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Immunophenotyping and Intracellular Staining of Fixed Whole Blood for Mass Cytometry (CyTOF)

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[Abstract] In this report, we present the implementation of mass cytometry for intracellular staining using fixed whole blood. In our assay described here, 250 µl of whole blood, is stimulated *in vitro* with PMA/ionomycin (or left unstimulated), in the presence of secretion inhibitors (brefeldin A and monensin), lysed-fixed using SMART TUBE buffers, barcoded (optional), surface stained, fixed, stained for intracellular markers, fixed and DNA stained. Using 250 µl of whole blood from a healthy donor, we show that the expression of major lineage populations such as T cells, B cells, NK cells and monocytes, as well as cytokines such as $CD4^+$ and $CD8^+$ IFNγ and TNFα across multiple batches (n = 27) is consistent, with the co-efficient of variation (CVs) ≤ 21%, implying minimum inter-variability. For each major cell type, the percentage is reported as a percent of singlets. The percentage of cytokine expression in response to stimulation is reported as a percent of the immediate parent cell type. This protocol has a number of benefits: from a biological perspective, it can be applied to clinical studies especially where blood draw volumes are limiting. Technically, the protocol can be adapted for barcoding, which adds the benefits of more uniform sample staining as well as antibody conservation especially for large study cohorts. Finally, for studies involving infectious diseases including the current global COVID-19 pandemic, this protocol permits infectious samples to be fixed prior to processing and staining, thereby reducing biosafety risks.

Keywords: CyTOF, Mass cytometry, Flow cytometry, Barcoding, Fixed whole blood, Immune profiling

[Background] Non-invasive methods to obtain biological samples and single cell technologies are much sought after, as they provide an opportunity to comprehensively study human diseases. Blood not only provides for a minimally-invasive, cost effective and readily accessible source of immunological sample, but whole blood stimulation also serves as the closest mimic of the *in vivo* condition. Single-cell Mass Cytometry (or Cytometry by Time of Flight mass spectrometry, CyTOF) is ideally suited to broad profiling of the immune system, since it allows for > 40 parameter panels, with little to no spillover between channels, which is a significant advancement over the procedural limitations of fluorescence flow cytometry (Leipold *et al.*, 2015). Conversely, cell acquisition speed is significantly lower, and cell loss significantly higher, for CyTOF compared to fluorescence flow cytometry. In this report, we show the establishment of a reference panel of 39-anti human antibodies for mass cytometry that broadly identifies the major immune cell types, well established T and B cell subpopulations, activation markers,

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cytolytic markers and cytokines. To characterize these immune cell lineages and their functional states, the panel of anti-human heavy metal-conjugated monoclonal antibodies was selected to target the epitopes shown in Table 3, and to be compatible with fixed whole blood samples (Fernandez and Maecker, 2015 and our unpublished data). The panel described here, comprises an almost equal number of pre-made and in-house conjugates. To build this panel, we implemented the recommended panel design guidelines for mass cytometry. Low-abundance targets were allocated to higher-sensitivity channels and antibodies were designated to channels to minimize potential spectral overlap (Takahashi *et al.*, 2017). All antibodies in the designed panel were then titrated to best discriminate the positive population from the negative. This panel can be further customized for specific hypothesis driven studies. In addition, by integrating barcoding (Behbehani *et al.*, 2014) within this framework, we also demonstrate the compatibility of this panel with large clinical studies, to minimize technical variability. The ability to freeze fixed blood samples is also convenient for assembly of sample sets for retrospective batched analysis, with decreased biosafety risks in the setting of infectious disease. With regard to SARS-CoV-2 or other infectious agents that might be present, whole blood fixation by the method described here has not been proven to be fully inactivating. But given the known effects of fixation on viral infectivity (Möller *et al.*, 2015), it provides an increased level of safety.

