sorting, this step is highly recommended.
10. All the instruments used for tissue dissociation should be sterilized either by autoclaving them or by using 70% ethanol.
11. Keep always tissues on ice for all the steps.
12. For tonsils, an enzymatic digestion is not necessary; however, if you are working with tissues that are harder and compact (e.g., lungs, intestine), perform an additional digestion step. For one piece of sample, 5 mL of digestion medium is needed (385 μL Liberase TL (Roche); 1.6 μL DNAse I (Invitrogen) in 4613.4 μL of 10% FCS (Gibco) RPMI 1640 medium). After preparing the medium, add 5 mL of the solution and incubate for 20 min at 37 °C, mixing regularly by inverting the tube. Depending on the samples, the digestion might take different incubation times. Obtained dissociated sample should be small enough to be aspirated using a 10 mL pipette and as close as possible to a single cell suspension.
13. Viability dyes like LIVE/DEAD™ must be prepared in plain PBS and not in staining buffer, in order to avoid quenching of the staining by the BSA contained in the buffer.
14. CD3 T cell contamination is really common when sorting human ILCs. Therefore, we suggest to prepare a Lineage cocktail excluding the anti-CD3 antibody, that will be added in a different fluorochrome. One option is to use the same staining panel for extracellular markers as the one presented in Subheading 2.3, but adding an anti-CD3 antibody conjugated to APCeF780 and use an anti-CD117 antibody labeled with APC instead of APC-Fire.
15. For some extracellular markers such as chemokine receptors it is recommended to incubate cells at room temperature instead at 4 °C.
16. To avoid very long sorting sessions, and to ensure high purity of sorted ILCs, a predepletion of $CD3^+$, $CD14^+$, and $CD19^+$ cells using magnetic cell sorting is highly recommended.

17. In our gating strategy we define ILC3 as lineage negative $CD127^+CRTH2^-cKit^+$ cells, as defined by Spits et al. [6, 7] However, in a recent publication, it has been shown that in human peripheral blood, lineage negative $CD127^+CRTH2^-cKit^+$ cells represent ILC precursors [8].
18. In order to obtain better transcription factor staining, 1 h room temperature incubation is recommended.
19. The stimulation of purified ILC subsets can be performed in 96-well plates according to the cell numbers obtained after sorting. If you are using cell suspensions from total human PBMCs or tissues, 48- or 24-well plates can be used, plating 1 or 2×10^6 cells/mL.
20. Stimulation periods can vary from an overnight incubation to up to 1 week, if the effect of compounds of interest is assessed on cytokine-stimulated human ILCs. For longer incubation times, add Brefeldin A only during the last overnight incubation period.
21. If you plan to concomitantly stain for cytokine production and transcription factor analysis, use the protocol described in Subheading 3.5 and the fixation/permeabilization kit (eBioscience™).
22. This supernatant will contain detectable amounts of the cytokines used to stimulate the different ILC subsets. Only cytokines specifically produced by ILCs should be assessed in the supernatant. For example, for ILC3, the stimulation media contains hrIL-2; hrIL-7; hrIL-1β and hrIL-23, so cytokines like IL-22; IFN-γ; TNF-α; IL17-A or IL-17F should be preferentially assessed.
23. Avoid repetitive thawing and freezing of the supernatants. If you plan measurements of cytokine in supernatants at different times, freeze several independent small aliquots of supernatants, for single thawing and use.
24. ILCs have a slow proliferation rate. If a fast proliferation rate is observed, this might indicate that the culture is contaminated with Lineage positive cells, most probably $CD3^+$ lymphocytes. Regularly check the phenotype of your cultured cells by flow cytometry (*see* Subheading 3.3). If necessary, perform an additional sorting to eliminate any $Lineage^+$ contaminants.
25. DMSO is a toxic compound. Perform all the manipulation on ice, in order to slow the DMSO entry into the cells.

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Chapter 11

Enumeration of Plasmacytoid Dendritic Cells in Peripheral Blood and Bone Marrow by Flow-Cytometric Analysis

Abdullah Alsuwaidan, Franklin Fuda, Weina Chen, and Mingyi Chen

Abstract

Dendritic cells (DCs) are powerful antigen presenting cells that are involved in regulating immune response. Plasmacytoid dendritic cells (pDCs) are subtype of DCs that present in small quantity in the bone marrow, peripheral blood, and lymph nodes. They are important component of our immune system in normal condition and diseases. They activate T cells and play a critical role in immune tolerance. In this chapter we review the immunophenotypic features of pDCs and provide a practical protocol for pDCs enumeration in the peripheral blood and bone marrow samples.

Key words Flow cytometry, Dendritic cells, Plasmacytoid dendritic cells, Immunophenotyping

1 Introduction

Dendritic cells (DCs) are highly specialized cells that link innate and adaptive immune response [1]. DCs are a heterogeneous population of cells that regulate the immune response through their function as potent antigen presenting cells. They have a special capacity to present the antigen to the naïve T cells which lead to T cell activation, and maturation. Additionally, depending on the inducing stimulus, they play a critical role in immune tolerance [2].

Two subtypes of DCs have been well described, conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) both derived from a common DC precursor arising from the CD34+ hematopoietic stem cell (HSC). Precursor pDC (pre-pDC) differentiate into pDCs in the bone marrow then released into peripheral blood where they reside in lymphoid tissues such as lymph nodes, tonsils, spleen, thymus, bone marrow, and Peyer's patches.

Mature pDCs stimulate distinct types of CD4 T helper (Th-1) cells in response to endogenous antigens. Likewise, pDCs can be stimulated by exogenous antigens like viruses [3]. Activated pDCs secret type I interferon that activate and enhance T cells and natural

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killer (NK) cells [4,–6]. Thus, pDCs play a critical role in variety of immune-mediated pathophysiological process such as graft-versus-host disease (GvHD), allograft rejection, and autoimmune disorders [2, 7].

Following hematopoietic stem cell transplantation (HSCT), pDC facilitate HSC engraftment and induce tolerance to prevent GvHD [5]. Several studies reveal the roles of pDC in immune reconstitution after HSCT for hematolymphoid malignancies and disease relapse, patient survival and risk of GvHD [1, 8]. The higher percentages of pDCs, after allogeneic HSCT for leukemia, are associated with successful bone marrow engraftment. On the other hand, lower pDCs count predicts relapse, death, and acute graft-versus-host disease [1]. In fact, it has been reported that the percentage of pDCs following HSCT is an independent indicator for adverse clinical outcomes [1, 8].

pDCs population are present in low quantity in bone marrow, peripheral blood and lymph nodes. They show plasmacytoid morphology and can be defined by their immunophenotype. In general, normal mature pDCs in the bone marrow express CD4, CD22dim, CD33, CD36, CD38, CD45, CD123, CD303, and HLA-DR, and they are negative for CD5, CD11b, CD13, CD15, CD34, CD56, and CD64 [8, 9]. Precursors of pDC often express CD34. Of note, subtle immunophenotypic variations have been described in pDCs in lymph nodes compared to bone marrow [9]. Furthermore, neoplastic pDCs may show immunophenotypic aberrancy as in cases of blastic plasmacytoid dendritic cell neoplasm (e.g., CD56 expression [10]).

Given the critical role of pDCs in the immune system and particularly in allogeneic HSCT, this chapter provides a practical approach to identify and quantify pDCs using multiparametric flow cytometry. Our protocol is designed to distinguish pDCs from other hematopoietic cells present in bone marrow.

2 Materials

2.1 Preparation of Bone Marrow Mononuclear Cells

Anticoagulated bone marrow or peripheral blood sample usually collected in heparinized or ethylenediaminetetraacetic acid (EDTA) tubes. It is ideal to analysis the sample within 48 h of collection.

2.2 Cell Staining Buffer (PBS/Azide/BSA (1% PAB))

500 mL Dulbecco's phosphate-buffered saline (DPBS) (10×); 22.75 g sodium azide; 50.0 g bovine serum albumin (BSA) (1%); Adjust the pH to 7.2–7.4 with HCl and final volume to 5 liters with additional deionized H_2O; Stored at 2–8 °C, brought to room temperature before use.

2.3 1% PFA Cell Fixation Solution

100 mL of paraformaldehyde (PFA) at 10% concentration; into 900 mL of phosphate-buffered saline (PBS, 10×); Stored at 2–8 °C, brought to room temperature before use.

2.4 RBC Lysis Buffer

80 g of NH_4Cl (ammonium chloride); 10 g of $KHCO_3$ (potassium bicarbonate); 3.7 g of EDTA (ethylenediaminetetraacetic acid); Adjust the pH to 7.2–7.4 with HCl and final volume to 1 liter with additional deionized H_2O; Use sterile filter, 0.22 μm polyethersulfone (PES) filter; Stored at 2–8 °C, brought to room temperature before use.

2.5 Monoclonal Antibodies

The listed antibody panels (Tables 1, 2, and 3) are suggested monoclonal antibodies combinations [11] to allow the enumeration of the pDCs among other myeloid and lymphoid cells using four- or ten-color flow cytometry, (*see* **Notes 1–3**).

Table 1
Suggested monoclonal antibodies for 10-color flow cytometry panels

Surface marker	Fluorochrome	Clone
CD2	FITC	S5.2
CD3	BV421	SK7 (Leu4)
CD4	PerCP-Cy5.5	SK3 (Leu3a)
CD5	PerCP-Cy5.5	L17F12
CD8	APC-H7	SK1
CD11b	APC	D12
CD13	PE	L138
CD15	FITC	MMA/HI98
CD22	APC	S-HCL-1
CD34	APC-R700	8G12
CD36	FITC	FA6.152
CD38	PE-Cy7	HIT2
CD45	V500-C	2D1
CD56	BV605	NCAM16.2
CD64	APC-R700	MD22
CD123	BV421	9F5/7G3
CD303	PE-Cy7	BDCA-2
HLA-DR	APC-H7	L243

Table 2
Antibodies panel for four-color flow cytometry

Tube	FITC	PE	PerCP	APC
1	CD36	CD123	HLA-DR	CD22
2	CD4	CD123	HLA-DR	CD56
3	CD34	CD123	CD45	CD303

Table 3
Antibodies panel for 10-color flow cytometry

Tube	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-R700	APC-H7	BV421	V500-C	BV605
1	CD15	CD13	CD4	CD38	CD11b	CD64	HLA-DR	CD123	CD45	–
2	CD36	CD34	CD4	CD303	CD22	–	HLA-DR	CD123	CD45	CD56

3 Methods

1. In general, it is advisable to stain 500,000 cells in each tube with appropriate volume of monoclonal antibodies and acquire at least 100,000 cellular events for analysis.
2. Several pretest factors that are critical and can affect the final result such as bone marrow collection method, anticoagulation, and transportation.
3. Testing the sample as early as possible is encouraged, ideally within 24 h or in less than 48 h of collection.
4. All testing should be carried out in sterile condition (*see* **Notes 4** and **5**).

3.1 Specimen Processing

1. Resuspend the anticoagulated specimen by inverting the sample several times.(*see* **Note 6**).
2. Decant the specimen into 50 mL conical tube.
3. Dissolve clots and remove particles.
4. Use filter to remove undissolved particles (70 μm nylon mesh) if needed.
5. Add 3 mL NH_4Cl (Ammonium chloride) lysis buffer into each tube and vortex.
6. Incubate for 10 min at room temperature (RT).
7. Centrifuge for 7 min 800 × *g* at RT.

8. Discard the supernatant.
9. Resuspend the cell pellet in 50 mL 1% PAB (PBS/Azide/BSA) and mix gently.
10. Centrifuge for 7 min 800 × *g* at RT.
11. Discard the supernatant.
12. Resuspend the cell pellet in 40 mL PAB (1%) and mix gently.
13. Centrifuge for 7 min 800 × *g* at RT.
14. Discard the supernatant.
15. Resuspend the cell pellet in 40 mL PAB (1%) and mix gently.
16. Count cells of the filtered bone marrow using the Coulter AcT diff 2 Analyzer.

3.2 Monoclonal Antibody Staining

1. Pipet appropriate quantity of patient sample to achieve 500,000 cells in each 12 × 75 mm tube.
2. Pipet 2–3 mL of PAB (1%) into each tube.
3. Add the precise amount of each monoclonal antibodies or antibody cocktail to the appropriate tube.
4. Vortex each tube gently.
5. Incubate for 15–20 min in the refrigerator (2–8 °C) in the dark.
6. Pipet 2–3 mL of PAB (1%) into each tube.
7. Vortex each tube gently.
8. Add 250 μL of 1% PFA fixative and vortex.
9. Run the tubes or store them in the refrigerator (2–8 °C) until they can be acquired on the flow cytometer, preferably within 1 h of the staining.

3.3 Data Acquisition

1. Sequential gating strategies are recommended to identify and quantify the pDCs (*see* **Notes 1**, **7** and **8**), (Fig. 1).
   (a) Gate out all doublets and nonviable cells (*see* **Notes 9** and **10**).
   (b) Gate all HLA-DR+/CD45+ and all CD4+ cells population.
   (c) Exclude T lymphocytes (CD3+).
   (d) Exclude monocytes (CD14+/CD64+/CD36+).
   (e) Exclude all events that are CD123– and/or HLA-DR–.
   (f) Report the percentage of pDCs as pDC% of total viable events.

3.4 Data Interpretation

1. Nonneoplastic pDCs in the bone marrow and peripheral blood frequently express CD4, CD22dim, CD33, CD36, CD38, CD45, CD123, CD303, and HLA-DR [12, 13], and lack

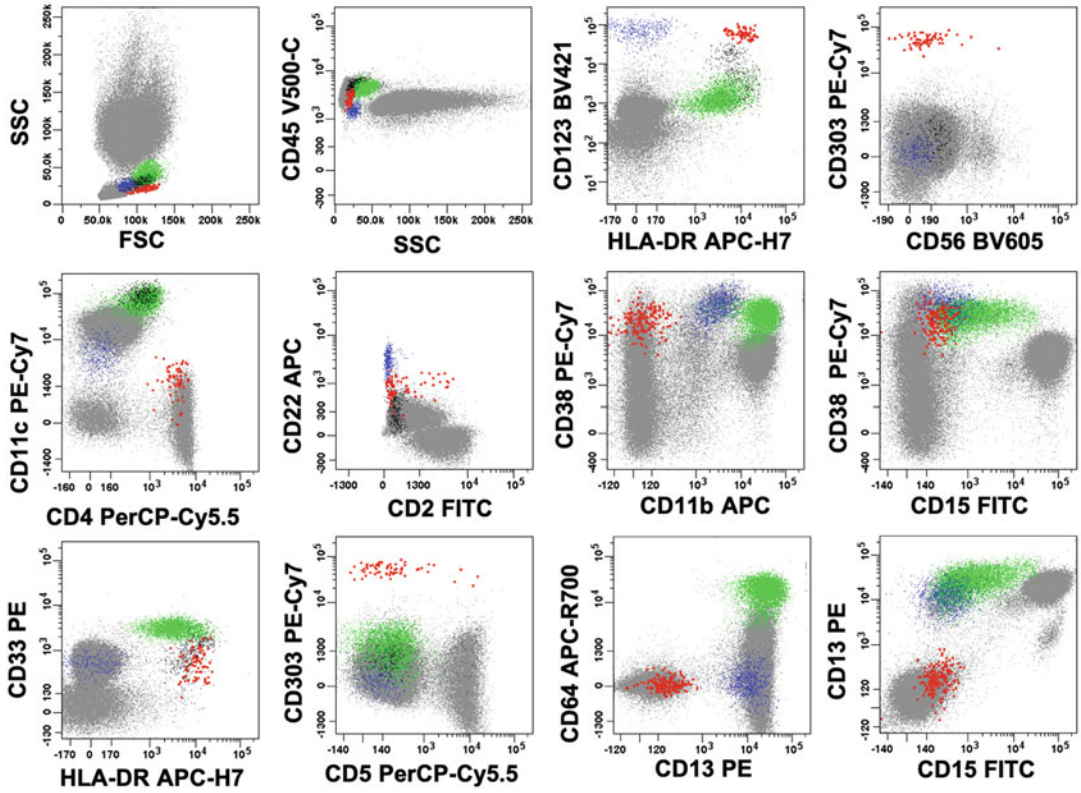

Fig. 1 Multiparametric flow cytometry analysis of a normal bone marrow specimen. The multicolor flow cytometry panel includes several monoclonal antibodies such as CD2, CD4, CD5, CD11b, CD11c, CD13, CD15, CD22, CD33, CD38, CD45, CD56, CD64, CD123, CD303, HLA-DR. pDCs (red population) are found just below monocytes (green population), that is, same size to monocytes but less SSC, in the FSC/SSC plot. Additionally, pDCs are slightly dimmer for CD45 with less SSC compared to monocytes in the CD45/SSC plot. In CD123/HLA-DR plot, pDCs are double positive for CD123/HLA-DR compared to basophils (blue population) that are CD123+/HLA-DR–. In this case, the nonneoplastic pDCs are characteristically positive for CD4, CD22, CD38, CD45, CD123, CD303, and HLA-DR, and largely negative for CD2, CD5, CD11c, CD13, CD15, CD33, CD56, and CD64

other myelomonocytic or lymphoid markers (*see* **Note 2** and **7** and **Notes 11–13**).

2. In the forward/side scatter (FSC vs. SSC) plot, pDCs are found just below monocytes in the two-dimensional scatter plot (i.e., same size to monocytes, but less SSC) [9].
3. In the CD45 vs. SSC plot, pDCs had slightly dimmer for CD45 and less SSC compared to monocytes [9].
4. In CD3 vs. CD4 plots, the cluster of nonmonocytic CD4+/CD3− are mainly pDCs providing no aberrant T cells are present.
5. In the CD45 vs. CD34 plot, pDCs may show maturation spectrum where early pre-pDCs (Stage I) are CD34+ and

CD45 dimmer than mature pDCs which are CD34− [14], (*see* **Note 14**).

4 Notes

1. A panel of monoclonal antibodies are used to better characterize the pDCs and discriminate these cells from other hematolymphoid cells that share some immunophenotypic findings.
2. It has been reported that a small subset of pDCs may express CD7 or CD2 particularly in the bone marrow samples. However, the majority of pDCs should be negative for these markers [7]. Also, CD33 can be negative in minority of cases.
3. The antibodies must be validated and titered for optimal signal-to-noise ratio under the settings to be used, particularly if the antibodies are pre-cocktailed.
4. Reagents should be used according to the manufacturer's instructions.
5. Daily QC, calibration controls, compensation procedure and preventive maintenance must be performed according to the manufacturer's recommendations.
6. It is crucial to evaluate the specimen for hemodilution which may affect the accurate pDCs count.
7. Clinical and morphologic correlation:
   (a) Knowing the clinical presentation and the indications for testing is essential in order to address the clinician concerns.
   (b) In cases with suspected increased pDCs, an examination of cytospin morphology of the corresponding sample is recommended. Additionally, correlation with the bone marrow biopsy and clot section is required to provide accurate assessment.
8. In normal condition, pDCs show reproducible staining pattern. They usually present within or close to blasts gate. While they can be detected using pregating strategy, we recommend the use of cluster analysis method that involves identifying multiple populations in ungated data
9. It is important to gate-out doublets "doublet discrimination" during the pDC analysis by examining forward scatter area over height (FSC-A and FSC-H) then exclude all events that do not represent single cells.
10. Excluding the nonviable cells or debris can be performed gating-out events with very low forward scatter in

FSC-A vs. SSC-A plot. The other option to remove the nonviable cells is to use a DNA-binding dye, such as 7-amino-actinomycin D (i.e., 7-AAD) which can provide a good assessment of sample integrity. However, it is essential to ensure that excluding nonviable cells do not interfere with the analysis of cell of interest.

11. Our protocol can be incorporated into routine evaluation of all bone marrow and peripheral blood samples for pDCs, particularly if there is increase in nonmonocytic CD4+/CD3− population.
12. The use of isotype controls is essential to exclude false-positive events (nonspecific staining).
13. It is advised to include Fluorescence Minus One (FMO) controls in multiparametric flow cytometry which help in identifying the proper gating boundaries.
14. Based on our experience, the early immature pDC may downregulate CD303 expression. This is mainly seen in bone marrow sample.

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Chapter 12

Immunophenotyping of Circulating Endothelial Cells and Endothelial Microparticles

Nicholas Wanner and Kewal Asosingh

Abstract

Flow-cytometric detection of circulating endothelial cells and endothelial microparticles is an essential tool in studies of vascular diseases. Here we describe the principles and detailed methods for human blood sample processing, storage, labeling, and gating of circulating endothelial elements.

Key words Endothelial, Flow cytometry, Microparticle, Immunophenotyping

1 Introduction

Studies of circulating endothelial cells and endothelium-derived microparticles are gaining interest in vascular biology. Enumeration and immunophenotyping of these circulating elements are becoming a standard flow cytometry application. Circulating microparticles and endothelial cells have been reported as markers of angiogenesis, endothelium damage, or vascular remodeling in several diseases [1–5].

Endothelial cells in the blood circulation are infrequent. Thus, all best practices for rare event flow cytometry are highly recommended to identify these populations successfully [6–10]. The signal-to-noise ratio in rare event detection requires the acquisition of many events and stringent elimination of artifacts and debris to gate for true endothelial cells. Nonspecific binding of antibodies must be reduced by the use of Fc-block or blocking serum before and during the incubation with test antibodies. This can also be facilitated by using a dump channel to exclude nonspecific and nonendothelial subsets. Another benefit of a dump channel is an enrichment of the rare event in the remaining population. A nuclear stain is included in the panel to discriminate true nucleated cells and cell fragments. DNA stain will also allow for the identification of hypodiploidy in cells representing dead and dying cells, which can

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display nonspecific antibody binding and should be excluded from analysis. In addition to the above steps all other general flow cytometry gating best practices apply. Time gating should be performed to select for events collected only during periods of laminar flow in the flow cell. Clusters of cells or cell doublets can be eliminated by analyzing the pulse height and width and excluding events that do not have a width in proportion to the height. Any remaining cell debris can be removed by discriminating events with light scatters too low to be cells.

Flow cytometry of circulating microparticles is an evolving technology. Guidelines for consensus best practices are still emerging [11–15]. As detailed below, special precautions should be considered to reduce background noise originating from particles in buffers and antibody aggregates. Antibodies titers should be determined using the microparticles of interest in titration experiments, and the presence of correct vesicles should be validated by treating samples with a detergent. Aggregation and swarming of microparticles should be prevented.

2 Materials

2.1 Blood Sample Processing

1. 10% Formaldehyde: 16% formaldehyde, 0.9% saline. A new 10 mL ampule of 16% electron microscopy-grade formaldehyde is diluted with 6 mL of saline to create a 10% solution. Store at room temperature for up to 2 weeks.
2. 10% Triton X-100: Triton X-100 detergent, 0.9% saline. Add 100 mL of 0.9% saline to a glass jar. Add 20 mL of Triton X-100 to the saline. Measure out an additional 80 mL of saline and with the pipet used to add the Triton X-100, add the saline. Add a magnetic stirring flea and allow all particles to dissolve while stirring on a magnetic stirrer. Aliquot 932 μL into cryovials and store in liquid nitrogen.
3. Lyse/perm Buffer: A vial of 10% Triton X-100 is thawed then added to 39 mL of 0.9% saline. The buffer is prewarmed to 37 °C before using.
4. Phosphate Buffered Saline (PBS): 137 mM sodium chloride, 2.7 mM potassium chloride, 9.8 mM phosphate buffer. Does not contain calcium or potassium.
5. Wash Buffer: 4% fetal bovine serum (FBS), PBS. 20 mL of FBS is added to 480 mL of PBS. The solution is filtered through a 0.22 μm filter. Store at 4 °C.
6. Freezing Medium: RPMI 1640, 20% FBS, 10% glycerol. Combine 35 mL of RPMI 1640 with 10 mL of FBS and 5 mL of glycerol. Store at −20 °C.

2.2 Cell Surface Staining for Flow Cytometry

1. Antibody diluting Buffer: 1% bovine serum albumin (BSA), PBS. Weigh and add 5 g of BSA to 500 mL of PBS with a stirring flea and stir until dissolved. Filter the solution through a 0.22 μm filter. Store at 4 °C.
2. Blocking Buffer: antibody diluting buffer, 10% normal goat serum (NGS) Abcam, Human Trustain Fc-block, Biolegend. Amount made is based on number of samples being stained. 250 μL made per sample; 25 μL of NGS and 6.25 μL of Fc-block added to 218.75 μL of diluting buffer.
3. 5 mL Polystyrene Round-Bottom Tubes.
4. Antibodies: CD3 PE-Cy7, Biolegend, use at 1/1600. CD19 PE-Cy5, Biolegend, use at 1/27. CD45 Alexa Fluor 700, Biolegend, use at 1/800. CD34 FITC, BD Bioscience, use at 1/5. All antibodies are diluted in antibody diluting buffer.
5. 4′,6-Diamidino-2-phenylindole (DAPI), Life Technologies, use at 1/400.
6. AbC Total Antibody Compensation Bead Kit, Life Technologies.
7. Ultra Rainbow Calibration Kit, Spherotech.
8. CS&T Research Bead, Becton Dickinson.

2.3 Microparticle Isolation and Staining for Flow Cytometry

1. Prostaglandin: 10 mM prostaglandin stock is diluted 1/1000 when added to plasma (1 μL of 10 mM prostaglandin to 1 mL of plasma).
2. Annexin-V Binding Buffer: 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$. Add 69 mg of $CaCl_2$, 2.5 mL of 1 M HEPES, and 7 mL of 5 M NaCl to 150 mL of diH_2O. Stir until the $CaCl_2$ has dissolved then use a graduated cylinder to bring the volume to 250 mL with diH_2O. Store at 4 °C.
3. r-hirudin: DSM Nutritional Products, 2000 ATU/vial, use at 1/1000.
4. Apogee Mix for Flow Cytometer Performance Assessment, Apogee.
5. SPHERO Rainbow Calibration Particles, 2.08 μm, Spherotech.

3 Methods

3.1 Blood Processing for White Blood Cells

1. Pipette 4 mL of whole blood per 50 mL tube. If less than 4 mL of blood is available, add saline to bring the volume to 4 mL.
2. Add 2.6 mL of 10% formaldehyde to the blood and incubate for 10 min at room temperature (RT).

3. After 10 min add 40 mL of lyse/perm buffer prewarmed to 37 °C and thoroughly vortex. Incubate for 20 min at RT (*see* **Note 1**).
4. Centrifuge for 10 min at 1000 × *g*. Aspirate the supernatant and wash the cells by adding 5 mL of wash buffer. Incubate cells in wash buffer for 15 min at RT.
5. Centrifuge again for 10 min at 1000 × *g* and repeat the aspiration, washing and incubation step.
6. During the second incubation, count cells to determine the number of vials to freeze in. There should be between 3.5×10^6 and 7×10^6 cells per vial.
7. Suspend cells in freezing medium and freeze in cryovials at −80 °C for 1–2 days before moving to liquid nitrogen. Alternatively samples can proceed with the staining process.

3.2 Cell Surface Staining for Flow Cytometry

1. Thaw aliquots of cells in cryovials at room temperature for 15 min.
2. Wash cells of freezing medium by diluting suspended cells with 4 mL of PBS in 5 mL polystyrene round bottom tubes.
3. Centrifuge samples at 900 × *g* for 5 min (all subsequent spins will be at 900 × *g* for 5 min), then aspirate the supernatant.
4. Resuspend each sample in 1 mL of blocking buffer by pipetting up and down. Incubate cells at RT in the dark for 20 min.
5. Remove an aliquot for the unstained sample.
6. Centrifuge all samples and aspirate.
7. The cells that are stained with DAPI are resuspended in 75 μL of diluted DAPI (1/400 in PBS). Vortex the cells and incubate for 15 min at RT in the dark.
8. Wash with 1 mL of diluting buffer then centrifuge.
9. Resuspend the DAPI stained cells in 300 μL of diluting buffer and divide into the DAPI comp, FMOs, and sample tubes (75 μL per tube) (*see* **Note 2**).
10. Add 200 μL of diluting buffer to all tubes except the DAPI comp tube. Put the DAPI comp tube aside and keep at 4 °C in the dark.
11. Centrifuge remaining samples and aspirate.
12. Add 100 μL of appropriate antibody dilutions to respective tubes and vortex (*see* **Note 3**). Incubate at room temperature on the platform lab shaker for 30 min.
13. Wash samples with 500 μL of diluting buffer and centrifuge.
14. Aspirate the supernatant and resuspend in 100 μL of BD FACSFlow, Becton Dickinson and keep at 4 °C until acquisition on the flow cytometer (*see* **Note 4**). *See* Fig. 1 for gating strategy of endothelial cells.

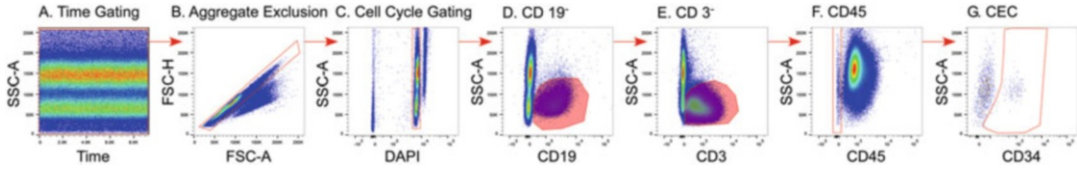

Fig. 1 Circulating endothelial cell gating strategy. Time gating (**a**), and aggregate exclusion (**b**) corrected for fluidic disturbances and cell doublets. DAPI was used to select cells in G0/G1 (**c**). $CD19^+$ (**d**) and $CD3^+$ (**e**) cells were excluded, then $CD45^-$ cells were selected (**f**) using the CD45 FMO. Finally CECs were gated using CD34 (**g**). The CD34 FMO was used to set gate boundaries

3.3 Isolation of Microparticles for Flow Cytometry

1. Blood is collected into cell preparation tubes with sodium heparin to prevent coagulation and platelet activation. Samples are processed as soon as possible.
2. Platelet-rich plasma is isolated by centrifuging whole blood at 150 × *g* for 20 min with the centrifuge brake off (*see* **Note 5**).
3. The supernatant is platelet-rich plasma (PRP) and it is collected from the tube and placed in a 15 mL tube. For each 1 mL of PRP, 1 μL of prostaglandin is added to prevent platelet activation.
4. Centrifuge PRP at 150 × *g* for 10 min. The same brake instructions from **step 2** are followed in this step.
5. The supernatant is again collected and placed in a separate 15 mL tube, then centrifuged at 1500 × *g* for 15 min with the acceleration and brake of the centrifuge at max.
6. Collect the supernatant and centrifuge at 2500 × *g* for 15 min (*see* **Note 6**).
7. Collect the supernatant again and centrifuge a second time at 2500 × *g* for 15 min.
8. The supernatant is collected then aliquoted in 250 μL portions before being frozen at −80 °C.

3.4 Staining of Microparticles for Flow Cytometry

1. Platelet free plasma (PFP) is thawed at room temperature and 18 μL of PFP per sample is transferred to an Eppendorf tube.
2. A reference sample containing 25 μL of PFP is also thawed and 24 μL of PFP is transferred to an Eppendorf tube.
3. AV binding buffer is prepared by adding r-hirudin to the buffer at a 1 to 1000 dilution then filtering the buffer twice through a 0.1 μm filter. R-hirudin will prevent aggregation of microparticles.
4. 400 μL of AV binding buffer is added to all tubes.
5. The tubes are centrifuged at 15,000 × *g* for 5 min (all subsequent spins are at this speed and length of time) in order to pellet microparticles (MPs).

6. A p1000 pipet is used to remove 403 μL of supernatant from the sample tubes which leaves a remainder of 15 μL of supernatant in the tubes. 404 μL of supernatant from the reference tube is removed which leaves a remainder of 20 μL of supernatant in the tube (*see* **Note 7**).
7. The residual volumes are mixed with a pipet to suspend the MPs, then 5 μL is aliquoted into separate tubes.
8. For the samples the tubes are unstained, AV only, and AV + CD144.
9. For the reference sample the tubes are unstained, AV only, CD 144 only, and AV + CD144.
10. To the CD144 only and AV + CD 144 tube, 20 μL of CD144 antibody (*see* **Note 8**) is added to the tube, pipetting up and down to thoroughly mix (*see* **Note 9**).
11. Samples incubate for 30 min at room temperature in the dark.
12. After the incubation, samples that were stained with CD144 are washed with 400 μL of binding buffer then centrifuged at 15,000 × *g* for 5 min.
13. For the samples that were washed, the supernatant is aspirated down to the 100 μL mark (*see* **Note 10**).
14. 95 μL of AV binding buffer is added to the unstained and AV only tubes to bring the volume to 100 μL.
15. 5 μL of AV is added to 100 μL of supernatant in the AV only and AV + CD144 tubes then pipetted up and down to mix (*see* **Notes 11** and **12**).
16. Samples incubate for 30 min at room temperature.
17. After the incubation, samples are washed with 400 μL of binding buffer then centrifuged at 15,000 × *g* for 5 min.
18. The supernatant is aspirated down to the 100 μL mark then the wash/centrifuge step is repeated.
19. The supernatant is aspirated to the 100 μL mark then 50 μL of AV binding buffer is added for a final volume of 150 μL.
20. Samples can be stored at 4 °C protected from light until they are run on the cytometer (*see* **Note 13**). *See* Fig. 2 for gating strategy of endothelial-derived microvesicles.

4 Notes

1. This protocol utilizes a one-step RBC lysis and fixation and permeabilization step [16, 17]. This procedure allows intracellular immunophenotying and preserves epitopes for phosflow. In addition, fixation before cryopreservation provides better light scatter resolution than unfixed cryopreserved cells

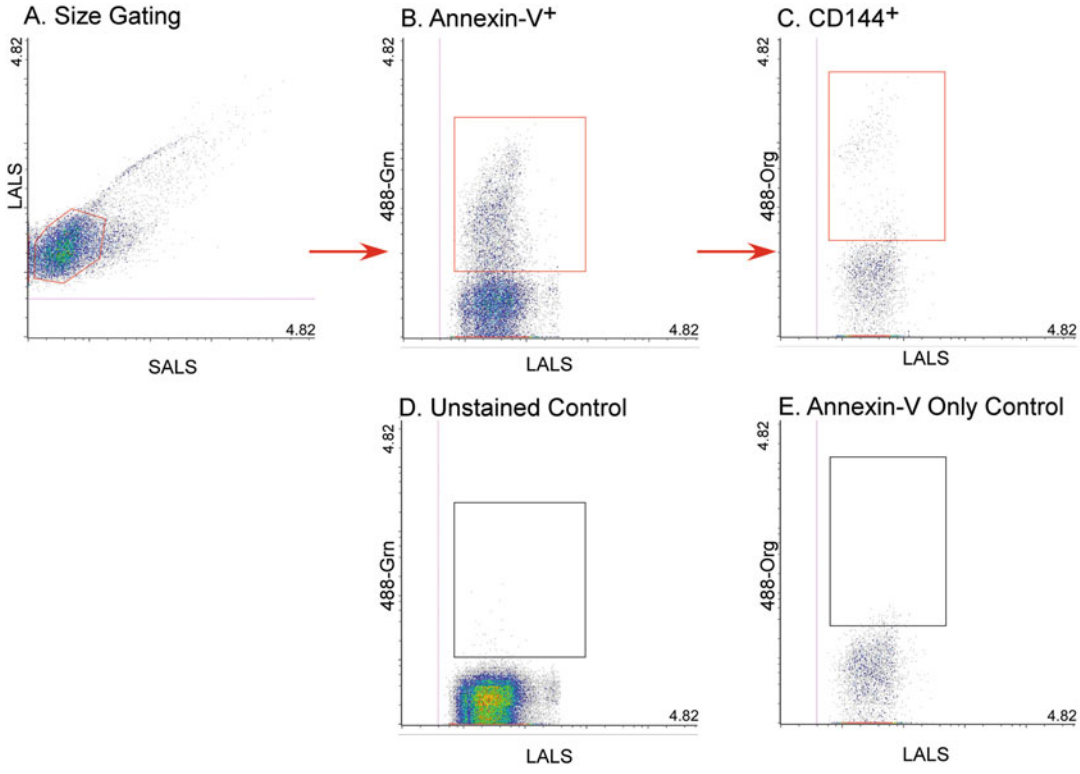

Fig. 2 Gating of endothelial-derived microvesicles in plasma. Small Angle Light Scatter (SALS) and Large Angle Light Scatter (LALS) were used to gate the microvesicle population based on size (**a**). Annexin-V$^+$ events were selected on a LALS/Green fluorescence channel plot (**b**). Annexin-V$^+$ region was gated based on the unstained sample (**d**). CD144 (VE-cadherin)$^+$ events in the annexin-V$^+$ gate were selcted on an LALS/orange fluorescence channel plot (**c**) based on an Annexin-V only control (**e**) Samples were acquired at a flow rate of 6.01 μL/min for 2 min. At least 5000 annexin-V$^+$ CD144-PE$^+$ microparticles were acquired

[16, 17]. Cryopreservation also allows for batch staining and analysis. Users can stain fresh samples, if desired.

2. The DAPI stained cells are aliquoted into four tubes. The tubes are DAPI comp, CD45 FMO, CD34 FMO, and complete panel.
3. 100 μL of antibody dilutions are made for each tube The CD45 FMO dilution will be have all antibodies (CD3, CD19, and CD34) except CD45. The CD34 FMO will be stained with all antibodies (CD3, CD19, and CD45) except CD34. The sample tube will be stained with all antibodies (CD3, CD19, CD45, and CD34). In this protocol CD34 is used to identify endothelial cells. Other antibodies have been cited in the literature as markers of endothelial cells and can be substituted for CD34 at the discretion of the user. In this case, it should be validated that the fixation and permeabilization did not compromise antigenicity.

4. Samples were run on a BD Fortessa. CS&T beads were run daily for cytometer set up. Six peak rainbow beads were run before each experiment to calibrate fluorescence channels.
5. If your centrifuge is able, the brake can be set to the minimum setting when the centrifuge is spinning at 50 × *g* or less.
6. Centrifuging at 2500 × *g* will pellet the platelets while keeping MPs in the supernatant.
7. It is important to not aspirate too much supernatant leaving less than 15 μL. 5 μL must be aliquoted 3 times so at least 15 μL is needed. More than 15 μL can remain, but it is important to note exactly how many μLs remain.
8. All antibody vials should be centrifuged at 15,000 × *g* for 10 min before used in the experiment in order to pellet aggregates.
9. Additional markers for endothelial derived MPs were tested including CD31 and CD34. CD144 was found to be the most highly expressed on vesicles.
10. Eppendorf tubes with 100 μL gradations should be used in order to aspirate to a specific volume of 100 μL.
11. AV is used as a general MP marker, but may not be binding to all MPs. Other MP markers can be used if the user determines it to be advantageous [18].
12. Presence of true MPs should be validated by dissolving vesicles by treating samples with detergent, such as Triton-X 100 [19].
13. Samples were run on the Apogee A50 Micro-cytometer. Size beads and rainbow calibration beads were run in order to standardize size resolution and fluorescence respectively.

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Chapter 13

Rare Event Phenotyping and Molecular Characterization: Circulating Tumor Cells

Moen Sen, Ling Wang, Liping Yu, and Erica L. Carpenter

Abstract

Noninvasive isolation of circulating tumor cells (CTCs) from patient blood samples allows for interrogation of valuable molecular and phenotypic information useful for disease diagnosis and monitoring response to therapy. However, CTCs are extremely rare relative to red and white blood cells (R/WBC), thus making CTC isolation from unmanipulated whole blood very time-consuming. Moreover, single CTC analysis often requires hand-picking, a step that can result in more CTC loss and compromised cell integrity. Here we describe an automated flow cytometry-based approach for isolation and analysis of single, viable CTCs that combines gentle RBC lysis and magnetic, no-wash negative-depletion of WBCs, followed by a highly adaptable sorting protocol for rare cells of interest. Multiparametric flow-cytometric panels allow probing of numerous extracellular markers for immunophenotyping, while whole transcriptome analysis contributes to molecular characterization of individual CTCs. Index sorting links single CTC proteogenomics information.

Key words Circulating tumor cells, Acoustic cell enrichment, Magnetic depletion, Flow-cytometric analysis, Molecular indexing, Phenotyping

1 Introduction

Circulating tumor cells (CTCs) are rare cells shed from the primary tumor into blood. The enumeration and characterization of CTCs isolated from the blood, also known as liquid biopsy, can serve as a valuable alternative to repeated invasive tumor biopsies for diagnosing and monitoring disease. However, isolation and characterization of CTCs has been challenging due to the fact that CTCs are extremely rare, present at 1–10 cells per ten billion blood cells, and also heterogeneous [1]. CellSearch®, the only US Food and Drug Administration (FDA) approved CTC characterization device has been used for the enumeration of CTCs in patients with metastatic breast, colorectal or prostate cancer. However, CellSearch-based phenotyping is restricted to one open channel, and additional steps are necessary for single cell isolation and molecular analysis [2–6].

J. Philip McCoy, Jr (ed.), *Immunophenotyping: Methods and Protocols*, Methods in Molecular Biology, vol. 2032, https://doi.org/10.1007/978-1-4939-9650-6_13,

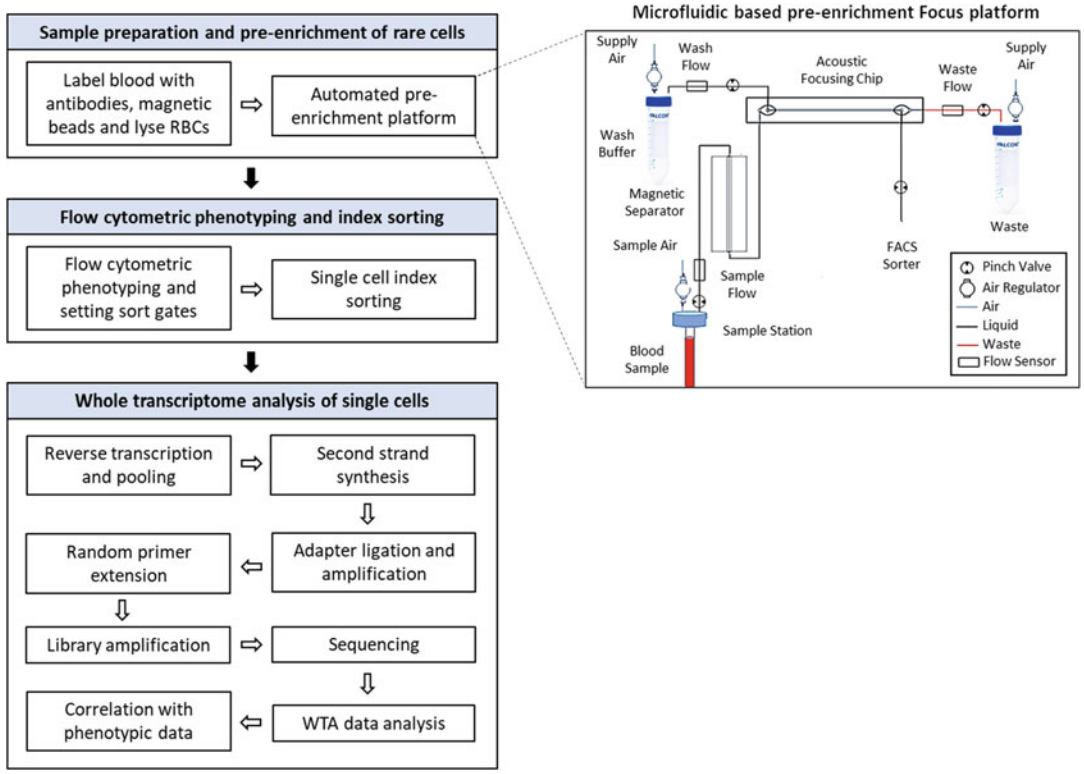

Fig. 1 Schematic overview of sample preparation, flow-cytometric phenotyping, and whole-transcriptome molecular analysis of circulating tumor cells

In this chapter, we describe an integrated workflow for the isolation, immunophenotyping and downstream molecular characterization of rare cells using Yellow Fluorescent Protein (YFP) positive CTCs isolated from the blood of tumor-bearing KPCY (Kras;p53;Cre;YFP alleles) mice as a model [7]. The YFP lineage is expressed in all cells of pancreatic origin, including CTCs, thus facilitating rare cell selection among YFP−/CD45+ WBCs. The workflow is simple and integrates isolation, phenotyping, and molecular characterization of single CTCs. The protocol is composed of several key steps, as illustrated in Fig. 1, including: (1) sample preparation involving staining cells for extracellular markers followed by magnetic labeling of WBCs, (2) gentle, no-wash RBC lysis, (3) magnetic depletion of WBCs, (4) acoustic focusing resulting in reduction of blood cells and debris and consequent enrichment of rare CTCs, (5) analysis and sorting of cells on a flow cytometer, and (6) whole transcriptome analysis of sorted single CTCs. While broad variations of the protocol have been described by other groups and us, the elimination of washes and a separate isolation step preceding molecular analysis greatly increases the workflow efficiency and minimizes cell loss [2, 8–16]. In addition, the combination of immunophenotyping with transcriptional

analysis at the single cell level affords truly high dimensional characterization of CTCs [16, 17]. This protocol increases sensitivity and provides better rare cell characterization by combining enrichment and index sorting of rare cells with downstream single cell whole transcriptome analysis [18–20].

2 Materials

2.1 Mouse Blood Sample Preparation

1. 70% ethanol.
2. 27 G 16 mm 1 mL insulin syringe.
3. Heparin.
4. 15 mL conical tube.
5. Aluminum foil.
6. Shaker.
7. Staining antibodies against WBCs (CD45) and known CTC markers such as EPCAM and ECAD.
8. IMag™ Magnetic beads.
9. G Biosciences RBC Lysis Buffer.
10. 5 mL polypropylene tube.