Materials and Reagents

1. Parafilm (PARAFILM, catalog number: H32207017002)
2. BD Vacutainer™ Plastic Blood Collection Tubes with Sodium Heparin: Conventional Stopper (Fisher Scientific, catalog number: 367874)
3. 96 Well, Square V-bottom deep well plate (Costar/Corning, catalog number: 3960)
4. Universal Lids for deep well plates (Corning, catalog number: 3099)
5. 5 ml polystyrene round-bottom tube with cell strainer cap (Falcon Coring, catalog number: 352235)
6. 1.8 ml cryotubes (NUNC, catalog number: 375418)
7. 50 ml polystyrene reservoir (Costar/Corning, catalog number: 4870)
8. 15 ml and 50 ml polypropylene conical tubes (Falcon, catalog numbers: 352096 and 352070, respectively)
9. 0.5 ml PCR tubes (Corning, catalog number: 3750)
10. 1.7 ml Microcentrifuge tubes (GeneMate, catalog number: C-3260-1)
11. 10 ml serological pipettes (Corning, catalog number: 4488)
12. 25 ml serological pipettes (Falcon, catalog number: 357525)
13. Freshly drawn whole blood in heparin green top tubes, from healthy donors or patients
14. EQ™ Four Element Calibration Beads (Fluidigm, catalog number: 201078)
15. Erythrocyte Lysis Buffer (Qiagen, catalog number: 79217)
16. Brefeldin A (Sigma-Aldrich, catalog number: B7651, stock concentration 5 mg/ml)
17. Monensin (Sigma-Aldrich, catalog number: M5273, stock concentration 5 mg/ml)

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18. Phorbol 12-myriate 12 acetate (PMA) (Sigma-Aldrich, catalog number: P8139, stock concentration 1 mg/ml)
19. Ionomycin (Sigma-Aldrich, catalog number: I0634, stock concentration 1 mg/ml)
20. Stable Lyse-V2 (SMART TUBE Inc., catalog number: 3L7080)
21. Stable Store-V2 (SMART TUBE Inc., catalog number: 3S1988)
22. BD FACS™ Lysing Solution (BD, catalog number: 349202)
23. Cell-ID™ 20-Plex Pd Barcoding Kit (Fluidigm, catalog number: 201060)
24. 16% paraformaldehyde (PFA) (Alfa Aesar, catalog number: 43368)
25. Phenotypic and intracellular antibodies (filtered through 0.1 µm spin filters) (Millipore, catalog number: UFC30VV00)
26. 10x phosphate-buffered saline (PBS) (ROCKLAND, catalog number: MB-008)
27. MilliQ water
    Note: Beakers or bottles used to store MilliQ water are not washed with soap, due to the barium content of most commercial soaps.
28. BSA (Sigma-Aldrich, catalog number: A7284)
29. 10% Sodium Azide (Teknova, catalog number: S0209)
30. 0.5 M EDTA (Gibco, catalog number:15575-038)
31. Cell-ID™ Intercalator Ir (Fluidigm, catalog number: 201192A)
32. 10x saponin-based permeabilization buffer (eBioscience, catalog number: 00-8333-56)
33. CyPBS (see Recipes)
34. CyFACS buffer (see Recipes)

Equipment

1. -80 °C freezer
2. Incubator at 37 °C, 5% CO_2
3. Biosafety Cabinet (BSL-2 rated) or appropriate biosafety cabinet recommended for the pathogen being tested
4. Centrifuge (Eppendorf Centrifuge 5810R)
5. p2, p10, p20, p100, p200, p1000 single-channel calibrated micropipettes (Rainin)
6. p200 and p1200 multi-channel calibrated micropipettes (Rainin)
7. 12 pin aspirator (V&P Scientific, Inc., catalog number: VP 187PC-3S)

Procedure

A. Whole Blood Stimulation and Fixation
   1. Use a 96-well deep well plate and label the wells with the unstimulated (US) and stimulated conditions (PMA/ionomycin). Keep PMA/ionomycin stimulated wells spatially separated (at least one well apart) from unstimulated or antigen-stimulated wells, to avoid cross-contamination and

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false positive signals.

2. Invert the heparin green top tube a few times to mix the freshly drawn whole blood.
3. Using a p1000 single-channel pipette, transfer 250 µl of whole blood (WB) into the labeled deep well plate.
4. Add secretion inhibitors Brefeldin A and Monensin to both unstimulated and stimulated conditions. Add PMA/ionomycin stimulants to the wells containing whole blood to be stimulated (Tables 1 and 2). Pipette up and down to mix well.

Table 1. Protein secretion inhibitors

Reagent	Stock concentration	Intermediate dilution	Final concentration in cell stimulation wells
Brefeldin A	5 mg/ml in DMSO (stored in aliquots at -20 °C)	1:10 in CyPBS	5 µg/ml (1:100), 2.5 µl in 250 µl
Monensin	5 mg/ml in ethanol (stored in aliquots at -20 °C)	1:10 in CyPBS	5 µg/ml (1:100), 2.5 µl in 250 µl

Table 2. Activators

Reagent	Stock concentration	Intermediate dilution	Final concentration in cell stimulation wells
Phorbol 12-myristate 13-acetate (PMA)	1 mg/ml in DMSO (stored in aliquots at -20 °C)	1:1,000 in CyPBS	10 ng/ml, 2.5 µl in 250 µl
Ionomycin	1 mg/ml in DMSO (stored in aliquots at -20 °C)	1:10 in CyPBS	1 µg/ml, 2.5 µl in 250 µl

5. Incubate the deep well plate for 4 h in an incubator at 37 °C, 5% CO_2.
6. At the end of the incubation, add 350 µl Stable-Lyse V2 (at room temperature) per 250 µl whole blood. Add the Stable-Lyse V2 in the same order as the addition of the secretion inhibitors and stimuli to the whole blood to maintain a consistent incubation time. Pipette up and down to mix the contents of the well. Incubate at room temperature (RT) for 15 min.
7. At the end of the 15 min incubation, immediately add 1,000 µl Stable-Store V2 (at room temperature) per 250 µl whole blood. Add the Stable-Store V2 in the same order to the wells as maintained in Steps A4 and A6 to maintain a consistent incubation time. Pipette up and down to mix well. Incubate at RT for 15 min. Total volume per well will be 1.6 ml (250 µl WB + 350 µl stable-lyse V2 + 1,000 µl stable-store V2).
8. At the end of the incubation, transfer 1.6 ml of the unstimulated and stimulated fixed whole blood sample to cryo-labeled tubes and place in -80 °C freezer until the samples are ready to be thawed and stained.

   Note: Samples are transferred to cryovials for freezing, to facilitate quick sample-thaw. Depending on the study design, samples, may be frozen in the deep well plate.

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B. Thaw Fixed Whole Blood Samples

1. Remove samples from -80 °C freezer and thaw in cold water for about 15 min.
2. Transfer the WB samples (1.6 ml) to a deep well plate.
3. Centrifuge cells at 974 *x g* for 10 min at 4 °C.
4. Aspirate the supernatant using the 8-12 pin aspirator.
5. (Optional step) If red blood cells (RBCs) are observed upon thawing the samples, add 1 ml of Erythrocyte Lysis Buffer (does not contain fixative). Incubate at RT up to a maximum of 10 min (watch for lysis). Stop the lysis by adding 0.8 ml of CyFACS buffer and proceed to Step B7.
6. If no RBCs are observed, add 1 ml CyFACS buffer.
7. Centrifuge cells at 974 *x g* for 10 min at 4 °C.
8. Repeat wash with 1 ml CyFACS buffer.
9. Centrifuge cells at 974 *x g* for 10 min at 4 °C.
   Note: Cell count/sample post 2 CyFACS washes should be ~1 x 10^6 cells.
10. Cells are now ready to be barcoded or surface stained as required.
    Note: If samples are not barcoded, skip Procedure C and proceed to Procedure D of surface staining.