2.2 Setting Up Pre-enrichment Platform (BD Focus)

1. Ficoll® Paque.
2. BD FACSFlow™ sheath solution or equivalent sheath solution for flow cytometry.
3. 50 mL Falcon conical tube.
4. Labview GUI.
5. Fluorescence-activated cell sorter (FACS) flow cytometer sorter.
6. BD Focus (prototype being developed by BD Biosciences, San Jose, California). The platform includes an analog pressure control system, a magnetic separator with plastic tubing for conducting magnetic separation, an acoustic-driven microfluidic device (chip) and fluidic tubing that passes sample from sample station through magnetic tubing, acoustic chip and sample inlet on FACS.

2.3 Single Cell Sorting

1. BD™ Precise WTA Single Cell Encoding Plate, 96 Well (BD Biosciences).
2. Flow-cytometric cell sorter (also used in Subheading 2.2, **item 5**).

2.4 Whole Transcriptome Analysis

1. Pipettes (1 μL–1000 μL volume capability).
2. RNase-free filter pipette tips.
3. Microcentrifuge tubes, 1.5 and 2.0 mL tubes.
4. 0.2 mL PCR 8-strip tubes.
5. UVP UV3 HEPA PCR Workstation (UVP) or equivalent.
6. PCR plate spinner (VWR) or equivalent.
7. Thermal cycler with heated lid.
8. Tube Magnet.
9. Magnetic separation stand for 0.2 mL tubes.
10. 2100 analyzer (Agilent Technologies) or equivalent.
11. Qubit Assay Tubes.
12. Qubit 3.0 Fluorometer (Thermo Fisher Scientific) or equivalent.
13. Illumina sequencer (Example: Illumina NextSeq 500 Sequencing System).
14. AMPure XP Reagent (Beckman Coulter).
15. Pure ethyl alcohol (ethanol), Molecular Biology Grade.
16. Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).
17. Agilent DNA High Sensitivity Kit (Agilent Technologies).
18. 80% (v/v) ethanol (ethyl alcohol): In a new 50 mL conical tube, add 4.0 mL of nuclease-free water and 16 mL of ethanol. Vortex and mix well. Prepare fresh and use $\leq$1 day.
19. BD™ Precise WTA Single Cell Kit (elution buffer included) (BD Biosciences).

3 Methods

3.1 Mouse Blood Sample Preparation

All steps involving preparation of blood sample should be performed at room temperature

1. Coat the syringe and 15 mL conical tube with an anticoagulant like heparin.
2. Euthanize the mouse according to appropriate ethical guidelines.
3. Spray 70% ethanol to disinfect the mouse.
4. Insert the 1 mL syringe right below the rib cage at a 45° angle and slowly draw blood out by cardiac puncture (*see* **Note 1**). Transfer the blood to a heparin coated 15 mL conical tube.
5. Set out lysis buffer (4× the volume of blood collected) at room temperature for use in **step 8**.

6. Add staining antibodies against markers of interest at appropriate concentrations and incubate for 20 min at room temperature covered in aluminum foil on a shaker (*see* **Notes 2** and **3**). This includes, for example, antibodies against known CTC markers like EPCAM and ECAD as well as the CD45-PE antibody that serves to both label and, together with the beads described in the next step, deplete unwanted CD45+ WBCs.
7. Add magnetic beads to deplete unwanted cells at a volume of 1:10. Thus, for 1 mL of whole blood, add 100 μL of magnetic beads. For example, beads conjugated to anti-PE are used to deplete unwanted WBCs labeled with CD45-PE (*see* **Note 4**). Incubate for 20 min at room temperature (RT) covered in aluminum foil on a shaker.
8. Add 4× RBC lysis buffer (4 times the volume of whole blood) and incubate for 20 min at room temperature covered in aluminum foil on a shaker (*see* **Note 5**).
9. Transfer the diluted, stained blood sample into a 5 mL polypropylene tube through a cell strainer cap (*see* **Note 6**).

3.2 Set Up BD Focus

The BD Focus should be set up while the blood sample is being processed. In our lab, the BD Focus set up and mouse blood sample preparation is conducted simultaneously by two people.

1. Prepare wash buffer for the BD Focus washing step by making 20% Ficoll® Paque in BD FACSFlow™ sheath solution.
2. Turn on the BD Focus.
3. Start the LabVIEW GUI (BD Biosciences, California) that monitors flow rates read from flow meters (Sensirion, Switzerland) on the sorter.
4. Push the magnet tubing into the magnetic field to engage magnetic separation.
5. Place a 50 mL Falcon conical tube containing 50 mL fresh wash buffer in the "wash port," and empty 50 mL Falcon conical tube on the "waste" port.
6. Connect the sample line of BD Focus to the sample line of the FACS system. Turn on to backflush BD Focus sample line with sheath fluid. The fluidic lines are primed when the wash buffer is pushed to the waste and sample tube.
7. Monitor air in the chip via MicroViewer, to make sure all air is pushed from the chip by the flow of saline from the sheath tank.
8. Place a 50 mL Falcon conical tube containing 50 mL fresh wash buffer in the "wash port," an empty 50 mL Falcon conical tube on the "waste port," and a 5 mL polypropylene test tube with FACS buffer on BD Focus (Fig. 1).

9. Adjust pressure regulators on BD Focus to achieve positive flow rates on sample line to FACS, wash buffer to chip, and waste line from chip to waste tube. Target values are sample line to FACS 60–70 μL/min, wash line 90–110 μL/min, and waste line 130–140 μL/min (*see* **Note 7**).
10. Check and set a frequency of 2.17 MHz and 15 volts on the pzt driver board to set the acoustic focusing chip at its optimal performance for cell focusing. The pzt frequency is the frequency at which the chip vibrates. Chip performance is sensitive to the frequency and amplitude. The frequency does not need to be changed until the chip is replaced. The manufacturer provides a suggested frequency and amplitude for each chip they fabricate.
11. Stop the pressure regulators when target flow rates are achieved. Replace the sample tube with the 5 mL polypropylene tube containing the experimental sample.

3.3 Run Sample on BD Focus

1. Start pressure regulators and let fluidics get to equilibrium state, which typically takes a minute. Target rare cells are enriched as the sample undergoes magnetic depletion of unwanted cells and acoustic focusing that washes RBC lysate/debris away.
2. Monitor event rate on flow cytometer and adjust pressure regulators to control sample flow rate.

3.4 Flow-Cytometric Analysis

1. Draw dot plots in FACS software and employ appropriate gating strategies to identify and characterize rare events. Gate out debris based on size (FSC v SSC). This is followed by gating on "live cells" that are DAPI negative. Following live/dead selection, live YFP+ CD45− rare CTCs are gated on for sorting. Gene expression profiling by whole transcriptome analysis is routinely performed by us to verify cancer cell identity in sorted CTCs [16]. As shown in the representative plots in Fig. 2, in one experiment 16 (0.11% of all events, 0.16% of live cells) YFP+ CTCs were detected in the blood of a tumor-bearing KPCY mouse (We have observed CTCs in the range of 1–254 cells/mL in KPCY mice with high metastatic burden) [16]. To better visualize the rare YFP+ events, the properties of the YFP gate were edited on FACS analysis software to show up as larger dots on the FACS plots (Fig. 2). Panels of multiple markers can be optimized to minimize spectral overlap to phenotype CTCs.

3.5 Single Cell Sorting

1. Set up the flow sorter to sort per the manufacturer's instructions.
2. Chill the 96 well plate holder of the sorter at 4 °C for 10 min.
3. Thaw the sealed 96 well Precise WTA plate at room temperature and spin at 377 × *g* for 10 s to collect the reagents in each

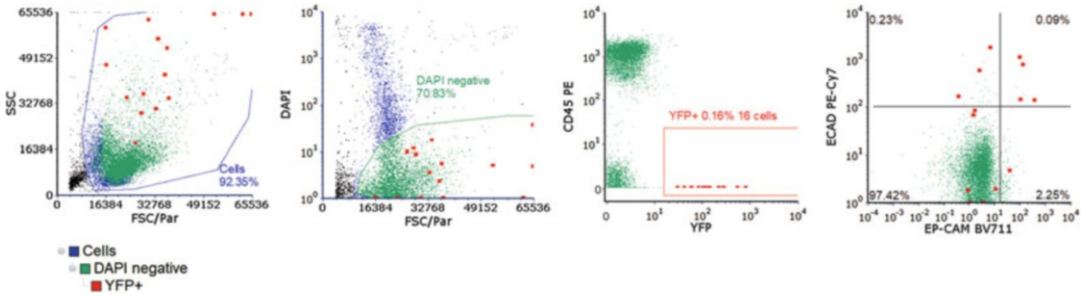

Fig. 2 Gating strategy for the detection of YFP+ CD45− circulating tumor cells

well. Place the plate on ice, slowly peel the seal from the plate and place it in the chilled plate holder of the sorter.

4. Sort single cells into the 96 well encoding plate and leave 3–6 wells empty for nontemplate controls. A few pools of 10 or more cells per well can also be included as sample input controls (*see* **Note 8**).
5. Immediately place the plate on ice, seal the plate with a plate cover, vortex and spin down at 4 °C, 377 × *g* for 10 s and freeze at −80 °C (*see* **Note 9**).

3.6 Whole Transcriptome Analysis

3.6.1 Perform Reverse Transcription, Pool and Purify the Product

Before conducting the procedure for WTA library prep, dedicate two isolated workspaces in the laboratory as preamplification and postamplification workplaces. In our laboratory, we used a PCR workstation as the preamplification workspace, and a separate clean space on the bench as the postamplification workspace.

In the preamplification workspace:

1. Briefly spin the WTA plate for 10 s at RT on the PCR plate spinner and incubate the plate at *65 °C for 3 min (Program 1* in the preamplification thermal cycler*)*. Cool on ice.
2. Prepare the RT enzyme master mix sufficient for a 96-well plate: 324 μL of Nuclease Free Water, 240 μL of Precise RT Buffer, 12 μL of RNase Inhibitor, and 24 μL Reverse Transcriptase, for a total volume of 600 μL. Use a multichannel pipette to dispense 5 μL of the mix into each of the 96 wells.
3. Seal the plate followed by a brief vortex and 10 s spin at 377 × *g* at room temperature (RT). Run *Program 2* in the preamplification thermal cycler: *42 °C for 30 min → 80 °C for 5 min → hold at 4 °C.*
4. Spin the plate for 10 s at 671 × *g* at RT and pool all reactions into one single 2 mL tube by using a multichannel pipette and then a single channel pipette. The final volume is approximately 900 μL.
5. Purify the pooled samples using 900 μL of AMPure XP beads.
6. Vortex to mix well and incubate at room temperature for 5 min. Place tube on magnet and wait 5 min until the liquid is clear and separating from the beads. Keep the tube on the

magnet and carefully remove the liquid and discard. Gently rinse the beads using 2 mL 80% (v/v) ethanol (prepare fresh and use ≤1 day). Immediately remove all residual ethanol solution with a pipette. Repeat this wash step once.

7. Remove the residual ethanol as much as possible and leave the tube open on the magnet to air dry the beads for 3 min. To elute the sample from the beads, remove the tube from the magnet and add Elution Buffer in an amount as instructed in the protocol. Wet the beads with the elution buffer and vortex well to mix. Wait 1 min and return the tube to the magnet. Wait 5 min until the solution is clear. Recover the purified product by carefully pipetting the solution into a new tube.
8. Leave the tube open to dry the beads at RT for 3 min before eluting with 72 μL of Elution Buffer in a new 0.2 mL PCR tube.

3.6.2 Perform Second Strand Synthesis and Purify the Product

In the preamplification workspace

1. Dilute the Precise SS Enzyme Mix in a 1.5 mL tube by adding 38 μL of the Precise SS Synthesis Buffer and 2 μL of the Precise SS Enzyme Mix. Gently mix and place on ice.
2. Prepare the second strand synthesis mix by adding 8 μL of the diluted Precise SS Enzyme Mix to 72 μL of the pooled samples in the 0.2 mL PCR tube from Subheading 2.4, bringing the volume up to 80 μL. Gently mix and place on ice.
3. Perform ss synthesis and run *Program 3* in the preamplification thermal cycler: *16 °C for 5 min → hold at 4 °C.*
4. Immediately put the second strand synthesis products on ice and add 5 μL of Precise SS Stop Solution. The total volume is 85 μL.
5. Purify the ss synthesis products using 100 μL of AMPure XP beads, and wash twice using 200 μL of 80% ethanol (v/v) on the magnet per Subheading 3.6.1, **step 6**.
6. Leave the tube open to dry the beads at RT for 3 min before eluting in a new 0.2 mL PCR tube with 55 μL of Elution Buffer per Subheading 3.6.1, **step 7**. Proceed immediately to next step.

3.6.3 Ligate the cDNA to the Precise WTA Adapter and Purify the Product

In the preamplification workspace:

1. Prepare the cDNA ligation master mix by slowly pipetting 15 μL of Precise Ligase to 55 μL of the ss synthesis products in the 0.2 mL PCR tube from Subheading 3.6.2 for a total volume of 70 μL. Briefly vortex and spin the tube at 671 × *g* for 10 s and keep it at room temperature.
2. Prepare the cDNA ligation mix by adding 3 μL of Precise WTA Adapter and 5 μL of Precise Ligase into the 70 μL of the cDNA ligation master mix bringing it to a total of 78 μL.

3. Perform ligation by running *Program 4* in the preamplification thermal cycler: *23 °C for 30 min → hold at 4 °C.*
4. Briefly centrifuge the products at 671 × *g* for 10 s, and then place them at room temperature.
5. Purify the 78 μL of ligation products using 75 μL of AMPure XP beads and wash twice using 200 μL of 80% ethanol (v/v) on the magnet per Subheading 3.6.1, **step 6**.
6. Leave the tube open to dry the beads at RT for 3 min before eluting with 55 μL of Elution Buffer in a new 0.2 mL PCR tube per Subheading 3.6.1, **step 7**. Proceed immediately to the next step.

3.6.4 Amplify the Ligation Products and Purify the Product

In the preamplification workspace:

1. Prepare the PCR master mix by adding the following components to the 0.2 mL tube containing the 55 μL of purified ligation products from Subheading 3.6.3: 25 μL of nuclease-free water, 20 μL of Precise WTA PCR Primer, and 100 μL of the Precise WTA PCR Mastermix, for a total volume of 200 μL.
2. Gently mix by pipetting (vortex may not be gentle enough) and place on ice. Pipette 50 μL of the PCR master mix into each of the three new 0.2 mL PCR tubes on ice for a total of four tubes with 50 μL each.

In the postamplification workspace:

3. Amplify the ligation products by running *Program 5* in the postamplification thermal cycler: *98 °C for 30 s → 20 cycles of (98 °C for 10 s → 58 °C for 15 s → 72 °C for 3 min) → 72 °C for 5 min → hold at 4 °C.*
4. Combine the four PCR reactions into a new 1.5 mL tube. This is the WTA product.
5. Purify the 200 μL of the WTA product using 200 μL of AMPure XP beads, and wash twice using 1 mL of 80% ethanol (v/v) on the magnet per Subheading 3.6.1, **step 6**.
6. Leave the tube open to dry the beads at RT for 3 min before eluting with 20 μL of Elution Buffer in a new 1.5 mL PCR tube per Subheading 3.6.1, **step 7**.

3.6.5 Perform Quality Control on the WTA Product

Store the purified WTA product at −20 °C for ≤6 months or perform QC on the WTA product before proceeding to Subheading 3.6.6.

In the postamplification workspace:

1. Use 2 μL of the WTA product to measure the concentration with the Qubit dsDNA HS Assay according to the

manufacturer's instructions. WTA product concentrations are typically ≥5 ng/μL.

2. Dilute the WTA product to about 1–3 ng/μL and use 1 μL to measure the WTA product size and purity by running a High Sensitivity DNA Chip on the Agilent Bioanalyzer per the manufacturer's instructions. The WTA product sizes are typically >300 bp and peak around 2000 bp.

3.6.6 Perform Random Primer Extension and Purify the Product

In the postamplification workspace:

1. Prepare the priming master mix: Calculate the volume (X) for 50 ng of WTA product from the estimated concentration, and then add 34-X μL of nuclease-free water, 10 μL of Precise WTA Library Random Primer Mix, and X μL of 50 ng WTA product in a new 0.2 mL PCR tube, for a total volume of 44 μL.
2. Denature and prime the WTA product by running *Program 6* in the postamplification thermal cycler: *95 °C for 2 min → 4 °C for 5 min → hold at 4 °C*. Place the denatured WTA product on ice.

In the preamplification workspace:

1. Prepare the primer extension reaction mix in a new 1.5 mL tube by adding 7.5 μL of Precise Reaction Buffer and 1.5 μL of Precise Extension Enzyme. Gently mix by pipetting (not vortex) and place on ice. Add 6 μL of the mix to the 0.2 mL PCR tube containing the 44 μL of the WTA product for a total of 50 μL primer extension reaction mix. Gently mix (not vortex) by pipetting and place on ice.
2. Run *Program 7* in the postamplification thermal cycler: *37 °C for 30 min → 80 °C for 20 min → hold at 4 °C* for Primer extension of the WTA product.
3. Purify the 50 μL of primer extension product using 35 μL of AMPure XP beads, and wash twice using 200 μL of 80% ethanol (v/v) on the magnet per Subheading 3.6.1, **step 6**.
4. Leave the tube open to dry the beads at RT for 3 min before eluting with 20 μL of Elution Buffer in a new 0.2 mL PCR tube per Subheading 3.6.1, **step 7**.

3.6.7 Amplify the Primer Extension Product and Purify the Amplified Library

In the preamplification workspace:

1. Prepare the library amplification master mix in a new 0.2 mL PCR tube by adding 25 μL of Precise WTA PCR Mastermix, 2.5 μL of Precise WTA Library Forward Primer, and 2.5 μL of Precise WTA Library Index Primer 1 or Primer 2 (*see* **Note 10**), for a total volume of 30 μL.

In the postamplification workspace:

2. Prepare the amplification mix by adding 20 μL of the primer extension product from Subheading 3.6.6 to the 30 μL of the library amplification master mix for a total of 50 μL. Gently mix and briefly spin the tube for 10 s at 671 × *g* at RT.
3. Amplify the amplification mix by running thermal cycler Program 8 in the postamplification thermal cycler: *98 °C for 30 s → 12 cycles of (98 °C for 10 s → 65 °C for 15 s → 72 °C for 20 s) → 72 °C for 2 min → hold at 4 °C.*
4. Purify 50 μL of amplified library using 35 μL of AMPure XP beads, and wash twice using 200 μL of 80% ethanol (v/v) on the magnet per Subheading 3.6.1, **step 6**.
5. Leave the tube open to dry the beads at RT for 3 min before eluting with 20 μL of Elution Buffer in a new 0.2 mL PCR tube per Subheading 3.6.1, **step 7**. The purified and amplified library can be stored at −20 °C for ≤6 months or proceed to Subheading 3.6.8.

3.6.8 Perform Quality Control on the Amplified Library

In the postamplification workspace:

1. Measure the concentration of the amplified library using 2 μL of the amplified library with the Qubit dsDNA HS Assay Kit according to the manufacturer's instructions. Library concentrations range from 10 ng/μL to 30 ng/μL.
2. Verify the amplified library size and purity by diluting the amplified library to 1–3 ng/μL and use 1 μL of the diluted amplified library to run a High Sensitivity DNA Chip on the Agilent Bioanalyzer according to the manufacturer's instructions. The amplified library consists of fragments ranging from 300 to 1000 bp.

3.6.9 Sequence the Amplified Libraries and Analyze the Data

Use an Illumina platform to sequence the libraries and generate 75 × 75 paired-end reads for a targeted range of 250,000–350,000 paired reads per cell (*see* **Note 10**). Use the BD™ Precise Whole Transcriptome Assay Analysis Pipeline on the Seven Bridges Genomics platform to demultiplex and map reads from the sequencing files, and calculate unique Molecular Indexes for each target with built-in correction algorithms. Use the BD Genomics Data View software (*see* **Note 11**) or open-source R statistical software for secondary analysis such as dimensionality reduction, differentiated gene expression, pathway analysis, and correlation of the Molecular Indexes measured by the BD Precise™ assay with the fluorescence intensity of the protein measured by flow cytometry at the single cell level.

4 Notes

1. Move the needle up and down or change the angle of the needle slowly if blood is not filling into the syringe. You should expect 0.6–1 mL of blood from a single cardiac puncture.
2. Titrations to determine appropriate antibody concentrations should be done using positive- and negative-control cell lines. In our laboratory, we use extracellular markers conjugated to fluorochromes to avoid an extra labeling step with a secondary antibody.
3. Fluorescence-minus-one (FMO) controls can be used to set gates [21]. If using FMOs, blood collected should be divided accordingly between the sample and FMO controls. FMO controls are then treated with magnetic beads and RBC lysis similar to the sample.
4. We titrated IMag™ magnetic beads to determine a concentration that maximizes depletion without causing rare cell loss on the magnet. Titrations may need to be done to optimize the best conditions for each laboratory. In our laboratory, we use CD45-PE (BD Pharmingen; Catalog 561087) at a concentration of 1:100 and BD IMag™ Anti-R-PE Magnetic Particles at a concentration of 1:10.
5. For our workflow, we use G Biosciences RBC lysis buffer and have demonstrated that our sample processing has minimal effects on gene expression [16].
6. Passing the blood through the cell strainer cap prior to running the sample on the BD Focus is important to prevent any clogging in the fluidic path, acoustic chip or nozzle.
7. Monitor the flow rates recorded by the flow meter. Stable laminar flow in the main flow channel of the acoustic chip is needed to achieve high cell recovery. If sudden change of any flow rate is observed the system needs to be stopped. Cleaning of fluidics (priming with DI followed by 1× PBS) needs to be performed before the analysis and sorting is resumed.
8. We suggest an event rate at or lower than ¼ of the pzt frequency (frequency at which the chip vibrates) of the sorter to make the sorting efficiency over 80%. Event rate can be optimized by adjusting sample concentration and sample flow rate. For example, with the 200 μm nozzle on the sorter and a pzt frequency of 2017 kHz (2.17 MHz), 400–500 per second event rate results in 90% efficiency. The sample flow rate on BD Focus can be tuned by adjusting the pressure regulator.
9. Use a cell sorter per the manufacturer's instructions to sort cells directly into the Precise WTA Single Cell Encoding Plate, preferably on a prechilled 96-well aluminum cooler block. The

sealed WTA Single Cell Encoding Plate with sorted cells can be stored at −80 °C for ≤6 weeks.

10. The Precise WTA Library Index Primers 1 or 2 are used to multiplex multiple libraries for sequencing. The plate indexes for the Precise WTA assay are equivalent to Illumina indexed adapters D701 (ATTACTCG) and D702 (TCCGGAGA). If running multiple plates, normalize all libraries to an equivalent concentration and pool them to prepare one sequencing run per Illumina guidelines.
11. External release of the BD Genomics Data View software is available at http://bitbucket.org/CRSwDev/dataview. Installation of the version 9.3 (R2017b) of the MATLAB Runtime is required for running the Data View v1.2.2.

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Chapter 14

Quality Control of Immunophenotyping

Bruce Greig

Abstract

Applications of immunophenotyping using flow cytometry offer precise and accurate means for providing information used to both diagnose and monitor disease; they serve as a standard platform for many research endeavors that study discrete populations of biological entities. The proper use of this highly sophisticated technology requires daily and ongoing monitoring of both the instrument and the methodology. Best practices for this begin with quality control (QC) procedures designed to set up and monitor the instrument performance, the reagents, and the results to ensure that they are working properly both on the day of use and over time. If the results of those QC procedures are outside of acceptable then recording the corrective action taken must also be included in the quality control records. Quality assurance (QA) is a way to know that the three phases of testing, namely, preanalytic, analytic, and postanalytic procedures, are being followed. This chapter describes the procedures used to assess quality control as it pertains to flow cytometry and immunophenotyping in all three phases of testing.

Key words Quality control, Quality assurance, Flow cytometry, Levey–Jennings, Immunophenotyping, Monoclonal antibodies, Immunofluorescence, Color compensation, FMO controls, Proficiency testing, Method validation, Accuracy, Precision

1 Introduction

Quality control defined: a set of procedures performed each day for the continuous and immediate monitoring of laboratory work to confirm whether the results are reliable and can be used to diagnose, monitor, or ascertain the presence of disease in clinical applications or to produce results that are accurate, precise, and reproducible in a research setting.

Why perform quality control in immunophenotyping? The reasons are as follows:

1. It insures that all measurands meet a standard.
2. It monitors day-to-day consistency and reliability.

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3. It provides confidence in results.
4. It insures reproducibility.
5. It addresses regulatory requirements.

Quality control (QC) and quality assurance (QA) as it pertains to immunophenotyping is a broad topic that covers many different components each of which is used to ensure that the results of a flow cytometry assay are reliable. QC is what you do to ensure your experiment or assay will do what it is supposed to do at each step from setup to acquisition to analysis to interpretation to the final answer. QA is the ongoing monitoring of all quality control procedures that reflect the overall acceptability of the results. QC procedures should demonstrate that everything being used to produce results is in proper working order prior to running patient samples in the clinical lab or biological specimens used in a research lab.

The results of the QC procedures on both the instrument and the reagents used by the laboratory should be readily available for inspection purposes [13, 14] as well as provide a means for long-term monitoring to detect trends and exceptions. Flow cytometry laboratories that perform clinical testing must meet and maintain several regulatory requirements found on the *College of American Pathologists (CAP) Flow Cytometry Inspection Checklist*. Figure 1: Quality control requirements of the CAP flow cytometry inspection checklist is a partial list of these requirements.

Flow cytometers used in immunophenotyping perform several different measurements and assurance of how well it is working is tantamount to being confident in the results it produces. Once the samples have been acquired additional analytic steps are necessary

FLO.23275	QC – Antibody Validation
FLO.23737	QC – Reagents/ Stain
FLO.23800	QC - Single/Dual Platform Tests
FLO.23925	QC Range Verification
FLO.24230	QC Corrective Action
FLO.24250	QC Testing of Controls
FLO.24300	QC Confirmation of Acceptability
FLO.24475	Monthly QC Review
FLO.24650	Comparability of Instrument/Method
FLO.25100	Instrument Function Checks
FLO.25150	Optical Alignment
FLO.25250	Instrument/Equipment Service Records
FLO.25300	Instrument/Equipment Maintenance Review
FLO.30250	Fluorescent Beads
FLO.30260	Color Compensation Settings
FLO.30270	Laser Current
FLO.30280	Calibration/Laser Performance

Fig. 1 Quality control requirements of the CAP flow cytometry inspection checklist [1]

to ensure that the interpretation of the results is accurate. All these considerations can be put into three categories:

1. Preanalytic quality control: quality control as it pertains to the steps taken to prepare an assay including the specimen requirements and reagents used to set up the assay [4].
2. Analytic quality control: quality control as it pertains to the instrument used to perform the immunophenotyping assay that ensures it is operating properly.
3. Postanalytic quality control: quality control as it pertains to the results generated by an immunophenotyping assay that ensures that they are accurate and precise.

Each of these steps is described in this chapter along with diagrams and references for further explanations.

2 Materials

1. QC beads, generally sourced from the instrument manufacturer or other commercial vendors.
   (a) Alignment/performance check beads.
   (b) Compensation beads.
2. PBS buffer with or without albumin
3. Monoclonal antibodies (various vendors).
4. 7-AAD viability dye
5. Ammonium chloride RBC lysing reagent
6. Quantitative commercial controls used in immunophenotyping procedures.
   (a) Normal control
   (b) Abnormal control
7. Qualitative controls for tests that use descriptive results i.e. “positive”, “negative”, “present”, “absent” and/ or “not detected”.
8. Miscellaneous laboratory equipment
   (a) Refrigerators and freezers
   (b) Centrifuges
   (c) Water baths and incubators
   (d) Biological hoods

3 Methods

3.1 Preanalytic Quality Control Procedures

3.1.1 Specimen Requirements

Proper specimen collection and timely processing are critical preanalytical quality control steps required for performing immunophenotyping procedures. Specimens used in flow cytometry testing can be obtained from several sources and in the clinical laboratory these may include peripheral blood, bone marrow aspirates, solid tissue biopsies, fine needle aspirates, and several types of body fluids such as CSF, pleural fluid, synovial fluid, and vitreous fluid. In a research setting this list may also include frozen tissue, animal samples, plant samples, sea water, and a variety of other sources. Each specimen has specific handling requirements that should be fully described in the procedures of the laboratory.

The following list includes the most widely used specimen types and their specific collection requirements for immunophenotyping.

3.1.2 Peripheral Blood and Bone Marrow Aspirates

All blood or bone marrow samples must be in an anticoagulant if the assay is for testing either white blood cells (WBCs) and/or red blood cells (RBCs) [25]. The most commonly used anticoagulants for immunophenotyping include EDTA, (sodium) heparin, and ACD (acid citrate dextrose). All are designed to prevent clotting while preserving the WBCs however, there are advantages and disadvantages to each and is discussed below. Another important consideration is the hold time between specimen collection and testing. This can be critical depending on the anticoagulant used. For the times listed in this section the laboratory should do their own validation of each anticoagulant to determine what is acceptable.

1. EDTA (ethylenediaminetetraacetic acid) preserves lymphocyte viability up to 72 h if refrigerated, 48 h at room temperature. This is the most common anticoagulant used in the clinical laboratory for testing lymphocyte subsets since it can also be used for performing white blood counts (WBCs) and differentials necessary for calculating the absolute lymphocyte counts. Granulocytes, however, may begin to deteriorate in less than 8 h due to the chelation of the calcium in the plasma by the EDTA which makes this anticoagulant less desirable for flow assays that focus on myeloid lineage populations. These tubes usually have a purple stopper [2].
2. Sodium heparin prevents the formation of fibrin from fibrinogen and thrombin activation thus preventing clotting of the sample. It may cause some RBCs to clump and, in some cases, impart a blue background on Wright-Giemsa stains so it is not suitable for hematology assays [3]; however, it works quite well on bone marrow aspirates and is the anticoagulant of choice for these samples. Samples in heparin are generally good for up to

48 h if refrigerated. These tubes usually have a light green stopper.

3. ACD (acid citrate dextrose) is the same anticoagulant used to collect whole blood donations for transfusion. It has many qualities that make it highly suitable for longer and more stabilized specimens and is more conducive to maintaining cell integrity and viability than either EDTA or heparin. However, it is a liquid additive and thus will make absolute counts inaccurate since it changes the volume of whatever is added to it, so it should not be used for either flow cytometry single-platform absolute counts or Hematology quantitative results. Samples in ACD are good for up to 48 h at room temperature and up to 4 days if refrigerated making it much preferred in laboratories that are not open all day every week. These tubes usually have a pale-yellow stopper.

3.1.3 Solid Tissue Biopsies and Fine Needle Aspirates

Tissue samples submitted for flow cytometry testing may be from many different sources. In a Hematopathology setting this would likely include a lymph node biopsy, soft tissue from an organ such as the gut, and even skin punches submitted by a dermatologist. If the sample is submitted from the same place as the lab doing the testing in most cases it will be reasonably fresh and intact. Care should be taken to insure good viability by using a transport media with the tissue such as RPMI with or without albumin that has nutrients and buffers that greatly improve the chances of maintaining viability. Using other media like plain PBS is discouraged because the samples will deteriorate much more quickly compared to a transport media like RPMI. The use of any fixative in the media is not acceptable since this will be toxic to the tissue and prohibit any possibility of doing a flow cytometry assay which requires viable cells. Some laboratories use frozen tissue banks that allow the lab to save rare or study samples in a way that they are not damaged by the subfreezing temperature. Previously frozen samples will need to be thawed and washed before processed to a single cell suspension. Follow published procedures that describe the steps necessary to gently bring the sample back to room temperature prior to making single cell suspensions. Making a single cell suspension of the solid tissue can be achieved several different ways including:

1. Grinding the sample with a mortar and pestle and rinsing with RPMI.
2. Using a tuberculin-sized needle that is filled with RPMI and repeatedly injecting it into the tissue in a petri dish to extract the cells of interest and leave behind the sample connective tissue. The cell suspension can then be washed with RPMI and concentrated prior to staining.

3. Adding an enzyme solution such as collagenase that digests the connective tissue leaving behind the cells of interest. Though effective in practice there may be higher cell damage using this procedure [6].
4. Using mechanical devices that disaggregate the connective tissue such as the Becton Dickinson Medimachine™. Small pieces of the tissue are put into a special cup called a Medicon™ which is then inserted into the Medimachine. RPMI is added to the Medicon, the cup capped, and the machine turned on. A spindle turns a raised metal lip that gradually forces the tissue through a bladed mesh as the spindle turns. This process then strips away the connective tissue leaving a single cell suspension in the RPMI at the bottom of the Medicon. The RPMI which now includes the separated cells is then withdrawn from the Medicon cup using a syringe, filtered, and then processed for staining [7]. Some laboratories perform cell counts after these steps to insure adequate staining based on concentrations that are appropriate to use with previously made antibody cocktails (a cocktail is two or more antibodies in the same tube).

3.1.4 Fine Needle Aspirates (FNAs)

FNAs are frequently submitted for flow cytometry testing in the context of hematopathology procedures. Tissue samples collected with this technique are a much less invasive procedure compared to obtaining a tissue biopsy. The samples are collected by using a very thin needle (22 gauge or smaller) that is inserted into an area of questionable tissue like a lymph node or other soft tissue. The cells are then aspirated into the syringe and added to a tube containing transport media such as RPMI. Since the cells are already in suspension very little manipulation of the sample is necessary prior to using it in an immunophenotyping procedure. It can then be washed, concentrated, and resuspended in RPMI prior to staining. These samples may have some peripheral blood contamination that happens during collection and thus will need to include a RBC lysing step as part of the staining procedure.

3.1.5 Body Fluids and Cerebrospinal Fluid (CSF)

Body fluids submitted for flow cytometry immunophenotyping can be collected from several different sources. These may include peritoneal, bronchial, pleural, synovial, pericardial, vitreous, and cerebrospinal fluids. In most situations the question for testing is to find and identify an underlying malignancy such as lymphoma or leukemia. Usually the body fluid itself if collected the same day will maintain the cell's viability if transported quickly and refrigerated. CSF however, is collected using a laparoscopic procedure and even though these specimens are usually sent to the lab with no additional media in the tube it is highly recommended that the sample be resuspended in fresh RPMI as soon as possible to prevent the rapid deterioration of any cells present. Studies have shown that CSF with RPMI added may have a much higher rate of recovery compared to samples that do not include a transport media [10].

3.2 Preanalytic Quality Control Procedures

3.2.1 Reagent Quality Control

Buffers and Lysing Solutions

All reagents used in an immunophenotyping experiment must be tested for efficacy prior to being used in a procedure [4]. For example, PBS (phosphate buffered saline) and RBC lysing agents such as ammonium chloride (NH_4Cl), can be tested quite easily using a sample type that is commonly submitted for testing such as peripheral blood. Set up a sample using the new reagents, then acquire on the instrument and review the results for acceptability. Is the light scatter okay? Are the RBCs lysed as expected? Record the results of testing in a Reagent QC log to track performance. Include the lot number if applicable, the testing specimen used and whether the results were as expected, usually by either "pass" or "fail" depending on criteria included in the procedure. As with all QC records, the supervisor or designee should review the results monthly.

3.2.2 Antibody Quality Control

Primary reagents like monoclonal antibodies require a more thorough control process than other test reagents [9]. Quality control of antibodies should include the following procedures:

1. Antibody specificity: does the antibody react with the intended antigen [12]? This aspect of antibody QC requires specific specimens to prove efficacy of the reagent. For example, if CD33 is being tested, the control material should include myeloid lineage cells such as granulocytes and monocytes, since that antigen is fully expressed on those cells. Lymphocytes present in those samples can serve as a negative control. Other antibody control materials include the following:
   (a) Commercial quality controls: These are samples that include a list of expected results for several antibodies in the package insert. These controls are generally used for daily testing of an antibody cocktail panel (a cocktail is two or more antibodies in the same tube), which is required for clinical applications like peripheral blood lymphocyte subsets or stem cell quantitation assays. These commercial controls will generally have known qualitative and quantitative values for most of the antibodies used for immunophenotyping in a clinical laboratory. *See* Fig. 2: Commercial control package insert taken from a commercial whole blood control listing.
   (b) In-house controls: Other control samples that can be used to check antibody specificity may be sourced from previously tested material that has been confirmed to be positive or negative for the antibody depending on the population of interest. If alternate testing is performed in the laboratory, such as immunohistochemistry (IHC), the flow cytometry antibody results can be compared to the results of the alternate assay such as a tissue section stained for CD20. However, this choice may only offer

Phenotype[3,a]	WBC Type[4]	%Positive Cell[1,b]	Expected Ranges[6]	Absolute Number[7,c]	Expected Range[4]
$CD2^+$	Lymphocytes	85.0	73.0 - 97.0	–	–
$CD3^{+a}$	Lymphocytes	78.4	68.4 - 88.4	1625	1275 - 1975
$CD3^+/CD4^{+a}$	Lymphocytes	51.1	45.1 - 57.1	1059	879 1239
$CD5^+$	Lymphocytes	74.6	64.6 - 84.6	-	-
$CD3^+/CD8^{+a}$	Lymphocytes	25.3	19.3 - 31.3	524	354 - 694
$CD3^-/CD16^+$	Lymphocytes	9.2	4.2 - 14.2	–	–
$CD3^-/CD16^+.CD56^+$	Lymphocytes	10.5	5.5 - 15.5	218	73 - 363
$CD19^+$	Lymphocytes	11.2	6.2 - 16.2	232	82 - 382
$CD20^+$	Lymphocytes	11.1	6.1 - 16.1	–	–
$CD3^-/CD56^+$	Lymphocytes	8.6	3.6 - 13.6	–	–
HLA · DR^+	Lymphocytes	15.8	9.8 - 21.8	–	–
$CD14^+$	Monocytes	85.0	70.0 - 100.0	–	–
$CD45^+$	Leukocyte Gate	····· CD45 is to be used for gating purposes only.[8] ·····			
$CD34^+$	Progenitor Cels	0.04	0.01 - 0.07	2.8	0.6 - 5.0
Lymphocyte[c]		31.4	21.4 - 41.4	2074	1674 - 2474
Monocyte		8.4	3.4 - 13.4	–	–
Granulocyte		57.1	47.1 - 67.1	–	–
Absolute[9]WBC[c]($x10^3/\mu L$)		-	-	6.6	6.1 - 7.1

Fig. 2 Example of a package insert from a whole blood commercial control listing reactivity and expected quantitative values for antibodies used in Immunophenotyping [28]

limited comparisons, since in many cases the number of antibodies used in Flow Cytometry applications may be greater than those found on the Histology test menu. The College of American Pathologists (CAP) also offers cell controls that can be used for a limited number of other rare antibodies such as CD103 and CD1a. Some laboratories with subzero freezers may cryopreserve samples that have previously been tested and later used for quality controlling new shipments of antibodies.

(c) Proficiency testing samples: If the laboratory participates in an external proficiency testing program such as the CAP Survey, there may be leftover samples that are still adequate for quality control testing purposes that can be stained with a variety of antibodies. Returned results of these surveys can then be compared to the antibodies being tested for quality in the lab. This may not be practical if there is a considerable time delay before the phenotype of the testing material is known, unless the lab has some way of preserving the cells.

3.2.3 Antibody Concentration and the Stain Index

Most antibody manufacturers provide directions that specify the amount of antibody to use for samples typically submitted for Immunophenotyping. Knowing the concentration of the sample is also necessary to insure saturation staining [11, 25]. For example, if a sample has a high concentration of cells, then the recommended amount of antibody per test may not be enough to insure adequate staining. In other cases, the amount of antibody listed may be

$$\text{Stain Index} = \frac{MFI_{pos} - MFI_{neg}}{2 \times SD}$$

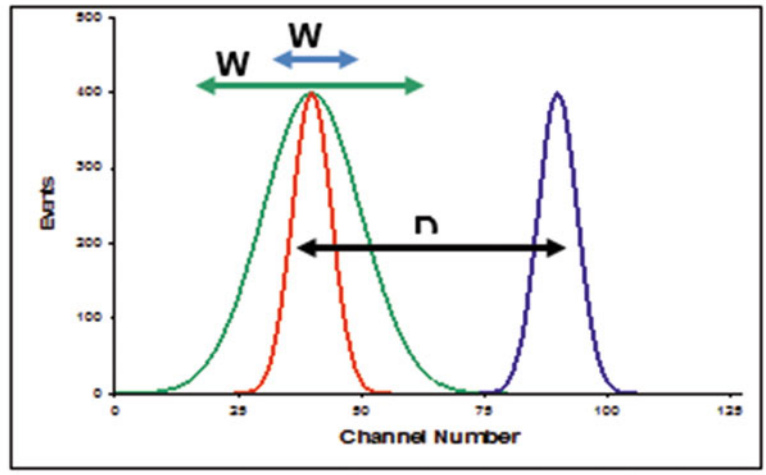

Fig. 3 The stain index (SI) calculation [22, 33]. The SI is the ratio of the difference between the positive mean intensity (MFI) and negative mean intensity (MFI) populations divided by two times the standard deviation (SD) of the negative population. The SI is used to compare fluorescence intensity of one antibody versus another or when comparing the concentration of one antibody versus a different dilution of the same antibody

several times more than is necessary and the excess antibody may negatively impact the staining by increasing the background signal. The best antibody concentration to use can be determined by calculating the stain index (SI). The SI is the ratio of the difference between the positive mean channel result and the negative mean channel result divided by two times the standard deviation (SD) of the negative population. *See* Fig. 3: Stain index calculation.

The stain index can be used to compare different antibodies and it can also be used to compare different concentrations of the same antibody. In some cases, the SI will improve using a lower concentration than what is recommended since the background signal width may be smaller than it is using a higher concentration. The higher the SI, the better the resolution between the positive and negative populations. Different SIs can often be seen with different concentrations. The difference can be due to one of two reasons: a lower intensity of the positive peak or a wider SD of the negative signal due to excess unbound antibody. Titrating the antibody is advantageous for determining the optimal

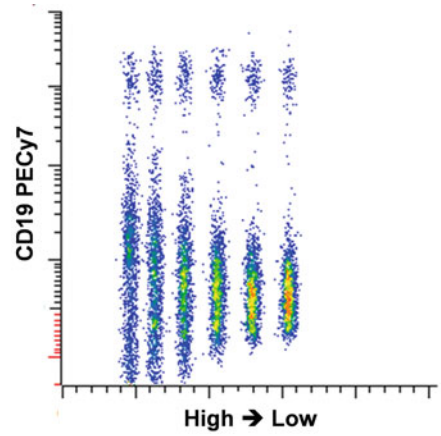

Fig. 4 Antibody concentration versus CD19 PEcy7 expression. Over the course of different titrations or dilutions the stain index (SI) improves using lower concentrations of the antibody. There is no noticeable loss in CD19 intensity (Y-axis); however, the background signal is much greater at higher concentrations of the antibody and thus has a lower stain index. Ideally, the standard deviation of the negative signal should be somewhere near that of unstained autofluorescence

concentration to use in the experiment. Different concentrations may have little effect on the overall intensity of the positive peak; however, in some cases the background or negative signal width decreases because of less unbound antibody and thus a better SI. An example of how the optimal antibody concentration can be calculated is seen in Fig. 4: Antibody concentration versus CD19 PEcy7 expression.

The titration procedure compares serial dilutions of the antibody after calculating the stain index of each dilution. The results are then graphed using the SI versus the concentration of the antibody. Determining the best titer of the antibody should be included in the quality control procedure for any new lot number or new shipment prior to using it in an assay. This is a requirement in many clinical applications especially for lab developed tests (LDT). It is okay to use a different amount than what is recommended by the manufacturer if you can document that it still performs as intended.

3.2.4 Antibody Titration Procedure

1. Make serial dilutions of the antibody beginning with the recommended volume followed by dilutions of 1:2, 1:4, 1:8, etc. The diluent used can be simple PBS buffer with or without calf serum which is reliable for antibodies that have a high expression such as CD45 and CD8. In some instances, especially very weak expressing antibodies should be diluted with unlabeled or unconjugated antibody to insure adequate antibody saturation staining.

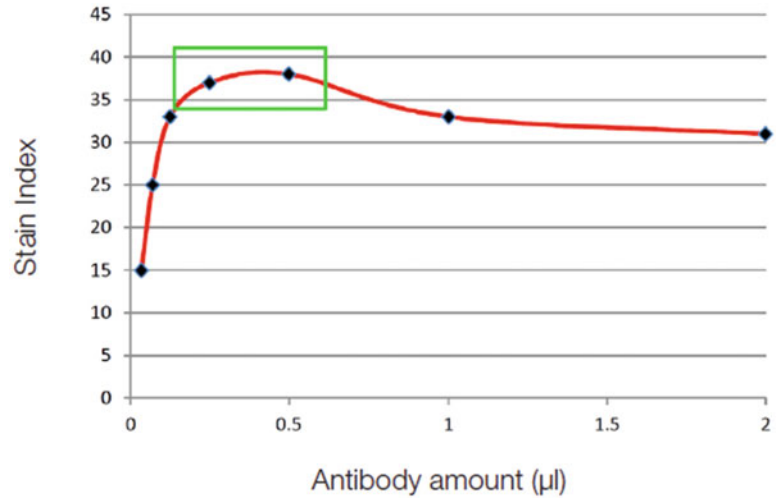

Fig. 5 Antibody concentration versus stain index. As the antibody concentration increases the stain index (SI) increases until it levels off. The optimal concentration should be somewhere close to the value of the stain index that begins to cease changing (in the example shown, approximately 0.5 µl/test)

2. Include a tube with no antibody (unstained) for comparative purposes of the negative peak of the population vs an unstained/ background signal.
3. Use the sample type that is typically tested such as peripheral blood, bone marrow aspirate, or tissues. It is important to have both positive and negative populations in each tube, so cell lines might not be the best choice if no negative populations are typically found in those samples.
4. Process all samples following the standard staining procedures used in your laboratory.
5. Run the samples on the flow cytometer with the same instrument settings used for actual testing purposes.
6. After acquiring the samples, graph the Stain Index versus the antibody concentration to determine the best overall concentration to use. *See* Fig. 5: Antibody concentration versus stain index as an example.