C. Barcoding

1. Barcode Perm Buffer: Prepare 4 ml for each sample to barcode by mixing 1 part Maxpar 10x Barcode Perm with 9 parts Maxpar PBS; store at 4 °C for up to one week.
2. Wash each sample with 1 ml Barcode Perm Buffer. Centrifuge cells at 974 *x g* for 10 min. Aspirate supernatant from the cells.
3. Repeat washes 2x with 1 ml Barcode Perm Buffer.
4. Resuspend each sample to be barcoded completely in 800 µl Barcode Perm Buffer.
5. Resuspend each barcode tube containing 10 µl of the pre-mixed barcode completely in 100 µl Barcode Perm Buffer.
6. Transfer 110 µl of barcodes to the appropriate samples.
7. Mix the sample with the barcodes immediately and completely by pipetting.
8. Incubate the samples with the barcodes for 30 min at RT.
9. Wash cells thrice with 2 ml of Maxpar Cell Staining Buffer. Centrifuge cells at 974 *x g* for 10 min. Aspirate supernatant from the cells.
10. Resuspend in 100 µl Maxpar Cell Staining Buffer.
11. Combine all barcoded samples into one well.
12. Centrifuge cells at 974 *x g* for 10 min. Aspirate supernatant from the cells.

D. Surface staining

1. Make the surface antibody cocktail in CyFACS buffer of metal-chelating polymer-labeled surface antibodies according to previously determined titration. Prepare sufficient volume of the antibody cocktail (Table 3) to add 200 µl of the cocktail for a barcoded pool of 10 samples or 70

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µl of the antibody cocktail/ sample for non-barcoded samples.

Table 3. CyTOF panel for the phenotypic and functional analysis of immune cell subsets in fixed whole blood

Metal Label	Specificity	Ab. Clone	Source	Staining step
89 Y	CD66b	G10F5	In-house	Surface
102Pd-110Pd	Barcode	optional		
113Id	CD57	HCD57	In-house	Surface
140Ce	Beads	n/a		
141Pr	HLA-DR	L243 (G46-6)	In-house	Surface
142Nd	CD19	HIB19	Fluidigm	Surface
143Nd	IL-10	JES3-9D7	In-house	Intracellular
144Nd	1L-4	MP4-25D2	Fluidigm	Intracellular
145Nd	CD4	RPA-T4	Fluidigm	Surface
146Nd	CD8	RPA-T8	Fluidigm	Surface
147Sm	CD20	2H7	Fluidigm	Surface
148Nd	CD40	5C3	In-house	Surface
149Sm	CD11c	Bu15	In-house	Surface
150Nd	CD123	6H6	In-house	Surface
151Eu	CD107a	H4A3	Fluidigm	Intracellular
152Sm	TNFα	Mab11	Fluidigm	Intracellular
153Eu	CD45RA	HI100	Fluidigm	Surface
154Sm	CD3	UCHT1	Fluidigm	Surface
155Gd	CD45	HI30	In-house	Surface
156Gd	CD38	HB-7	In-house	Surface
157Gd	CD25	M-A251	In-house	Surface
158Gd	CD33	P67.6	In-house	Surface
159Tb	GM-CSF	BVD2-21C11	Fluidigm	Intracellular
160Gd	CD14	M5E2	Fluidigm	Surface
161Dy	IFNγ	4S.B3	In-house	Intracellular
162Dy	CD69	FN50	Fluidigm	Intracellular
163Dy	TCRγδ	B1	In-house	Surface
164Dy	IL-17	N49-653	Fluidigm	Intracellular
165Ho	CD127	A019D5	Fluidigm	Surface
166Er	IL-2	MQ1-17H12	Fluidigm	Surface
167Er	CD27	L128	Fluidigm	Surface
168Er	CD154 (CD40L)	24-31	Fluidigm	Intracellular
169Tm	CCR7	150503	In-house	Surface
170Er	PD1	EH12.1	In-house	Surface
171Yb	Granzyme B	GB11	Fluidigm	Intracellular
172Yb	FcRL5	509f6	In-house	Intracellular
173Yb	Perforin	B-D48	In-house	Intracellular
174Yb	CD21	Bu32	In-house	Surface