3.2.5 Multiantibody Cocktails

An antibody cocktail is whenever two or more antibodies are combined to make a multiuse reagent. As part of panel design the antibody cocktail is constructed using combinations that are used for immunophenotyping applications. There are several advantages to using cocktails. They can save time when setting up multiple experiments and are likely to lower or prevent pipetting errors that could happen if the single cocktail was made up each time the experiment was performed. Another advantage to using cocktails is that you can standardize delivery volumes. This makes test setups

easier since many single reagents use different volumes and in a multicolor cocktail this can be very labor intensive and prone to error. The cocktail can be made in advance and for enough tests to last a few days or weeks.

1. Initial QC testing: Any cocktail must be proven to be accurate before using it for testing or reporting purposes. Initial quality testing of the cocktail should include setting it up on a fresh sample along with a second tube of the in-use cocktail. Results should agree for both reactivity and quantitation if applicable. Shelf life of the cocktail must be validated as part of implementation of the cocktail. There are recommended practice guidelines [4, 8] for use however each lab should determine its own pass or fail requirements.
2. Ongoing testing: If the cocktail is kept over several days, it should be tested each week for acceptability using positive and negative controls. Previously tested samples are good sources of QC material. Loss of any antibody or a definitive change in intensity means that the cocktail should be discarded. Criteria for acceptability should be included in the QC procedure. Document results of the quality control and keep records of the testing.

3.2.6 Antibody Cocktail Procedure (Example)

The full cocktail procedure can be found in Notes section. The example shown in the recipe is for a 10 test, 4-color cocktail for T-lymphocyte subset testing using titrated antibodies and buffer that is aliquoted using 100 μl cocktail/test. Use a previously determined titer amount for each antibody in the cocktail and add enough buffer to create a standardized aliquot for testing such as 100 μl/test. The additional volume of the buffer will also allow better mixing.

3.2.7 Conclusion, Preanalytic Quality Control

Every experiment used in Immunophenotyping uses specific samples from a wide range of sources. Each laboratory should develop their own specific sample requirements that include the instructions for collection, the media used to transport the sample, and the storage and conditions required for testing. In addition, the use of any reagents to perform the test must also be checked for suitability using controls that confirm each of the preparation steps of the assay. Records of all quality controls must be kept in a concise way that ensures easy retrieval for inspection purposes as well as to monitor performance over time. Upon successful completion of these preanalytical steps the Immunophenotyping testing can now proceed to the analytic phase.

3.3 Analytic Quality Control Procedures

3.3.1 Instrument Quality Control

Introduction: Quality control of the immunophenotyping assay continues with consideration of the testing that includes the flow cytometer itself. These very sensitive and highly adaptable platforms are rather simple in terms of how they work. Basically, a fluidics system consisting of either buffered saline or deionized water provides a means for the sample to be introduced into a flow cell chamber. The flow cell has a very specific shape that through the principle of hydrodynamic focusing takes the cells or other objects in a random mixture and puts them into a single file by forcing them through a small opening as they exit the flow cell. Upon exiting the flow chamber the cell stream is bisected by one or more light sources, typically lasers with monochromatic emissions. The intersection of the cells with the light produces several specific light signals that are then directed by fiber optic cables or mirrors and filters into electronic detectors commonly known as photomultiplier tubes (PMTs). The PMTs then convert those signals into a digital format for data analysis using software programs.

Despite the simplicity of its operation the quality of the results can be greatly affected by the performance of any or all these components of acquisition: fluidics, optics, and electronics. Daily instrument maintenance and quality control is paramount to ensuring reliable results. This section of Immunophenotyping Quality Control will consider and describe the steps to perform on the instrument as preparation for running test samples. *See* the Notes section for a step-by-step guide for setting up a flow cytometer for immunophenotyping assays. Details of the procedures and explanation of each step in the analytic QC of the instrument is found in the following section.

3.3.2 Instrument Quality Control: Flow Cytometer Startup and Maintenance

1. Fluidics system startup: The flow cytometer fluidics system is truly the lifeblood of the instrument. Proper care and cleaning of the fluidics system is synonymous with good quality control and helps ensure high quality results. Prior to turning on the instrument check the levels of the fluidics containers. They are usually found either on a fluidics cart or other storage system near the instrument. This includes the sheath fluid, cleaning solutions, or DI water, etc. that may be a part of the whole fluidics system. If the instrument has a waste container dispose of anything from the last time it was used. Be sure to add bleach to the container to minimize the danger of any hazardous waste that may result from using biological samples. Most flow cytometers have built-in programs that provide a means for the sheath fluid and other solutions to be primed and distributed throughout the system including the flow cell and ultimately into the waste container. As part of the instrument startup a series of priming and flushing cycles takes place to be sure that everything is working and that all the fluidics lines are free of air bubbles. Sometimes when the instrument is shut down for

several hours bubbles can come out of the sheath fluid solution still in the instrument much like what is observed on the side of a glass of water left standing overnight. If those bubbles are not adequately removed as part of the fluidics startup they may cling to the inside of the flow cell and create focusing issues much like any other obstruction in the fluidics system.

2. Fluidics clean cycle: Samples routinely run on the instrument such as tissue cell suspensions and bone marrow specimens may have high amounts of debris or fibrin or other nonintact cell residue that can build up on the inside of the sample insertion rod, fluidics tubing, or flow cell. After starting up the instrument the fluidics system should be put through a short cleaning cycle that includes running dilute bleach (10%) for 5 min followed by a DI water sample for 5 min. Running a series of cleaning samples helps to remove the buildup and prevent clogs or other obstructions that might be present in the insertion rod or fluidics tubing. Some labs prefer to do this cleaning sequence one or more times a day. A good rule of thumb is that for every 1 min you run dilute bleach it should be followed for the same amount of time by running a tube of DI water. This is to adequately remove any residual bleach from the fluidics system. A full system clean of 60 min (30/30) or more should be performed at least once a month to more thoroughly clean the fluidics lines and flow cell chamber.
3. Fluidics function checks as part of bead QC.

   As mentioned before, issues with fluidics are probably the most common cause of instrument-related problems. Most every instrument has a quality control program that uses latex beads to measure and standardize each parameter of the instrument operation. Usually performance issues are first indicated by out of range bead QC results including diminished resolution of either or both light scatter parameters, forward scatter (FSC) and side-scatter (SSC). A well-maintained instrument that has been set up and optimized should demonstrate that the FSC/SSC dot plot of a lysed blood sample has at least three distinct WBC populations corresponding to the lymphocytes, monocytes, and granulocytes. On the other hand, if the instrument has population resolution issues seen by light scatter due to fluidics problems the populations tend to drift downward toward the threshold on the FSC/SSC dot plot (*see* Fig. 6: Good light scatter resolution versus poor light scatter resolution).

   If the flow cytometer has debris buildup over time in the fluidics lines or the sample insertion rod or if the stream is obstructed in some way the Forward Scatter Channel (FSC) signal is usually the first to show this problem. There will either be a gradual loss of the signal resulting in a lower location on forward scatter of a population or an increase in the detector

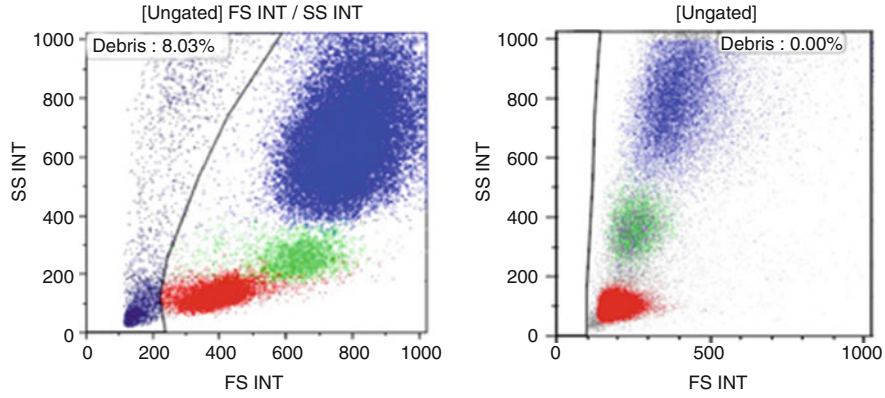

Fig. 6 Good light scatter resolution versus poor light scatter resolution: Left histogram: RBC-lysed peripheral blood sample with optimized and finely tuned FSC/SSC signals shows granulocyte (blue) populations. Right Histogram: FSC/SSC dot plot with poor optical alignment, possibly due to fluidics issues. Notice the loss of FSC output versus SSC resolution

gain if you are using settings that are autoadjusted by the bead QC software to meet a target range for each peak channel. Eventually these changes to the detector settings will exceed the defined limits of the bead QC program and the results will have one or more parameter failures.

4. Laser performance: The solid-state lasers found on most flow cytometers are considerably more dependable and less prone to failure than the high-power water-cooled lasers that were used on many of the research-type instruments years ago. However, they still need time to warm up and equilibrate. Usually the time required to run the fluidics start-up cycle and cleaning steps is typically long enough for the lasers to warm up. In most cases the instrument will have an indication of when it is ready to be used following the startup time for the laser.
5. Flow cell, optics, and electronics QC using commercial beads.
   (a) Introduction to the Bead QC procedure: Following instrument and fluidics startup, initial cleaning, and laser warm-up the flow cytometer must then be checked for proper operation. This is typically accomplished using latex beads or other materials, usually sourced from an instrument vendor, to ascertain the overall function of the fluidics, electronics, and optical alignment. These quality controls checks are where the "quality" of Quality Assurance begins in flow cytometry. The CAP checklist requires that performance of function checks on all instruments follow a defined schedule [1]. For results from a flow cytometer to be reliable and accurate, the setup and monitoring of the flow cytometer performance should always be checked when the instrument is started up each day. Some labs may require this as often as once each shift if the instrument is used continuously.

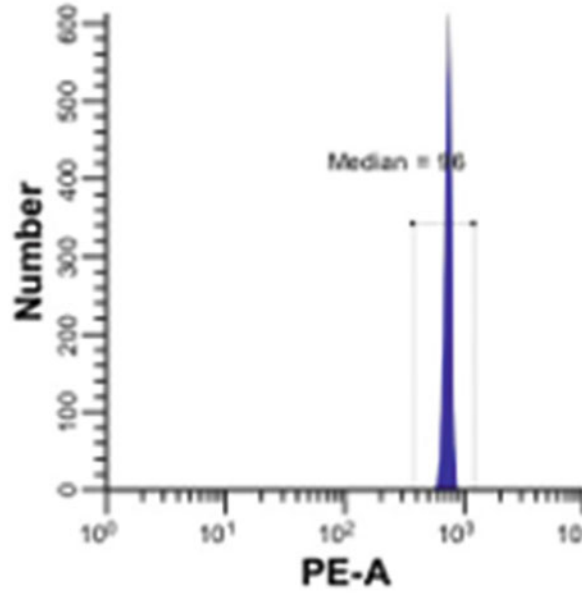

Fig. 7 QC bead signal. QC Beads run each day are placed in a specific channel of each detector by the software automatically adjusting the detector gain or voltage to match the baseline target value

To help ensure that the flow cytometer is fully functional and each event signal is meeting a set standard, most instrument manufacturers use bead QC programs that provide information on all the major components of the instrument. After establishing a baseline, the performance is then monitored on an ongoing basis to be sure that it is still operating properly. These latex beads are polychromatic and thus can be detected across all the PMTs and light scatter detectors. The beads have different sizes and signal intensities so that linearity and precision can be calculated along with PMT performance. If for instance the PMT voltage required to place the bead signal in the previously established baseline channel is outside the software limits, then that particular parameter will be flagged as failing (*see* Fig. 7: QC bead signal).

6. Focal point/alignment check: The alignment of the laser to the flow cell stream is critical and has a direct impact on the quality of the results. Most flow cytometers have one or more laser light sources that are directed by a series of lenses and prisms to a fine focal point at the exit point of the flow cell chamber. If the laser or the flow cell is not properly aligned or focused, then the resolution of one or more signals may be suboptimal. An instrument with good alignment means that the laser light intersecting the sample stream is precise and finely tuned. A bright, sharp signal provides better resolution and sensitivity

Fig. 8 Optical alignment and fine focusing. Good optical alignment equates to a fine focus which can provide a sharper, clearer signal that gives better resolution and higher sensitivity (right image) compared to an instrument with poor optical alignment (left image) that is blurred or out of focus

compared to a signal that is not quite as precise (*see* Fig. 8: Optical alignment and fine focusing).

Running QC beads each day does not actually change the alignment of the light with the flow cell but provides a way of knowing just how the performance compares to the baseline standard established earlier. Performing an actual alignment of the instrument laser or flow cell is not something done routinely, especially in a clinical setting. That procedure is typically performed by a service engineer as part of an initial setup of the instrument or as part of regularly scheduled preventative maintenance or whenever a major component like a laser or a PMT is replaced. Some research flow cytometers may allow the user to change or adjust the optical alignment, but this is the exception, not the rule since in most instruments the flow cell itself may be semipermanently attached to the optical bench.

7. PMT performance: Besides checking the alignment of the laser and the flow cell the results of running QC beads are indicators of PMT performance. After creating a baseline setup for each signal, the daily performance of the QC beads is measured against the results of the baseline measurements. Each of these performance checks provides information about the detector efficiency, sensitivity, resolution, precision, linearity, background (noise) level, and laser delay. By definition:

   (a) Detector efficiency is the maximum signal output with minimum PMT voltage. A failing PMT will have a loss

of efficiency over time and subsequently require more voltage to put the emitted signal within the program's baseline range of the scale. An increase in PMT voltage may then result in a higher background signal and subsequent loss of sensitivity.

(b) Sensitivity is the ability to distinguish true positive signals from true negative signals. One way to determine PMT sensitivity is to run polychromatic beads and calculate the stain index (SI). The SI equation uses the fluorescence intensity of the positive peak versus the negative peak and can be calculated using this equation:

$$\text{SI} = \text{MFI of positive bead} - \text{MFI of the unlabeled bead}/2 \times \text{SD of unlabeled bead}$$

(SI = stain index, MFI = mean fluorescence intensity, SD = standard deviation)

The standard deviation (SD) of the negative peak is a reflection of the width of the background signal. Some fluorochromes have a broad negative signal, while others may have one that is much narrower and thus have a lower SD and in many cases a higher SI.

The SI calculation on some instruments is performed by the bead QC software, but it can also be put into and calculated on a spreadsheet if you know the result values to use in the equation. Knowing the relative stain index of each detector and monitoring it on at least a weekly basis is highly recommended. Each laboratory should establish an acceptable range, usually ±2 SD of the mean SI for each PMT. If over time, the bead sensitivity results show a downward trend it may be due to instrument issues such as faulty fluidics, dirty optics, a failing laser or even electronic noise. If this trend continues for a certain amount of time the service engineer should be notified if minor troubleshooting does not resolve the issue.

(c) Signal resolution is the ability to detect discrete signals in a mixture where differences are very small. Good resolution can also lead to higher sensitivity. One way of determining how well the instrument can resolve multiple populations with small differences is to run beads that have varying intensities. There should be good separation between each of the populations throughout the range of the detector including very weak signals versus negative signals and very bright signals being on scale and not crushed against the right axis. If the signals are not clearly differentiated it may indicate an alignment/focal point problem often seen when there are fluidics issues especially if it happens in more than one channel. Cleaning the flow

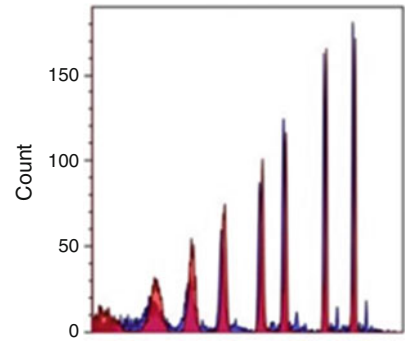

Fig. 9 Multiple fluorescent intensities demonstrating high resolution. A finly tuned flow cytometer with proper alignment and PMT settings can resolve multiple populations which demonstrate discrete differences in intensity

cell using a manufacturer recommended cleaning solution can oftentimes improve the quality of the results (*see* Fig. 9: Multiple fluorescent intensities demonstrating high resolution).

(d) Precision is the ability to detect the same signal repeatedly within a defined range of the baseline. The measurement of precision is the coefficient of variation (CV) of multiple results. High CVs mean poor precision which negatively impacts the reproducibility of your results.

(e) Linearity is the property of bead signals to be detected in a straight line over time. If the detector has good linearity, a signal that has twice the output of the first signal should then be 2× brighter than the first signal and so on for subsequent results with increasing positive peaks [21]. This is very important for experiments that measure DNA ploidy where the G2 + M population should be 2× the G1/G0 population [5, 23, 24]. If it is not, then the results might be misinterpreted as being aneuploid. Good linearity means that the reliability of multiple signals is expectedly proportional.

(f) Background level is sometime referred to as the noise level. It is the extraneous signal detected when no other signal is expected. It is present on all PMT outputs, but it may be higher in some compared to others. Knowing the background signal is important because it may impact the sensitivity if it is higher than the otherwise negative signal produced by the cells being measured. High background levels that increase over time are often seen in PMTs or other instrument electronics that are beginning to fail or need recalibration. If routine troubleshooting does not improve the background signal it may require attention by a service engineer.

(g) Laser delay is the time difference between the bead/cell being excited by the first laser followed by signal output from the second or subsequent lasers. It is used to unite the signals produced by multiple lasers exciting different fluorochromes on the same cell. If the delay is too long it can result in missing or abbreviating detection of the second and following signals. Generally, laser delay issues can be traced back to fluidics problems. When something slows or impedes the fluidics stream such as a partially obstructed flow cell, it may sometimes cause laser delay errors. Performing regular instrument maintenance like cleaning will usually prevent this problem.

8. Levey–Jennings charts.

Besides the bead QC indicating the overall operation of the instrument on a specific day it also provides a way to monitor the performance of several parameters over the course of days or weeks to demonstrate reliability and consistency. Most flow cytometers are rather stable from day to day however, the alignment or precise focal point can drift or deteriorate over time due to any number of reasons including a fluidics issue, faulty electronics, laser failure, or some other unknown cause. There may be a gradual decrease in performance in any of the electronics or lasers that may predict a future failure. The bead QC program used should include graphical displays of all measured results plotted over time. These are known as Levey–Jennings (L-J) charts and they allow the user to observe or detect changes, trends, or outliers that can in some cases predict future problems that can be addressed before they totally fail. *See* Fig. 10: Levey Jennings Charts.

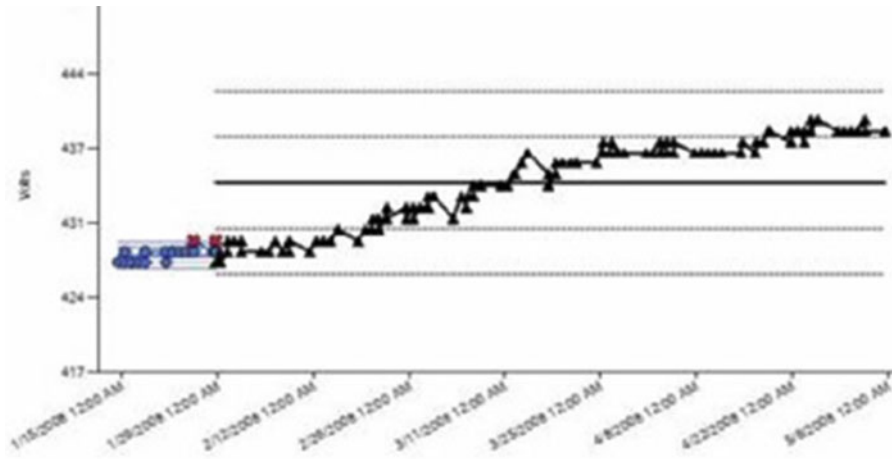

Fig. 10 Levey–Jennings graph of daily PMT QC settings over time. The voltage of the FL8 detector required to meet the baseline specifications is gradually rising over time which may indicate an impending failure of that PMT

If only one detector shows a trend, then the problem may be a faulty PMT. If more than one detector from the same laser shows this problem, it may indicate that the laser performance is the potential problem. In some cases, the issue may a damaged fiber-optic cable that transmits the signals from the flow cell to the PMTs. Generally, a problem like this must be addressed by a service engineer. If more than one parameter is out over multiple lasers, then the problem is more likely to be the fluidics or an impending failure of the laser or even the instrument electronics or circuit boards or interface of the instrument and workstation. Comments on L-J charts about outliers or trends should be included in the results as well as any action taken to troubleshoot the issue observed.

9. QC Bead results summary.

   Performing routine maintenance and running QC beads to check fluidics, alignment, and sensitivity is the first step in preparing the instrument to run experiment samples. Each lab should determine how and when to respond to a QC failure. In some cases, performing a system clean of the fluidics and flow cell will often fix the problem and in other cases more detailed troubleshooting is required. If the problem continues then a service call to the instrument vendor may be necessary to find and fix the issue. Documentation of the QC results and monitoring performance over time helps ensure that testing will be successful. After successfully completing the QC beads procedure the instrument is almost ready for test samples; however, an important next step is to optimize the instrument settings using actual samples. This step is described in the next section.

10. Optimizing the instrument settings.

    Optimizing the instrument settings means getting the maximum performance or the best signal–noise ratio for each detector [22]. The signal–noise ratio is the difference between the median positive signal and the median negative signal. Think of optimizing as fine-tuning the instrument settings as compared to a course adjustment that happens when running only the QC beads. Do not confuse optimizing the settings with setting up compensation. Color compensation setup follows optimizing the instrument. In the clinical lab think of this as moving the instrument from being "bead-ready" to one that is "sample-ready." Why? Because in some cases, bead signal intensity will be different from cell signal intensity and using stained cell samples to optimize the bead settings may improve the performance of the instrument. This is especially important for experiments designed to identify discrete populations which are sometime present with both normal and abnormal cells. If the default setting is too low there may be a loss of

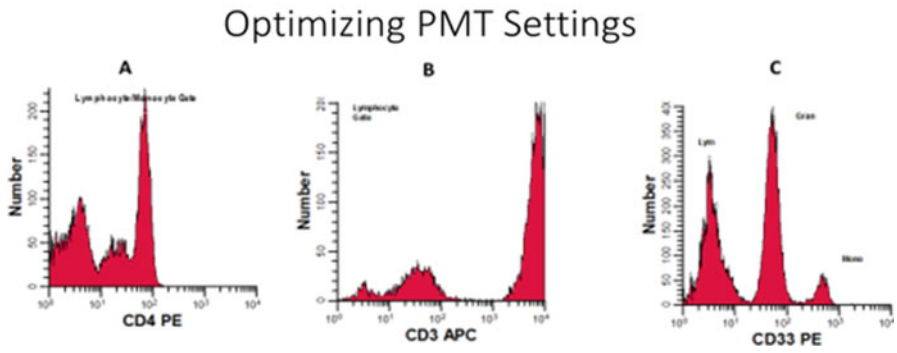

Fig. 11 Optimizing PMT settings. In Histogram **a**, WBCs gated on lymphocytes and monocytes stained with CD4, the PMT setting is too low and as a result the intermediate population (monocytes) is difficult to resolve. In Histogram **b**, the default PMT setting is too high so CD3+ T cells gated on lymphocytes appears to be off-scale when in fact are still on scale; however, the brightest events are piled up against the right axis and may be visually underestimated. In Fig. **c**, a lysed whole blood sample, the PMT setting is optimal. CD33 expression shows clear resolution between the bright (monocytes), intermediate (granulocytes), and negative (lymphocytes) WBC populations

sensitivity in picking up the weak signal or there may be poor separation between the positive signal and the negative signal which might be hiding an intermediate signal. On the other hand, if the bead PMT setting is too high the positive signal might be only partially observed if it is pushed up against the far axis. This makes the upper end of the signal look like it is off scale when instead it is simply piled up in the last channel and may be erroneously underestimated. *See* Fig. 11: Optimizing PMT Settings, for examples of PMT settings that are too low, too high, and then optimal using a stained cell sample with bright, intermediate, and negative populations present.

How often is it necessary to optimize? Most flow cytometers are stable and give reproducible results day after day so the frequency of performing optimization really depends on a few specific situations:

(a) Whenever an instrument is initially installed.

(b) After any major maintenance such as replacing a laser, realigning the flow cell, changing out a PMT, or changing the configuration of the PMTs.

(c) Change the QC Bead Baseline configuration or after setting up a baseline using a new lot number of QC beads.

A good rule of thumb is to check the settings using a fully stained sample and observing each PMT for optimal performance at least once a month.

11. Optimization methods.

Forward Scatter (FSC)/ Side Scatter Optimization: Start by running a cell sample such as lysed whole blood (LWB)

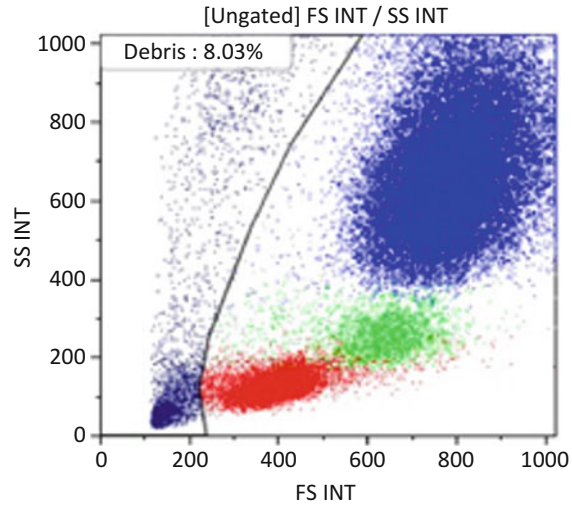

Fig. 12 FSC versus SSC with good separation and resolution. Forward scatter versus side scatter of a lysed whole blood sample demonstrating good separation between all 3 WBC populations with the noise threshold set just below the lymphocytes to allow visualization of intact cells versus debris

using the default settings from the bead QC. The first difference you will notice is that the light scatter settings and possibly the threshold will need to be adjusted since beads are often much smaller than cells. Adjust the FSC/SSC light scatter settings until you can see good separation between the lymphocyte, monocyte, and granulocyte populations. Be sure to set the threshold to just below the lymphocyte population to minimize the noise and debris events but still show the entire population of lymphocytes present. Keep the granulocyte population on scale however, take advantage of the entire FSC/SSC area to see each population clearly like that seen in Fig. 12: FSC versus SSC with good separation and resolution.

PMT Optimization: Optimizing the fluorescence PMTs can be performed several different ways and each has advantages and disadvantages, but they should all allow the user to see an improvement in signal separation between the bright positive, intermediate, and negative populations present. Two of the most popular methods used to optimize the PMT settings are the Unstained Sample Method and the Single-stained Sample method. Note: optimization should be performed with the **compensation settings turned off.** That step follows optimizing the instrument settings.

(a) Unstained sample method: This method has been used for several years and is relatively easy to perform and not very expensive since no antibodies are required. Set up the instrument starting with the default PMT settings from

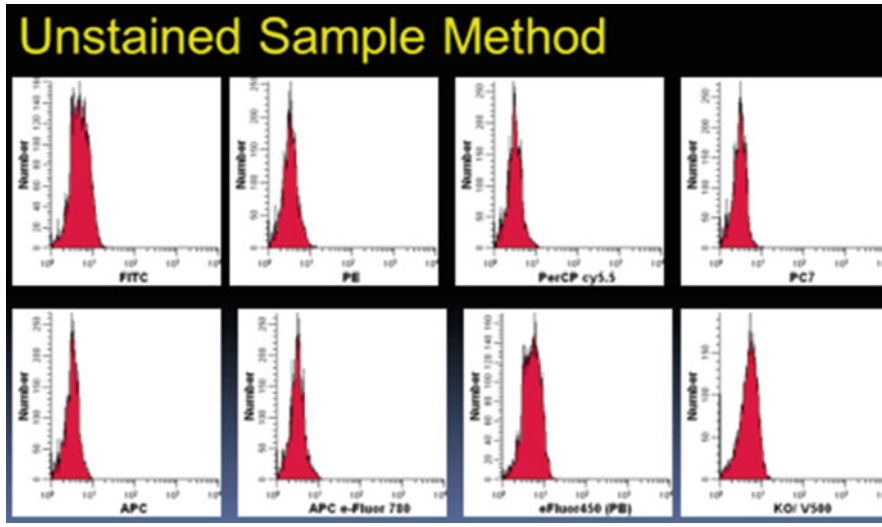

Fig. 13 Unstained sample method for optimizing PMT settings. Unstained sample optimization method gated on lymphocytes from a lysed whole blood specimen. The PMT settings are adjusted so that the leading edge of the negative signal is just beyond the background signal in each detector

the bead QC. Run an unstained lysed whole blood sample and draw a gate on the lymphocyte population on forward versus side light scatter. One at a time, adjust the PMT settings for each parameter in your sample testing panel until the leading edge of the negative signal goes just beyond the background noise signal. Continue until each PMT has been set. *See* Fig. 13: Unstained sample method for optimizing PMT settings.

One advantage to this method is that it does not require any antibodies, so it is rather easy to set up. A disadvantage to using this method, however, is that you will not know until you run a positive-stained sample whether the positive peak will be on scale if the setting is too high, or whether a weak positive population can be differentiated from a negative or bright positive population if the setting is too low. Use the unstained results as a starting point but also include a fully stained sample that shows both positive and negative populations to be sure that the all of the populations are visible on the scale of each PMT.

(b) Single-stained sample method: This method uses a panel of lysed whole blood samples that are single-stained with each conjugate to be used in the experiment. The advantage of this method is that you can visualize the full range of signal intensities of multiple populations: bright, intermediate, and negative for each PMT. For the single-stained samples, choose an antibody that has multiple

levels of expression depending on the populations present. Three antibodies that are useful for this method are:

- CD4: gated on lymphocytes and monocytes, stains positive for T-helper lymphocytes (bright), monocytes (intermediate), and is negative for CD8+ T cells and granulocytes.
- CD33: Another good marker for visualizing three or more populations. Monocytes are strongly positive, granulocytes stain with an intermediate intensity, and lymphocytes are negative.
- CD8: gated on lymphocytes, stains T-cytotoxic suppressor cells very bright and natural killer cell lymphocytes with a heterogeneous mid-intensity, and CD4+ T cells are negative. CD8 is one of the brightest antibodies available and gated on lymphocytes it will usually express intensity at the highest decade possible for that particular conjugate; however, CD8 may not be the best antibody to use for optimization simply because the intermediate population (NK lymphocytes) is oftentimes not very distinct compared to the intermediate populations observed using CD4 or CD33. *See* Fig. 14: Stained cell method for optimizing PMTs

  Adjust the PMT setting so that the bright peaks are on scale, the intermediate peak can be clearly seen, and the negative population is also clearly defined, just beyond the background signal. This includes seeing a

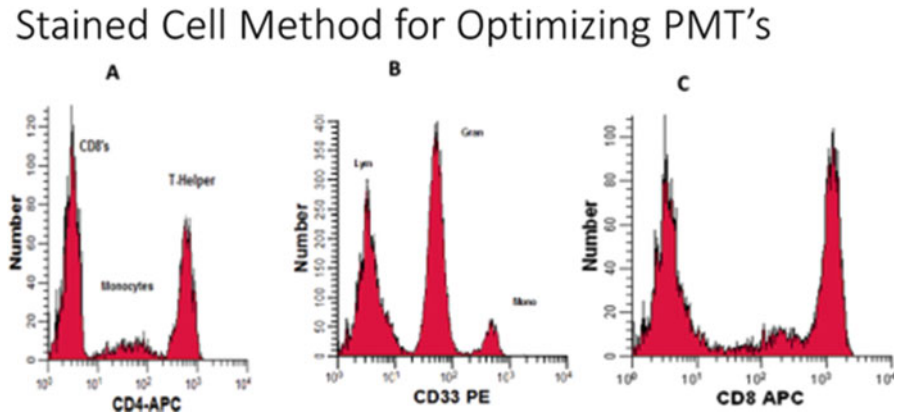

Fig. 14 The Stained cells method for optimizing PMTs. In Histogram **a**, WBCs gated on lymphocytes and monocytes stained with CD4, three separate populations can be clearly visualized: T-Helper cells stain bright, monocytes have an intermediate intensity, and the T-cytotoxic suppressor lymphocytes are negative. In Histogram **b**, CD33 is used to optimize the PMT. Ungated WBCs stained with CD33 show monocytes are bright, granulocytes intermediate, and lymphocytes are negative. In Histogram **c**, WBCs gated on lymphocytes express bright CD8, intermediate positive for NK cells, and are negative for T-helper lymphocytes

difference between discrete, intermediate populations versus bright or negative populations. The panel you choose to optimize with should include as many of the same single color-conjugates as you have available using the antibodies previously suggested or use others that will give you the three separate populations. CD45 is not a good choice for this method simply because in normal peripheral blood there is no negative population of intact WBC's. Defining the difference between negative and positive is an important aspect of the method. If an antibody is not found in every color used in the experiment, then use a combination panel that includes one or more of the other choices in the missing colors of the primary antibody conjugate. Of course, you can also use antibodies that you will be testing specimens with to see if they look okay; however, be sure that the results are on scale and discrete differences can be detected between negative and positive results. *See the Notes section for complete instruction of this procedure*: Single-stained Sample Optimization Procedure, which describes this method of optimizing the instrument.

12. Color compensation.

Once the instrument PMT settings are established the next step in Analytic Quality Control is to set up the color compensation. Compensation is the mathematical correction for spectral overlap between different fluorophores. If two or more color conjugates are used in an experiment, especially if they are excited by the same laser, there is a high probability that some of each signal will overlap into the adjacent signal. This overlap must be accounted for and adjusted to insure signal fidelity, each color independent of the adjacent or overlapping signal. Compensation correction is especially important in distinguishing weakly expressing antigens from negative populations since the bright signal of one fluorochrome might spill over and be detected as a dimly positive population of an adjacent fluorochrome. Even though band pass filters allow specific wavelengths of light to pass through to the detector, while blocking others, there may still be a signal from one fluorophore overlapping with the signal of another fluorophore. This can incorrectly make a single positive or even negative signal appear to now have a weak positive signal. *See* Fig. 15: PE versus PC7, uncompensated versus compensated.

For the sample that is positive for one antibody to not be incorrectly counted as positive for the other antibody a correction calculation, also known as *compensation*, must be applied to the signal spillover. Now, when the compensation is applied

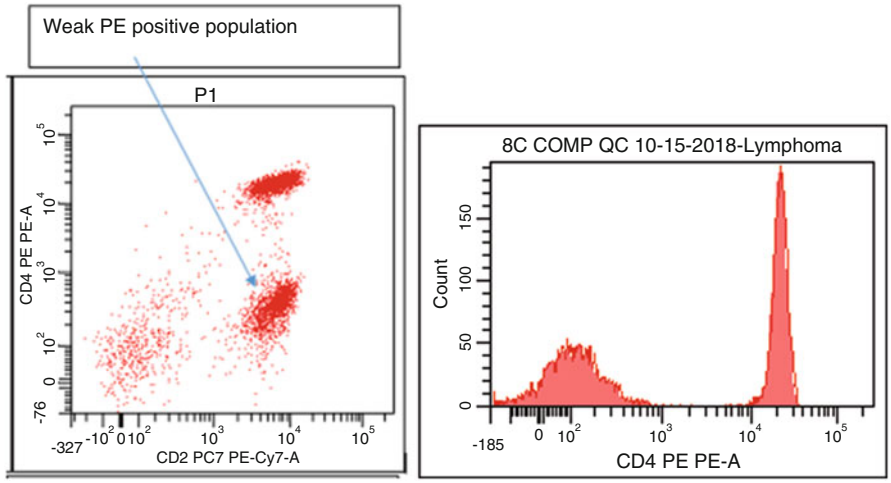

Fig. 15 PE versus PC7 undercompensated and showing a weak PE positive population. PE and PC7-stained cells appear to express both PC7 and two populations of PE + PC7 dual-positive populations incorrectly due to undercompensation of PE-PC7 in PE

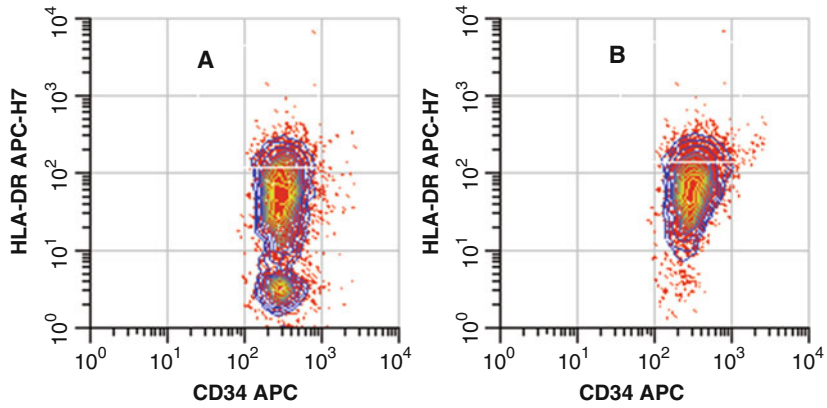

Fig. 16 Different compensation settings of APC-H7 versus APC. Histogram **a**: Overcompensation between APC-H7 and APC appears to show two separate populations and Histogram **b**: APC-H7 and APC, correctly compensated, shows a single population that is positive for both CD34 and HLA-DR

there should be no false-positive populations present. Conversely, if the compensation correction is too much it could potentially cause a weak dual-positive population to disappear and instead be only positive for one marker by being drawn into the axis of the adjacent conjugate. For example, *see* Fig. 16, CD 34 versus HLA-DR (*see* Fig. 16: Different compensation settings of APC-H7 versus APC).

This dot plot is from a clinical flow cytometry leukemia panel. The CD34 versus HLA-DR appears to have both a dual-positive and a CD34 exclusive population implying that there are two different blast populations; however, the signals are

actually overcompensated. Why does this matter? Because immature cells that are CD34 positive and HLA-DR negative may be mistaken for promyelocytes. Promyelocytic leukemia (PML) is a very dangerous disease that, if not identified early, can, in many cases, cause disseminated intravascular coagulation (DIC), an internal bleeding disorder, so identifying it is imperative. Correctly compensated, the population in question in this example is actually dual positive for both CD34 and HLA-DR and turns out to be AML not APL.

13. Setting up compensation on a flow cytometer.

In some cases, the same beads used for checking instrument operation and alignment might also include a setup for color compensation so that both procedures can be done simultaneously. There are advantages and disadvantages to this approach depending on the application. If the same antibodies and conjugates are used all the time, then this method may be preferred. However, if there are multiple panels with a wide variety of antibodies and conjugates in the experiment, separately running QC beads from compensation beads may be a better option to insure more specific compensation settings for each panel.

Begin by setting up and running a panel that includes both unstained and single-color stained beads or cells with one tube for each conjugate that will be used in the experiment [15]. After acquiring that panel compensation is then calculated by a compensation software program. A portion of the first signal is subtracted from the total of the overlapping signal and vice-versa for each of the colors where there is spillover. The portion of signal subtracted from the total signal is known as the compensation value and the total compensation calculated for the experiment is called a compensation matrix. *See* Fig. 17: Compensation matrix for an 8-color experiment

In early versions of flow cytometers that could detect 4 or fewer colors setting up the compensation could be done just by looking at one signal versus another and making the necessary adjustments to correct for any under- or overcompensation. More recent flow cytometers can detect upward of 10 or more colors; some research platforms can detect 20 or more colors. This means calculating the compensation now requires software programs to take into consideration every possible combination of fluorochromes. For instance, to set up compensation for a four-color experiment could require up to 12 calculated values versus the number for a 10-color experiment which could be up to 90 different calculated values.

(a) Label-specific compensation: Most compensation software programs allow the user to include both a generic as well as label- or lot-specific antibodies in experiments

Compensation Matrix for an 8-Color Experiment

Signal	into FITC	into PE	into PerCP-cy5.5	into PE-cy7	into APC	into APC-H7	into BV421	into BV 510
FITC	100.00%	14.50%	3.00%	0.20%	0.00%	0.02%	0.00%	3.12%
PE	1.00%	100.00%	22.00%	0.70%	0.02%	0.02%	0.02%	0.03%
PerCP-cy5.5	0.01%	0.00%	100.00%	12.00%	1.61%	7.03%	0.03%	0.05%
PE-cy7	1.00%	5.50%	7.50%	100.00%	0.03%	10.00%	0.03%	1.00%
APC	0.00%	0.11%	1.00%	0.09%	100.00%	8.50%	0.02%	0.03%
APC-H7	0.01%	0.02%	0.06%	0.53%	1.06%	100.00%	0.04%	0.07%
BV421	0.01%	0.02%	0.06%	0.00%	0.00%	0.02%	100.00%	16.50%
BV 510	0.17%	0.16%	0.11%	0.01%	0.00%	0.01%	10.00%	100.00%

Fig. 17 Compensation matrix for an eight-color experiment. Each fluorochrome in the experiment is shown with its respective compensation setting versus the other fluorochromes being used. The diagonal values are all 100% because a color cannot be compensated against itself. The higher the value the more overlap each signal has with the opposing signal. When designing an experiment, the use of color-conjugates with very high spectral overlap should be avoided for those markers that are used to find very discrete populations since they might be lost due to the high compensation correction value

where there is more than one color being detected in the same PMT in a multitube panel. For instance, PE-cy5 and PerCP cy5.5 are both acquired using the same PMT; however, the spillover of PE-cy5 into the adjacent PE channel will be different from the spillover of PerCP 5.5 into PE and will therefore need a separate compensation value. Another reason to consider using label-specific settings is when tandem dyes are used in the experiment. In some cases, the tandem dye will have a different background signal compared to the same antibody/ conjugate from a different lot number. This changes the signal–noise ratio along with the compensation needed to account for that difference. These settings are now lot-specific as opposed to label specific. Label- and lot-specific settings allow both antibodies to be used in the same experiment since the compensation matrix now includes calculations for both antibodies.

The compensation panel can be set up a few different ways. Some instrument software compensation programs use multicolor beads along with a nonlabeled (unstained) bead. Compensation software programs require a negative/ unstained population of some kind. This establishes the background signal from which everything beyond this signal is counted as positive. The unstaineded bead/ cells are first acquired using the PMT voltages previously determined during optimziation. Single colored/stained beads

are subsequently acquired, one for each conjugate/lot number in the experiment. After all the tubes are acquired the software then calculates and applies mathematical compensation to each signal until there is no spillover from another conjugate. The file format used for flow cytometry results is designated with ".fcs" as part of each file name.

All results acquired as *fcs* files version 3.0 and above are acquired uncompensated; however, the compensation calculation is included as a keyword in the file which can then be applied during analysis or even turned off. This gives the user the option of using the calculated compensation values that are included with the file or even adjusting it with the analytical software to a more suitable setting if desired. FCS versions earlier than 3.0 have fixed compensation values that cannot be adjusted post-acquisition.

Most instrument vendors supply compensation beads that are either surface coated with the colors of the conjugates being used or beads that are coated with anti-IgG, also known as "capture" or "comp" beads,". Capture or comp beads include both an unstained bead and another bead which is coated with a surface anti-mouse IgG-kappa that makes it possible to use any antibody/ conjugate to setup compensation in your experiment as long as it is an IgG subclass. The beads are setup and stained just like cells however they do not require a lysing step, just washing 1x after the primary incubation (*see* Fig. 18: Compensation setup using capture beads).

The advantage of using capture/comp beads is that unlike cells they are nonspecific and will react with almost any antibody. The disadvantage of using beads is that they do not always demonstrate the same intensity as a stained cell. If the comp bead signal is very bright or is at the end of the histogram scale turning down the PMT voltage to lower the signal is not an option since it changes the setting you worked hard to optimize. Another disadvantage of using comp beads is that they will not react with some panel reagents such as 7-AAD or Syto 6 which are dyes, not antibodies. Reactive cells on the other hand, may be difficult to come by especially for rare antigens. They should, however, more closely resemble the signal intensity of cells used in your experiment so a compensation setup using stained cells may be more accurate than using beads.