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Table 3. Continued

175Lu	IgD	IA6-2	In-house	Surface
176Yb	CD56	NCAM16.2	Fluidigm	Surface
191Ir	DNA1	n/a		
193Ir	DNA2	n/a		
209Bi	CD16	3G8	Fluidigm	Surface

2. Transfer the surface antibody cocktail into a 0.1 µm spin filter and centrifuge using a tabletop microcentrifuge (RCF = 14,000 or max speed) for 2 min at room temperature.
3. For a barcoded sample pool (x 10 conditions) add 200 µl surface antibody cocktail.
4. Add 70 µl surface antibody cocktail to each non-barcoded sample.
5. Incubate the cells at RT for 30 min.
6. Wash cells with 1 ml CyFACS buffer. Centrifuge cells at 974 *x g* for 10 min. Discard supernatant by aspiration.
7. Repeat CyFACS wash. Centrifuge cells at 974 *x g* for 10 min. Aspirate the supernatant.
8. Fix: Add 1.8 ml of BD FACS Lysing solution (stock diluted 1x with MilliQ water) to all samples.
9. Incubate cells overnight at 4 °C.

E. Intracellular staining
1. Centrifuge cells at 974 *x g* for 10 min at 4 °C. Discard supernatant by aspiration.
2. Prepare 1x Perm Buffer (1:10 dilution with MilliQ water). Add 1 ml Perm buffer.
3. Centrifuge cells at 974 *x g* for 10 min at 4 °C.
4. Repeat wash with Perm buffer.
5. Centrifuge cells at 974 *x g* for 10 min at 4 °C.
6. Make the intracellular antibody cocktail (Table 3) in CyFACS buffer of metal-chelating polymer-labeled surface antibodies according to previously determined titration. Prepare sufficient volume of the antibody cocktail to add 200 µl of the cocktail for a barcoded sample pool of 10 samples and 70 µl of the antibody cocktail/sample for non-barcoded samples.
7. Transfer the intracellular antibody cocktail into a 0.1 µm spin filter and centrifuge using a tabletop microcentrifuge (RCF = 14,000 or max speed) for 2 min at room temperature.
8. For a barcoded sample pool (x 10 conditions) add 200 µl of intracellular antibody cocktail.
9. Add 70 µl intracellular antibody cocktail to each non-barcoded sample.
10. Incubate the cells at RT for 30 min.
11. Wash cells with 1 ml CyFACS buffer. Centrifuge cells at 974 *x g* for 10 min. Discard supernatant by aspiration.
12. Repeat 2 x CyFACS washes. Centrifuge cells at 974 *x g* for 10 min. Discard supernatant by aspiration.
13. Fix: Prepare 2% PFA from 16% PFA in CyPBS.
14. Add 200 µl of 2% PFA in CyPBS to each sample.
15. Incubate at 4 °C overnight.

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F. DNA staining

1. Add 1 ml CyFACS buffer to each sample.
2. Centrifuge cells at 974 *x g* for 10 min at 4 °C. Discard supernatant by aspiration.
3. Prepare 1:1,000 dilution in 2% PFA (in CyPBS) of Ir-intercalator.
4. Add 300 µl of diluted Ir-intercalator to each sample and pipet to mix thoroughly.
5. Incubate at RT for 20 min.
6. Wash 2 x in CyFACS buffer. Centrifuge cells at 974 *x g* for 10 min at 4 °C. Discard supernatant by aspiration.
7. Wash 2 x with MilliQ water. Centrifuge cells at 974 *x g* for 10 min at 4 °C. Discard supernatant by aspiration.
8. Using a cell strainer, dilute cells with EQ™ Four Element Calibration Beads (1:10 dilution with MilliQ water). Aim for a cell concentration of 0.6-1.0 million cells/ml.
9. Acquire samples (250k events per sample) on the CyTOF machine, after standard instrument set up.