An additional consideration when setting up compensation using stained cells is deciding what sample types to use. The stained control samples need to be at least as bright as any that will be used in the experiment since it is

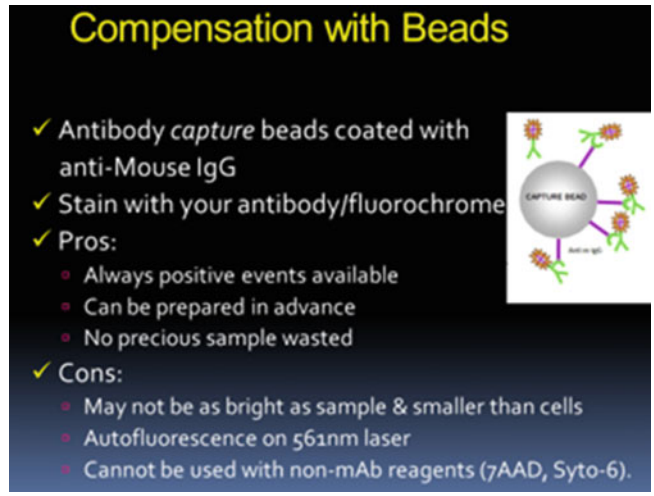

Fig. 18 Compensation setup using capture/ comp beads which are beads coated with anti-mouse IgG can be used to set up a compensation panel. The beads react with any antibody as long as it is IgG-class. The advantages are that they will work almost universally, be prepared in advance, and can be used with rare antibodies. However, the bead staining may show a different intensity compared to stained cells, and autofluorescence may be an issue with certain lasers. Beads cannot be used for nonantibody stains like 7-AAD or Syto-6

the brightest color signals that can spill over into the adjacent channels. One of the best sample types and antibodies to use in the compensation panel is CD8-stained lymphocytes. CD8 is a high-density antigen on lymphocytes and thus whatever conjugate is used CD8 will be as bright or even more bright than other common lymphocyte antigens. It is also negative on CD4+ T-lymphocytes so now there is both a positive and negative population present for calculating the compensation. If CD8 is not available in every conjugate of your panel, choose a different one that will have a positive signal intensity of at least the third decade on a log scale. Other antibodies to consider using in a stained cell compensation panel would be either/or both CD33 and CD14 gated on monocytes, and CD16 gated on granulocytes. CD33 gated on all WBCs in LWB should demonstrate a bright positive monocyte population, intermediate positive granulocytes, and be negative on lymphocytes. CD14 will be very bright on monocytes and negative on lymphocytes. CD16 will be very bright on neutrophils, moderately positive on NK lymphocytes, and negative on monocytes. Avoid using cell cultures in the comp panel unless your experiment uses these cell sources, due to the increased background or noise or autofluorescent signal they often have compared to normal lymphocytes.

In most cases the calculated compensation from the controls will be acceptable for most if not all the parameters in your experiment. An example Compensation setup procedure is found in the Notes section of this chapter.

14. Compensation controls: Before using the optimized and now compensated instrument settings to test actual samples from your experiment additional controls should be run using the optimized and now-compensated PMT settings to be sure that the compensation is correct. This requires running an additional panel for demonstrating and adjusting the compensation if necessary. The compensation controls setup procedure is found in the Notes Section of this chapter (Section 4.5).

3.3.3 Compensation Controls

Once compensation is set up it is important to test the settings using single-stained cells. Cells are preferred over beads since the samples in your experiment will in most cases be cell-based, and it is okay to use the same cells you used to create the compensation to check the new compensation settings. There are different approaches to how best to do this using stained samples [16, 17]. The next section describes a procedure that many flow labs use.

1. *F*luorescence-*M*inus-*O*ne (FMO) controls

   FMO controls are one of the most widely used ways for checking compensation settings. For this procedure stained cell samples are set up that include every conjugate found in the experiment *minus 1 color*. For instance, for a 10-color experiment using FMO-controls see the example below. "FL" stands for flourescence color; the number stands for the PMT:

   (a) Tube 1: (blank)/FL2/FL3/FL4/FL5/FL6/FL7/FL8/FL9/FL10.

   (b) Tube 2: FL1/(blank)/FL3/FL4/FL5/FL6/FL7/FL8/FL9/FL10.

   (c) Tube 3, 4, etc. until each color has been omitted once. Continue building the panel for all 10 tubes omitting a different color in each tube.

   (d) Run the FMO panel on the flow cytometer using the PMT and compensation settings previously determined.

   (e) After acquiring each tube in the FMO panel analyze and create two-parameter dot plots that include the offset or biexponential display of each negative/blank channel versus each of the other positive channels. *See* Fig. 19: Log versus offset/biexponential dot plots which shows the difference between a dot plot with and without an offset display.

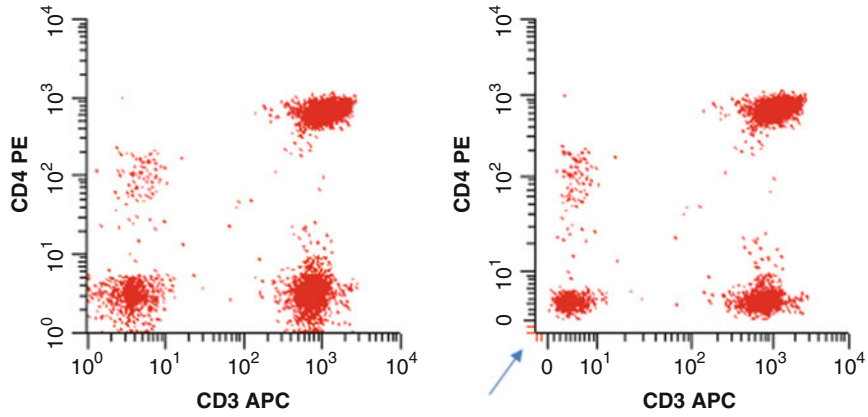

Fig. 19 Log versus offset/biexponential dot plots. The dot plot on the left shows the origin of each axis at the bottom. The dot plot on the right is the same dot plot with the axis offset/biexponential setting turned on allowing you to see events both above and below the origin (blue arrow) for demonstrating compensation

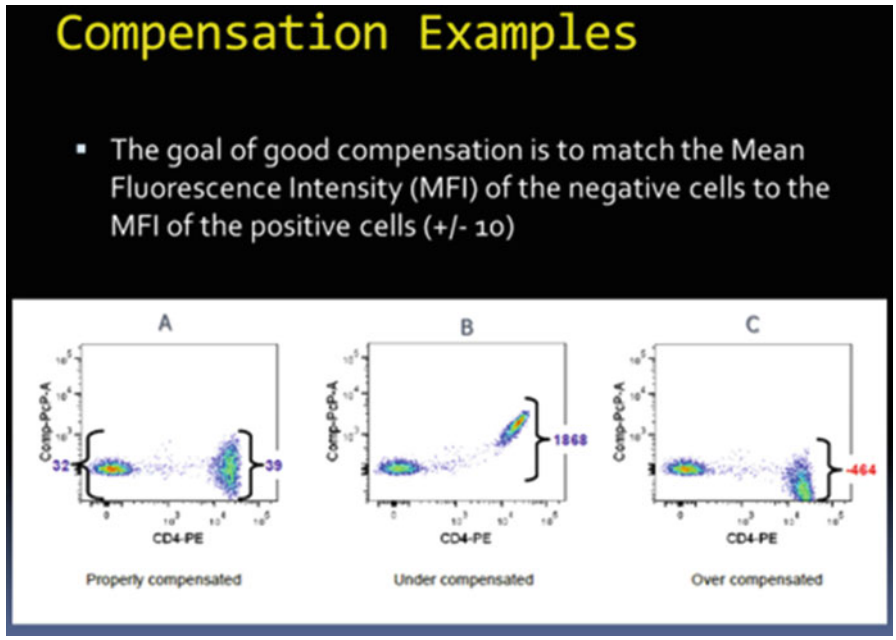

Fig. 20 Compensation examples using FMO controls. The positive CD4 result is shown versus an unlabeled detector as a way of demonstrating compensation between the two PMTs. The ideal compensation setting (dot plot **a**) shows the mean fluorescence intensity (MFI) of the positive peak to be very close to the MFI of the negative peak. Undercompensation in the middle figure (**b)** shows the MFI of the positive peak to be significantly greater than the negative peak MFI. The MFI of the right dot plot (**c**) shows the positive MFI to be significantly less than the negative peak MFI and thus is overcompensated

The next example (Fig. 20: Compensation examples using FMO controls) illustrates what (a) correct, (b) undercompensation, and (c) overcompensation look

like using three different settings. Adjust the compensation settings to get an even distribution of positive and negative spread on the baseline otherwise known as the "fishtail" pattern.

Once you have reviewed and made any final adjustments to the compensation the settings can now be used in your experiment.

2. Alternate method for checking compensation settings: *FMX controls*

The *FMO* panel is a good method for checking compensation settings but it has some drawbacks. It can be an expensive (up to 90 antibodies in a 10 tube FMO panel) and time-consuming approach to checking compensation. However, another similar but less expensive compensation control procedure is to set up the same panel of 10 tubes but instead of leaving out one conjugate in each tube, leave out *all but one* so that you have a positive result in one conjugate that can be compared to the all of the other negative parameters. Include an additional tube that has reactive antibodies for each parameter in your experiment. It will be used to make any final adjustments to the previously calculated compensation and should now demonstrate how well the compensation settings work. Again, run the FMX control tubes using the previously determined instrument settings, create offset dot plots of every possible combination and compare the positive MFI result in each file to the other unstained/ negative parameters. An example of the FMX-panel would look something like this:

(a) Tube 1: *CD8 FITC/*FL2 blank/FL3-blank, FL4-blank, FL5-blank, etc.

(b) Tube 2: FL1 blank/*CD8 PE/*FL3-blank, FL4-blank, FL5-blank, etc.

(c) Tube 3: FL1 blank/FL2 blank/*CD8 PerCP/*FL4-blank, FL5-blank, etc.

(d) Tubes 4 or more, until each color has been used once.

(e) Include a fully stained all-color control tube as the last tube in the panel. See the example below.

CD20 FITC/CD2PE/CD5 PerCPcy5.5/CD8 PE-cy7/ CD3 APC/CD7 or CD14∗ APC-H7/CD4 BV421/CD19 BV510.

(f) Acquire the FMX panel on the instrument using the previously calculated compensation settings. Analyze each file one at a time and look at each positive result versus the other fluorescence parameters used in the panel and be sure that the biexponential or offset setting is turned on. Now you should be able to recognize compensation

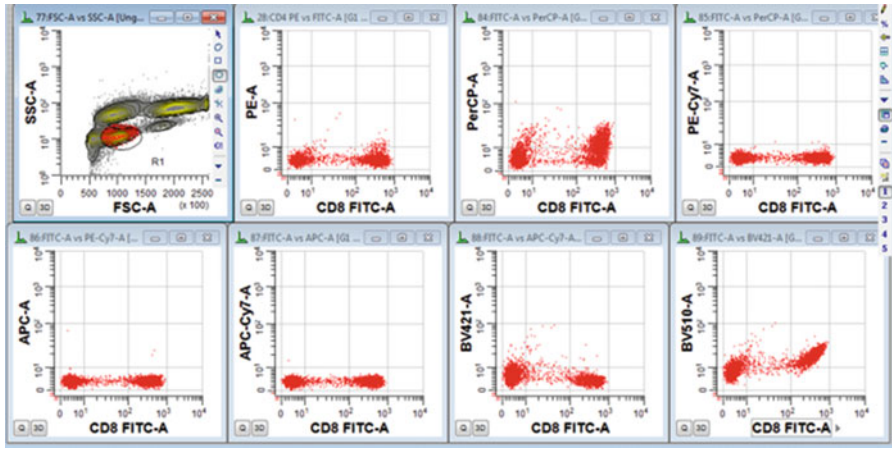

Fig. 21 FMX CD8 FITC control versus Each PMT. CD8 FITC versus all other colors. In this display, CD8 FITC versus each other color shows good compensation settings except for the CD8 FITC versus BV421 which is slightly overcompensated and the same versus BV510 which requires additional compensation

that is acceptable between two colors versus compensation settings that need adjustment (*see* Fig. 21: FMX CD8 FITC Control vs each PMT). This display demonstrates that the CD8 FITC versus PerCP and the same versus BV510 require additional compensation.

Follow this same example for each FMX control and change the *X*-axis to whatever parameter is positive versus the negative channel on the *Y*-axis for all subsequent tubes. Hint #1: If you set this up once and save the analysis that includes each possible color combination it can be used again for checking the compensation of a fully stained sample in your experiment. That compensation control is described in the next section.

3. Fully stained compensation control.

The last compensation control tube should, if possible, include all antibody conjugates used in the experiment. The eight-color cocktail used in the example includes several lymphocyte and monocyte antibodies found in normal peripheral blood. Some will coexpress and some will be exclusive. Use the results of this tube to do one last check of each combination to insure no under- or overcompensation exists. Hint #2: This cocktail can also be used to monitor compensation each day or periodically without setting up and staining multiple tubes. Keep a copy of the analysis worksheets showing all the fluorescent combinations for future reference so that if questions arise about the compensation in an experiment, analyze the results in question and use the previously saved dot-plot combination worksheet to quickly identify any compensation issues. By observing each conjugate versus the others, you should be

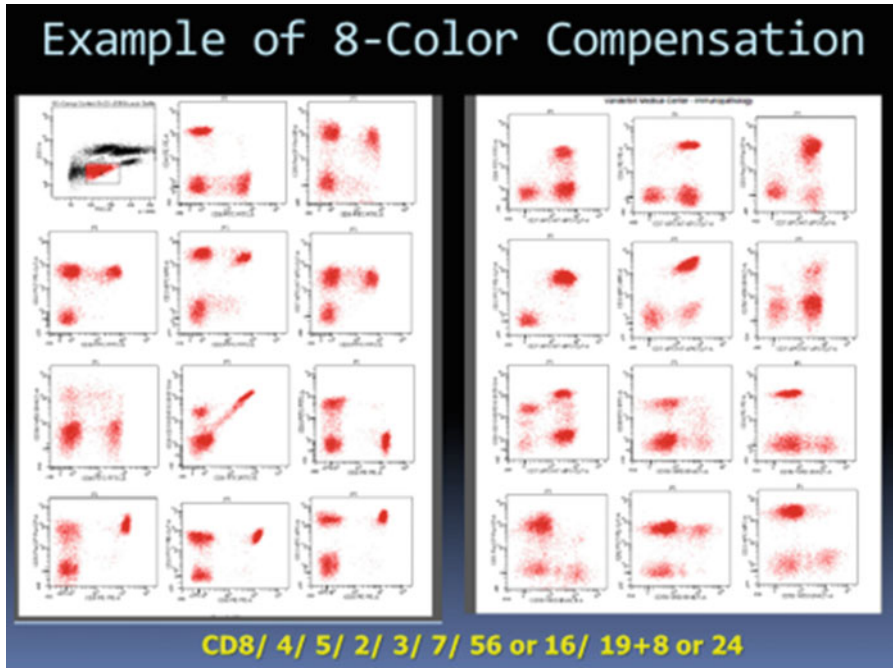

Fig. 22 Example of eight-color compensation. Multiple dot plots of every combination in an eight-color experiment using a cocktail that includes CD8/4/5/2/3/7/56 or 16/19 + 8. CD19 and CD8 are paired with the same conjugate to observe both coexpression and single staining. This example only shows two of the possible combinations, FITC versus X and PE versus X. Instrument compensation settings can now be monitored daily using a normal whole blood control with the multicolor cocktail

able to identify any compensation issues and make minor adjustments if necessary. *See* Fig. 22 Example of 8-Color compensation shows 2-parameter dot plots of multiple detectors in an experiment.

This example only shows half of the possible combinations, FITC versus X and PE versus X. Instrument compensation settings can now be monitored daily using a normal whole blood control stained with the multicolor compensation control cocktail.

4. Compensation settings summary

Multicolor flow cytometry experiments are only as good as the compensation settings used to correct for spectral overlap of all the color conjugates found in the panel. Once the compensation is set up initially, daily/ weekly monitoring is recommended to ensure that those settings are still valid. Minor adjustments can then be made to the compensation to correct for any drift. If on the other hand the PMT settings change by more than 10%, or there is major maintenance performed, the compensation setup should be repeated since either of those situations will likely change the spectral overlap of some or all

of the conjugates being used. An experiment that has been set up on a flow cytometer that is properly QCed, optimized, and color-compensated helps insure that results are accurate, precise, and dependable.

3.4 Miscellaneous Laboratory Equipment QC

Immunophenotyping laboratories have other instruments besides the flow cytometer that are required for setting up an assay. Examples include refrigerators and freezers, centrifuges, biological flow hoods, and manual or automatic pipettes to name a few. These devices must be maintained to insure proper operation and suitability for use. If the refrigerator or freezer that you store antibodies in has wide fluctuations in temperature over time it could damage the reagents. A centrifuge has rotors, safety latches, and timers that should be checked regularly to insure safe and proper operation. Biological flow hoods are safety devices that should have regular scheduled maintenance to ensure that potential hazardous material is contained. Most laboratories use private companies who specialize in maintaining this sort of equipment. Records of regular maintenance of miscellaneous equipment and daily monitoring of the temperatures is considered a good laboratory practice and is required by some laboratory regulation agencies. [29]

3.4.1 Analytic Quality Control Conclusion

Daily monitoring of the flow cytometer performance including alignment checks, background and sensitivity limits, fluidics, PMT performance, and laser output, is a standard of best practice in flow cytometry laboratories and may also be required for regulatory compliance. It helps insure consistency and reliability on a day-to-day basis. Tracking the results of QC over time may reveal trends that could predict future instrument problems. Understanding how each component of the instrument is performing: fluidics, optics, alignment, and electronics helps insure that results are reliable, accurate, and precise. Besides the flow cytometer, keeping maintenance and operation records of other miscellaneous equipment in the laboratory such as refrigerators, freezers, and centrifuges is also important for meeting quality assurance best practice standards [30].

3.5 Postanalytic Quality Control Procedures

3.5.1 Quantitative Assay Quality Controls

Once the instrument has successfully passed the analytic quality control procedures the final step of verification is to run sample controls. This includes both quantitative and qualitative controls [18]. For example, clinical laboratories that report quantitative results such as lymphocyte subset percent and absolute counts, or stem cell percent and absolute counts, are required to run at least two levels of commercial or verified controls each day the test is performed. The controls should include both a normal control as well as an abnormal control so that the full range of reportable results is represented. Quantitative commercial controls come with package insert values that must also be verified by the laboratory

prior to being put into use. Oftentimes these control values have a wide range of acceptable limits and therefore the laboratory should not only verify those limits but calculate ranges of their own based on multiple runs. Using the mean ±2 standard deviations after 10 times will usually be enough to generate statistical ranges that satisfy the requirement. The laboratory calculated control ranges should now reflect what the reports on both normal and abnormal results will be and should not be below the lower limit of detection (LLOD).

For those assays in which a commercial control is unavailable the use of a known positive sample verified by other means or by another laboratory that performs similar testing can be an acceptable substitute. Records of the controls and how the results were calculated are required for verification and acceptability for use.

3.5.2 Qualitative Assay Quality Controls

Qualitative controls, also known as method controls, test not only the instrument but the staining and processing procedures from setup to acquisition to analysis. Every antibody in an experiment should be tested for accuracy prior to being put into use whether as part of a cocktail or as a standalone reagent. This quality testing was mentioned previously in the preanalytic quality control section. Some commercial controls used for quantitative testing also include a list of additional antibodies that will react with the control for qualitative purposes. Method controls on the other hand are those controls used in an experiment that prove that the setup and staining procedures are working as indicated. As was also mentioned in the preanalytic QC section, material tested using alternate methods such as histology and hematology can be used as method controls. If proficiency testing material is available from a recent survey, it too can be used as a method control.

3.5.3 Isotype Controls

Isotype controls are basically antibodies of the same immunoglobulin class as the primary antibody including both the heavy and light chain molecules but lacking the specific component of that antibody to react with the target antigen or are directed against an irrelevant target. Isotype controls can serve as experiment controls and many labs use these to demonstrate the background staining or nonspecific staining present when using the test antibody. Setting a statistical gate on the positive population was based on where the negative signal ended for the isotype control. But there are some inherent limitations to using isotype controls for this purpose [19]. In some cases, the isotype control may not exactly match the target antibody and may react with an unexpected antigen, what is known as nonspecific binding. This may introduce uncertainty about what is positive versus what could be nonspecific staining. Another difference in using an isotype control is that it may have a different fluorescence to protein (F/P) ratio versus the target antibody. If the F/P ratios are different then the background

staining may be different and now the negative vs positive boundary becomes subjective.

There are situations, however, where the use of an isotype control can be helpful. Immunophenotyping panels designed to study intracellular antigens can be challenging due to the presence of more Fc- receptors inside the cell than on the cell surface which may result in more nonspecific binding of the antibodies and thus a higher background signal. An isotype control can be used to know what that nonspecific staining looks like to more accurately know where to gate the positive population. Another useful application of isotype controls is when a fluorochrome is being used for the first time and you want to see what typical background staining looks like. This provides a starting point for optimizing the signal as part of setting up the instrument.

Rare event detection is another situation in which the isotype control may serve as a useful reference for determining positive versus negative especially in those samples that may have weak expression or if a low density antigen is being investigated. However, a better strategy is to create a gating scheme that with successive structure, such as counting stem cells using the ISHAGE protocol [31], can preclude the need for an isotype control. As the population of interest is refined, the sequential gates used in the analysis may provide a more accurate assessment of determining positive versus negative results rather than running a separate isotype control.

3.5.4 Postanalytic Quality Controls Internal/Intraassay Controls

Internal/intraassay Controls are method controls that occur naturally in a sample and demonstrate what positive versus negative looks like for each same cell types being studied. For instance, lysed whole blood WBC's include lymphocyte, monocyte, and granulocyte populations. An antibody like CD33 reacts with monocytes and granulocytes but is negative on lymphocytes. CD19 is positive on B cells but is negative on T and NK cells as well as other myeloid populations present. In each of these examples you have one or more positive populations and one or more negative populations. In most bone marrow specimens other examples can be used within the tube to confirm different antibody reactivities, both positive and negative, using the same approach. *See* Fig. 23: Using intraassay controls.

An advantage of using internal/intraassay controls is that now the test antibody's reactivity can be described because the conditions, F/P ratio, and isotype are all in one place compared to a using a separate isotype control tube.

Each lab should establish their own criteria for describing the use of internal controls as a method and cocktail quality control check. Abnormal populations typically found in malignancy may aberrantly express antigens or be missing antigens that are expected

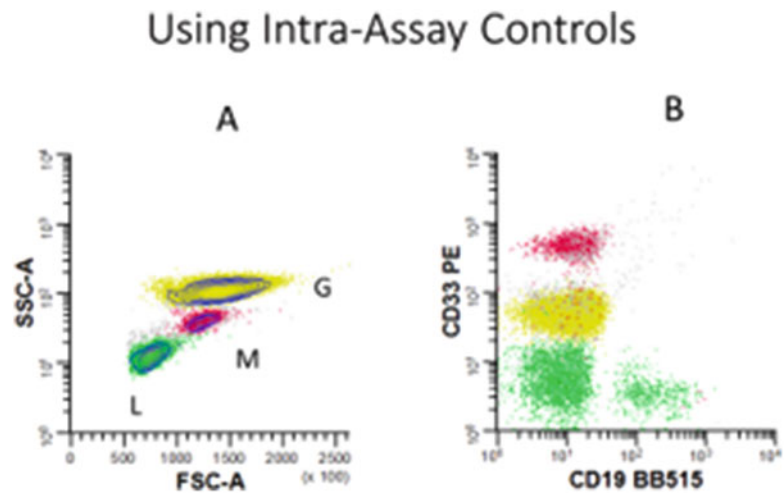

Fig. 23 Using intraassay controls. Antibody reactivity can be assessed using intraassay controls. Histogram A shows lymphocytes (L) in green, monocytes (M) in red, and granulocytes (G) in gold on forward/side light scatter. In Histogram B, lymphocytes can be used to assess antibody performance since in the example shown CD19 is positive for B cells and negative for T cells, monocytes, and granulocytes. CD33 is correctly positive on monocytes (bright) and granulocytes (weak) and is negative on lymphocytes

to be present on normal cells. In these cases, clear descriptions of normal reactivity should be included with each antibody combination used in the experiment as part of the procedure Interpretation of Results section.

3.5.5 Postanalytic Quality Control Methods Using Time as a Parameter

Flow cytometer list mode data is stored in a specific format that includes not only a record of every signal or event but also the time point at which that event occurred. This additional measurement is known as the Time parameter. The Time parameter can be a useful quality control indicator to alert you to problems with the sample acquisition, especially the fluidics [20]. For example, when an acquisition is either interrupted by something briefly blocking the fluidics or if the sample runs dry, these incidents can result in a loss of signal or the inclusion of bubble events in the list mode file results. Bubbles will look like collected events especially on light scatter. Looking at a histogram of time versus another parameter should normally show a steady sequence of events, but if there is an interruption during the acquisition or bubbles present, the histogram will demonstrate a noticeable change in the pattern or shift of a parameter. An interruption in the acquisition can be identified using the Time dot plot. Results before the interruption should look continuous; however, the results after an interruption may produce a false signal that could change the appearance of your data. The good news is that you can still salvage an otherwise faulty

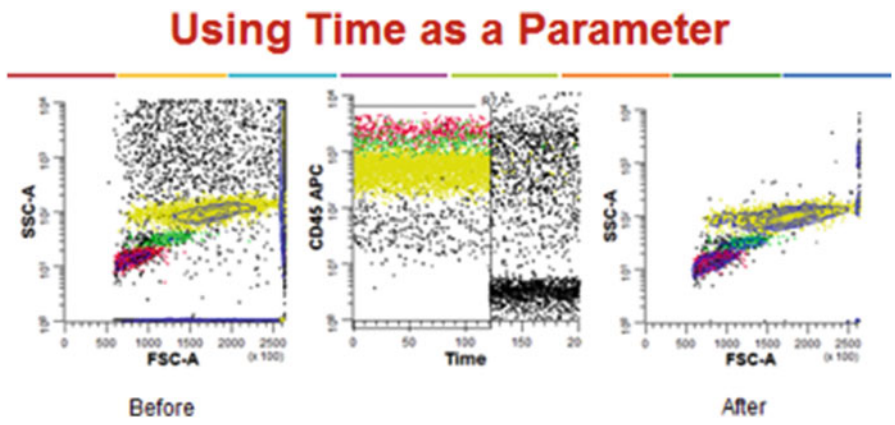

Fig. 24 Using Time as a gating parameter. The FSC/SSC histogram on the left shows what happens when a sample is run until empty. This introduces air bubbles, which appear as the multiple black dots in the upper area of the histogram. The middle histogram, Time versus CD45 shows a clear break where the bubbles started being acquired. A gate is then drawn around the "clean" events to omit the bubble events. The bubble-free-gated FSC/SSC histogram on the right no longer shows any bubble events

acquisition without having to set up or acquire it again by gating away from the problem. For example using the Time versus FL4 or other fluorescence parameter, draw a gate that now includes only the events before the interruption or when air was aspirated. Now the newly gated dot plots should be clear of the previously acquired interruption or air bubbles. Subsequent dot plots should now be based on the Time gate (*see* Fig. 24: Using time as a gating parameter).

3.5.6 Postanalytic Quality Control Methods Using Singlet Gates

Another issue that can impact the quality of your results is when the sample contains doublets or cellular aggregates. Doublets are when two or more cells for whatever reason are bound together as they pass through the flow cell chamber and are counted as one event. Using a singlet gate is an important consideration especially in experiments that measure DNA ploidy. If two cells with a 2N DNA content (resting, G1/G0 phase) are measured as one cell they could be mistaken as one cell having twice as much (proliferating, G2 + M) content. Samples with numerous doublets would then falsely skew the number of proliferating cells versus resting cells. Doublets can also be seen when specimens undergo several centrifugation steps such as preparing bulk lysed samples. Bulk lysing is often used in experiments that look for rare events, especially minimal residual disease (MRD) leukemia cases. To capture these rare events more specimen is required than that used under routine circumstances. In a bulk lysing procedure, using 1 ml of sample instead of 100 μl increases the number of cells tenfold. Now you have significantly more WBCs than before, and here is where the problem of doublet aggregate combinations is likely to occur

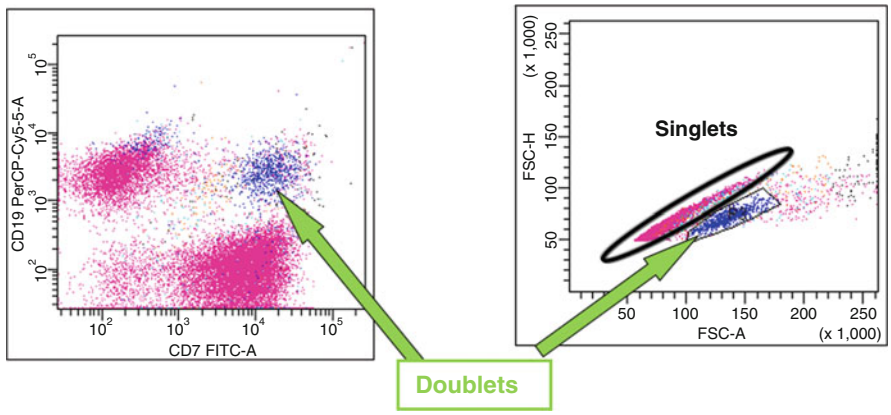

Fig. 25 Using a singlet gate to omit doublet events. In this example, the blue population before singlet gating appear to be positive for both CD19, a pan-B cell marker and CD7, a T cell marker. Normal B cells should be negative for CD7 and normal T cells should be negative for CD19. Using a Singlet gate that excludes doublets shows the red populations to be as expected for both CD7 and CD19. Singlet gating is a good practice regardless of the sample type since this phenomenon may happen quite frequently

especially if there are multiple centrifugation steps during the sample lysing and washing. If doublets are included in the analysis, the results may be misidentified as something abnormal such as a cell appears to coexpresses both a T cell antibody and a B cell antibody when in fact it is two separate cells that are stuck together also known as a doublet. To eliminate doublets from the analysis, draw a dot plot of forward scatter (FSC) *area* versus forward scatter *height*. Please note that some instruments measure width instead of height and if so, use that signal instead versus the FSC-Area. Doublets have a larger surface area than single cells and can be seen falling toward the FSC-Area axis. Draw a gate around the singlet population which is found along the diagonal axis of the dot plot to now exclude the doublet events. This should be the first gate used in the analysis so that subsequent events are only those found within the singlet gate (*see* Fig. 25: Using a singlet gate to omit doublet events).

3.5.7 Postanalytic Quality Control Methods Using a Viability Gate

Samples received for immunophenotyping if collected locally or within a day's distance will usually have high levels of viability especially if the recommended temperature and transport media are used to deliver the samples. However, in some circumstances even though all efforts are made to ensure that the sample is viable the sample by its very nature of collection may contain nonviable cells. Apheresis specimens submitted for stem cell counts is a prime example. The mechanical means by which the product is collected as well as the length of time to perform the apheresis procedure can result in some portion of the cells dying. Since CD34+ stem cell counts are typically less than 1% the need for accurate gating to

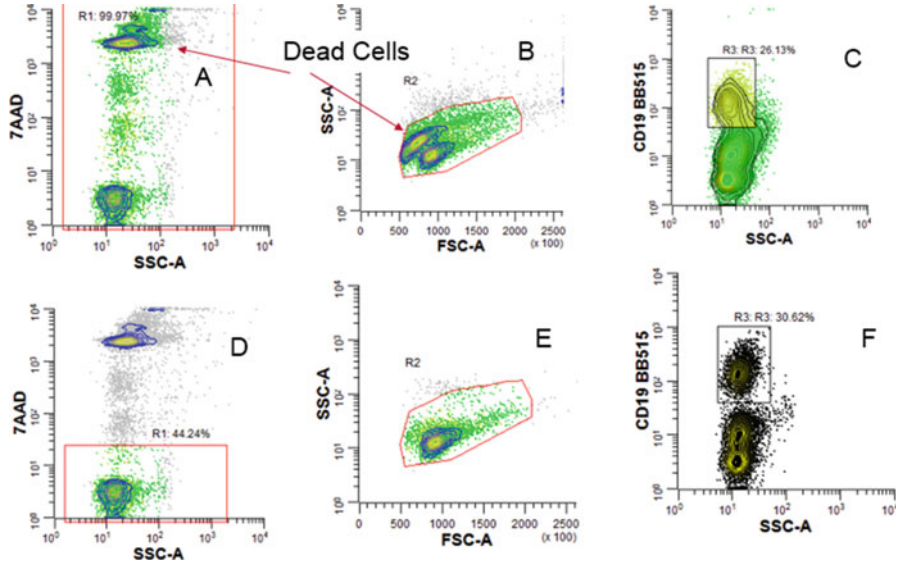

Fig. 26 Using 7-AAD to identify and gate out nonviable cells. Events in dot plot A have a high number of nonviable cells which take up the 7-AAD dye, demonstrated by the very bright expression of the 7-AAD on the Y-axis. The dead cells are seen in dot plot **B**, FSC/SSC as the secondary population on the SSC axis above the lymphocytes. Dot plot **C**, SSC/CD19, includes the dead cells which dilute the actual percentage of B cells present in the sample. Gating on just the cells that are 7-AAD negative in dot plot **D** cleans up the analysis by now omitting the dead cells. The SSC/CD19 dot plot F () shows only the viable cells with a more accurate CD19 result

omit the nonviable cells is crucial to the experiment. Other sample types with the potential for increased numbers of dead cells would include CSF and tissue biopsies especially if they lack adequate or appropriate transport media like RPMI. Using a viability dye such as 7-AAD in the immunophenotyping panel can be very beneficial for identifying the dead cells which take up the 7-AAD dye since the cell membrane of dead cells is usually permeable[26]. Including a viability gate in your gating scheme is highly recommended and even required if you perform clinical testing on samples over 24 h old (*see* Fig. 26: *Using 7-AAD to identify and gate out of the nonviable cells* for an example of how to remove dead cell events from the analysis).

3.6 External Quality Assessment (EQA)

Another regulatory requirement for clinical labs performing immunophenotyping is to participate in an external quality assessment program (EQA) also known as proficiency surveys. Laboratory regulatory agencies such as the College of American Pathologists require that a laboratory reporting clinical results of any assay participate in an EQA that is designed to test each aspect of the testing and reporting procedure. The laboratory's performance is then compared and graded against other laboratories performing the same testing. Proficiency surveys typically occur 2–3 times per

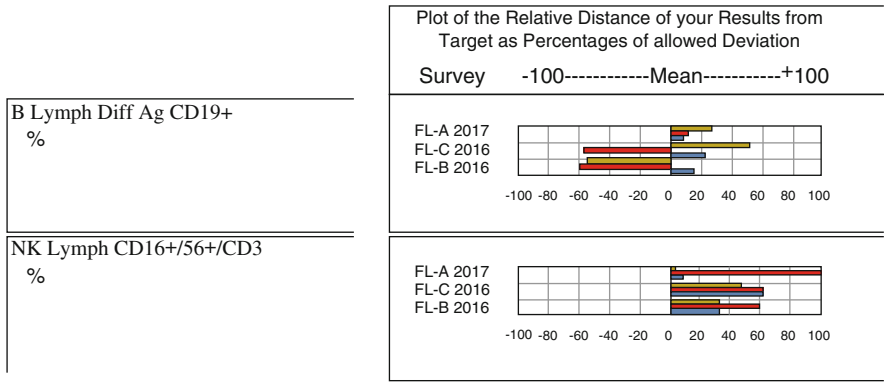

Fig. 27 Proficiency results over time. The CD19% over three different surveys shows results that fall both above and below the mean of the acceptable results. Conversely, the results of the NK Lymphocytes consistently run above the mean during the same period. This may be indicative of an analysis gating issue or antibody that might need to be further investigated for suitability

year and will usually include two or more samples with each mailing. Proficiency testing must follow the laboratory's written procedures for all the steps used to test and report patient samples. This not only provides information about the validated procedures but also measures the performance of the instruments used, acceptability of the reagents, and should over time even demonstrate potential issues that may not be as obvious on a day to day basis. For instance, if the returned results for CD19 in an immunodeficiency panel are acceptable but consistently trend higher than the mean of the other participants it may indicate either a reagent, gating issue, or instrument problem that might otherwise have gone unnoticed (*see* Fig. 27: EQA results over time).

3.7 Quality Control Records

All testing in Immunophenotyping requires that records of quality control performance be maintained and retained for a certain period of time[27]. There is a saying that goes, "If you did not document what happened then it did not happen." Quality assurance and quality control records are the proof of that purpose. Each step in the process of preanalytic, analytic, and postanalytic performance must be documented for acceptability. Best practices in quality control management include having an overall quality assurance plan along with the records of procedure and instrument validations, reagents used in testing, daily instrument performance and control results, assay records of specimen analysis that include the gating diagrams, and any proficiency testing results. For a summary of records that should be readily accessible and up to date *see* the Notes section of this chapter.

3.8 Conclusion

Quality control (QC) and quality assurance (QA) in immunophenotyping spans all three phases of laboratory testing: preanalytic, analytic, and postanalytic procedures. As part of an overall quality

program performing and documenting each result of these controls helps ensure the best outcome of an experiment. Good laboratory practice requires that every aspect of the assay should have measurable methods for monitoring the quality of the reagents, the instrument's performance, and the assay's expected results. Records of QC for each procedure should be well maintained and up to date and include the signature of the person or persons who oversee the testing.

4 Notes

4.1 Reagent Recipes Used in Immunophenotyping

1. PBS buffer, pH 7.2, with sodium azide. Some laboratories include bovine calf serum in their PBS buffer as an additional stabilization agent for the cells. This recipe does not include that additive.

   Caution: Sodium azide is an irritant and a poison. Avoid all direct skin contact, wear gloves, and wash hands thoroughly after handling.

   Materials:

   (a) PBS, 1×, Mediatech, Inc., Cat. # 21-040-CM.

   (b) Sodium azide, Fisher Scientific, #S227-500.

   Working solution:

   (a) Add 1 g sodium azide to 1 l of plain PBS.

   (b) Mix until dissolved.

   (c) Check that the pH is between 7.2 and 7.4.

   (d) Label and date (date made and date expired).

   (e) Store at 2–8 °C when not in use. Expiration = 3 months.

2. Ammonium chloride (NH_4Cl) RBC Lysing Agent:

   Materials:

   (a) Ammonium chloride, Fisher Scientific, #A661-500.

   (b) Potassium bicarbonate, Fisher Scientific, P184-500.

   (c) Disodium EDTA, Fisher Scientific, #S311-500.

   Working solution:

   (a) Place the following into a 1-l volumetric flask:

   - 8.26 g ammonium chloride.
   - 1.0 g potassium bicarbonate.
   - 0.037 g (37 mg) disodium EDTA.

   (b) Add deionized water to 1-l line on flask. Mix until all ingredients are dissolved.

   (c) Check pH: 7.2–7.4. Discard, if >7.4.

   (d) Dispense into 100 ml bottles. Fill bottles to near the top so that there is no air space*.

(e) Cap tightly. Put Parafilm around the cap to prevent air exposure.

(f) Label and Date (date made, and date expired). Expiration = 1 week. Store at 4 °C until ready to use.

∗NOTE: There should be as little air on top of the lysing solution as possible. Air will cause a decrease in the pH over time and lower the effectiveness of the lyse solution.

3. 7-AAD Viability Dye.
Materials:

(a) 7-Aminoactinomycin D, Molecular Probes, Cat.#A-1310, 1 mg.

(b) Dimethyl sulfoxide (DMSO), Fisher Scientific, Cat. #D128-500.

(c) 500 ml Flow PBS, stock solution.
7-AAD Stock Solution:

(a) Add 1 ml DMSO to 1.0 mg 7-AAD.

(b) Store at room temperature or in the dark for up to 1 month.
7-AAD Working Dilution

(a) Use 5 μl of 7-AAD stock solution/1–two million cells.

4.2 Four-Color T-Cell Subsets Cocktail for a 10- Test Aliquots Recipe

Antibody	Volume/test∗	Antibody total	Buffer volume
CD3 FITC:	10 μl/test	100 μl	650 μl
CD4 PE	10 μl/test	100 μl	
CD8 PerCPcy5.5	10 μl/test	100 μl	
CD45 APC	5 μl/test	50 μl	

∗Volume/test is based on titration results of initial antibody quality control

4.3 Instrument Quality Control Workflow

1. Prepare to use the instrument by turning it on and allowing sufficient time to warm up the lasers and electronics, usually 10–15 min.
2. Check or Refill Fluids for Operation: Sheath tank, cleaning solutions, etc. and empty waste containers; add bleach or other neutralizing hazardous waste solutions to a waste container.
3. Perform any scheduled maintenance following the manufacturer's guidelines: clean interior of flow cell using an approved medical device cleaning solution, change fluidics filters, once or twice a month perform an extended whole instrument clean ("long clean").

4. Open Operation Software and initiate instrument fluidics startup if applicable.
5. Prepare QC material: beads, stained cells, and controls.
6. Open the Quality Control software program and run beads or some other vendor-suggested material according to the Instrument Setup procedure to check the: alignment, sensitivity, precision, accuracy, electronics/PMT settings, laser power output, and fluidics performance.
7. (Optional step, *see* **step 12** for details) Optimize the instrument settings using stained cells to produce the highest signal–noise ratio for each PMT as well as the forward versus side light scatter to insure good population separation and resolution. Include this step whenever a new QC bead lot number is put into use or baseline performance is set up, or when there has been major maintenance such as a scheduled PM, laser or PMT replacement, or laser/flow cell alignment. If you add new parameters to your assay optimizing those detectors is suggested.
8. Perform compensation monitoring daily or weekly using either beads or stained control cells that includes all of the fluorochromes used in that day's experiments to assure proper compensation for running cell samples.
   (a) If three or more parameters need adjustment to the compensation settings, then the full Compensation Setup procedure (*see* **step 13**) should be performed using either single-stained beads or single-stained cells.
9. Run quantitative commercial controls if those assays are performed in your laboratory. Two levels including a normal and an abnormal are required for regulatory purposes [30]. Package insert ranges must be validated before putting into daily use.
10. Record QC results and comment on any out-of-range results. If three or more parameters are out of range or fail specifications or if a single parameter is out three consecutive times, proceed to troubleshooting.
11. If troubleshooting is required, document all steps taken to resolve the issue. Follow the instructions found in the troubleshooting section of the instrument operation procedure. If results are still out, further action such as contacting the instrument operational support, or the vendor service engineer may be required. Document any troubleshooting or service performed along with records of the instrument performance after any repairs are made or otherwise addressed.
12. Optional steps: Optimize the Instrument Settings.
    (a) Optimization is a procedure for adjusting the instrument settings to achieve maximum performance and is not required daily but should follow the initial installation

and/or any major maintenance such as a scheduled PM or repair. Optimize the instrument PMT settings using stained controls.

(b) If any instrument PMT settings are changed by more than 5 volts, compensation setup should also be performed and should follow the instrument optimization procedure as well as:

- After any major changes in PMT settings as part of bead QC.
- Whenever a new antibody panel is introduced.
- If there are color-conjugate changes or additions in an established panel. For example, changing CD45 FITC to CD45 APC or adding PE-cy7 to replace PC7.

13. Setting up compensation.

(a) The Compensation Setup procedure uses either single-stained beads or cells with a high-density antibody such as CD8, gated on lymphocytes that are acquired with the optimized PMT settings and no compensation applied.

(b) Run one tube/single color for each conjugate used in the experiment as well as a tube with unstained cells. Use label-specific tubes if more than one conjugate is used for a single detector. For example, a panel that includes FITC in one tube and Alexa 488 in a different tube would require separate compensation settings for each color used for the FL1 detector. Label-specific settings are also recommended for different lot numbers of tandem conjugates.

(c) After acquiring the panel of tubes, the compensation software program creates a matrix and calculates the amount of compensation to apply to each PMT signal to correct for any spectral overlap between fluorochromes to ensure signal fidelity.

(d) Compensation controls such as FMO (fluorescence minus one color) or FMX (fluorescence minus all but one color) should follow the compensation setup procedure to fine tune if necessary any overcompensation or undercompensation observed with the calculated values.

(e) The compensation settings are then saved with the PMT settings and used to run the experiment samples.

(f) After an initial setup, compensation must then be monitored and demonstrated to work daily using controls to ensure the settings are still valid for use.

14. For clinical laboratories that perform quantitative testing, at least two levels of controls with known acceptable ranges must be run every day the flow cytometer is used. Controls with

both normal and abnormal results demonstrate that the instrument's accuracy and precision limits are still valid.

15. For laboratories that perform qualitative testing, running known positive samples should be included daily to insure reliability of the antibodies and cocktails being used.
16. Document all QC results in the laboratory records and comment on any results that are out of range and any corrective action taken.
17. Perform scheduled maintenance on any ancillary lab equipment such as centrifuges, fume hoods, water baths, refrigerators and freezers, pipettes, or other miscellaneous laboratory equipment used for sample testing. Temperature-dependent devices should be monitored for meeting the acceptable temperature range as determined by the laboratory.
18. The laboratory supervisor or designee should review and sign off on all QC results monthly.
19. Keep records of all QC results, maintenance, troubleshooting, and repairs for at least 2 years.
20. Proceed with testing samples.

4.4 Instrument Optimization Procedure Using Single-Stained Cells

Set up single tubes and stain with one antibody-fluorochrome for each detector on the instrument and add an extra tube for an unstained sample. Follow your normal surface staining procedure. A typical 8-color Optimization Panel would include the following tubes:

Tube 1	Unstained sample
Tube 2	CD4 FITC
Tube 3	CD4 PE
Tube 4	CD4 PerCP cy5.5 or PE-cy5
Tube 5	CD4 PE-cy7 or PC7
Tube 6	CD4 APC
Tube 7	CD4 APC-cy7 or APC-H7
Tube 8	CD4 BV421 or Pacific blue™
Tube 9	CD4 BV510 or Krome™ Orange

1. Start with the PMT settings from the bead QC. Be sure to turn off the Compensation for this exercise. Compensation will be set up after the Optimization procedure.
2. Run the unstained tube-1 and adjust forward and side scatter light signals to display all three WBC populations clearly. Adjust each PMT setting so that the leading edge of the negative signal extends just beyond the background or noise, as performed in the previous method.

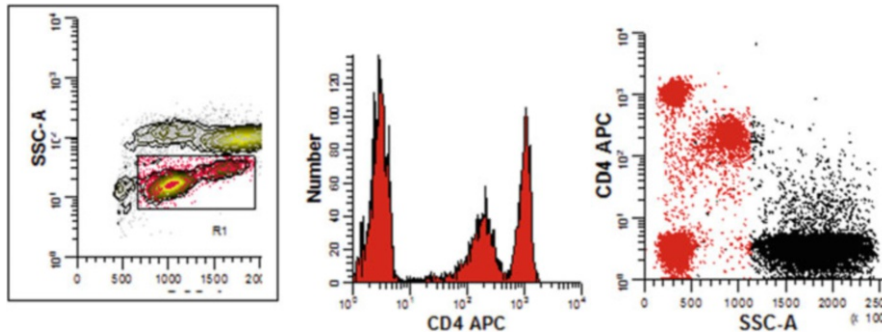

Fig. 28 Optimized Gating setup that demonstrates CD4 gated on Lymphocytes and Monocytes (R1). The bright population are CD4+ lymphocytes, the intermediate population are monocytes, the negative population are CD4- lymphocytes and granulocytes. Using this setup is a way to optimize PMT settings to clearly show all three populations.

3. After running the unstained sample run the first tube of the stained sample. If using CD4 draw a gate that includes both the lymphocytes and monocytes. *See* Fig. 28 : Optimized Gating setup that includes lymphocytes and monocytes.
4. Next, draw either a single-parameter histogram of the conjugate being acquired, that is, CD4 FITC = FL1, etc. or a two-parameter dot plot of SSC/FL1. Adjust the PMT voltages for each detector until the three separate populations can be clearly seen like that of the CD4 histogram and dot plot of the figure.
5. Continue acquiring each single-color tube adjusting the PMT voltages to achieve the best separation between the three populations until each PMT is optimized.
6. Save the now optimized PMT settings. Once the PMT settings are established the instrument is ready to set up the compensation.

4.5 Compensation Setup Procedure Using Cells or Beads

1. Set up Single Stained Controls:
   (a) If using the stained-cell method, select a whole blood specimen that has a normal WBC and a normal distribution of lymphocytes.
2. Antibodies and fluorochromes.
   (a) Use one for each fluorochrome (CD8 FITC, CD8 PE, etc.) in the experiment.
   (b) Use one for each tandem lot number.
   (c) Include an unstained tube that will have sample only.

   Below is an example of an eight-color compensation panel that uses CD8 with a different conjugate in each tube.

Tube 1	Unstained sample
Tube 2	FITC, Alexa 488, BB 515, or other green wavelength conjugate
Tube 3	PE
Tube 4	PerCP, PE-cy5, or other yellow/orange conjugate
Tube 5	PE-cy7, PC7, or other
Tube 6	APC
Tube 7	APC-cy7, APC-H7™, or other
Tube 8	Pacific blue, V450, BV421, or other
Tube 9	Krome Orange™, V500, BV512, or other

(d) Set up the panel using the same volume/titer of each antibody that is used in the experiment and follow the laboratory staining procedure incubation times, RBC-lyse time (cell method only), wash cycles, and resuspension media such as PBS buffer.

(e) Follow the procedure for whatever Compensation program your instrument is designed to use and afterwards save the updated calculated compensation settings.

(f) Continue to the Compensation Controls procedure found in Section 3.3.3.

4.6 Quality Control Documents and Records

1. The Immunophenotyping Laboratory should have a Quality Assurance Plan—a statement that explains all aspects of quality control including preanalytic, analytic, and postanalytic procedures, and results of those procedures.
2. Preanalytic Records.
   (a) Reagent Usage—a written procedure consistent with the manufacturer's instructions OR records of method accuracy evaluation if alternative procedures are used such as for a lab developed test. These records should be kept for at least 2 years.
   (b) Antibody Validation—Antibodies used are validated on the cell subpopulation of interest in the context of the antibody combination used in an assay.
   (c) New Reagent Lot/Confirmation testing for suitability.
   (d) Procedure Validation—written records of any procedure validation including signature approval. These records should be kept as long as the procedure and/or instrument stay in use.

3. Analytic Records.

   Instrument Maintenance—records of all maintenance procedures as well as any maintenance performed by a service engineer including scheduled maintenance.

   Instrument Performance including all daily QC results and signed monthly summary should be kept for at least 2 years.

4. Postanalytic Records.

   (a) Gated dot plots and histograms should be saved either electronically or as a hard copy. Clinical laboratories are required to keep these records for at least 10 years.

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Chapter 15

Immunophenotyping of Acute Myeloid Leukemia

Pallavi Kanwar Galera, Chunjie Jiang, and Raul Braylan

Abstract

Immunophenotyping by multiparameter flow cytometry is a rapid and efficient technique to simultaneously assess and correlate multiple individual cell properties like size and internal complexity along with antigen expression in a population of cells. This method is utilized for rapid characterization of the blasts and classification of acute myeloid leukemia (AML), in both the peripheral blood (PB) and bone marrow (BM). This technique is not only useful in the initial diagnosis but also in monitoring and determining prognosis of the disease through minimal residual disease (MRD) testing. This chapter provides an overview of procedures for specimen processing, staining, and immunophenotyping of AML and describes the principles of data analysis for AML classification and MRD testing.

Key words Immunophenotype, Acute myeloid leukemia, Myelodysplastic syndrome, Minimal residual disease, Flow cytometry

1 Introduction

AML is a clonal myeloid precursor neoplasm characterized by an increased number of blasts or blast equivalents in the BM and PB. Myeloid blasts include myeloblasts, monoblasts, erythroblasts, megakaryoblasts, and blast equivalents include promyelocytes and promonocytes. AML is currently being subdivided into six categories by the 2017 Revision of WHO classification [1], including AML with recurrent genetic abnormalities; AML with myelodysplasia-related changes (AML-MRC); therapy-related myeloid neoplasms; AML, not otherwise specified (AML, NOS); myeloid sarcoma and myeloid proliferations associated with Down syndrome. These categories are based on morphological, immunophenotypic, genetic, and clinical data with immunophenotype of the blasts being an important component. Historically, AML was defined by the presence of greater than 20% blasts or blast equivalents in a BM or PB specimen; however, this prerequisite is not

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required anymore for some of the genetically defined AML categories such as cases harboring the t(15;17), t(8;21), or inv(16).

Flow cytometric immunophenotyping can be utilized for rapid identification and characterization of the blasts and blasts equivalents, in both the PB and BM [2, 3] and, at the same time, it allows us to assess the maturation patterns of the various BM elements, further refining the classification of AML [4, 5]. With adequate population selection (gating) strategies and appropriate antibody panels, flow cytometry can also be very useful in the enumeration of blasts, especially in PB. However, while flow cytometry is an excellent means for detection and characterization of blasts, it may not be as accurate in quantitating blasts in BM specimens primarily due to uncontrolled hemodilution [6], leading to underestimation of blast numbers and making morphology/immunohistochemistry still the gold standard for blast counting in BM samples. Other confounding factors like loss of nucleated red blood cells (RBC) during lysis and processing, may artificially increase the blast percentage [7, 8]. These issues do not apply to blast counts in PB.

Flow cytometry is also of paramount importance in monitoring disease and prognosticating it through minimal residual disease (MRD) testing [9–11]. MRD is the presence of leukemic blasts at levels lower than the limit of detection by conventional morphologic evaluation. Multiple studies have demonstrated that detection of leukemic blasts following induction and consolidation therapy identifies patients who are at a higher risk of relapse and thus a worse outcome [12–15]. Due to its affordability, wide spread availability and quick turnaround time, flow cytometry is extensively used for MRD testing [16–18].

This chapter outlines the protocol used in our laboratory, providing an approach for diagnosing, and classifying AML using a 10-parameter (eight-color) measuring flow cytometer (FACSCanto II) with FACSDiva data acquisition software (both from Becton Dickinson (BD)). It also describes the various strategies utilized for data analysis using FCS Express software (De Novo). It should be emphasized that there are plethora of approaches and guidelines that can be utilized for these purposes [19, 20], and other laboratories may use different procedures to obtain equivalent results.

2 Materials

2.1 Reagents

1. Phosphate buffered saline (PBS) (KD Medical)/bovine serum albumin (BSA) (EMD Millipore Corp.) buffer: 500 mL 1× PBS and 0.5 g bovine serum albumin, fraction V (fatty acid

free). Mix well and filter through a 45 μm Millipore filter. Store at 4 °C for up to 1 month.

2. FACSLyse Buffer (BD): Prepare a 1:10 dilution of the FACSLyse buffer using deionized water. Store at room temperature in a glass bottle for up to 1 month.
3. Stabilizing Fixative (BD): Prepare a 1:3 dilution of the Stabilizing Fixative using deionized water. The solution can be kept for 1 month when stored in a polystyrene container at room temperature.
4. Erythrocyte Lysis Buffer (Qiagen).
5. Fix Perm Reagent A and B (Caltag).

Precautions

All PB and BM products should be considered biohazardous and treated with proper precautions as if they were capable of transmitting infection. The Stabilizing Fixative contains 3% paraformaldehyde. Appropriate disposal methods should be used.

2.2 Monoclonal Antibody Panels

Table 1 describes the antibodies utilized in our laboratory for detection, characterization, and classification of AML with appropriate corresponding fluorochromes (*see* **Note 1**).

2.3 Specimens

1. PB (anticoagulated in EDTA or Heparin).
2. BM Aspirate (anticoagulated in EDTA or Heparin).

Table 1
Antibodies and fluorochromes utilized in our laboratory*

FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC- Alexa 750	APC-H7	Pacific Blue	V500
CD16	CD10	CD5	CD19	CD10	CD34	CD14	HLA-DR	CD45
CD42b + CD61	CD13	CD11c	CD56	CD56	CD38		CD2	
CD36	CD19	CD117	CD34	CD11b	CD71		CD4	
cMPO#	CD64	CD34	CD15	CD7	CD3		CD19	
	CD5	CD33		CD123			cCD3#	
	cCD79a#	CD3		cTDT#				

Abbreviations: *APC* Allophycocyanin, *APC-Alexa 750* Allophycocyanin Alexa Fluor 750, *APC-H7* Allophycocyanin H7, *FITC* Fluorescein isothiocyanate, *MPO* Myeloperoxidase, *PB* Pacific blue, *PE* Phycoerythrin, *PE-Cy7 (PC7)* Phycoerythrin cyanine-7, *PerCP-Cy5.5* Peridinin chlorophyll protein cyanine 5.5, *TDT* Terminal deoxynucleotidyl transferase, *V500* BD Horizon dye V500

*Other laboratories may utilize different antibody and fluorochrome combinations

#c: Intracytoplasmic antibodies

3 Methods

3.1 Labeling with Monoclonal Antibodies Against Surface Antigens

It is also critical to appropriately titrate the amount of antibody to get an optimal antibody dilution for maximum signal-to-noise ratio [21, 22].

1. Label 12 × 75 mm disposable polystyrene tubes and add the antibodies.
2. Wash the PB or BM sample at least twice as follows: To wash the sample, in a 15 mL disposable conical tube add at least 2 mL of PBS/BSA to the sample and spin at 311 × *g* for 8 min. Aspirate the supernatant and repeat again by adding at least 2 mL of PBS/BSA, mixing, and spinning at 311 × *g* for 8 min. Aspirate the supernatant. Resuspend by adding PBS/BSA to the sample to make a final white cell concentration of 5000–10,000 cells/μL.
3. Add 100 μL of cell suspension (white cell concentration 5000–10,000 cells/μL) to each 12 × 75 mm disposable polystyrene tube with antibodies.
4. Incubate the tubes that contain the antibodies and the sample for 15 min to 1 h at room temperature in the dark (*see* **Note 2**).
5. Add 2 mL 1× FACSLyse Buffer to each tube to lyse the red blood cells. Vortex.
6. Incubate for 10 min at room temperature in the dark.
7. Spin at 216 × *g* for 5 min.
8. Decant supernatant. Mix.
9. Add 2 mL PBS/BSA to each tube.
10. Repeat **steps 7–9** (*see* **Note 3**).
11. Add 400 μL 1× Stabilizing Fixative to each tube (*see* **Note 4**).
12. The cells are now ready for analysis on the flow cytometer.

3.2 Labeling with Monoclonal Antibodies Against Combined Surface and Intracellular Antigens

This method allows the detection of cytoplasmic antigens, along with the assessment of expression of surface antigens. It utilizes a cross-linking fixative to stabilize cellular contents prior to labeling with antibodies against cytoplasmic antigens. The procedure outlined here utilizes Erythrocyte Lysis Buffer (Qiagen) and Fix Perm Reagent A and B (Caltag), but other products may be used. It is also critical to appropriately titrate the amount of antibody to get an optimal antibody dilution for maximum signal-to-noise ratio [21, 22].

1. Lyse RBCs from sample using Erythrocyte Lysis Buffer: To lyse the RBCs add 2 mL of the Erythrocyte Lysis Buffer to 200 μL of cell suspension in a 12 × 75 mm disposable polystyrene tube. Allow to lyse for approximately 10 min. Spin at 216 × *g* for 5 min. Decant supernatant.

2. Wash the sample: To wash the sample add 2 mL of PBS/BSA and then spin at 216 × *g* for 5 min. Decant supernatant.
3. Add appropriate antibodies to the 12 × 75 mm disposable polystyrene tube containing the cells for cell surface staining.
4. Incubate for 15 min.
5. Wash the sample: To wash the sample add 2 mL of PBS/BSA and then spin at 216 × *g* for 5 min. Decant supernatant.
6. Add 100 μL of Fix Perm Reagent A.
7. Incubate for 10 min.
8. Wash the sample: To wash the sample add 2 mL of PBS/BSA and then spin at 216 × *g* for 5 min. Decant supernatant.
9. Add 100 μL of Fix Perm Reagent B.
10. Add intracellular antibodies.
11. Incubate for 15 min.
12. Wash the sample: To wash the sample add 2 mL of PBS/BSA and then spin at 216 × *g* for 5 min. Decant supernatant.
13. Resuspend in 400 μL of Stabilizing Fixative.

3.3 Instrument Settings

A variety of instruments are available and different laboratories may use different instruments to perform flow cytometric analysis. Settings and calibrations are instrument-dependent, and manufacturer's guidelines should be employed. The number of cells acquired for analysis per tube may vary depending on the level of detection sensitivity desired. In our laboratory we acquire at least 100,000 cells/tube for routine analysis; however, higher numbers are necessary for MRD testing.

3.4 Data Analysis

3.4.1 Data Quality and Viability

The initial evaluation of results consists of an assessment of the quality of data and viability of the specimen. One strategy to evaluate quality and consistency of the acquired data is to observe data over time to identify air aspiration artifacts due to sample exhaustion and fluidic instabilities (*see* **Note 5**) (Fig. 1). The second step in ensuring the quality of data collected is to make certain that the data analyzed is an actual measure of a single cell at a time. Frequently, high cell concentration, high flow rates or nonspecific antibody bindings can induce cell aggregates and clump formations. To analyze exclusively single cells, a combination of forward light scatter area (FSC-A) and forward light scatter width (FSC-W) and/or height (FSC-H) is utilized to exclude cell doublets and aggregates from the data (Fig. 1).

Finally, to evaluate the viability of the specimen and to exclude debris and degenerated cells, viability dyes (propidium iodide, 7AAD) can be utilized. Alternatively, for practical purposes, nonviable cells may be excluded utilizing light scatter characteristics [19, 23, 24].

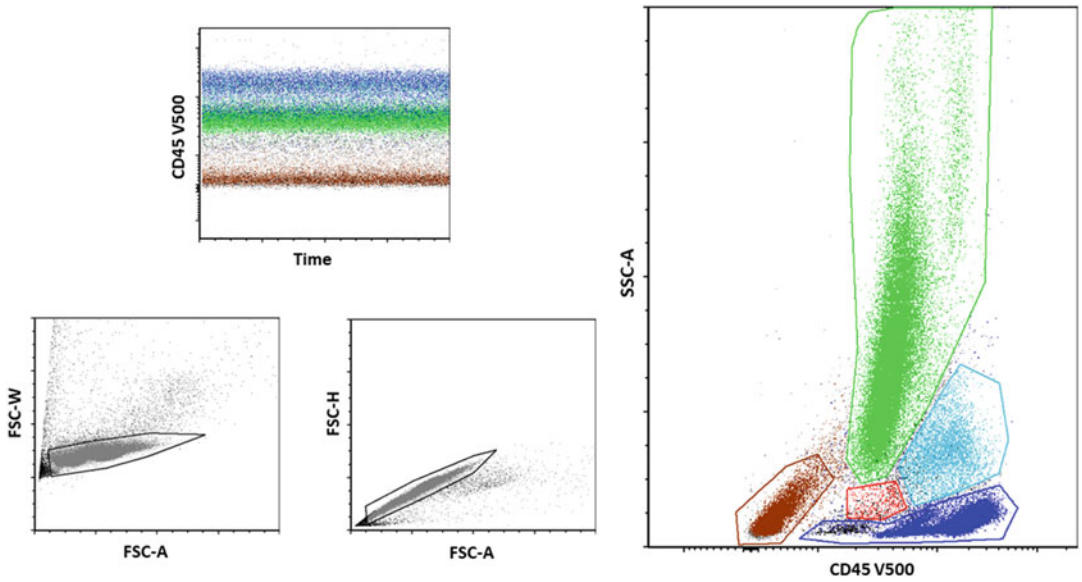

Fig. 1 Normal bone marrow. Data quality: Data is observed over time to identify and exclude fluidic instabilities such as air aspiration artifacts (upper left graph). A combination of forward light scatter area (FSC-A) and forward light scatter width (FSC-W) (lower left graph), and/or forward light scatter height (FSC-H) (lower middle graph) is utilized to exclude doublets and to ensure that the data analyzed is a measure of a single cell at a time. Initial gating (right graph): Based on the side light scatter (SSC-A) and CD45 expression the marrow populations can be broadly selected as red blood cells (brown), granulocytes (green), lymphocytes (blue), monocytes (aqua), and cells in the blast gate (red)

3.4.2 Initial Cell Population Gating

The initial gating, after excluding all elements that are not single viable cells, is performed to define broad categories of cell populations. This is done by examining side light scatter (SSC-A) and CD45 signals (*see* **Note 6**) [25]. Using this combination, the following populations can be broadly identified: erythroid (low SSC-A and negative CD45), granulocytes (moderate to high SSC-A and moderate CD45), lymphocytes (low SSC-A and bright CD45), monocytes (intermediate SSC-A between lymphocytes and granulocytes and bright CD45) and blasts (low SSC-A and dim CD45) (Fig. 1) [26]. Though an effective initial gate, the "blast gate" based on SSC-A and CD45 may be inaccurate [27] since there may be other populations that fall in this gate, namely basophils, hypogranular myeloid cells, plasmacytoid dendritic cells, and immature monocytes. Also, there would be instances where the blasts may have a brighter or dimmer CD45 and a varied SSC-A. Other populations may also, to a certain extent, be relatively ill-defined using this initial gating approach. Therefore, a panel of additional markers is required to further delineate the blasts as well as other distinct cell populations.

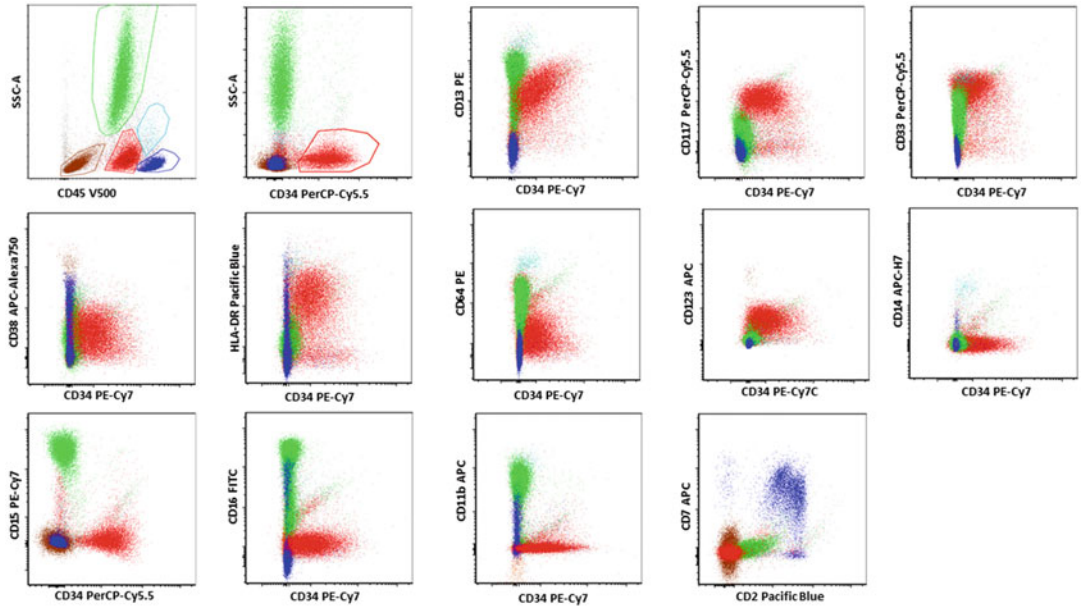

Fig. 2 Bone marrow with increased, phenotypically abnormal myeloblasts, comprising 23% of total cells. The blasts (red dots) express CD45, CD34, CD13, CD117, CD33 (bright), CD38 (dim/negative), HLA-DR, CD64 (dim/negative), and CD123 (dim). Blasts are negative for CD14, CD15, CD16, CD11b, CD7, and CD2

3.4.3 Strategies for Blast Identification

To assess the presence of myeloblasts, commonly used antigens include CD34 (Fig. 2) and CD117 since blasts usually express these markers. However, some myeloblasts may be negative for CD34 and/or CD117 and not even fall within the typical location in the CD45 and SSC-A graphs, making their recognition difficult. In these instances, other reagents and strategies are necessary for their detection. For example, blasts in acute promyelocytic leukemia are CD34 dim or negative and may mimic normal granulocytes but they usually have distinct phenotypic features [28]. Also, monoblasts usually express no or dim CD34, requiring the use of reagents for monocyte-associated markers for their recognition.

3.4.4 Strategies for Lineage Assignment

Myeloid lineage markers commonly used include CD13, CD15, CD33, MPO, and CD16 along with markers expressed during monocytic differentiation such as CD11b, CD64, CD14, and CD4 (Fig. 2). Erythroid precursors express CD71, CD105, CD117, Glycophorin A (CD 235a), and CD36, and megakaryocytic precursors express platelet-associated glycoproteins such as CD61 and CD42b. Assessment of lymphoid markers (CD3, CD2, CD4, CD5, CD7, CD19, CD56, CD22, and CD79a) should be included at the time of initial diagnosis, to assign lineage or to look for aberrant antigen expression. Non-lineage-specific markers (CD38, CD56, CD123, and HLA-DR) are also helpful to further delineate blasts and aberrancies.

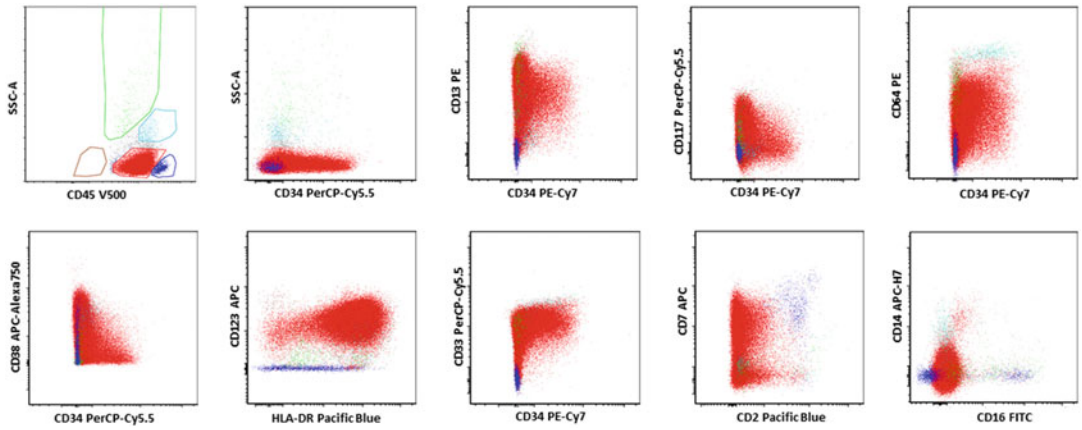

Fig. 3 Bone marrow involved by AML with *FLT3* mutation and phenotypically abnormal myeloblasts comprising 95% of total cells. The blasts (red dots) express CD45, CD34 (dim to negative), CD13, CD117 (variable), CD64 (variable), CD38 (partial), HLA-DR, CD123, CD33, CD7 (partial aberrant). Blasts are negative for CD16, CD14, and other T-cell and B-cell markers (not shown)

Immunophenotypic aberrancies include over or under expression of antigens, or absence of antigen expression (e.g., dim or absent CD38, CD13, or CD33 expression on myeloblasts), asynchronous antigen expression (expression of markers of mature elements in early precursors such as CD11b, CD64, or CD15 in myeloblasts), abnormally homogenous expression of certain markers (e.g., of CD38 or CD33) and expression of nonmyeloid antigens on myeloblasts (CD2, CD7, CD56, and CD19) (Figs. 3, 4, and 5) [7, 29].

3.4.5 Background Myelopoiesis

In acute myeloid leukemias, it may be useful to evaluate the background myelopoiesis to determine the presence of possible dysplastic changes. For this purpose, knowledge of normal maturation patterns utilizing various combinations of antigens is essential. Patterns generated by CD45 and SSC-A (SSC-A reflecting granularity), CD13 and CD16, CD11b and CD64, and CD11b and CD16 have been well studied for assessment of myeloid maturation and are reproducible [4, 5] (Fig. 5). Alterations of these patterns and other aberrancies like loss of CD10 on mature granulocytes, or expression of CD56 on myeloid and monocytic cells are helpful clues into probable underlying myelodysplasia [7, 19, 30].

3.4.6 AML with Recurrent Genetic Abnormalities, Special Considerations

Immunophenotyping can be helpful as an adjunct in classifying AML with recurrent genetic abnormalities; however, cytogenetic and molecular studies remain the mainstay in the diagnostic algorithm. Although the association between the immunophenotype and the recurrent genetic abnormality is not absolute, a few of the AML with recurrent genetic abnormalities show a strong correlation with the immunophenotype including AML with t(8;21)(q22;

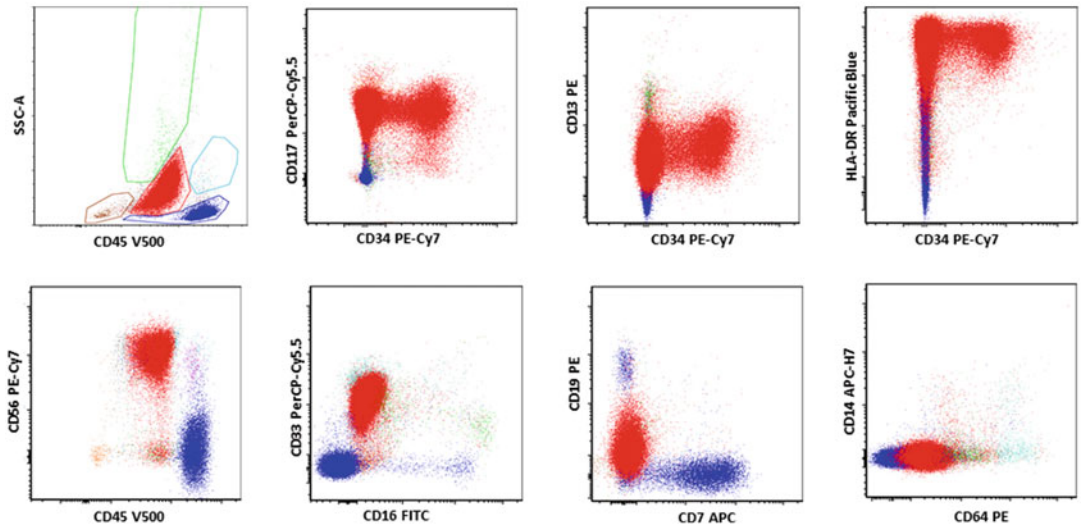

Fig. 4 A patient with history of autoimmune lymphoproliferative syndrome with *FAS* mutation. Bone marrow aspirate shows two populations of myeloblasts (red dots) based on CD34 expression with the minority of the blasts (approximately 25%) expressing CD34 and the majority of the blasts are negative for CD34. The blasts also express CD45, CD117, CD13, HLA-DR (bright), CD56 (aberrant), and CD33. A small subset with dim expression of CD19 is noted. The blasts are negative for CD7, CD14, and CD64. They were also negative for CD10, CD20, CD3, and CD5 (not shown)

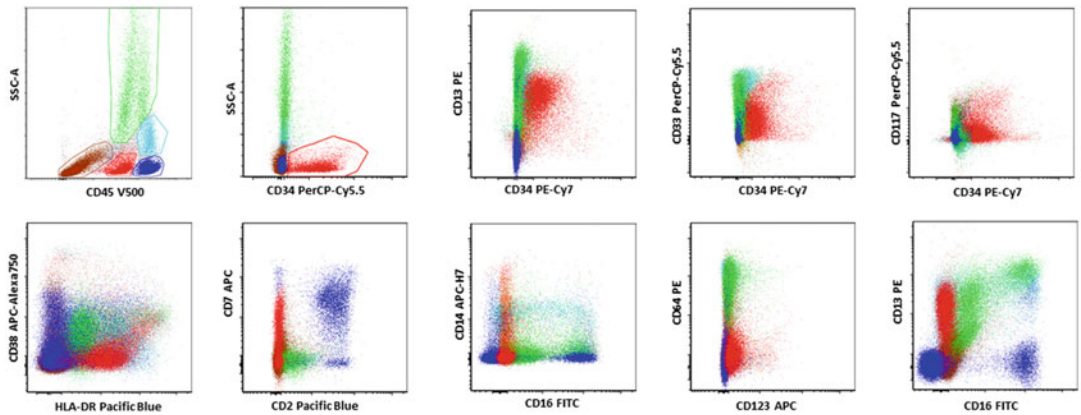

Fig. 5 A patient with history of BRCA1 and BRCA2 mutations and therapy for breast cancer 7 years prior to presentation. Bone marrow aspirate shows abnormal myeloblasts (red dots), comprising 13% of total cells. The blasts express CD45, CD34, CD13, CD33, CD117 (dim to negative), HLA-DR, CD7 (partial & aberrant) and are negative for CD38 (aberrant), CD2, CD16, CD14, CD64, and CD123. Granulocytes (green dots) reveal disrupted maturation pattern based on the expression of CD13 and CD16

q22.1), acute promyelocytic leukemia, t(15;17)(q22;q11-12), and AML with t(9;11)(p21.3;q23.3). Some salient immunophenotypic features of AML with recurrent genetic abnormalities are described below:

1. AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*: Blasts usually show high expression of CD34, HLA-DR, MPO, and CD13, and weak expression of CD33. Neutrophilic differentiation and maturation as assessed by expression of CD15 and/or CD65, maturation asynchrony (coexpression of CD34 and CD15) and frequent coexpression of B-cell markers (CD19, and cytoplasmic CD79a) [31, 32] are noted (*see* **Note** 7). CD56 expression is seen in a subset of cases, and this may portend an adverse prognosis [33, 34]. The adverse prognosis could be secondary to the fact that cases with *KIT* mutations have higher CD56 expression [35].
2. AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*: Blasts express CD34 and CD117 (KIT) intensely. Blast populations differentiating toward granulocytic lineage (positive for CD13, CD33, CD15, CD65, and MPO) and the monocytic lineage (positive for CD14, CD4, CD11b, CD11c, CD64, and CD36) are seen. Maturation asynchrony (coexpression of CD34 and CD15) and coexpression of CD2 with myeloid markers are frequently noted [1, 36].
3. Acute promyelocytic leukemia (APL) with t(15;17)(q22;q11-12); *PML-RARA:* Abnormal promyelocytes (blast equivalents) demonstrate relative high SSC-A, homogeneous bright expression of CD33, heterogeneous expression of CD13, CD117 and MPO, and low or absent expression of HLA-DR, CD34, CD11a, and CD11b. CD64 expression is common. In the microgranular variant there is frequent expression of CD34 and CD2 by at least some cells [37]. CD2 expression in APL has been associated with *FLT3*-ITD [38]. CD56 expression is seen in a subset of cases and has been associated with a worse outcome [39].
4. AML with t(9;11)(p21.3;q23.3); *KMT2A-MLLT3*: Most adult AML cases reveal monocytic differentiation (CD14, CD4, CD11b, CD11c, CD64, and CD36,) with a variable expression of markers of immaturity (CD34, CD117), and CD56 [40] (Fig. 6). In children, there is a high expression of CD33, CD65, CD4, and HLADR, with a lower expression of CD13, CD34, and CD14 [41].
5. AML with t(6;9)(p23;q34.1); *DEK-NUP214*: The blasts have a nonspecific myeloid immunophenotype, with expression of MPO, CD9, CD13, CD33, CD38, CD123, and HLA-DR. Most of the cases show an expression of CD34, CD117, and CD15. A subset of cases is TdT positive. Associated basophilia is often present and can be detected with basophils uniformly expressing CD123, CD33, and CD38 but no HLA-DR [42–45].

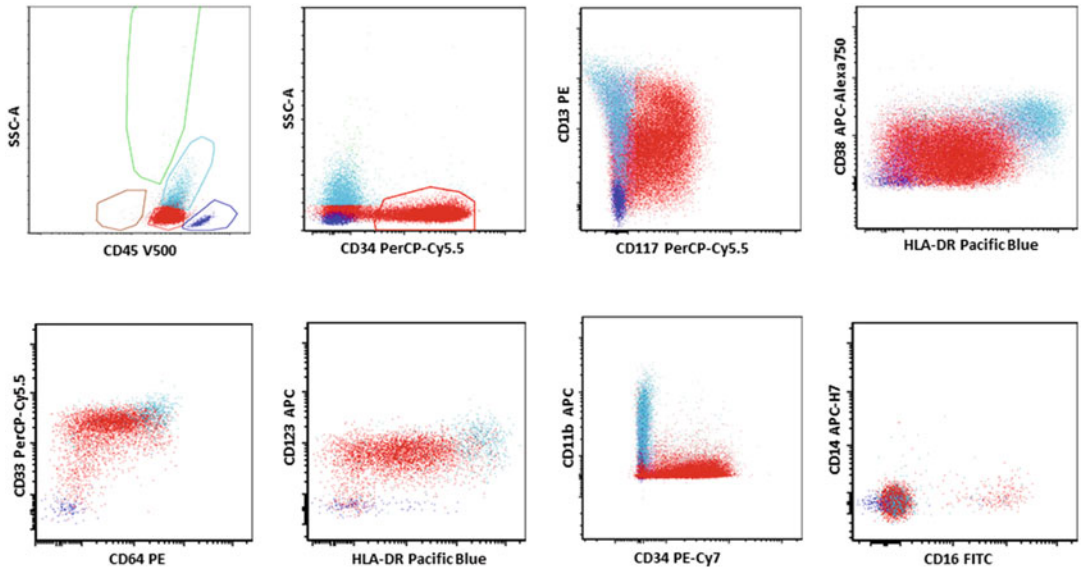

Fig. 6 AML with t(9;11)(p21.3;q23.3); *KMT2A-MLLT3*. Bone marrow aspirate reveals over 90% blasts, with two different immunophenotypically abnormal populations: The first population is of immature myeloblasts (red dots), representing approximately 81% of total cells and expressing CD45(dim), CD34 (partial, 72% of total cells), CD117, CD13, HLA-DR, CD33 (bright), CD123, and CD64 (dim to negative). These blasts are negative for CD14, CD16, and CD11b. They are also negative for CD15, CD61/CD42b, T-cell and B-cell markers (not shown). The second population is of monoblasts (aqua dots), representing approximately 10% of total cells with intermediate SSC-A and expressing CD45 (brighter than first population), CD13, HLA-DR (bright), CD38, CD33 (bright), CD64 (bright), CD11b (variable), CD36 (not shown). These are negative for CD34, CD117, CD16, and CD14

6. AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2, MECOM*: The blasts express CD34, CD33, CD13, CD117, HLA-DR, and CD38 (commonly), and a frequent aberrant CD7. A subset of cases may express megakaryocytic markers such as CD41 and CD61 [46, 47].
7. Acute megakaryoblastic leukemia with t(1;22)(p13.3;q13.1); *RBM15-MKL1*: The blasts express one or more of the platelet glycoproteins: CD41 (glycoprotein IIb/IIIa), CD61 (glycoprotein IIIa), and CD42b (glycoprotein Ib) (*see* **Note 8**) along with variable expression of myeloid markers CD13 and CD33. They also express CD36. CD34, CD45, and HLA-DR are often negative [48–50].
8. AML with mutated *NPM1*: The blasts show high expression of CD33 and variable (often low) CD13 expression along with CD117 and CD123 [51]. CD34 and HLA-DR are often negative (*see* **Note 9**). The blasts can either have an immature myeloid or a monocytic immunophenotype with expression of CD36, CD64, and CD14 [52]. A blast population with expression of CD34, CD25, CD123, and CD99 is associated with *FLT3*-ITD mutations [53].

9. AML with biallelic mutation of *CEBPA:* The blasts usually express HLA-DR and CD34. Expression of CD7 is also commonly seen. The cases with biallelic mutation are reported to have a higher frequency of HLA-DR, CD7, and CD15 expression and a lower frequency of CD56 expression in comparison to cases with a single mutation [54, 55]. Monocytic markers such as CD14 and CD64 are usually absent.
10. AML with mutated *RUNX1*: The blasts usually express CD13, CD34, and HLA-DR, with variable expression of CD33, monocytic markers, and MPO [56, 57].

3.4.7 Minimal Residual Disease

The detection limit of leukemic blasts by flow cytometry ranges from 0.1% to 0.01% of leukocytes. The detection limit is dependent on various factors, including the number of cells collected, antibody panel, flow cytometer, and distinct immunophenotypic properties of the leukemic blasts. For a detection sensitivity of 0.01%, at least 1,000,000 cells should be analyzed.

Two strategies are commonly employed in MRD testing for AML by flow cytometry. The first strategy is identifying a combination of aberrant markers and their expression levels on leukemic blasts that are usually absent in normal hematopoietic precursors. This strategy is widely known as "leukemia-associated immunophenotype" or LAIP. At diagnosis a broad, extensive antigen panel is utilized to define the LAIP. Subsequently after treatment, the selected antibody panel is used to identify blasts with pretreatment LAIP. The advantages of this approach are that the data analysis is relatively simple, and sensitivity for each LAIP (defined based on the background expression in nonleukemic specimens) is known. The disadvantages include the requirement to know the complete phenotype of the pretreatment leukemic sample, the need for customized antibody panels for subsequent evaluations after the evaluation of the initial diagnostic specimen and, finally, the immunophenotype of the leukemic blasts might change after treatment [3, 9, 11, 13, 20, 23].

The second strategy known as "difference from normal" identifies leukemic blasts based on immunophenotypic differences between the leukemic blasts and normal hematopoietic precursors. There is no requirement of defining or having prior knowledge of LAIPs at diagnosis (which is a hindrance in many tertiary or referral centers that do not have this information available); the same standardized panel can be used for all evaluations and since this method is based on differences between blasts and normal myeloid precursors, immunophenotypic shifts and change of LAIPs after treatment do not interfere with the assessment. However, extensive knowledge of antigen expression patterns of normal hematopoietic precursors and their maturation patterns is essential for an accurate evaluation [3, 9, 11, 20, 23]. In practice, a combination of the two strategies is often utilized.

4 Notes

1. A variety of antibodies with a variety of fluorochrome conjugates are available by various manufacturers. The antibody and fluorochrome selections and combinations may vary depending upon the instrument, number of detectors and the approach of the particular lab. Additional antibodies that are not frequently used in our laboratory such as CD99, CD25, and CD41 can also be added to aid in the diagnosis of AML [46–50, 53].
2. Prolonged fixation and exposure to fluorescent light may degrade fluorochromes. To minimize fluorochrome degradation, labeling of the specimen with antibodies prior to red cell lysis along with limited fixation and decreased light exposure is recommended.
3. Addition of the wash step reduces background fluorescence due to unbound antibody.
4. The use of a fixative helps in better preservation of the light scatter properties of the cells and provides a better separation between viable and nonviable cells.
5. In case of air bubble artifact secondary to aspiration of air due to sample exhaustion, the error can be corrected by simply excluding the data during those time periods.
6. The advantage of using SSC-A and CD45 for initial gating is that by just adding a single common antibody across all tubes the various populations can be compared with ease across all tubes.
7. AML with t(8;21) can be misclassified as mixed phenotype acute leukemia due to frequent expression of B-cell markers (CD19, and cytoplasmic CD79a) on the blasts. Presence of CD56 and CD19 positive myeloid blasts should raise a possibility of AML with t(8;21). Cases of acute monoblastic leukemia, especially with t(8;16), may show a higher expression of CD14 [58].
8. In AML (megakaryoblastic) with t(1;22) cytoplasmic expression of CD41 or CD61 is more specific and sensitive than surface staining.
9. Cases of AML with *NPM1* mutations are often negative for CD34 and HLA-DR; similar to blasts equivalents seen in APL; however, they have a lower SSC-A and fall in the blast gate. CD34 positive cases do occur and have been associated with an adverse prognosis [59, 60].

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Chapter 16

Immunophenotyping of Acute Lymphoblastic Leukemia

Joseph A. DiGiuseppe and Jolene L. Cardinali

Abstract

Immunophenotyping by flow cytometry is an important component in the diagnostic evaluation of patients with acute lymphoblastic leukemia. This technique further permits the detection of minimal residual disease after therapy, a robust prognostic factor that may guide individualized treatment. We describe here laboratory methods for both the initial characterization of lymphoblasts at diagnosis, and the detection of rare leukemic lymphoblasts after treatment. In addition to antibody combinations suitable for diagnosis and detection of minimal residual disease, we describe procedures for peripheral blood and bone marrow sample preparation, procedures for labeling of cell-surface and intracellular proteins with fluorochrome-conjugated antibodies, and approaches to analysis of immunophenotypic data.

Key words Immunophenotyping, Flow cytometry, Acute lymphoblastic leukemia, Minimal residual disease, Antibodies

1 Introduction

Acute lymphoblastic leukemia (ALL) comprises a group of malignant neoplasms of committed B-lineage or T-lineage precursor lymphoid cells termed lymphoblasts [1]. Diagnosis and classification of ALL require an integrated approach incorporating morphology, immunophenotype, cytogenetics, and molecular genetics [1, 2]. Immunophenotyping by flow cytometry is an important component in the diagnostic evaluation of patients with ALL [3], enabling its rapid distinction from morphologic mimics such as acute myeloid leukemia (AML) [4] and acute leukemia of ambiguous lineage [5], including mixed phenotype acute leukemia (MPAL). In addition, immunophenotyping by flow cytometry permits the detection and quantification of rare leukemic blasts that may persist after treatment of ALL (reviewed in [3, 6, 7]). The presence of such minimal residual disease (MRD) following therapy is among the strongest prognostic factors in ALL [8], and may potentially inform subsequent individualized therapies. Here we describe methods for immunophenotyping of ALL by flow

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cytometry. We describe antibody combinations suitable for diagnosis and detection of MRD, procedures for peripheral blood and bone marrow sample preparation, procedures for labeling of cell-surface and intracellular proteins with fluorochrome-conjugated antibodies, and approaches to analysis of immunophenotypic data.

2 Materials

2.1 Reagents

1. *PBS/azide (Phosphate-buffered saline with 0.1% sodium azide)*: Add 120.0 g NaCl, 3.0 g KCl, 17.25 g anhydrous Na_2HPO_4, 3.0 g KH_2PO_4, and 15.0 g of sodium azide to a 15 L container, and fill to 15 L with distilled water. Mix thoroughly, allow to sit overnight, and verify that pH is between 7.2 and 7.4. Store at room temperature for up to 3 months.
2. *0.5% PBS/BSA/azide (Phosphate-buffered saline with 0.5% Bovine serum albumin (w/v) and 0.1% sodium azide)*: Add 7.5 g bovine serum albumin (BSA) to 1500 mL PBS/azide (Reagent 1) in a 1500-mL bottle. Mix thoroughly until the BSA is completely dissolved. Store at 2–8 °C for up to 3 months.
3. *2.5% PBS/BSA/azide (Phosphate-buffered saline with 2.5% Bovine serum albumin (w/v) and 0.1% sodium azide)*: Add 5.0 g BSA to 200 mL PBS/azide (Reagent 1) in a 250-mL Erlenmeyer flask. Mix thoroughly and store at 2–8 °C for up to 3 months.
4. *5% PBS/BSA/azide (Phosphate-buffered saline with 5% Bovine serum albumin (w/v) and 0.1% sodium azide)*: Add 10.0 g BSA to 200 mL PBS/azide (Reagent 1) in a 250-mL Erlenmeyer flask. Mix thoroughly and store at 2–8 °C for up to 3 months.
5. *RPMI/FCS (Roswell Park Memorial Institute 1640 medium with 10% fetal calf serum (v/v))*: Incubate 50 mL of fetal calf serum at 37 °C for 30 min, and add to 450 mL of RPMI. Store at 2–8 °C for up to 1 month.
6. *2% Formaldehyde solution*: Add 200 mL of 10% methanol-free formaldehyde to a 1000-mL volumetric flask. Bring to volume with 0.5% PBS/azide, and adjust pH to 7.2–7.4. Store at 2–8 °C for up to 6 months.
7. *FACS™ Lysing Solution (BD Biosciences)*: Add 20 mL of 10× FACS™ Lysing Solution to a 200-mL volumetric flask. Bring to volume with distilled water. Store in a light-resistant bottle at 2–8 °C for up to 1 month.