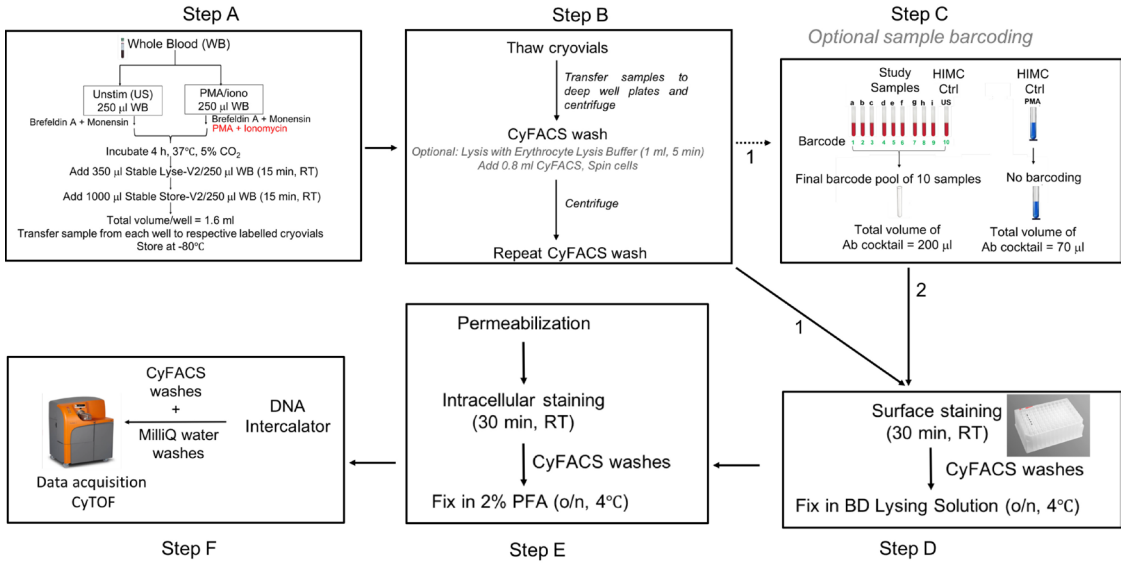

Figure 1. Schematic of the steps described in the procedure

Data analysis

Identification of Cell Lineages and Functional Subsets

After acquisition on the Helios instrument, FCS files are obtained for downstream analysis. If EQ beads (Fluidigm) are added, the files can be normalized based on EQ bead intensity, using the Nolan Lab MATLAB normalizer available freely on github.com (https://github.com/nolanlab/bead-normalization/releases). The normalized files can then be uploaded to Cytobank (www.cytobank.org) or analyzed in another software such as FlowJo (BD Biosciences) for manual gating. A gating schematic for both barcoded and non-barcoded samples (Figures 2A, 2B, respectively) is shown to

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demonstrate the application of the panel to both approaches. Hierarchical gating is performed using ^{191}Ir and ^{193}Ir DNA intercalator, ^{140}Ce beads and the event length parameter to discern intact singlets from debris and cell aggregates. All other major immune cell populations are sequentially identified using the lineage surface protein markers as indicated in the respective lineage plots (Figures 2A and 2B). Our panel also enables the identification of immune cell subpopulations such as memory T subsets (CD45RA, CD27 on $CD4^+$ and $CD8^+$ T cells), Tregs ($CD4^+CD25^{hi}CD127^{low}$), activation markers (HLA-DR and CD38 on $CD4^+$ and $CD8^+$ T cells), plasmablasts ($CD27^{hi}CD38^{hi}$ on $CD19^+CD20^+$ B cells) and stages of isotype-switched naïve and memory B cells (CD27, IgD on $CD19^+CD20^+$ B cells) as indicated in the respective defined plots (Figures 2A and 2B). Figure 2C shows the cytokine expression of TNFα and IFNγ upon PMA/ionomycin stimulation on $CD4^+$ and $CD8^+$ T cells.