2.2 Antibody Combinations

Antibody combinations suitable for the diagnosis and detection of minimal residual disease (MRD) in acute lymphoblastic leukemia (ALL) are presented in Table 1. These combinations were developed for use on flow cytometers equipped with three lasers (excitations at 405 nm, 488 nm, and 633 nm), and capable of detecting at

Table 1
Antibody combinations suitable for diagnosis and detection of minimal residual disease in acute lymphoblastic leukemia

Tube	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-AF750	PB, V450, or BV421	V500
1	Kappa	Lambda	CD20	CD19	CD10	CD38	CD5	CD45
2	CD20	CD22	CD34	CD19	CD13 + CD33	CD38	CD10	CD45
3	CD20	CD49f	CD34	CD19	CD58	CD38	CD10	CD45
4	CD24	CD304	CD34	CD19	CD86	CD38	CD10	CD45
5	CD16	CD56	CD5	CD3	CD7	CD8	CD4	CD45
6	CD7	CD1a	CD3	CD2	CD5	CD8	CD4	CD45
7	cyMPO	cyCD3	CD34	–	CD7	–	HLA-DR	CD45
8	cyMPO	cyCD22	cyCD79a	CD19	CD34	–	HLA-DR	CD45
9	nTdt	cyCD3	cyCD79a	CD19	CD34	–	HLA-DR	CD45

FITC fluorescein isothiocyanate, *PE* phycoerythrin, *PerCP-Cy5.5* peridinin-chlorophyll-Cy5.5, *APC* allophycocyanin, *APC-AF750* APC-Alexa Fluor 750, *PB* Pacific Blue, *V450* BD Horizon™ V450, *BV421* Brilliant Violet™ 421, *V500* BD Horizon™ V500

least eight different fluorochromes. Not all the antibody combinations in Table 1 are required in each case. Tubes 1, 2, and 7–9 would suffice to establish a diagnosis of B lymphoblastic leukemia, and tubes 5–9 would be sufficient for a diagnosis of T lymphoblastic leukemia; in both instances, the assessment of intracellular antigens (tubes 7–9) permits exclusion of MPAL [5]. For MRD assessment in B-lineage ALL (tubes 2–4) and T-lineage ALL (tubes 5, 6), more focused panels are appropriate.

3 Methods

Cell suspensions can be prepared from peripheral blood or bone marrow samples anticoagulated with either EDTA or heparin (*see* **Note 1**). Universal precautions should be observed in handling specimens.

3.1 Preparation of Cell Suspension from Peripheral Blood

1. Pipet an anticoagulated peripheral blood specimen into a 10-mL specimen tube labeled with two patient identifiers, and denote the meniscus with a permanent marker.
2. Dilute the peripheral blood with 0.5% PBS/BSA/azide to 10 mL.
3. Centrifuge at 800 RCF for 5 min.
4. Remove the supernatant, and reconstitute to 10 mL with 0.5% PBS/BSA/azide.

5. Centrifuge at 800 RCF for 5 min.
6. Repeat **steps 4** and **5**.
7. Resuspend in 5.0% PBS/BSA/azide to designated initial volume.
8. Adjust to a WBC concentration of 10,000/μL or less (*see* **Note 2**). For experiments in which cell-surface immunoglobulin light chains will not be labeled (*see* Tubes 2–9, Table 1), this cell suspension is used in Subheadings 3.3 and 3.4.
9. If cell-surface immunoglobulin light chains will be labeled (*see* Tube 1, Table 1), aliquot 1 mL of the cell suspension prepared above into an empty 10-mL specimen tube labeled with two patient identifiers, denote the meniscus with a permanent marker, dilute to 10 mL with FCS/RPMI, and incubate at 37 °C for 30 min (*see* **Note 3**). Centrifuge, wash twice, and resuspend the incubated specimen as in **steps 3–8**, prior to use in Subheadings 3.3 and 3.4.

3.2 Preparation of Cell Suspension from Bone Marrow

1. Filter anticoagulated bone marrow specimen through a 53-μM nylon mesh into a labeled specimen cup.
2. Gently dislodge cells from marrow particles collected on the mesh using the bottom of a polystyrene tube or bulb of a transfer pipette, and wash any freed cells through the mesh with 2.5% PBS/BSA/azide.
3. Transfer the filtered specimen to an appropriately labeled 10-mL specimen tube. Cap and mix well. Denote the meniscus with a permanent marker. Obtain a cell count of the suspension.
4. Centrifuge at 800 RCF for 10 min.
5. Remove the supernatant, resuspend in 0.5% PBS/BSA/azide, centrifuge at 800 RCF for 5 min, and decant the supernatant. Wash once more in this manner.
6. Resuspend in 5.0% PBS/BSA/azide to achieve a final WBC concentration of 10,000/μL. For experiments in which cell-surface immunoglobulin light chains will not be labeled (*see* Tubes 2–9, Table 1), this cell suspension is used in Subheadings 3.3 and 3.4.
7. If cell-surface immunoglobulin light chains will be labeled (*see* Tube 1, Table 1), aliquot 1 mL of the washed cell suspension prepared above into an empty 10-mL specimen tube labeled with two patient identifiers, denote the meniscus with a permanent marker, add 10 mL of RPMI/FCS, and incubate at 37 °C for 30 min (*see* **Note 3**).
8. Centrifuge, wash twice, and resuspend the incubated specimen as in **steps 3–8** of Subheading 3.1, prior to use in Subheadings 3.3 and 3.4.

3.3 Labeling Procedure for Cell-Surface Antigens

1. Label 12 × 75-mm polystyrene tubes with two patient identifiers and the antibody combination to be added.
2. Deliver 100 μL of the appropriate antibody combination (cocktail) to each tube (*see* **Note 4**).
3. Deliver 100 μL of the cell suspension (or preincubated cell suspension in the case of immunoglobulin light chain studies) as prepared in Subheadings 3.1 and 3.2 to each tube and vortex (*see* **Note 5**).
4. Incubate the tubes for 15 min at room temperature in the dark.
5. Pipet 2 mL of FACS™ Lysing Solution into each tube and vortex.
6. Incubate for 10 min at room temperature in the dark.
7. Centrifuge for 5 min at 800 RCF, and remove the supernatant.
8. Dilute to a volume of 2 mL with 0.5% PBS/BSA/azide and vortex.
9. Centrifuge for 5 min at 800 RCF and remove the supernatant.
10. Fix with 500 μL of 0.5% PBS/BSA/azide and 500 μL of 2% formaldehyde.
11. Acquire the labeled cells on the flow cytometer (*see* **Note 6**). Acquisition of 100,000 viable cells is sufficient for initial diagnosis; for MRD detection, acquisition of 1,000,000 cells is attempted (*see* **Note 7**). To mitigate carryover during acquisition for MRD detection, tubes containing distilled water should be positioned between sample tubes.

3.4 Combined Labeling Procedure for Cell-Surface and Intracellular Antigens

Abnormal patterns of cell-surface antigen expression (including expression of antigens that are normally absent, loss of antigens that are normally expressed, and expression of antigens or combinations of antigens at inappropriate developmental stages) are common among the acute leukemias, including B-lineage and T-lineage acute lymphoblastic leukemia. By comparison, cytoplasmic expression of certain antigens confers improved lineage specificity; incorporation of intracellular studies into the diagnostic panel is therefore useful. The following procedure, which permits simultaneous detection of cell-surface and intracellular antigens, should be used for antibody combinations 7–9 in Table 1 (*see* **Note 8**). We use the Fix & Perm cell fixation and cell permeabilization kit (ThermoFisher), but similar products are manufactured by others.

1. Label 3 12 × 75-mm polystyrene tubes with two patient identifiers, and add 100 μL of cocktail containing antibodies directed against the cell-surface antigens designated in Tubes 7–9 (Table 1).
2. Add 100 μL of cell suspension to each tube, and vortex.

3. Incubate for 15 min at room temperature in the dark.
4. Add 100 μL of Reagent A to each tube, and vortex.
5. Incubate for 15 min at room temperature in the dark.
6. Add 2 mL of 0.5% PBS/BSA/azide and centrifuge for 5 min at 800 RCF.
7. Decant the supernatant, add 100 μL of Reagent B to each tube, and vortex until the cell pellet is thoroughly resuspended.
8. Add the appropriate volumes of antibodies against the intracellular epitopes designated in Tubes 7–9 (Table 1), and vortex.
9. Incubate for 15 min at room temperature in the dark.
10. Add 2 mL of 0.5% PBS/BSA/azide and centrifuge for 5 min at 800 RCF.
11. Decant the supernatant, and add 500 μL of 0.5% PBS/BSA/azide and 500 μL of 2% formaldehyde.
12. Acquire the labeled cells on the flow cytometer (*see* **Note 6**). Acquisition of 100,000 viable cells is sufficient for initial diagnosis; for MRD detection, acquisition of 1,000,000 cells is attempted (*see* **Note 7**). To mitigate carryover during acquisition for MRD detection, tubes containing distilled water should be positioned between sample tubes.

3.5 Data Analysis

At both diagnosis and assessment of MRD after therapy, the goal of data analysis is the identification of normal and abnormal cell populations on the basis of their immunophenotypic and light-scatter properties. Data analysis is an iterative process, which requires a thorough understanding of normal and abnormal maturation patterns [6, 9], and familiarity with the technical artifacts that may produce spurious immunophenotypic patterns [10]. The magnitude of this challenge is underscored by a recent study of MRD detection in B lymphoblastic leukemia, in which analytic precision among a cohort of experienced practitioners using identical data sets was disappointing [11]. Although the development of algorithms capable of analyzing complex multiparametric data sets holds the promise of improving analytic precision [12], such approaches have yet to be widely adopted in routine practice.

3.5.1 Exclusion of Artifacts

Assessment of immunophenotypic patterns is facilitated by exclusion of artifacts due to fluidic perturbations during acquisition, and focusing the analysis on viable singlet cells. A display of all events acquired over time (Fig. 1) may reveal sample exhaustion or fluidic perturbations; if present, such compromised data may be excluded from further analysis. Coincident events may yield unexpected combinations of antigen expression, and thereby simulate abnormal populations. A display of peak area vs. peak height for a single parameter enables gating on singlet events, and exclusion of

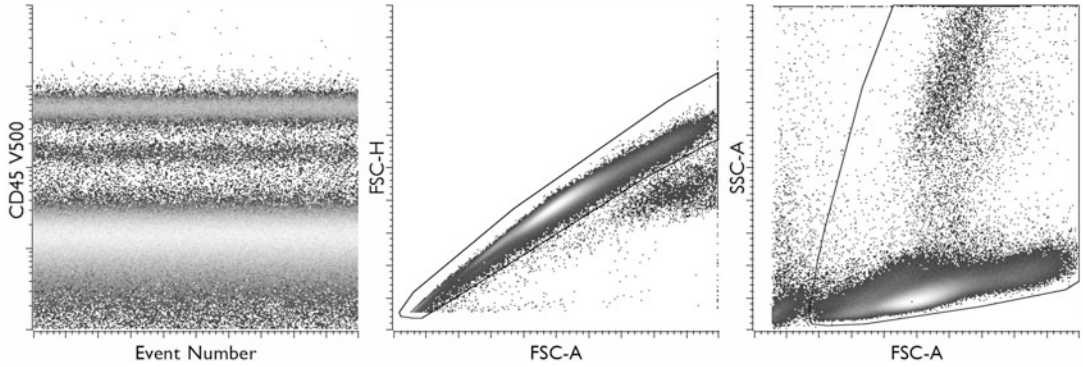

Fig. 1 Gating strategies to exclude overt data artifacts. (*Left*) Assessment of acquisition using event number to detect potential fluidic perturbations. (*Center*) Exclusion of coincident events (cellular doublets or higher-ordered aggregates) by gating on singlets defined by FSC-A and FSC-H. (*Right*) Exclusion of debris and nonviable cells by gating on cells with the FSC and SSC properties of viable cells

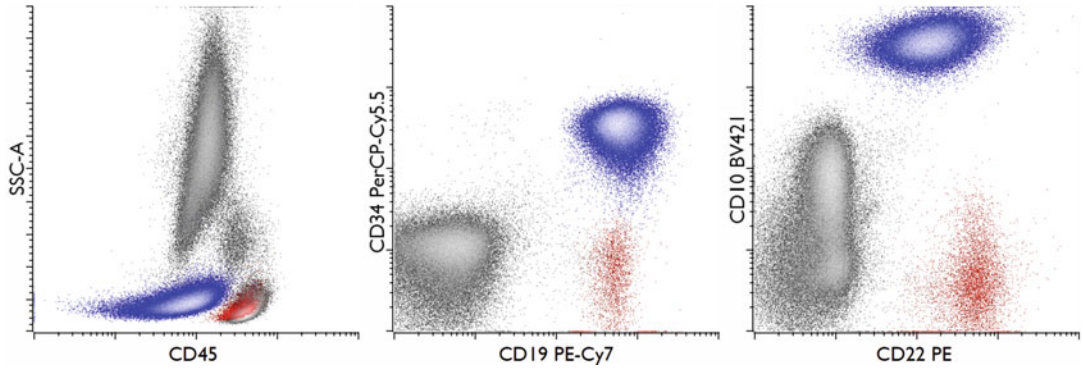

Fig. 2 Identification of blasts at diagnosis in B lymphoblastic leukemia (peripheral blood). The B-lymphoblasts (blue) have dim CD45 relative to mature B cells (red), and low SSC. In addition to B-lineage antigens (CD19, CD22), the blasts express CD10 and CD34 (All viable singlets are shown)

coincident events from subsequent analysis (Fig. 1). Finally, since degenerating or nonviable cells may be bound nonspecifically by fluorochrome-conjugated antibodies, the analysis may be facilitated by gating on cells with the light-scatter properties of viable cells (Fig. 1; *see* **Note 9**).

3.5.2 Identification of the Abnormal Population: Diagnosis

The next step in data analysis is identification of the abnormal population. A display of CD45 vs. SSC is very useful in this regard, as lymphoblasts commonly occupy a region defined by dim CD45 and low SSC (Figs. 2 and 3). In the case of B lymphoblastic leukemia, CD45 expression may be virtually undetectable (Fig. 1), while in the case of T lymphoblastic leukemia, overlap of CD45 expression between the blasts and mature T cells is more common (Fig. 2). Bivariate plots of antigen vs. light scatter, or antigen vs. antigen are then used to refine phenotypic

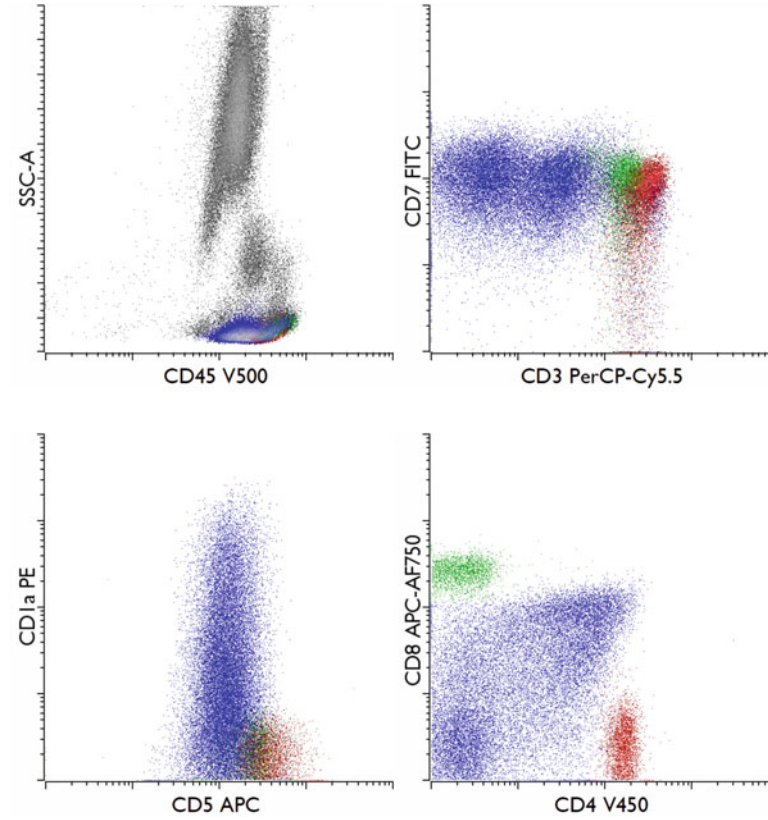

Fig. 3 Identification of blasts at diagnosis in T lymphoblastic leukemia (peripheral blood). The T-lymphoblasts (blue) express CD45 at levels slightly dimmer than (but partially overlapping with) those of the normal CD4+ (red) and CD8+ (green) T cells. The blasts are phenotypically heterogeneous, with absent or dim surface CD3, and partial, variable CD1a. Subsets of the blasts are CD4–/CD8–, CD4–/CD8(dim)+, CD4(dim)+/CD8–, or CD4+/CD8+ (All viable singlets are shown in the upper left panel; only T cells are shown in the remaining panels)

characterization of the blast population (Figs. 2 and 3). Identification of the abnormal population is usually straightforward at the time of diagnosis, since blasts typically comprise a substantial fraction of all cells.

3.5.3 Identification of the Abnormal Population: MRD

By comparison, identification of a rare persistent blast population after treatment may be challenging. In many cases, knowledge of the patient's diagnostic immunophenotype may facilitate subsequent MRD detection. However, the patient's diagnostic studies may not be available at the time of MRD assessment. Moreover, alterations in phenotype during therapy are well described in both B-lineage [13] and T-lineage ALL [14]. In contrast, identification of populations whose composite immunophenotypes differ from those of normal cells is not subject to these

limitations. An important caveat in the identification of MRD using either approach is the recognition of normal precursor lymphoid cells. In the case of MRD detection in B lymphoblastic leukemia, in particular, the presence of normal B-cell precursors [15] in bone marrow may complicate the analysis [11].

In analyzing the data for potential abnormal populations after therapy, it is helpful to draw an initial gate using CD19 vs. SSC for B lymphoblastic leukemia (Fig. 4; *see* **Note 10**) or CD7 vs. SSC for T lymphoblastic leukemia, as these antigens are expressed in virtually all cases. This initial gate is then refined in an iterative fashion using various combinations of antigen expression to isolate a cluster of events whose multiparametric phenotypic properties differ from those of normal precursor cells (Figs. 4 and 5). In the illustrated example of MRD detected in a patient treated for B lymphoblastic leukemia (Fig. 4), the initial CD19+ gate includes a preponderance of normal B-cell precursors (blue), with dim CD45, little or no CD20, and substantial CD34, all of which are immunophenotypic properties shared by the patient's MRD (red, highlighted).

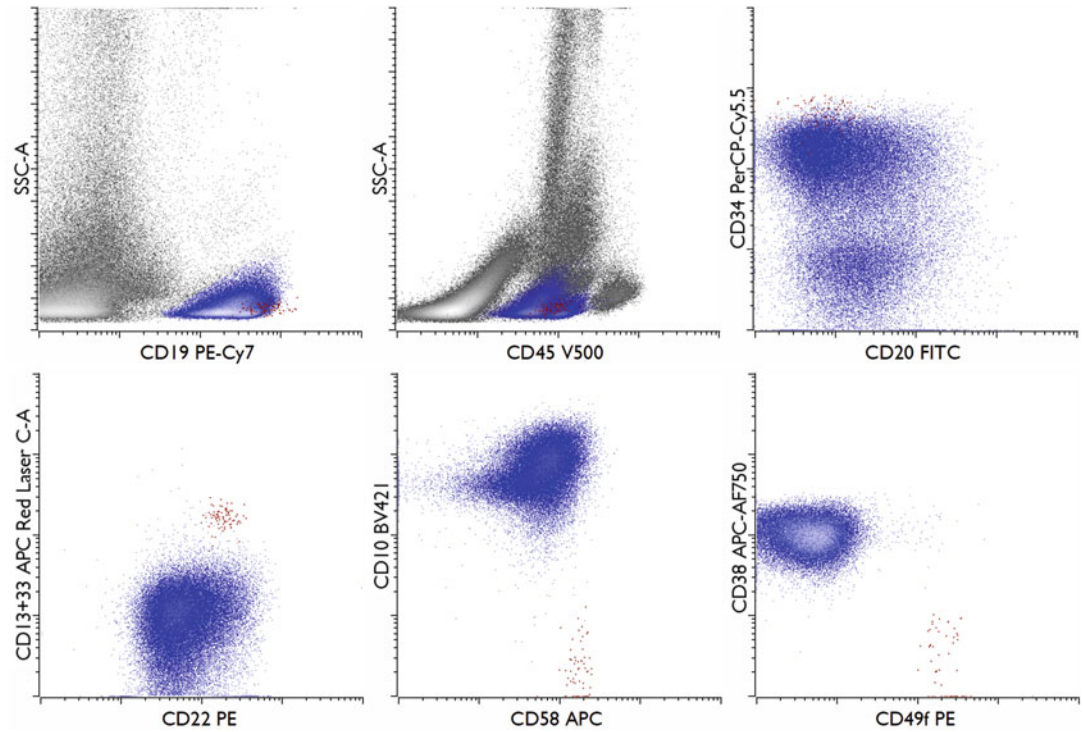

Fig. 4 Identification of MRD in B lymphoblastic leukemia (bone marrow). The initial CD19+ gate includes a preponderance of normal B-cell precursors (blue), with dim CD45, little or no CD20, and substantial CD34; in contrast, the MRD (red, highlighted) differs from the normal B-cell precursors in its expression one or more myeloid antigens (CD13 + CD33), lack of CD10 and CD38, and overexpression of CD49f and CD58 (All viable singlets are shown in the upper left and center panels; only B cells are shown in the remaining panels)

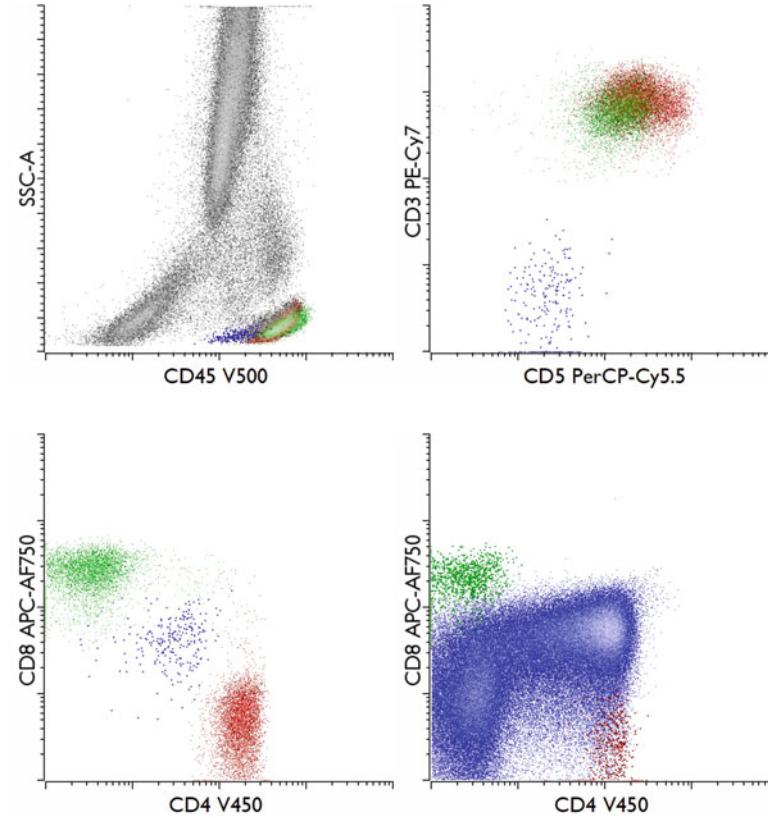

Fig. 5 Identification of MRD in T lymphoblastic leukemia (bone marrow). An abnormal T-cell population (blue, highlighted) is identified with dim CD5 and CD45, absent surface CD3, and coexpression (albeit weak) of CD4 and CD8. These phenotypic features differ from those of the normal CD4+ and CD8+ T cells (red and green, respectively), but resemble those of a subset of blasts seen in the patient's diagnostic specimen (*Lower Right*) (All viable singlets are shown in the upper left panel; only T cells are shown in the remaining panels)

However, the MRD in this case differs from the normal B-cell precursors in its expression of one or more myeloid antigens (CD13 + CD33), lack of CD10 and CD38 expression, and over-expression of CD49f (*see* **Note 11**) and CD58 (Fig. 4). In the example of MRD detected in a patient treated for T lymphoblastic leukemia (Fig. 5), iterative gating discloses an abnormal T-cell population (blue, highlighted) with dim CD5 and CD45, absent surface CD3, and coexpression (albeit weak) of CD4 and CD8. These phenotypic features differ from those of the normal CD4+ and CD8+ T cells (red and green, respectively) but resemble those of a subset of blasts seen in the patient's diagnostic specimen (Fig. 5, lower right panel).

4 Notes

1. In general, samples should be processed as soon after collection as is feasible to minimize loss of cell viability. However, if a delay in preparation of the cell suspension cannot be avoided, the specimen may be diluted 1:2 with RPMI and held overnight at 2–8 °C.
2. Ideally, the final WBC concentration of the cell suspension will be 10,000/μL, enabling the delivery of 1,000,000 cells for labeling upon pipetting a volume of 100 μL.
3. Preincubation at 37 °C allows for Fc-receptor-mediated endocytosis of bound immunoglobulin molecules, thereby reducing background staining in studies with fluorochrome-conjugated antibodies directed against immunoglobulin light chains.
4. The individual fluorochrome-conjugated antibodies that comprise previously characterized antibody combinations (*see* Table 1) may be pooled to produce "cocktails" with reagents sufficient for use in multiple (e.g., 50) assays. These are prepared such that the appropriate final concentration of each antibody will be achieved upon addition of 100 μL of cell suspension to 100 μL of cocktail. However, the laboratory should verify that the performance of the pooled reagents does not differ significantly from that observed with individually added reagents, and determine the stability of each cocktail. We aliquot cocktails into light-resistant glass vials, and have found these aliquots to be stable for up to 4 weeks at 2–8 °C. To verify that the correct reagents have been added during its preparation, each new cocktail is tested with an appropriate normal specimen. Fluorescence intensities and patterns of antigen expression observed using the new cocktail should be visually indistinguishable from those seen with the previous cocktail (i.e., lot-to-lot comparison).
5. For MRD analysis, in which the goal is to collect up to 1,000,000 labeled cells, 200 μL of washed cell suspension (containing a total of 2,000,000 cells) should be added to 200 μL of cocktail.
6. Immediate acquisition of fixed samples is desirable, since breakdown of tandem fluorochromes is enhanced by exposure to heat, light, and fixatives. In our experience with the antibody combinations listed in Table 1, storage of fixed samples overnight at 2–8 °C in the dark prior to acquisition on the flow cytometer does not result in substantial tandem degradation. However, tandem dyes and their conjugates vary widely in their propensity to degrade, and each laboratory should verify that storage of fixed, labeled samples prior to acquisition does not yield significant tandem degradation.

7. Visual recognition of a rare abnormal population comprising fewer than 20 cellular events is difficult, and the threshold for positivity in most studies demonstrating the clinical significance of MRD in ALL has been 0.01%, or 1 in 10,000 cells [8]. At a minimum, then, 200,000 viable cells would be required to recognize a cluster of 20 abnormal cells at a frequency of 0.01%. The ability to recognize a potentially abnormal cluster as a distinct cellular population is also influenced by the magnitude of antigenic deviation from normal, the extent to which antigen expression is homogeneous, and the number of admixed normal events; in certain instances, populations comprising greater than 20 cells may not be apparent. We therefore attempt to acquire 1,000,000 events for each antibody combination in MRD assays.
8. By enabling the simultaneous detection of a relatively myeloid-specific antigen (myeloperoxidase) and T-lineage (cytoplasmic CD3-ε; Tube 7) or B-lineage (cytoplasmic CD22 and CD79a; Tube 8) antigens, Tubes 7 and 8 facilitate the identification of mixed phenotype acute leukemia (MPAL), T/myeloid and B/myeloid, both of which should be distinguished from ALL. Tube 9 includes the nuclear antigen, TdT, in conjunction with T-lineage and B-lineage antigens; TdT expression (though not specific for ALL) distinguishes precursor lymphoid neoplasms from their mature counterparts.
9. By eliminating much of the background/nonspecific staining associated with degenerating or nonviable cells, selective gating on events with the light scatter properties of viable cells may facilitate recognition of normal and abnormal cellular populations during data analysis. However, care must be taken to ensure that cells of interest are not inadvertently excluded from subsequent analysis.
10. Caution is advised in using CD19 as a gating target for MRD detection in B lymphoblastic leukemia because of the increasing use of therapeutic agents directed against this molecule (e.g., blinatumomab [16], anti-CD19 chimeric antigen receptor (CAR)-T cells [17]), which may result in loss of the epitope. In these cases, antibody combinations that do not rely on detection of CD19 should be used [18].
11. In our experience [19, 20], overexpression of CD49f is among the most common recurrent phenotypic aberrations in B lymphoblastic leukemia, though we have observed an apparent diminution in CD49f expression in samples held for extended periods (e.g., greater than a day) prior to processing. Extended delays in sample processing should therefore be avoided.

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Chapter 17

Clinical Flow-Cytometric Testing in Chronic Lymphocytic Leukemia

Dalia A. Salem and Maryalice Stetler-Stevenson

Abstract

Flow-cytometric demonstration of the typical chronic lymphocytic leukemia (CLL) immunophenotype is vital for diagnosis. CLL has a characteristic immunophenotype, expressing CD5, CD19, dim CD20, dim CD22, CD23, bright CD43, dim CD45, dim to negative CD79b, dim CD81, CD200, and dim monoclonal surface immunoglobulin. This characteristic immunophenotype allows a definitive diagnosis and the ruling out of another leukemia or lymphoma. Flow cytometry also provides important prognostic information and accurate assessment of response to therapy. Here we describe optimal specimen collection, red cell lysis, appropriate panel, cell staining, acquisition on a flow cytometer, and analysis for CLL specimens.

Key words Flow cytometry, Immunophenotype, Chronic lymphocytic leukemia, Minimal residual disease

1 Introduction

Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) is a neoplasm of mature B-lymphocytes involving peripheral blood (PB), bone marrow (BM), and secondary lymphoid tissues (spleen, lymph nodes). CLL is the most common leukemia of adults in western countries and it accounts for 7% of non-Hodgkin lymphomas. The diagnosis is established by blood count, morphology, and immunophenotyping by flow cytometry (FC) of circulating B-lymphocytes [1].

FC demonstration of the typical CLL immunophenotype is vital for diagnosis. CLL typically displays a characteristic immunophenotype, expressing CD5, CD19, dim CD20, dim CD22, CD23 (*see* **Note 1**), bright CD43, dim CD45, dim-to-negative CD79b, dim CD81, CD200, and dim monoclonal surface immunoglobulin (Ig) (Fig. 1) [2] but negative for CD10, CD103, and CD123 as well as other T-cell and myeloid antigens [3, 4]. Diagnosis of CLL requires $\geq 5 \times 10^3/\mu l$ circulating monoclonal B-lymphocytes with a CLL immunophenotype in the PB. The term SLL designates the

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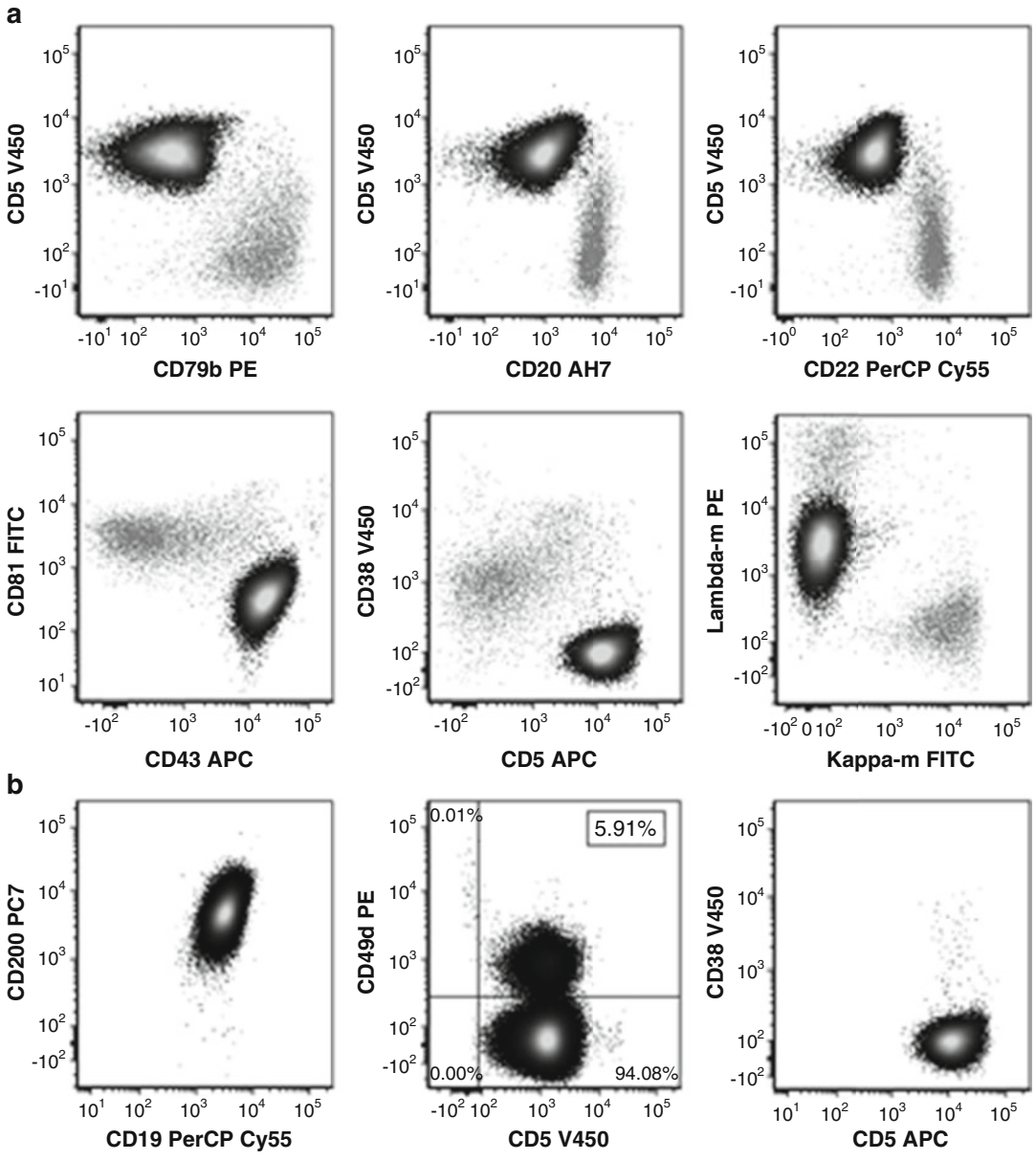

Fig. 1 (**a**) Gated on CD19 positive lymphocytes. Abnormal CLL cells (black) with normal B-cells (gray) in background. CLL cells are positive for CD5, have bright CD43, and have dim CD79b, CD20, CD22, and CD81. CD38 is negative. The CLL cells are monoclonal, dim positive for lambda surface light chain and negative for kappa surface light chain. (**b**) Gated on abnormal CLL cells. Cells are CD200 positive. Prognostic markers CD49d (less than 30% positive) and CD38 are negative

cases with a circulating CLL cell count $<5 \times 10^3/\mu l$ and recognized nodal, splenic, or other extramedullary involvement. The differential diagnosis of CLL/SLL primarily includes monoclonal B-cell lymphocytosis (MBL) and mantle cell lymphoma (MCL).

MBL is the presence of $<5 \times 10^3/\mu l$ circulating monoclonal B-lymphocytes in absence of any associated lymphadenopathy, organomegaly, extramedullary involvement, or any other feature of B-cell lymphoproliferative disorders [5]. MBL CLL-type (75% of MBL cases) has an immunophenotype identical to CLL. The expression of CD200 and CD23 in combination with dim CD20, CD22, CD45, CD79b, and CD81 as well as bright CD43 differentiates CLL from MCL [4]. Infrequently CLL cases may have an atypical immunophenotype such as lack of CD5 and CD23, normal intensity of CD20, CD22, and CD79b, aberrant expression of T-cell, myeloid or other B-cell antigens [6–8].

In addition to its role in diagnosis of CLL, FC also provides important prognostic information. CD38 expression in 30% or greater of the CLL cells was found to be associated with aggressive clinical course and to correlate with Ig heavy chain mutational status [9–11]. CD49d expression by 30% or greater of the CLL cells is an independent indicator of CLL prognosis and is superior to CD38 in predicting clinical progression in CLL patients. CD49d in CLL is associated with unfavorable cytogenetic profile and is particularly associated with trisomy12 [12, 13]. CD49d expression is stable over time and determination of a positive or negative status is straightforward [14, 15]. FC detection of minimal residual disease (MRD) levels during and after therapy has also been shown to be an independent predictor of progression-free and overall survival in CLL [16, 17].

2 Materials

2.1 Lysing Solution (1×)

1. Weigh 8.29 g ammonium chloride (NH_4Cl) and 1 g potassium bicarbonate ($KHCO_3$) and place them into 1000 ml glass or clear plastic bottle.
2. Add 4 ml 0.5 M EDTA solution, and fill to a volume of 1000 ml with distilled water (H_2O). Cap and mix well.
3. Store at room temperature for up to 6 months.

2.2 Wash Solution: Phosphate Buffered Saline (PBS) with 10% Bovine Serum Albumin (BSA) and Sodium Azide

1. Weigh 4 g BSA and add to 4 l of 1× PBS with 0.1% sodium azide. Cap and mix well by inverting several times. Allow wash solution to sit at room temperature for 30 min, mix again, and check to be sure the BSA has dissolved.
2. Perform pH testing after BSA is in solution and while the solution is at room temperature. Adjust pH to 7.3–7.5 by adding small amounts of concentrated HCL (if the pH is >7.5) or concentrated NaOH (if the pH is <7.3) (*see* **Notes 2** and **3**).
3. Store at 4 °C for 2 months from preparation.

2.3 Fixative: 0.5% Formalin Fixative Solution

1. Mix 50 ml of 10% buffered formaldehyde with 950 ml of PBS in a bottle, cap and mix well (*see* **Note 4**).
2. Wrap bottle with foil and tape to protect from light. Check PH and adjust to 7.3–3.5 (*see* Subheading 2.2).
3. Store at room temperature for up to 1 year.

3 Method

3.1 Cocktails and Panels

The panel in Table 1 is an example of an 8 color panel that can be used to diagnose CLL and monitor disease post therapy. Each cocktail is made by addition of the appropriate volumes of different Antibody (Ab) as recommended by the vendor and/or titration. The amount of the cocktail to be added during staining tube (Subheading 3.6, step one below) is based on the sum of the different Ab volumes in this cocktail. The cocktail should be stored at 4 °C and light exposure minimalized. The stability of these cocktails should be determined by each clinical laboratory.

3.2 Specimen Collection

PB and BM are the primary samples to be examined, although lymph node (LN) biopsy or fine needle aspiration (FNA) may be indicated. PB and BM specimens collected in sodium heparin are stable at room temperature for up to 72 h and 24 h respectively while EDTA PB and BM specimens are stable for 24 and 12 h respectively. Improper or lengthy specimen storage can negatively impact results. Overnight shipment of specimens with the possibility of exposing the specimen to temperature extremes has been shown to alter antigen expression [18]. An LN biopsy must be made into a cell suspension using mechanical tissue disaggregation (using commercial devices or manual tools). FNA samples usually do not require additional disaggregation. FNA or cell suspension can be stored in RPMI with 10% fetal bovine serum for up to 18 h at 4 °C.

Table 1
Antibody panel for CLL diagnosis/follow-up

	FITC	PE	PerCP	PC7	APC	AH7	v450	v500
1	CD103	CD25	CD123	CD19	CD23	CD20	CD11c	CD45
2	CD81	CD79b	CD22	CD19	CD43	CD20	CD5	CD3
3	Kappa-m	Lambda-m	CD20	CD19	CD5	CD14	CD38	CD45

3.3 Pre-lysis, Cell Count, and Viability Assessment [18, 19]

Minimizing sample treatment before staining and timely sample processing greatly reduces the risk of losing cells of interest (especially in case of CLL MRD) and maintains cell viability and integrity.

1. Transfer ≤5 ml of either PB or BM into labelled 50 ml conical tube(s).
2. Add ammonium chloride lysing Solution (*see* Subheading 2.1) to fill each conical tube. Cap and invert several times to mix. Incubate 10 min room temperature, inverting to mix occasionally and at end.
3. Centrifuge tubes at 500 × *g* for 5 min, check for pellet and aspirate or decant supernatant into waste container (*see* **Note 5**).
4. Cap and vortex tube(s) gently for less than 5 s to dislodge pellet (s). Fill the tube with PBS, cap, and invert to mix. Repeat **step 3** above (*see* **Note 6**).
5. Cap and vortex tube gently to dislodge the entire cell pellet. Add PBS to the cell pellet according to the pellet size (0.2–2 ml for small pellets and 3–5 ml for larger pellets), and flick or vortex gently to mix. Record the final cell suspension volume to be used later to adjust the cell count.
6. Assess viability and perform a cell count by using a viability dye that is excluded from viable cells and automated cell counters or a hemocytometer [20]. Samples with less than 75% viability should be rejected unless the sample is irreplaceable.

3.4 Cell Concentration Adjustment

1. Divide the total WBCs count (obtained by multiplying the cell count in the "count" tube by the dilution factor multiplied by the preparation volume in Subheading 3.3, **step 5**) by the target WBCs count to get the amount of the required final volume. Example: if the total WBCs count = 15.0×10^6 and the target count is 20×10^6/ml, then the final volume should be 0.75 ml.
2. Adjust the final volume of the cell suspension to reach the target cell concentration of 20×10^6 cells/ml (*see* **Note 7**).

3.5 Staining Cells with Antibodies [18, 19]

1. Deliver the appropriate volume of the premade cocktail (Subheading 3.1) to the bottom of the labeled 12 × 75 mm tubes using automatic pipette.
2. Add 100 μl patient cell preparation to each tube and vortex briefly to mix. Incubate 30 min at room temperature protected from the light.
3. Wash stained tubes by adding 3–4 ml wash solution to each tube; vortex to mix then centrifuge at 500 × *g* for 5 min, check for pellet and aspirate or decant tube into waste container, flick or vortex tube to dislodge entire cell pellet.

4. Repeat wash **step 3** above.
5. Add 200–300 μl formalin fixative and vortex to mix and store stained and fixed cells at 4 °C until acquisition (*see* **Note 8**).

3.6 Acquisition

Data is acquired on a cytometer. Care should be taken not to exclude low FSC events. Acquisition of ungated data is stored as list mode data. 1,000,000 to 2,000,000 events (dependent on level of MRD detection required) are acquired when cellular content is adequate. In specimens with low cell count (e.g., FNA) acquire as many events as possible. Acquiring a high number of events is crucial for MRD detection. At the minimum all fluorescence channels, time, FCS-A, FSC-H and SSC-A should be acquired.

3.7 Analysis

Sequential gating of the list mode data is performed to distinguish abnormal cells of interest from normal cells based on light scatter and antigen profile. An example of a typical sequence of hierarchical analysis gating follows:

1. The FSC-A vs. time plot of ungated cells is evaluated for discontinuity indicating fluidic disturbances (e.g. bubbles, clogs, or in the case of low cell number the tube being acquired dry). A time gate is drawn to exclude such events (Fig. 2).
2. A viable gate is drawn on the time gated SSC-A vs. FSC-A plot to exclude debris with very low scatter properties (Fig. 2) and is placed under the time gate.
3. A singlets gate is drawn on the viable gated FSC-H vs. FSC-A plots to exclude doublets (Fig. 2) and is placed under the viable gate.
4. Antigen vs. SSC gates are drawn to define T-cells (CD3+) and B-cells (CD19+) (Fig. 2) and placed under the singlet gate.
5. The antigen based gates are displayed on a FSC-A vs. SSC-A plot to create a scatter gate that includes all lymphocytes (Fig. 2). This lymphocyte gate is placed under the singlet gate.
6. The antigen profile of the lymphoid cells is evaluated under the lymphocyte and B cell antigen gates to identify the CLL immunophenotype (Fig. 1).
7. For MRD assessment, low level CLL involvement must be detected among a predominately polyclonal B-cell background. There are two general analysis approaches.
   (a) Method 1: Based upon European Research Initiative in CLL (ERIC) method [2, 16, 21]. The ERIC method utilizes cells stained with cocktail 2 in Table 1 and a Boolean gating strategy in which CD19 positive B-cells are evaluated and a series of analysis gates are created in areas where normal B-cell populations are not observed (Fig. 3): A lymphocyte scatter gate that includes all CD19

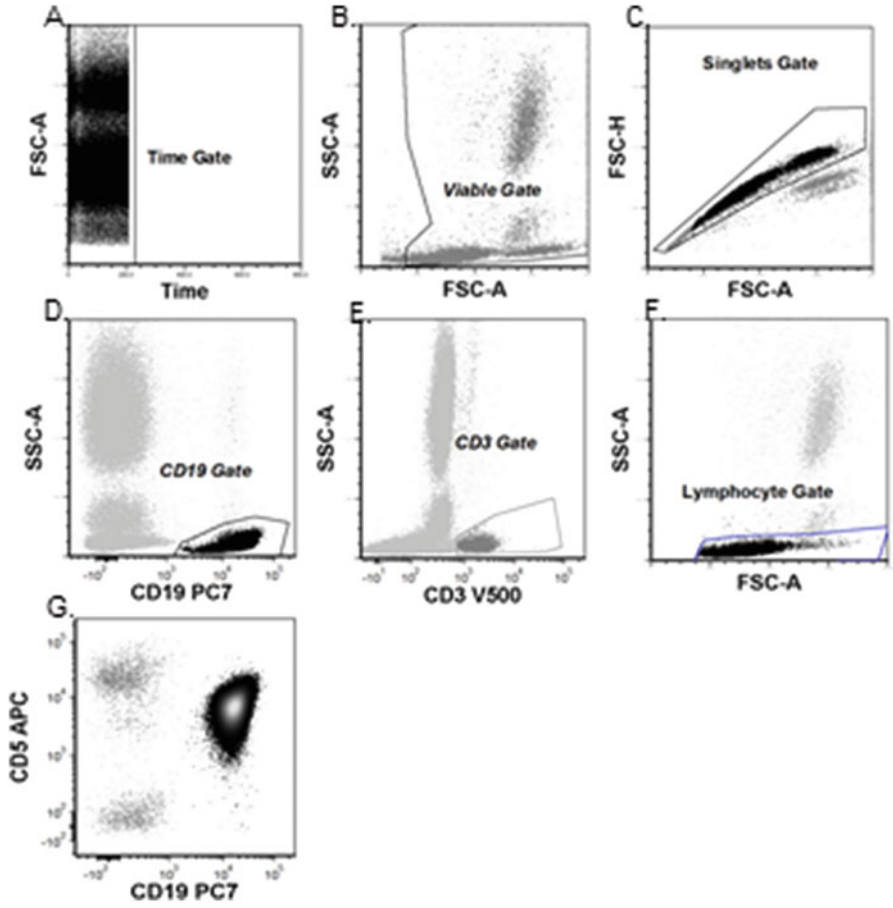

Fig. 2 Basic analysis for CLL: (**a**) Time gate. (**b**) Time gate displayed. Viable gate to exclude debris. (**c**) Time gate displayed. Singlets gated to exclude doublets. (**d**) Gated on Time, Viable, and Singlets gates. Antigen vs. SSC gates to define T-cells (CD3+) (**e**) Antigen vs. SSC gates to define B-cells. (**f**) Gated on time, viable, and singlets gates. Antigen-based gates are displayed on a FSC-A vs. SSC-A plot to ensure lymphocyte gate includes all lymphocytes. (**g**) Final analysis gate: Gated on time, viable, singlets, and lymphocyte gates

positive cells is the first gate. A gate of CD19 positive and CD3 negative cells is placed under this gate. Then four gates are created: gate 1; CD5 positive CD79b dim to negative, gate 2; CD5 positive CD20 dim to negative, gate 3; CD5 positive CD22 dim to negative, and gate 4; CD43 positive and CD81 dim to negative. The CLL analysis gate consists of all cells in the lymphocyte scatter gate, CD19 positive, CD3 negative, and present in gates 1, 2, 3, and 4. Normal B-cells do not meet all of these criteria.

(b) Method 2: Hierarchical gate based upon abnormal antigen expression to define a monoclonal B cell population (Fig. 4). **Steps 1** through **5** are performed. A CD19

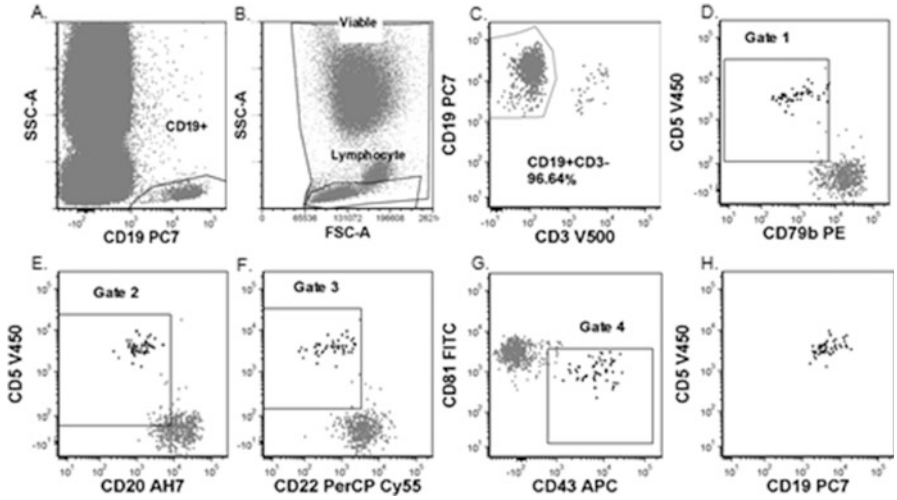

Fig. 3 CLL MRD Method 1: (**a**) Viable and Lymphocyte gates. (**b**) CD19+ gate. (**c**) CD19+CD3– gate. (**d**–**g**) Gated on viable, singlet, lymphocyte and CD19+CD3– gates. The four gates of this method. (**h**) Minimal residual CLL. Gated on Viable, Singlet, Lymphocyte, CD19+CD3– gates and gates 1–4

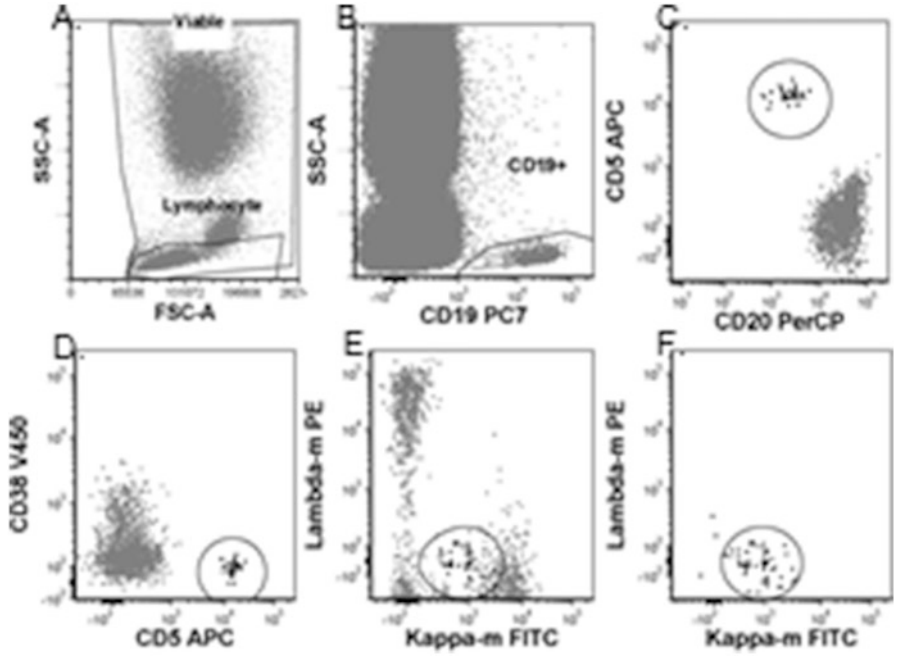

Fig. 4 CLL MRD analysis method 2: (**a**) Viable and lymphocyte gates. (**b**) CD19+ gate. (**c**) Gated on viable, singlet, lymphocyte, and CD19+ gates. Abnormal CLL cells CD20 dim and CD5+ in ellipse. (**d**) Gated on viable, singlet, lymphocyte and CD19+ gates. Abnormal CLL cells CD5+ and homogeneously CD38 negative in ellipse. (**e**) Gated on viable, singlet, lymphocyte and CD19+ gates. Abnormal CLL cells in ellipse masked by polyclonal B cells. (**f**) Gated on viable, singlet, lymphocyte, CD19+, and abnormal CD5, CD20, and CD38 gates. Abnormal CLL cells in ellipse are monoclonal, dim positive for kappa and negative for lambda

positive gate is drawn within the lymphocyte scatter gate. The B-cells in the CD19 gate are interrogated and light chain expression is evaluated in the cells expressing CD5, dim CD20, and atypical CD38.

4 Notes

1. CD23 is labile and can decrease with specimen storage prior to processing.
2. The wash solution should be checked with a fresh PB sample before use in patients (*see* Subheading 3). The supernatant from the lysed control blood should be clear. If there is evidence of hemolysis, discard solution and prepare a new one.
3. As this solution contains sodium azide, its vapor should not be breathed, and it should not be poured down the sink where explosive conditions may develop.
4. Formaldehyde is a known carcinogen and acutely hazardous; do not inhale and upon skin contact flush with water.
5. Post removal of supernatant during red cell lysis, gauze or a cotton swap can be used to carefully wipe debris from the insides of the tube(s); do not disturb the pellet before wiping inside the tube.
6. Washing cells with room temperature PBS prior to staining removes cytophilic antibody (bound immunoglobulin from the serum). As cytophilic antibody may be still bound to NK and T-cells, kappa and lambda expression is analyzed in association with a B-cell marker, such as CD19, CD20, or CD22, (besides exclusion of CD3 and or CD14 positive events) in order to remove the monocytes, T and NK cells with bound extrinsic Ab from the analysis.
7. Preparation of target cell concentration:
   (a) If greater than 20×10^6/ml (Increase the final PBS volume).
      - Divide WBC concentration by 20, multiply by cell volume to get the final PBS volume.
      - Add PBS up to the new volume and note on Flow QA sheet.
      - Example 1: If cell count is 20×10^6/ml in 5 ml, $20/20 = 1 \times 5 = 5$ ml; add PBS to 5 ml.
      - Example 2: If cell count is 80×10^6/ml in 3 ml, $80/20 = 4 \times 3 = 12.0$ ml; add PBS to 12.0 ml.

(b) If less than 10×10^6/ml (Re-concentrate to decrease the final PBS volume):

- Centrifuge specimen at $300 \times g$ for 10 min.
- Divide WBC concentration by 20, multiply by cell volume to get the final PBS volume.
- Remove supernatant down to the new final.
- Example: If cell count is 3.0×10^6/ml in 3 ml, $3/20 = 0.15$ ml $\times$ 3 = 0.45 ml final volume.

8. For best results, acquire stained fixed cells within 24 h.

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Chapter 18

Immunophenotyping of Paroxysmal Nocturnal Hemoglobinuria (PNH)

Andrea J. Illingworth, Iuri Marinov, and D. Robert Sutherland

Abstract

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare but often debilitating disease which may lead to death in up to 35% of patients within 5 years if unrecognized and untreated. Detection of PNH and assessment of PNH clone size in RBC and WBC lineages by flow cytometric analysis has increased in importance due to the availability of novel therapies. These therapies typically block the hemolysis of red blood cells and thus significantly lower the morbidities and mortality associated with this disease. This chapter describes validated, state-of-the-art, high-sensitivity flow cytometric methodologies based on latest published testing guidelines for PNH.

Key words Paroxysmal nocturnal hemoglobinuria (PNH), Glycophosphatidylinositol (GPI)-anchored protein, Aplastic anemia (AA), Myelodysplastic syndrome (MDS), CD59, FLAER

Reagents and Materials
Flow cytometer capable of at least five-color analysis
Test tubes
Antibodies (*see* Tables 1, 2, 3, and 4 for recommended antibody combinations).
Lysing solutions: BC Immunoprep, BC OptiLyse, BC VersaLyse, BD FACSLyse, or other validated lysing solution.
Buffers: Phosphate Buffered Saline (PBS), PBA (PBS and 0.1% sodium azide and 0.1% bovine serum albumin) refrigerated.

1 Introduction: Clinical Utility of GPI-AP Deficiency Testing in PNH and BMFS

Glycophosphatidylinositol-anchored protein (GPI-AP)-deficient hematopoietic stem cells (HSC) occur following somatic mutations of the X-linked phosphatidylinositol glycan complementation Class A gene (PIG-A), [1, 2]. Mutated HSC are able to escape immune attack mediated by CD4+ Th1, Th17 and autoreactive CD8+ cytotoxic T cells, resulting in clonal expansion followed by additional genetic or epigenetic events [3]. The proinflammatory bone

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marrow environment is characterized by reduction of CD4+CD25+FOX3 regulatory T cells with an altered cytokine profile, expansion of CD4+ helper Th1 and effector Th17 cells involved in organ-specific autoimmunity and expansion of CD8+ cytotoxic T cells. Such responses represent a well-documented, common pathophysiological mechanism for disorders comprising paroxysmal nocturnal hemoglobinuria (PNH) and bone marrow failure syndromes (BMFS). Such disorders present with clonal hematopoiesis, exhibit overlapping clinical symptoms and propensity to develop myelodysplastic syndrome (MDS) and/or acute myeloid leukemia (AML) [4–6]:

1. The distinction between the rare inherited forms of bone marrow failure syndromes (IBMFS) such as congenital neutropenia (CN), Diamond–Blackfan anemia (DBA), Schwachman–Diamond Sy (SDS), amegakaryocytic thrombocytopenia (AT), reticular dysgenesis (TAR), Fanconi anemia (FA), dyskeratosis congenita (DC) and the more frequent acquired forms of bone marrow failure syndromes (ABMFS) such as aplastic anemia (AA) and hypoplastic MDS (h-MDS) is not usually straightforward: approximately 5% of patients younger than 40 years with diagnosed AA have been reported to carry germline mutations [7]. GPI-AP-deficient clones are never present in IBMFS, thus high sensitivity flow cytometry (HS FCM) in addition to chromosome breakage studies, lymphocyte telomere length analysis and molecular methods is useful for the differential diagnosis, due to high negative predictive value [8].
2. The presence of GPI-AP-deficient clones in ABMFS has been reported in 25–45% of patients with AA, 5.5–9.8% of patients with MDS, 5.7% of patients with unexplained cytopenia, 6% of patients with pancytopenia [9–11]. The GPI-AP-deficient clone size can vary from <0.1% (BMFS, subclinical PNH, and aplastic PNH) to >90% in hemolytic and/or thrombotic PNH forms. The clinical significance of middle to large PNH clones is related to hemolysis and/or thrombosis in PNH patients, while the presence of rare GPI-AP-deficient cells (<0.1%) in AA and h-MDS is contributive for exclusion of IBMFS, predictive for better response to immunosuppressive therapy (IST) and lower incidence of transformation to MDS/AML [12].
3. GPI-AP-deficient clones can grow with time: approximately 15–20% of patients with BMFS could develop subclinical or clinically relevant PNH clone and 20–30% of patients with classical PNH could develop pancytopenia [13–15].
4. Current international recommendations for GPI-AP deficiency testing by HS FCM comprise all cases with unexplained isolated anemia, unclear iron deficiency anemia, unexplained isolated neutropenia or thrombocytopenia, cytopenias, any

subtype of MDS with evidence of hemolysis, venous or arterial thrombosis with atypical location (portal vein, hepatic vein, mesenteric vein, splenic vein, sinus vein) and signs of hemolysis, cytopenia or in young patients as well as cases with acquired DAT-negative hemolysis, intravascular hemolysis or recurrent abdominal pain or dysphagia. For patients with AA and no detectable PNH phenotypes the current guidelines recommend reassessment every 6 months for 2 years and yearly thereafter as long as they remain negative for PNH phenotypes. For patients with AA and detectable PNH phenotypes, reassessment is recommended every 3 months for 2 years, with subsequent yearly testing if level of PNH phenotypes remains stable [16, 17].

While the ability to rapidly detect GPI-deficient cells by flow cytometry has led to improved diagnosis, patient management, and prognosis in PNH and related disorders, early, simple CD55/CD59 based approaches were neither accurate nor sensitive below the 1–4% clone size, rendering them inadequate to detect small PNH clones present in PNH+ AA and MDS cases [16, 18]. Since 40% of samples positive for the presence of PNH cells contain GPI-deficient cells at a level of 1% or less (*see* Fig. 1 in [19]), the development and validation of sensitive, standardized methodologies is essential to reliably detect small populations of GPI-deficient PNH phenotypes. Once established, the frequencies of PNH phenotypes in red and white blood cell lineages in multiple normal samples must be determined [18–20]. Additionally, the sensitivities of the assays have to be determined by titrating ("spiking") a PNH sample into a normal sample [18–24]. Furthermore, there is much variability between different antibody clones/conjugates used in the analysis of PNH in RBC and WBC lineages. To successfully develop highly sensitive, accurate and reproducible assays, careful selection and titration of antibody clones/conjugates for lineage-specific gating (RBCs, neutrophils, and monocytes) and specific GPI-antigen detection within each cell lineage is required [18, 23, 24].

The detection of PNH phenotypes by flow cytometry represents a unique challenge in that the assays are designed to detect "negatives," that is, PNH phenotypes have "lost" the antigens of interest, namely, glycophosphatidylinositol (GPI)-linked structures. This has considerable implications for how instruments are set up and compensated because the target GPI-negative PNH cell populations (both RBCs and WBCs) need to be visible and fully on scale.

2 Materials

1. Antibody clone/conjugate selection for high-sensitivity PNH RBC assay
   (a) *Gating antibody*: CD235a (glycophorin A) is the only RBC-specific gating reagent available for delineating RBCs from other events (WBCs, platelets, debris, etc.). Since CD235a (in particular) conjugates can cause massive aggregation of RBCs when used at anything close to saturating levels, careful selection and extensive titration is required to minimize aggregation while retaining good signal-to-noise characteristics of the assay. Screening of a large number of CD235a clones/conjugates showed that negatively charged FITC conjugates generated significantly less aggregation than their PE-conjugated counterparts [18, 25].
   (b) *GPI-linked antibodies*: While loss of the GPI-linked CD55 and CD59 structures was traditionally used to detect PNH RBCs, CD55 is inferior to CD59 due to its dim expression and inability to delineate Type II and Type III PNH RBCs [16, 18, 22]. Thus, CD59PE conjugates offer the best separation of Type I, Type II, and Type III RBCs. Screening and titration of a large number of CD59PE conjugates was used to identify the best-performing CD59 reagents [18, 24]. A summary of recommended CD235a and CD59 clones/conjugates is shown in Table 1. To avoid the generation of false negatives, it is highly recommended that "cocktails" of the chosen CD235aFITC and CD59PE reagents be premixed and diluted with clean PBS to aid the accurate addition of reagents for this assay [18, 24].

Table 1
Recommended CD235a-FITC and CD59-PE conjugates for high-sensitivity PNH RBC assay

Target	Antibody conjugates	Purpose	Clone and vendor
RBC	CD235a-FITC	Gating on RBC	10F7MN (eBio) YTH 89.1 (Cedarlane) KC16 (BC) JC159 (DAKO)
	CD59-PE	GPI-linked for RBC	OV9A2 (eBio) MEM-43 (Invitrogen) MEM-43 (EXBIO/Cedarlane)

2. Antibody clone/conjugate selection for WBC analysis
   (a) *Gating antibodies*:
   - *CD45*: The inclusion of a pan-CD45 conjugate in the WBC staining cocktail is highly recommended. Along with a light scatter plot, CD45 is highly effective at removing subcellular debris, platelets and unlysed RBCs and stains peripheral blood samples in a highly predictable manner allowing for "pattern recognition."
   - *Lineage-gating reagents—neutrophils and monocytes*: The development of high sensitivity assays is critically dependent on the use of good lineage-specific "gating" reagents for neutrophils and monocyte. In the past, CD33 was widely used to "gate" both neutrophils and monocytes [25] but for a variety of reasons was a suboptimal choice for high sensitivity detection of PNH neutrophils and monocytes [18]. As detailed elsewhere [18, 23, 24, 26], appropriately selected, validated, and titrated conjugates of CD15 and CD64 outperform the ability of CD33 conjugates to accurately delineate neutrophils and monocytes. Occasionally, CD64 may be upregulated in activated neutrophils making the separation between monocytes and neutrophils challenging in such cases.
   - *GPI-linked reagents—neutrophils and monocytes*: As outlined previously [16, 18, 22–24], two GPI-linked structures must be analyzed per WBC lineage assessed. Combinations of FLAER and CD24, or FLAER and CD157 represent the most tested combination of reagents to detect GPI deficient neutrophils, while FLAER in combination with either CD14 or CD157 represent the most validated combination of reagents to detect GPI-deficient monocytes [23, 24].

After much empirical testing of multiple clones and instrument-specific conjugates, recommended clones/conjugates of GPI-specific reagents for use on instruments equipped with 4-, 5-, and 6- (or more) PMTs are shown in Tables 2, 3 for Beckman Coulter and BD Biosciences platforms respectively [24], and in Table 4 for recently developed cross-platform assays [27].

3 Methods

3.1 Instrument Setup; Key Points

1. Standard setup instructions from both major manufacturers are optimized for specific applications and do not transfer directly to PNH testing in either red or white cell assays.

Table 2
Recommended clones/conjugates for high-sensitivity detection of PNH WBC on Beckman Coulter cytometers

Target	Antibody conjugates	Purpose	Clone and vendor
WBC	FLAER-Alexa 488	GPI-linked (Neuts + Monos)	NA (Cedarlane)
	CD24-PE	GPI-linked (Neuts)	SN3 (eBio), ALB9 (BC)
	CD24-APC		SN3 (eBio, EXBIO)
	CD14-PE	GPI-linked (Monos)	61D3 (eBio), RMO52 (BC)
	CD14-APC700		Tuk4 (Invitrogen) RMO52 (BC)
	CD157-PE	GPI-linked (Neuts + Monos)	SY11B5 (eBio, EXBIO, BD, BC, Sysmex)
	CD64-PC5	Gating on Monocytes	22 (BC)
	CD64-ECD		22 (BC)
	CD64-PC7		22 (BC), 10.1 (EXBIO)
	CD15-PC5	Gating on Neutrophils	80H5 (BC)
	CD15-PerCP-eF710		MMA (eBio)
	CD15-PerCPCy5.5		MEM158 (EXBIO)
	CD45-PC7	Debris/unlysed RBC exclusion + pattern recognition	J33 (BC)
	CD45-KO		J33 (BC)
	CD45-eF450		2D1 (eBio)

Table 3
Recommended clones/conjugates for high-sensitivity detection of PNH WBC on Becton Dickinson cytometers

Target	Antibody Conjugates	Purpose	Clone and vendor
WBC	FLAER-Alexa 488	GPI-linked (Neuts + Monos)	NA (Cedarlane)
	CD24-PE	GPI-linked (Neuts)	SN3 (eBio), ML5 (BD)
	CD24-APC		SN3 (eBio, EXBIO)
	CD14-PE	GPI-linked (Monos)	61D3 (eBio),
	CD14-APC		Tuk4 (Invitrogen) MoP9 (BD)
	CD157-PE	GPI-linked (Neuts + Monos)	SY11B5 (eBio, EXBIO, BD, BC, Sysmex)
	CD64-APC	Gating on Monocytes	10.1 (BD, eBio)
	CD64-PECy7		10.1 (EXBIO), 22 (BC)
	CD15-APC	Gating on Neutrophils	HI98 (BD)
	CD15-PerCP-eF710		MMA (eBio)
	CD15-PerCPCy5.5		MEM 158 (EXBIO)
	CD45-eF450	Debris/unlysed RBC exclusion + pattern recognition	2D1 (eBio)
	CD45-PerCP		2D1 (BD)
	CD45-APC-H7		2D1 (BD)

Table 4
Recommended clones/conjugates for cross-platform three-laser B-C Navios/BD Canto II High sensitivity PNH WBC 5-, 6-, and 7-color assays

Target	Antibody conjugates	Purpose	Clone and vendor
WBC	FLAER-Alexa 488	GPI-linked (Neuts + Monos)	NA (Cedarlane)
	CD157-PE (all)	GPI-linked (Neuts + Monos)	SY11B5 (eBio, EXBIO, BD, BC, Sysmex)
	CD24-APC (6C, 7C)	GPI-linked (Neuts)	SN3 (eBio)
	CD14-APCeF780 (7C)	GPI-linked (Monos)	61D3 (eBio),
	CD64-APC (5C)	Gating on Monocytes	10.1 (BD, eBio)
	CD64-PECy7 (6C, 7C)		10.1 (EXBIO), 22 (BC)
	CD15-PerCP-eF710 (all)	Gating on Neutrophils	MMA (eBio)
	CD15-PerCPCy5.5 (all)		MMA (eBio)
	CD15-PerCPCy5.5 (all)		MEM 158 (EXBIO)
	CD45-eF450 (all)	Debris/unlysed RBC exclusion + pattern recognition	2D1 (eBio)

2. FLAER is an Alexa 488 conjugate and specific compensation settings are required for this conjugate. Settings optimized for FITC must not be used because of the different spillover of Alexa 488 and FITC.
3. On instruments equipped with biexponential or logicle displays it is important to ensure "negative cell populations," the very targets of testing in PNH (*see* below) are fully on scale with median channels above the zero axis. When optimally set up for the detection of PNH phenotypes, such populations should appear similar when viewed in either logarithmic or biexponential/logicle mode.
4. The appropriate target population must be used for PMTv settings. For the red cell assay, unstained red blood cells are appropriate; for the white cell assay, it is important to ensure the unstained lymphocyte population is clearly on scale.

3.2 Sample Preparation and Instrument Setup for RBC Analysis

For instruments that do not automatically save "TIME" as a parameter, this parameter must be collected so that fluidics can be monitored if needed. Instrument setup for the RBC assay is performed using a fresh normal blood sample, diluted 1:100 with clean Phosphate buffered saline (PBS). Light scatter voltages, photomultiplier tube voltages (PMTv) and compensation are optimized as detailed in several previous publications [18, 23, 24, 28], and recent examples can be viewed in Figs. 1 and 2 of [24]).

3.3 Recommended Staining Protocol for RBC

Anti-coagulated (EDTA is preferred, but heparin can also be used) peripheral blood samples should be less than 48 h old. If possible, these samples should be kept refrigerated for this may extend the stability of these samples.

1. Prepare a 1:100 dilution of the sample with clean PBS.
2. Pipet 100 μl of diluted blood sample into the bottom of a test tube using reverse-pipetting to avoid aerosol generation, and remove pipette carefully to avoid leaving blood trails on the inside of the tube.
3. Prepare CD235aFITC/CD59PE antibody cocktail as described in previous publications [18, 24] Note that specific clones should be validated/titrated by each laboratory to confirm optimal performance.
4. Pipet appropriate cocktail amount directly into the diluted blood sample and mix by gently swirling the sample using a low speed vortex. Note: For the "immature RBC (iRBC)" staining protocol, 1 μl of CD71-APC (clone MEM-75, EXBIO) is also added at this point.
5. Incubate the sample in the dark for 20 min at room temperature (incubation up to 60 min has been validated).
6. Wash twice with PBS by centrifugation and resuspend in 0.5–1.0 ml of PBS.
7. "Rack" the sample (drag across a hard plastic or metal test tube rack several times) to disrupt any RBC aggregates generated by the staining/washing procedure immediately before acquisition on the cytometer.
8. It is generally good to acquire sample immediately, as the binding of some CD235a conjugates has been shown to be unstable after washing/racking. Delay can also allow for the reformation of some RBC aggregates.
9. Acquire a minimum of 100,000 RBCs (gated on FS log versus CD235a. If two or more events were displayed in the Type III PNH RBC region, data acquisition should be continued until one million events are acquired.

3.4 Sample Preparation and Instrument Setup for WBC Analysis

For instruments that do not automatically save "TIME" as a parameter, this parameter must be collected so that fluidics can be monitored if needed. To analyze white blood cells, 100 μl of anticoagulated peripheral blood is stained with the reagent cocktail of choice and subject to an RBC lysis step. Regardless of lysing agent used, the cells need to be washed thereafter with phosphate buffered saline supplemented with 1% bovine serum albumin before acquisition. A multistep Boolean gating approach has been adopted to efficiently gate neutrophils and monocytes that begins with setting light scatter voltages in linear mode to ensure that all cells of interest (neutrophils and monocytes for PNH "clone" detection and lymphocytes used as internal staining controls) are properly on-scale and above the threshold/discriminator, used to remove subcellular debris. Light scatter voltages and PMTv are optimized as detailed in several previous publications [18, 23, 24, 28].

3.5 Establishing the Compensation Matrix

Light scatter voltages, photomultiplier tube voltages (PMTv) and compensation for the PNH WBC assay are optimized as detailed in several previous publications [18, 23, 24, 28] and examples are shown in Figs. 6 and 7 for a FLAER-based 6-color assay on a 10-color Navios cytometer.

3.6 Recommended Staining Protocol for Five- and Six-Color WBC Assays (Neutrophils and Monocytes)

All individual antibodies were verified for appropriate reactivity with target cells and titrated to optimize specific staining performance prior to being cocktailed for use in the single tube five- or six-color assays. It is recommended that samples be stained fresh or after a maximum storage time of 48 h at 4 °C [19, 23, 24].

1. Using reverse-pipetting, pipet 100 μl of fresh peripheral blood carefully into the bottom of a test tube without touching the side of the tube.
2. Add an appropriate volume of premixed and validated cocktail directly to the blood aliquot in the bottom of the tube and mix very gently but thoroughly as described above for the RBC assay.
3. Incubate for 20 min in the dark (incubation times for up to 60 min have been validated).
4. Lyse the RBCs using an appropriate lysing agent. ImmunoPrep, VersaLyse (BC), FACSLyse (BD) or ammonium chloride-based lysing agents following the manufacturer's recommendations. While all are acceptable, those containing fixatives may help retain cellular integrity better than those that do not.
5. After lysing, wash cells once with PBS, resuspend in 1 ml of PBS and acquire on the cytometer. Note: Samples should be acquired immediately, as delays can cause light-scatter changes, especially when fixative-free lysing agents are used.
6. Acquire a minimum of 50,000 neutrophils and 5000 monocytes in listmode for clinical test samples.
7. If small numbers of GPI-deficient cells are observed, acquisition times should be increased until a statistically reliable number of "PNH phenotypes" are acquired (20–50 cells).

4 Analysis

A correct diagnosis of paroxysmal nocturnal hemoglobinuria (PNH) is essential for effective patient management and possible treatment if clinically indicated [10, 11, 16]. Although flow cytometry has become the gold standard for the diagnosis and monitoring of patients with PNH and related diseases [16, 23], it is important to emphasize that flow cytometry by itself does not establish the clinical diagnosis of overt/clinical PNH as this is

determined by a number of other clinical factors in addition to the finding and quantification of glycosylphosphatidylinositol (GPI)-deficient cells by flow cytometry [1–3]. HS FCM analysis can identify the presence of PNH phenotypes and is able to quantify the PNH clone size in RBCs and WBCs (both neutrophils, and monocytes) down to a lower limit of quantification (LLOQ) of 0.01% for RBC and 0.05–0.1% for neutrophils. The PNH clone size in WBCs is typically used to determine the extent of disease. Historically, the neutrophil clone size was used for this purpose based on the absence of at least two GPI-linked structures from neutrophils. However, more recently many laboratories have noted the additional value of assessing and reporting the PNH clone size in the monocytes as well [19].

4.1 RBC Analysis

For high-sensitivity RBC analysis, most previously published Guidelines recommend the use of CD235a-FITC for lineage-specific RBC gating and CD59-PE to detect GPI-deficient cells [18, 24]. RBCs are analyzed by a series of gating dot plots and diagnostic dot plots:

1. *TIME (x axis) versus SS log (y axis) dot plot*. TIME is collected as a parameter (as is recommended for all clinical flow assays) and monitored during acquisition so that if fluidics problems are encountered, the sample can be reacquired if possible, or if not, data acquired prior to the fluidics hiatus can be "gated" and only that portion of the data file subsequently analyzed (Fig. 1, top row left).
2. *FS log (x axis) versus SS log (y axis) dot plot* to gate out debris (Fig. 1, top row middle). It is important to adjust the threshold (discriminator) for the forward scatter so that no RBCs are excluded from acquisition. Make sure that gate includes the RBC cluster and excludes any cellular debris on the left.
3. *CD235a-FITC (x axis) versus FS (y axis) dot plot* to gate singlet RBCs and to quantify and exclude any remaining RBC aggregates (Fig. 1, top row right).
4. *CD59-PE (x axis) versus CD235a-FITC (y axis) dot plot* (Fig. 1, bottom row left). Check the Type III area for cells, which are CD235a brightly positive/negative for CD59 and record the percentage. Check the Type II area for cells, which are also brightly positive for CD235a with intermediate CD59 expression and record the percentage.
5. *CD59-PE (x axis) versus CD235a-FITC (y axis) density plot* (Fig. 1, bottom row middle). Use this plot if the separation is not clear based on the previous dot plot.
6. *CD59-PE single parameter histogram* (Fig. 1, bottom row right). Bivariate dot plots and/or density plots are typically recommended over single-parameter histograms, especially

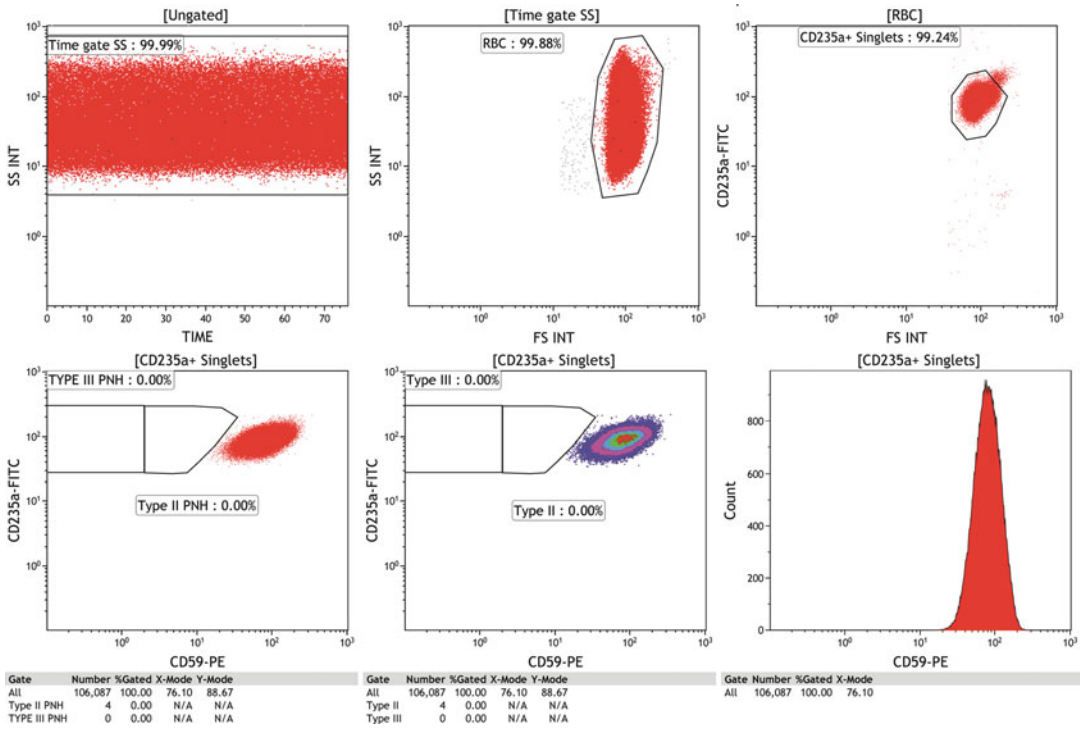

Fig. 1 Normal patient with no PNH RBC clone. Sample stained with CD235a-FITC/CD59-PE

for samples containing small numbers of PNH phenotypes, for identifying poorly stained samples that need to be restained, and for detecting media contamination and troubleshooting instrumentation issues (*see* Supplementary Data of [18]). However, while data regarding clone sizes comes predominantly from the two-dimensional plots, in which the gating regions are linked across the dot plot and density plots, the single parameter histogram can also be useful in some situations, and when comparing RBC reagent cocktails lot-to-lot. All three plots work in concert for optimal adjustment of the regions for Type III PNH cells and Type II PNH cells (*see* Fig. 2).

4.2 Separation Between Normal Type I RBC, PNH Type II and PNH Type III RBCs

In most cases the PNH clusters are evident and the initial region settings can be used for the quantification of the Type II and Type III PNH RBC based on a combination of bivariate dot plots, density plots and the single parameter CD59 histogram (Fig. 2). However, in some patients the regions may need to be adjusted (dynamic region setting) based on slight patient-specific differences in the CD59 expression rather than strictly adhering to the initial region setting. Occasionally there is no clear distinction (clustering) between PNH Type II and PNH Type III or between PNH Type II and normal Type I cells which renders the interpretation

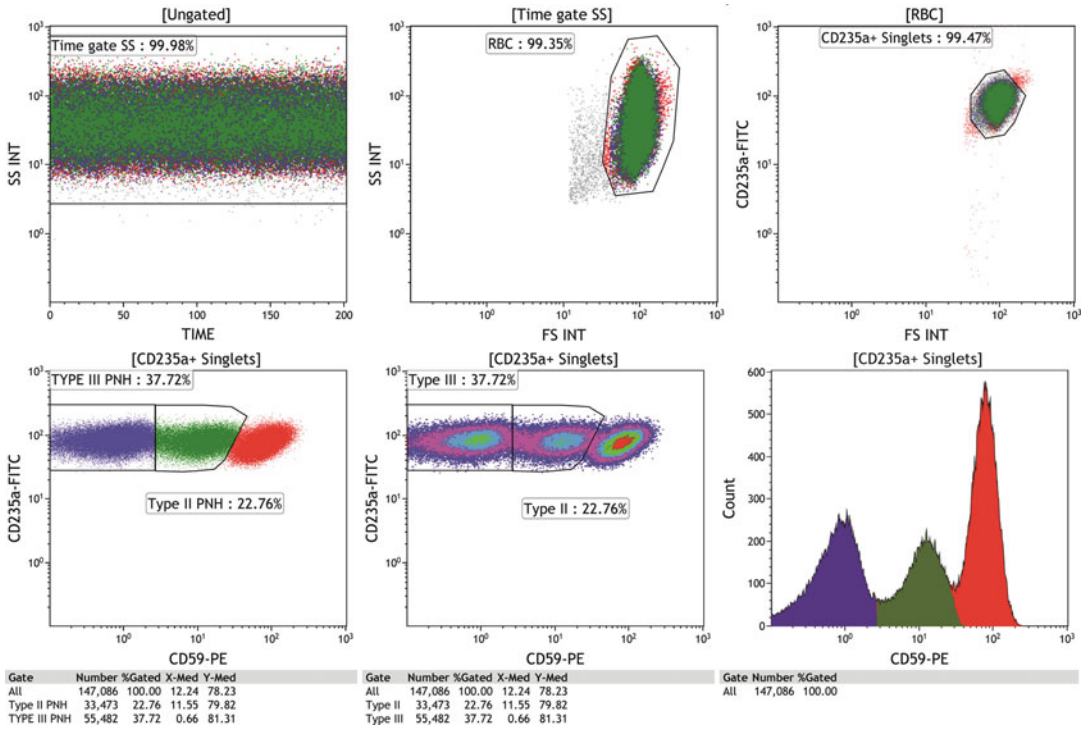

Fig. 2 Patient with PNH RBC Type II and PNH RBC Type III clone. Sample stained with CD235a-FITC/CD59-PE

challenging and the PNH clone size assessment and reporting potentially problematic. In such cases it may be appropriate to include a descriptive comment in the report and state that the percentages provided are an estimate only. In cases with no separation between Type III PNH cells and Type II PNH cells, it is recommended to report a combined population of RBCs with CD59 deficiency rather than setting arbitrary regions for Type II and Type III. Patients with poor separation between PNH Type II and normal Type I RBCs are especially challenging as the region setting between these two populations will affect the total combined PNH clone size. Reasons for this poor separation may include the presence of microcytes (smaller RBCs) in certain patients, transfusion (transfused RBCs typically have slightly decreased CD59 expression) and other unexplained decrease in CD59 expression. In these cases, a static region setting (based on the initial region setting) would not be optimal and a more flexible region setting based on the delineation of cell clusters is recommended. Considering the challenges of setting gating regions for accurate assessment of normal Type I RBC, PNH Type II, and PNH Type III populations, the addition of CD71, that stains immature RBCs (iRBC) allows for a much more objective assessment (*see* below).

4.3 Assessment of PNH in immature RBCs (iRBCs)

We have recently performed a study of over 25 large-clone PNH cases with the regular two-color RBC assay supplemented with CD71-APC [29]. This assay can be performed on any cytometer equipped with blue and red lasers. While mature RBCs can be assessed for PNH clone content, the CD71 is used to gate immature RBCs (iRBC). The latter can then be assessed for PNH iRBC clone content. The results demonstrated that the CD71-based RBC assay is better able to delineate Type III, Type II and normal iRBCs in PNH cases with WBC clones of 5% or larger. Total iRBC clone sizes (Type II plus Type II) were in almost all cases, very similar to the WBC clone sizes (whether measured in neutrophils or monocyte lineages). The assay retained the ability to detect minor PNH RBC clones in other BMFS cases in which the lack of sufficient reticulocytes does not allow for the assessment of the latter.

1. Figure 3 shows the advantage of the iRBC assay in a case in which delineating PNH Type III and Type II RBCs from normal cells was impossible. However, the use of CD71 to gate iRBCs (bottom left plot) allowed for the delineation of the Type III (7.4%) and Type II PNH RBCs (83.7%) from the normal RBCs. The WBC clone size in this sample was 94.2% and 94.8% for neutrophils and monocytes respectively (data not shown) compared to the total iRBC PNH content of 91.1%.
2. In the second example (Fig. 4), from another patient with a large WBC clone who was receiving regular RBC transfusions, there were relatively easy-to-delineate populations of Type III and Type II PNH RBCs although the Type II cells were not well separated from the normal RBCs (middle left plot). However, analysis of the CD71+ iRBC population demonstrated virtually all the PNH iRBCs (lower left) to be of the Type III phenotype (93.5%) with very few (0.7%) Type II iRBCs. Thus, even in some cases in which the PNH content of mature RBCs can apparently be relatively easy to analyze with confidence, the assay is in fact generating totally misleading data.
3. In a third example shown in Fig. 5 (top row), large WBC clones were detected (99.25% and 99.03% in neutrophils and monocytes respectively) while no discernable PNH clones could be detected in mature RBCs (lower left plot). Only when the Type II gate was moved (lower middle plot) to include the major population of RBCs (that expressed slightly lower levels of CD59 staining than bona fide normal Type I cells, data not shown), was it apparent that in this very unusual case, the Type II RBCs expressed almost as much CD59 as normal RBCs in a non-PNH sample. This interpretation was confirmed by the analysis of CD71+ iRBCs (lower right plot) in which the iRBCs also expressed CD59 at slightly lower levels than those from non-PNH samples (not shown). This example again highlights

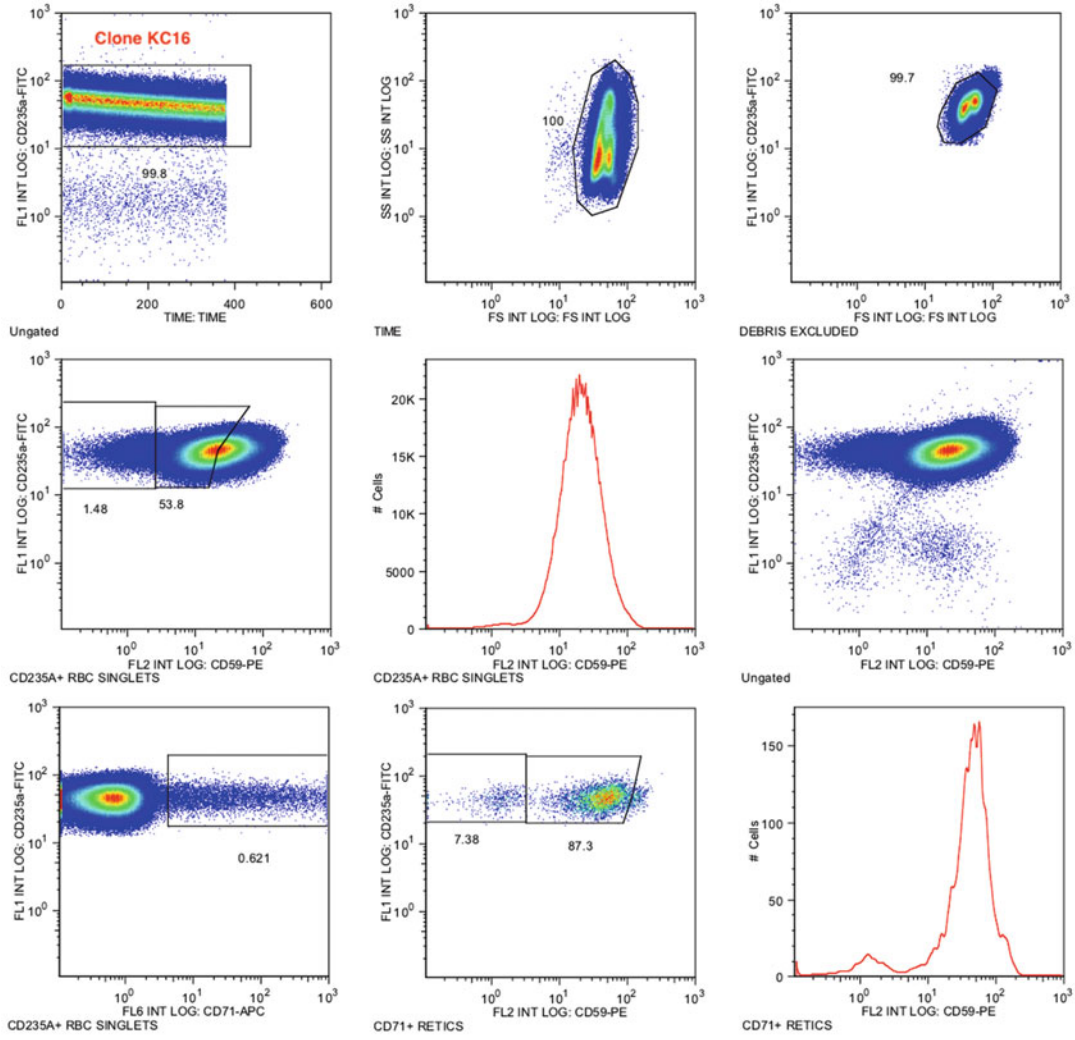

Fig. 3 PNH sample containing 94.2% PNH neutrophils and 94.8% PNH monocytes (data not shown) stained with CD235aFITC/CD59-PE/CD71-APC

the utility of the CD71-based RBC assay and also underscores the necessity of performing flow analysis on both WBC and RBC lineages in the detection of bona fide PNH phenotypes.

Overall, this study demonstrated (Figs. 3, 4, 5) that in PNH patients with large PNH clones, it is not possible to derive accurate Type III and Type II clone sizes from the mature (CD235a+) RBC component. Attempts to do so even by experts are often futile and can lead to the generation of completely misleading data. Misleading Type II RBC data is the biggest culprit and even when it appears straightforward to "gate" Type II RBCs (Fig. 4), such populations are usually artifacts with prior transfusions representing a major source of "false" Type II RBC phenotypes. In the majority of

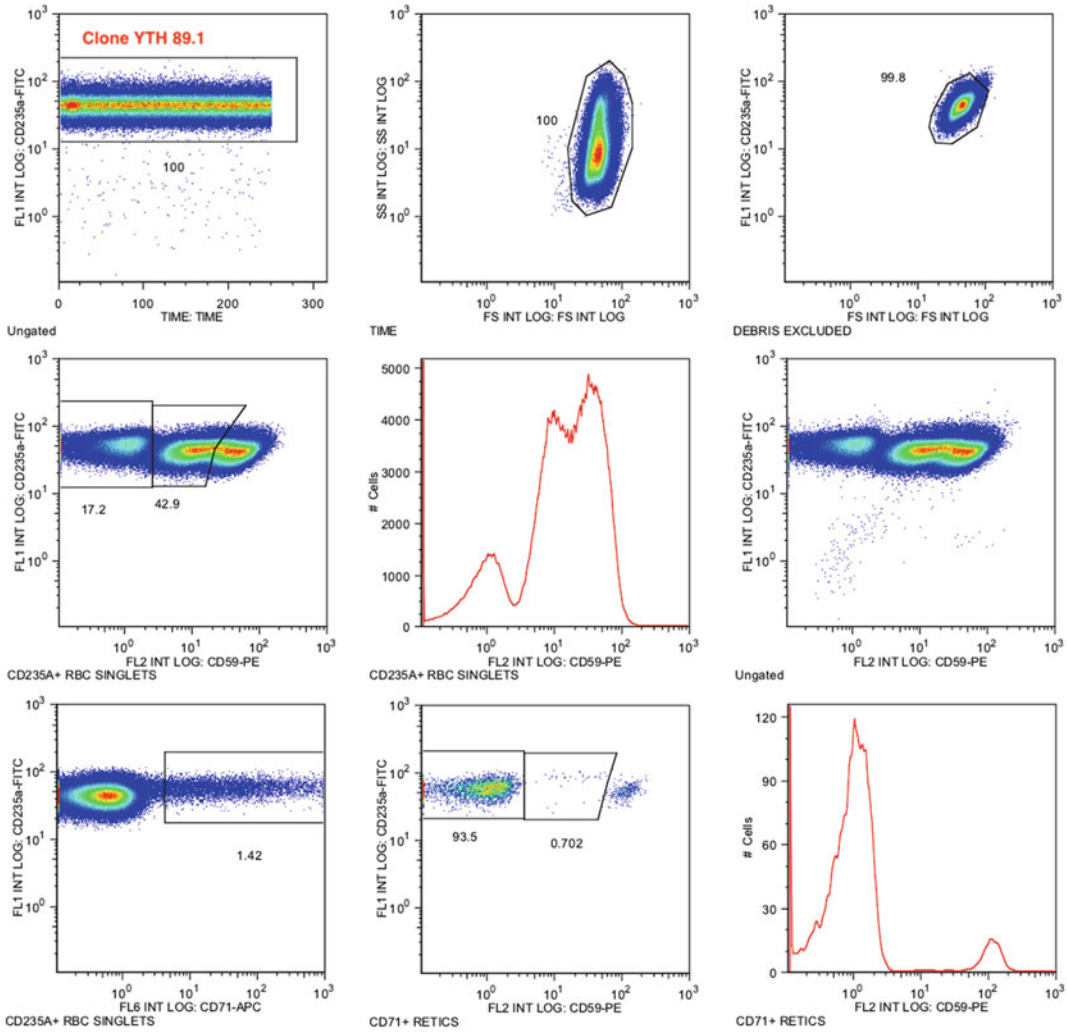

Fig. 4 PNH sample containing 97.3% PNH neutrophils and 99.6% PNH monocytes (data not shown) stained with CD235aFITC/CD59-PE/CD71-APC

large-clone PNH cases, Type III PNH iRBCs are the predominant PNH population although striking examples of predominantly Type II PNH iRBCs can also be observed (Fig. 5).

This study has also shown that if analysis of the red cell lineage is to be clinically useful, it needs to be accurate. For large-clone PNH cases, accurate data cannot be derived from mature RBCs (due to transfusion and hemolysis). For large-clone PNH cases, accurate data can be derived only from immature RBCs (iRBCs). For small clone PNH cases (clone sizes <1%) and BMFS cases containing small numbers of PNH RBC phenotypes, it is difficult to collect enough iRBCs for statistically precise data. However, in the latter, sufficient mature PNH RBCs can usually be identified to generate reliable and precise data.

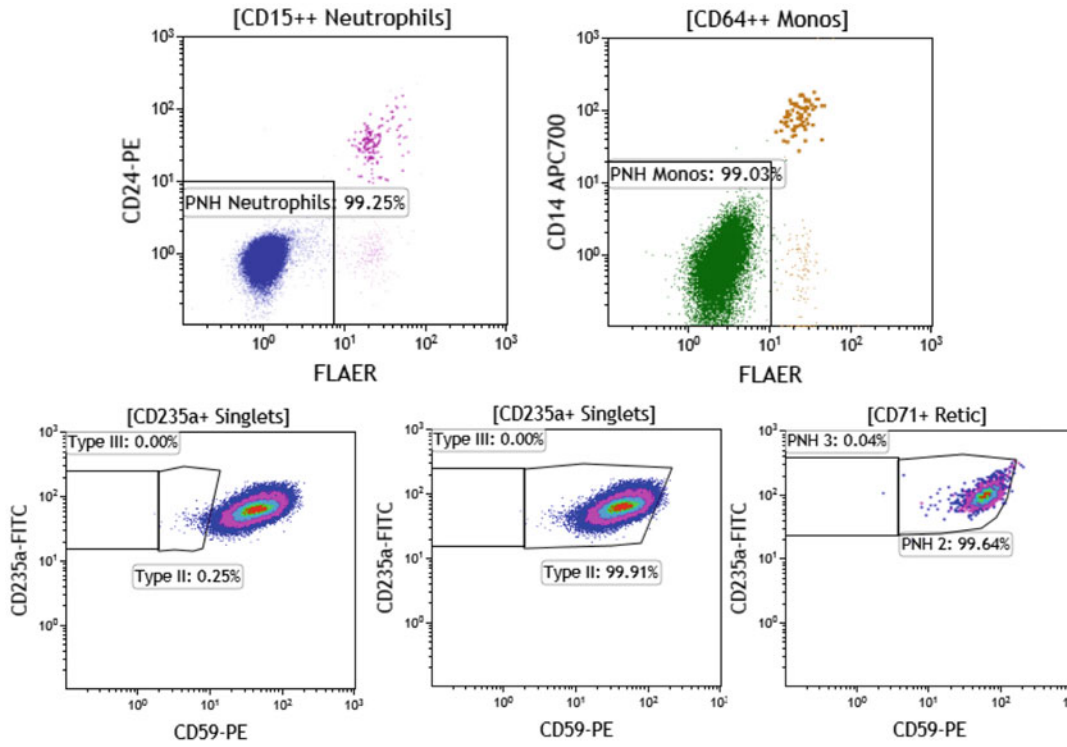

Fig. 5 PNH sample containing 99.25% PNH neutrophils (top right plot) and 99% PNH monocytes (top left plot) stained with CD235aFITC/CD59-PE/CD71-APC700

4.4 WBC Analysis

High-sensitivity methodologies to detect PNH phenotypes in neutrophils and monocytes have been published extensively [18, 22, 23]. Initial methods were based on a four-color neutrophil tube (FLAER, CD24, CD15, and CD45) with a reflex monocyte tube (FLAER, CD14, CD64, and CD45). Newer flow cytometers with 5, 6, or more PMTs allow for the simultaneous detection and quantification of both neutrophils and monocytes in a single tube. Currently recommended WBC panels based on established PNH guidelines include a FLAER/CD24/CD14-based panel and a FLAER/CD157-based panel [19, 24].

1. *6-color FLAER/CD24/CD14-based single tube Assay for Neutrophils and Monocytes*: Fig. 6 shows the sequence of bivariate gating dot plots from a Navios-specific reagent set comprising FLAER-Alexa 488, CD24-PE (clone ALB9), CD15-PC5 (clone 80H5), CD64-PC7 (clone 22), CD14-APCA700 (clone RMO52), and CD45-KO (clone J33). In this panel (Figs. 6 and 7) neutrophils are gated on CD45 and CD15 and a PNH clone can be detected based on the absence of the GPI markers FLAER and CD24. Monocytes are gated on CD45 and CD64 and a PNH clone can be detected based on the absence of FLAER and CD14.

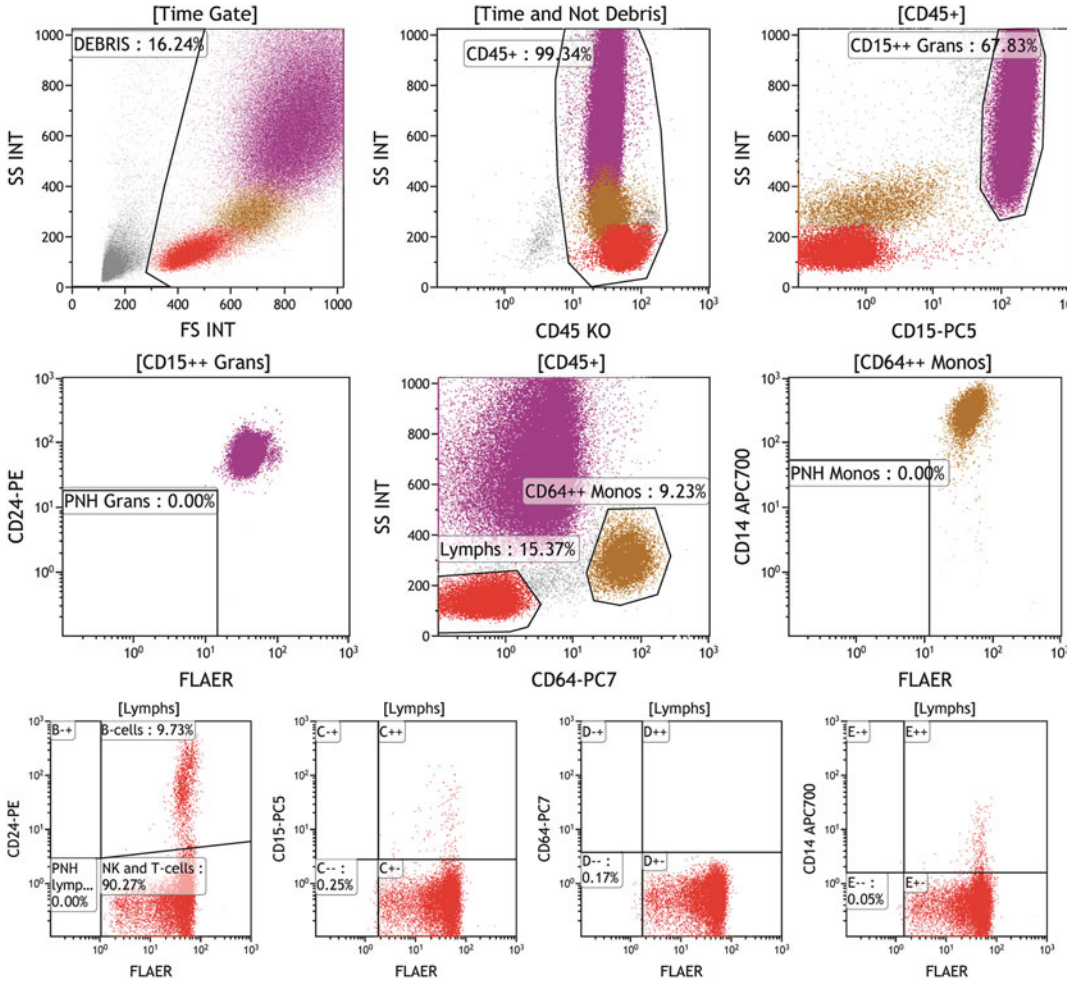

Fig. 6 FLAER/CD24/CD14-based WBC assay with no PNH clone in neutrophils and monocytes. Sample stained with FLAER-Alexa 488/CD24-PE/CD15-PC5/CD64-PC7/CD14-APC700/CD45-Kr0

(a) *TIME (x axis) versus CD45 or SS (not shown) on y axis dot plot* with a "TIME" gate to confirm that no fluidics irregularities are present. This time gate can be adjusted horizontally to include only the events without fluidics issues if necessary.

(b) *Optional dot plot containing FS INT/FS W (x axis) versus FS peak/FS H (y axis)* with a singlet gate (not shown) to be gated on in subsequent plots. (This is not mandatory but may be useful if a large amount of cellular aggregates is present in the sample.)

(c) *FS linear (x axis) versus SS linear (y axis) dot plot* allows for confirmation of all WBC clusters and debris exclusion. All WBC subsets should be clearly visible and optimally separated. There are two approaches, either a "live" gate to be

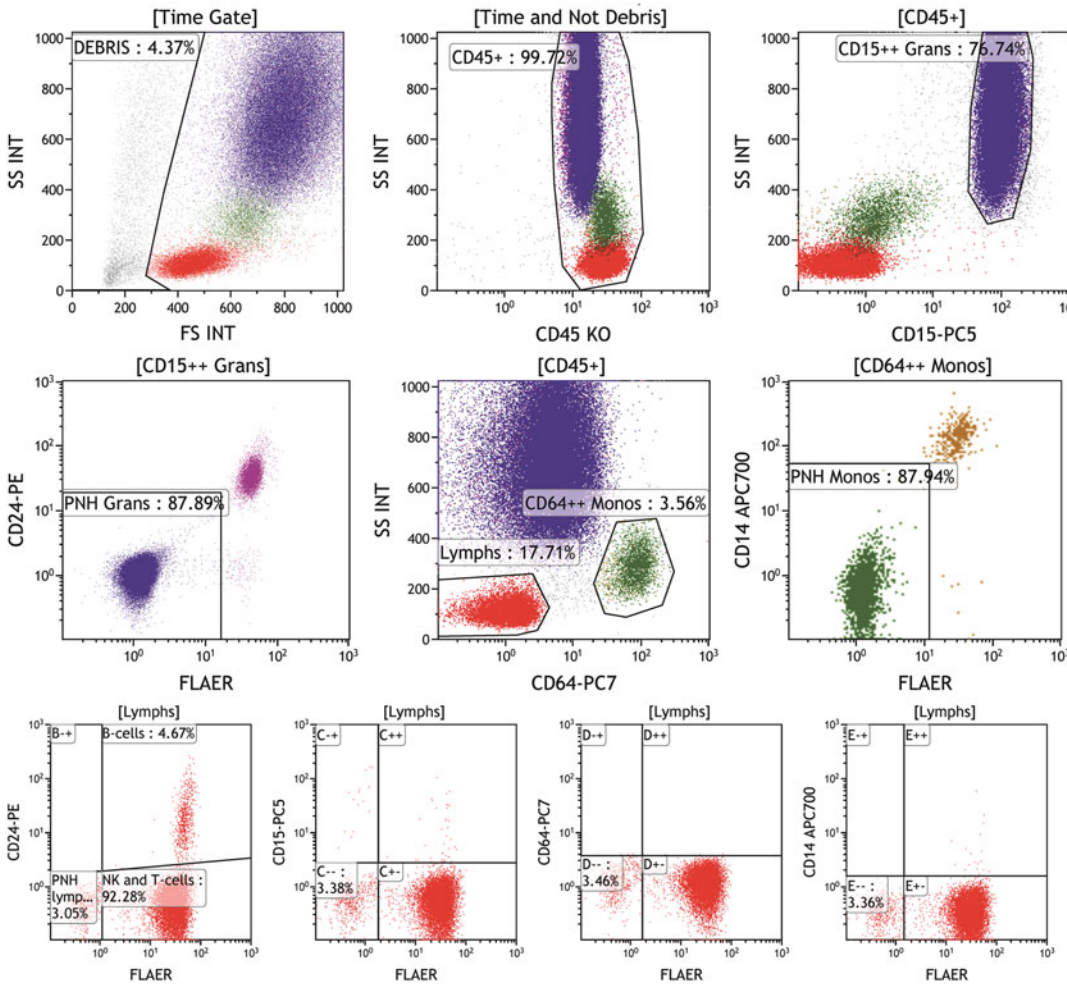

Fig. 7 FLAER/CD24/CD14-based WBC assay with PNH clone present in neutrophils and monocytes. Sample stained with FLAER-Alexa 488/CD24-PE/CD15-PC5/CD64-PC7/CD14-APC700/CD45-KrO

gated on in subsequent plots (shown in Fig. 8) or a "debris" exclusion gate (shown in Figs. 6 and 7) which can be used to gate out the debris in subsequent plots). Make sure that the debris gate excludes the debris (platelets and degenerating granulocytes).

(d) *CD45 (x axis) versus SS (y axis) dot plot* with a "CD45+" gate. Set gate around all CD45+ cell populations (lymphocytes, monocytes, and granulocytes) and exclude CD45-negative events. Gate all subsequent plots on this CD45+ gate. The CD45 vs. SS plot is not only useful for pattern recognition but also useful for excluding any unlysed RBCs and other debris not removed by the debris exclusion gate in the light scatter plot.

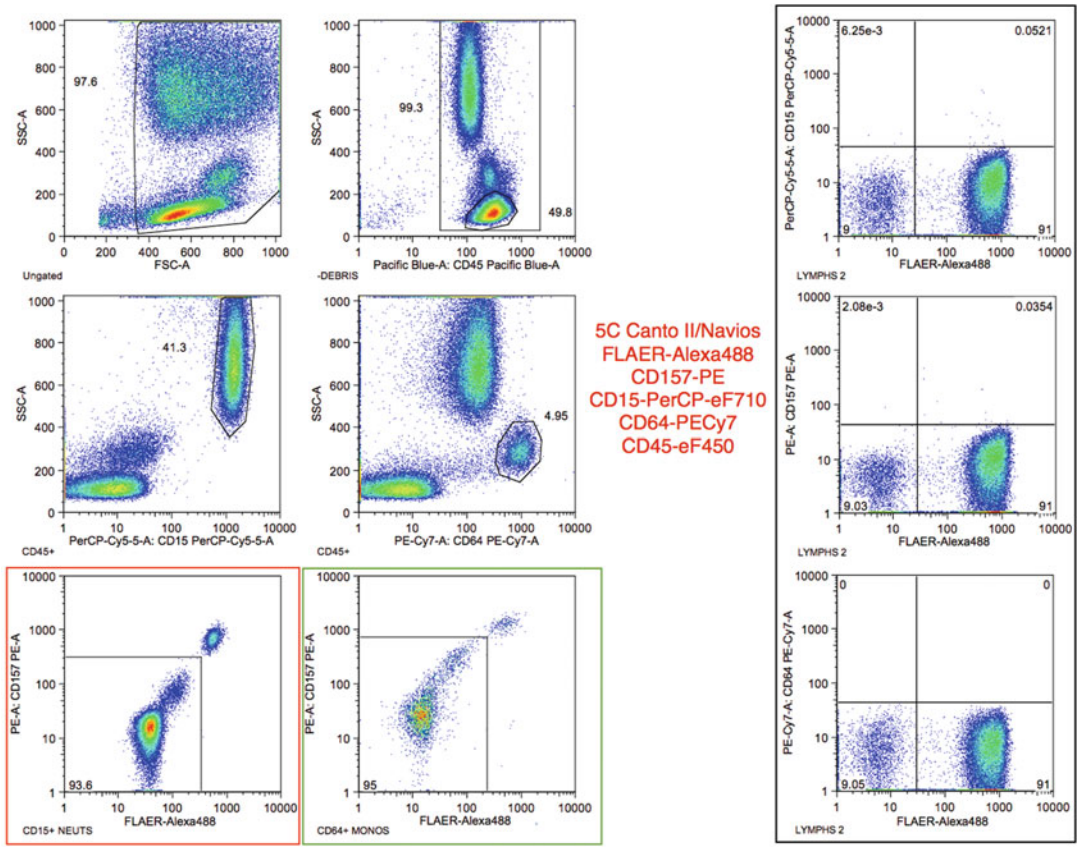

Fig. 8 CD157 and FLAER-based cross-platform assay with PNH clone present in neutrophils and monocytes. Sample stained with FLAER-Alexa 488/CD157-PE/CD15PerCPeFluor710/CD64-PECy7/CD45-eFluor450

(e) *CD15 (x axis) versus SS (y axis) dot plot* is gated on the CD45+ populations and includes a gate drawn around the CD15++ neutrophils excluding as well as possible the CD15 dim+ eosinophils visible to the left of the neutrophil population.

(f) *FLAER (x axis) versus CD24 (y axis) dot plot* is gated on the CD15++ neutrophils, and a region is drawn to encompass the FLAER-negative/CD24-negative cells, which represent the PNH neutrophils. In most cases, the PNH clone in neutrophils is composed of the so-called Type III neutrophils (complete absence of GPI markers FLAER and CD24), but occasionally there may be "Type II" neutrophils (intermediate staining for FLAER and CD24). It is recommended to include both Type III and Type II neutrophils into one combined PNH neutrophil gate.

(g) *CD64 (x axis) versus SS (y axis) dot plot* is gated on the CD45+ cells, and a region is drawn around the CD64++ monocytes.

(h) *FLAER (x axis) versus CD14 (y axis) dot plot* is gated on the CD64++ monocytes, and a region is drawn to delineate the FLAER-negative/CD14-negative cells, which represent the PNH monocytes. As previously described for the neutrophils, typically the PNH clone in monocytes is composed of the so-called Type III monocytes (complete absence of GPI markers FLAER and CD14), but occasionally there may be "Type II" monocytes (intermediate staining for FLAER and CD14). It is recommended to include both Type III and Type II monocytes into one combined PNH monocyte gate. Make sure to exclude the monocytes with bright FLAER but scattered decreased CD14 expression; these events may represent dendritic cells or immature monocytes and should not be included into the PNH clone gate for monocytes.

2. *Internal controls for six-color FLAER/CD24/CD14-based panel*: Optimal instrument setup and standardization in terms of PMT voltage optimization, daily monitoring and computer assisted spectral overlap compensation is a prerequisite for a validated flow cytometry assay. The use of a normal control sample is necessary as an external control to verify the assay performance for PNH RBC and WBC testing. Lymphocytes are not a suitable target population for the PNH clone quantification due to their long life span, but they serve as internal controls for verification of antibody staining and compensation settings in the patient sample. For protocols using FLAER in combination with CD24 for neutrophils and CD14 for monocytes as GPI-specific markers, the following plots should be created and used for analysis:

   (a) *FLAER (x axis) versus CD24 (y axis) dot plot*, which is gated on lymphocytes based on lymphocyte gate set on the CD64 vs. SS plot. This allows for the clear distinction of T lymphocytes (FLAER++/CD24−), NK cells (FLAER+/CD24−), and B lymphocytes (FLAER++/CD24+). Figure 6 demonstrates these internal controls and also confirms that no PNH lymphocytes (FLAER−/CD24−) are seen in this normal sample. Figure 7 shows the same Boolean gating strategy on a PNH patient with the diagnostic plots clearly demonstrating the presence of PNH phenotypes in both neutrophil and monocyte lineages. The lymphocytes (internal controls) also show a much smaller PNH clone in the dual-negative region, thus verifying that the instrument settings and compensation are optimally set.

   (b) *FLAER (x axis) versus CD14 (y axis), CD15 (y axis) and CD64 (Y axis) plots* (Figs. 6 and 7, bottom row) verifies the instrument settings with all subsets visible on scale and clustered in the expected locations.

3. *5-color FLAER/CD157 based single tube cross-platform assay for neutrophils and monocytes*: In this panel neutrophils are gated on CD45 and CD15 and monocytes are gated on CD45 and CD64. A PNH clone can be detected in both the neutrophils and monocytes based on the absence of the GPI markers FLAER and CD157. CD157 is highly expressed on both mature neutrophils and monocytes [30] leading to the possibility that CD157 could replace both CD24 and CD14, allowing for the development of a single tube, high sensitivity 5C assay to identify and quantify both PNH neutrophils and PNH monocytes on cytometers with five or more PMTs [31]. It is important to note that several CD157-negative, non-PNH cases have been observed in the authors' laboratories [23, 24]. For these rare cases, the inclusion of the second GPI reagent (FLAER) as part of the built-in robustness of the assay prevents the misinterpretation of the data as a PNH clone-containing sample; the loss of two GPI-linked structures is required to identify bona fide PNH phenotypes. Furthermore, in keeping with current state-of-the-art guidelines [19, 22–24] the RBC lineage should also be analyzed on every sample tested for the presence of PNH WBCs. As these rare CD157-negative non-PNH samples only contain normal (Type I) RBCs, there is even less chance of misinterpretation. For protocols using FLAER in combination with CD157 for neutrophils and monocytes as GPI-specific markers, the following plots should be created and used for analysis:
   (a) *TIME (x axis) versus CD45 or SS (not shown) on y axis* with a "TIME" gate to confirm that no fluidics irregularities are present. This time gate can be adjusted horizontally to include only the events without fluidics issues if necessary.
   (b) *Optional dot plot containing FS INT (x axis) versus FS peak (y axis)* with a singlet gate (not shown) to be gated on in subsequent plots. (This is not mandatory but may be useful if a large amount of cellular aggregates is present in the sample.)
   (c) *FS linear (x axis) versus SS linear (y axis) dot plot* allows for confirmation of all WBC clusters and debris exclusion. All WBC subsets should be clearly visible and optimally separated. There are two approaches, either a "live" gate to be gated on in subsequent plots (shown for this panel) or a "debris" exclusion gate (as previously shown in Figs. 6 and 7) which can be used to gate out the debris in subsequent plots). Make sure that the debris gate excludes the debris (platelets and degenerating granulocytes).
   (d) *CD45 (x axis) versus SS (y axis) dot plot* with a "CD45+" gate. Set gate around all CD45+ cell populations

(lymphocytes, monocytes, and granulocytes) and exclude CD45-negative events. Gate all subsequent plots on this CD45+ gate. The CD45 vs. SS plot is not only useful for pattern recognition but also useful for excluding any unlysed RBCs and other debris not removed by the debris exclusion gate in the light scatter plot.

(e) *CD15 (x axis) versus SS (y axis) dot plot* is gated on the CD45+ populations and includes a gate drawn around the CD15++ neutrophils excluding as well as possible the CD15 dim+ eosinophils visible to the left of the neutrophil population.

(f) *FLAER (x axis) versus CD157 (y axis) dot plot* is gated on the CD15++ neutrophils, and a region is drawn to encompass the FLAER-negative/CD157-negative cells, which represent the PNH neutrophils (Fig. 8, bottom left). In most cases, the PNH clone in neutrophils is composed of the so-called Type III neutrophils (complete absence of GPI markers FLAER and CD24), but occasionally "Type II" neutrophils (intermediate staining for FLAER and CD157) may also be seen (as shown in Fig. 8). It is recommended to include both Type III and Type II neutrophils into one combined PNH neutrophil gate.

(g) *CD64 (x axis) versus SS (y axis) dot plot* is gated on the CD45+ cells, and a region is drawn around the CD64++ monocytes.

(h) *FLAER (x axis) versus CD157 (y axis) dot plot* is gated on the CD64++ monocytes, and a region is drawn to delineate the FLAER-negative/CD157-negative cells, which represent the PNH monocytes (Fig. 8, bottom middle). As previously described for the neutrophils, typically the PNH clone in monocytes is composed of the so-called Type III monocytes (complete absence of GPI markers FLAER and CD157), but occasionally there may be "Type II" PNH monocytes (intermediate staining for FLAER and CD157). It is recommended to include both Type III and Type II monocytes into one combined PNH monocyte gate. In some cases, the FLAER/CD157 expression of Type II monocytes may be almost as bright as the staining for normal monocytes and careful interpretation and knowledge of the position/staining of normal monocytes is necessary to separate PNH Type II monocytes from normal monocytes.

4. *Internal controls for FLAER-CD157-based panel*: For protocols including FLAER in combination with CD157 for both neutrophils and monocytes as GPI specific markers, the FLAER versus CD157 dot plot, gated on lymphocytes can be less

informative, since CD157 is not expressed on mature T and B lymphocytes. However, basophils are usually present in gated lymphocyte populations with this assay and they provide an internal control in situations where PNH clone sizes in neutrophils and monocytes approach 100%. Meanwhile, the delineation of PNH and non-PNH lymphocytes based on FLAER staining still provides reliable evidence of proper instrument setup (PMT voltage and compensation) and antibody specificity (Fig. 8). Gated lymphocytes are displayed on control plots of FLAER versus CD157, CD15 and CD64 (far right plots) to demonstrate optimal instrument setup and compensation has been established. Technical details of the assay design and reagent selection can be found in [31], and for cross-platform variants in [27].

5 Minor PNH Clones

Regardless of lineage, when less than 1% of the target population is GPI-deficient (*see* Fig. 9), PNH clone sizes have been historically referred to as "minor" [22]. Such small populations of GPI-deficient cells are often encountered in patients with AA and some subsets of MDS [16]. Clinically, these patients do not usually show symptoms of hemolysis but the presence of minor clones in AA has been associated with better response to immunosuppressive therapy [15]. AA patients must be monitored at specific intervals for possible clone size expansion to identify patients, who progress to clinical PNH [17]. From a technical perspective, the same PNH RBC and WBC reagent panels can be used to screen all patient samples. However, for those containing only small numbers of PNH phenotypes, the number of events acquired will need to be increased depending on the sensitivity of the assay as established by the laboratory. The generally accepted smallest number of events required to reproducibly detect a PNH population and determine

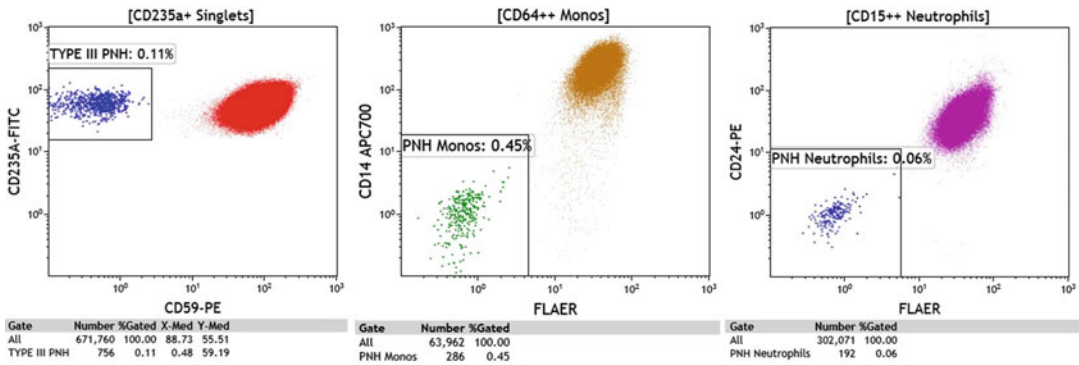

Fig. 9 Minor clone in all three lineages showing a quantifiable PNH clone (>50 PNH cells)

the limit of detection (LOD) is 20 PNH events, the generally accepted smallest number of events required to reproducibly quantify a PNH population and determine lower limit of quantification (LLOQ) is 50 PNH events. Lower levels should be validated in each laboratory.

The characteristics of quasi-quantitative assays and the different relevant parameters are described in more detail in the ICCS/ESCCA PNH Consensus Guidelines, data analysis [19] and validation section [20].

6 Reporting

Based on the recent ICCS/ESCCA PNH consensus guidelines [19], the following components are recommended for a PNH report:

1. Report if a *PNH clone present or absent*. It is important to be clear and to avoid potentially misleading ambiguous terminology. A report stating that a CD59 test is "negative" may imply to some providers that the target population is negative for the GPI marker CD59 (thus indicating a PNH clone) or that no CD59 absence is seen (thus indicating the absence of a PNH clone).
2. Report the *PNH clone size in the RBCs* (total PNH clone size as well as the percentages for Type II and Type III PNH populations). There is a clinical significance associated for Type II and Type III RBCs. Type I RBCs are normal red blood cells with bright CD59 expression and a lifespan of approximately 120 days. Type III PNH RBCs have complete CD59 deficiency, which results in no protection from complement-mediated lysis and a shortened lifespan of 10–15 days. Type II PNH RBCs have partial CD59 deficiency resulting in partial protection from complement mediated lysis. Just as the expression of CD59 on Type II RBCs varies considerably from patient to patient, the lifespan of Type II cells reflects this being intermediate between Type I normal RBCs and Type III PNH RBCs. Since the clinical significance of Type II PNH RBCs and Type III PNH RBCs is well established, it is recommended to report them separately and combined as the total PNH RBC clone.
3. Report the *PNH clone size in both lineages for the WBCs* (neutrophils and monocytes). The PNH monocyte clone is often larger than the neutrophils PNH clone and reporting only the PNH neutrophil clone may underestimate the PNH clone size in the WBCs. Neutrophils and monocytes may also show the presence of Type II populations but the clinical and biological significance of these populations has not been established at

this time. It is therefore recommended to report only the total PNH clone size in the neutrophils and monocytes.

4. *Interpretive terminology of reporting PNH clones based on CSLI H52-A2* [21]:
   (a) PNH population >1%: "PNH clone".
   (b) PNH population 0.1–1%: "minor population of PNH cells" or "minor PNH clone".
   (c) PNH population <0.1%: "rare cells with GPI deficiency" or "rare cells with PNH phenotype.
5. List *all gating and diagnostic markers* used for the PNH assay.
6. State the *lower limit of quantification (LLOQ) for the neutrophil/granulocyte assay and the RBC assay* on the report, stating the recommended LLOQ of 0.05% or better for RBCs (100,000 gated cells) and 0.1% or better for neutrophils (50,000 gated cells). It is important to include this information to the provider as an LLOQ of 1% means that the possibility of a minor clone (less than 1%) cannot be excluded based on this LLOQ.
7. *Retesting recommendations*: see current recommendations and requirements based on International Guidelines for retesting frequencies in PNH [16] and related diseases [17]. Examples of informative PNH reporting templates are shown in reference 19 and Appendix A of its Supplementary Data. An interactive reporting template that contains the above recommendations has recently been developed under the auspices of the Canadian PNH Network and can be downloaded at http://www.pnhnetwork.ca/pnh-resources.

7 Assay Validation

The high-sensitivity PNH assay is a quasi-quantitative assay and the intended use is to evaluate for the presence or absence of a PNH clone with respect to LOD as well as to quantify the PNH clone size if present with respect to LLOQ. The validation process should meet criteria for accuracy, specificity, sensitivity, repeatability, reproducibility and stability [32–34]:

1. The accuracy of a measurement, which refers to the closeness of agreement between the average value of a large number of test results and the true or accepted reference value [33] cannot be directly determined for PNH assays, because of unavailable reference sample. External quality assessment and/or inter-laboratory comparison represent the only available option for validation and mandatory step for ISO accreditation [35].

2. The analytical specificity of the assay reflects the validation of all antibodies/reagents and corresponding fluorochromes, which was discussed in previous section.
3. The clinical specificity represents the ability to exclude abnormal specimen, defined by true negatives/true negatives + false positives and should be determined by assay of a series of samples and scoring for abnormality in comparison to a suitable reference method, such as clinical diagnosis [36].
4. The analytical sensitivity of the assay is determined by the limit of blank (LOB) defined by the highest apparent signal detected in replicates of a sample containing no measurand and the LOD defined by the lowest level of measurand that can be reliably distinguished from the LOB [37]. For the high-sensitivity PNH assays the LOB could be determined by measuring a few replicates of a few negative specimens run over a few separate days and calculating the mean and standard deviation (SD) according to the formula: LOB = mean of blank + 1.645 SD of blank assuming, that 95% of negative values will be below this limit. Typically, the LOB for well-established PNH assays is <0.001% (<10 PNH phenotypes out of 1000,000 acquired events). LOD is closely related but usually greater than LOB and could be determined by measuring a few replicates of a few negative specimens run over a few separate days and calculating the mean and SD according to the formula: LOD = mean of blank + 2SD (3SD) of blank or by measuring a few replicates of a few low positive specimens run over a few separate days and calculating the SD according to: LOD = LOB + 1.645 SD of low positive. It is highly recommended for each lab to perform a "spiking experiment" in order to evaluate the sensitivity of the assay. This is performed by making a serial dilution of PNH blood "spiked" into normal blood which provides various levels of PNH clone sizes down to very low numbers of PNH cells [21]. Alternatively, target LOD could be estimated by measuring a few replicates of a few low positive specimens run over a few separate days and calculating the reproducibility (inter-assay imprecision) expressed as coefficient of variation (CV%) or by confirming, that no more than 5% of the values for a target LOD fall beyond the LOB. The generally accepted smallest number of events required to reproducibly detect a PNH population and determine LOD is 20 PNH events, lower levels should be validated individually.
5. The functional sensitivity of the assay is determined by the LLOQ, representing the lowest level of measurand that can be reliably detected at predefined levels of bias and imprecision [37]. LLOQ is usually greater than LOD and for PNH assays could be determined by measuring a few replicates of a few positive (near the expected LLOQ) specimens run over a few

separate days and calculating the reproducibility (inter-assay imprecision) expressed as CV%, which should be acceptable at levels below 10%. The generally accepted smallest number of events required to reproducibly quantify a PNH population and determine LLOQ is 50 PNH events, lower levels should be validated in each laboratory.

6. The clinical sensitivity or the ability to detect an abnormal specimen and distinguish from normal specimens, defined as true positive/true positive + false negative should be determined by assay of a series of abnormal samples and scoring for abnormality in comparison to a suitable reference method, such as clinical findings [37].
7. The assay performance characteristics are determined by repeatability (intra-assay imprecision) and reproducibility (inter-assay imprecision). It is recommended to assay a few replicates from at least 5 samples within a single analytical run for repeatability and a few replicates from at least 5 samples in separate analytical runs for reproducibility [38]. For confirmation of good performance characteristics CV% below 10% should be obtained for samples with more than 1% target PNH cells, and below 20% for samples with minor clones (<1%).
8. The validation of specimen, processed specimen and reagent stability has been reviewed in previous sections.

8 Additional Options for PNH Testing

8.1 Reagent Selection

Since publication of the 2010 Guidelines [16] a large number of specific clones/conjugates have been assessed for lineage-specific gating and detection of GPI-deficient neutrophils and monocytes.

1. Optimal titration ranges must be established for selected conjugates and validated both individually and in combination with other reagents on both normal and PNH samples.
2. Using this information, high-sensitivity assays were developed using four-color cocktails based on FLAER, CD24, CD15 and CD45 (for neutrophils) and FLAER, CD14, CD64 and CD45 (for monocytes) and (Supplementary Data, 23, 24).
3. Platform-specific versions of these robust four-color assays were developed for clinical cytometers with four or more PMTs [18, 23] and were capable of successful deployment beyond the laboratories of original developers [39]. These assays and reagent sets were found to be highly reliable across FC500 and FACSCalibur instrument platforms in International studies performed under the auspices of the UK NEQAS organization using whole blood stabilized samples [40].

4. Subsequently, five-color single tube assays capable of simultaneous high-sensitivity detection of both PNH neutrophils and PNH monocytes based on FLAER, CD157, CD15, CD64, and CD45 were developed for a variety of clinical cytometers with 5 or more PMTs [23, 31, 41], and again independently validated [42].
5. Instrument-specific CD24/CD14-based 6-color [19, 24, 28, 39, 41] assays have been developed for clinical instruments with six or more PMTs (Beckman Coulter Navios and BD FACSCanto).
6. Most recently, 7-, 6-, and 5-C reagent sets have been developed that can be analyzed on either B-C Navios or BD Biosciences Canto II cytometers or on both [27]. Thus, for large reference flow laboratories that are often equipped with instruments from multiple manufacturers, a single reagent set can be employed and analysis performed on whichever instrument platform is available at the time. Because these reagent sets utilize all three lasers in Navios and Canto II platforms, instrument setup and compensation are extremely simple with such an approach, and maximal separation of PNH WBC phenotypes from their normal counterparts is thereby optimized. As indicated above, it is advisable to evaluate both neutrophils as well as monocytes as the latter may reflect the size of the PNH clone more accurately in some cases (*see* Fig. 12 in [19]).

8.2 Instrument-Specific Reagent Cocktails for Four-, Five-, and Six-Color WBC Assays

1. Note that cocktailing of reagents is very important for WBC assays, just as it is for the RBC assay. If reagents are added one at a time, there is increased risk of failing to add individual reagents, or adding too much or too little, especially where very small volumes of some individual reagents need to be accurately pipetted. Such errors run the risk of generating "laboratory-induced PNH" if the GPI-specific reagents are inadvertently omitted. For laboratories that run the assay infrequently, there is an increased requirement to ensure that the reagents used in the assay are working as specified. In this setting, it is recommended that the laboratory stain a normal sample at least once per month to validate the performance of all reagents used in the assay [18, 22].
2. As detailed elsewhere, the Practical Guidelines [18] defined two 4-color reagent cocktails for the high sensitivity detection of PNH neutrophils and PNH monocytes for both Beckman FC500 and BD FACSCalibur platforms. Subsequent studies using these assays/reagent sets have shown them to be reproducible and robust using fresh samples across multiple laboratories [39] and in International studies performed with stabilized whole blood samples under the auspices of the UK NEQAS organization [40].

3. As also detailed elsewhere [23, 24, 31], the availability of a bright PE conjugate of the SY11B5 CD157 clone allowed for the development of a five-color (FLAER, CD157-PE, CD64-ECD, CD15PCy5, CD45-PC7), single tube, high-sensitivity assay for both neutrophils and monocytes that could be performed on the Beckman FC500 and Navios instruments. A variant taking advantage of all three lasers of the BD FACSCanto II (FLAER, CD157-PE, CD15-PerCPeFluor710, CD64-APC, and CD45eFluor450) was also developed that could also be deployed on the three-laser Navios [23]. The five-color assays were also robust and reproducible as evidenced by other studies comparing a six-color (FLAER, CD24, CD14, CD15, CD64, and CD45) cocktail with the CD157-based five-color assay [42]. It should be noted that the neutrophils and monocytes of occasional non-PNH patient samples analyzed with CD157/FLAER based assays fail to express CD157 [23, 24]. While a polymorphism in the CD157 gene has been shown to be responsible for some of the CD157-negative cases [43], this explanation does not account for all of them [44]. While the biological cause(s) of this remains under study, the mandatory inclusion of the second GPI reagent (FLAER) is part of the built-in robustness of the assay and prevents the misinterpretation of the data as a "PNH clone-containing sample"; the loss of two GPI-linked structures per lineage is required to identify bona fide PNH phenotypes. Furthermore, in keeping with current state-of-the-art guidelines [19, 22–24, 41], the RBC lineage was also analyzed and in every CD157-negative case there was no evidence of any PNH phenotypes confirming the finding as an isolated single (CD157) GPI deficiency. An example of a CD157-negative case using a seven-color combination (FLAER, CD157-PE, CD15-PerCPeFluor710, CD64-PECy7, CD24-APC, CD14-APCeFluor780, and CD45-eFluor450) is shown (Case 14) in the Supplementary data from [19]. Such CD157-negative, non-PNH cases should simply be reported as "No evidence of PNH in RBCs and WBCs" [24].
4. A number of six-color, single tube, high-sensitivity assays have been developed whose gating strategies are also derived from the original four- and five-color assays. As shown below, a Navios-specific cocktail comprising FLAER, CD24-PE, CD15-PC5, CD64-PC7, CD14-APC700, and CD45-KO was used to stain a normal (and PNH sample (Figs. 6 and 7). Neutrophils and monocytes are gated by a combination of light scatter, CD45 expression and either CD15 staining (neutrophils) or CD64 staining (monocytes). PNH phenotypes are detected and/or quantified based on their loss of FLAER and CD24 binding (neutrophils) or FLAER and CD14 binding

(monocytes) [41]. Similar six-color reagent sets have also been developed previously for the BD FACSCanto platform [39, 42].

8.3 Reagent Cocktails for Cross-Platform Assays

1. Recently, six-color cocktails comprising FLAER, CD157-PE, CD15-PerCP-eFluor710 (or CD15-PerCPCy5.5) CD64-PECy7, CD24-APC, and CD45-eFluor450 have been developed [27] that can be performed on both Navios and Canto II instruments (*see* Fig. 16 of [24]).
2. Samples can be stained with this 6-C reagent set and be analyzed on either Navios or three-laser Canto variants or on both.
3. In addition, a five-color version of this cross-platform assay (FLAER, CD157-PE, CD15PerCPCy5.5, CD64-APC, and CD45eFluor450) has also been developed [27]. Specific reagent cocktails, recommended clones and conjugated forms used in these 5C, 6C, and 7C cross-platform (3-laser Navios and Canto II instruments) assays are detailed in [27] and shown in Table 4.

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