Data Reproducibility across Different Batches

To test the reproducibility of the panel, we analyzed data from replicate frozen vials of a fixed control blood sample, previously unstimulated or stimulated with PMA+ionomycin. These control sample replicates were stained and run in 27 separate batches. For each batch, we processed the unstimulated sample replicate in the context of 10 barcoded samples that included other donors and stimulations (Figure 2A), whereas the PMA+ionomycin stimulated replicate was stained and run independently in the same batch (without barcoding). This was to prevent false positive signals due to potential contamination of barcoded samples with the highly-stimulated PMA+ionomycin sample (Figure 2B). We calculated the frequency of the lineage populations using singlets as the parent population (Figure 3A) as well as frequency of TNFα and IFNγ for both $CD4^+$ and $CD8^+$ T cells (Figure 3C) (Leipold *et al.*, 2018). The percent co-efficient of variation (CV) was calculated to determine the degree of variability of expression across batches (Leipold *et al.*, 2018). Our analysis showed that the frequency of parent is comparable (CV ≤ 21%) across the 27 batches for both the lineage populations (Figure 3B) and cytokine expression (Figure 3D), suggesting minimum technical variability.

In summary, this study demonstrates the establishment and assessment of an ICS antibody panel for fixed whole blood that can be adapted to a barcoding approach.

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Representative Data

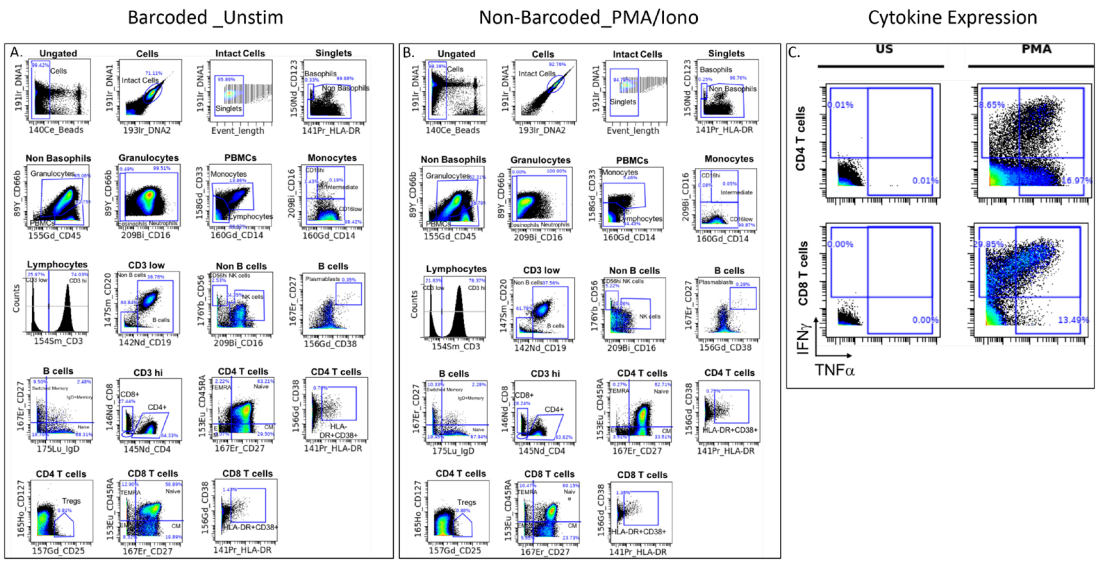

Figure 2. Schematic gating of fixed whole blood. The gating scheme is shown for fixed whole blood from a healthy donor processed in the same batch. The unstimulated condition (A) was included in a barcoded pool of 10 samples, while the PMA/ionomycin stimulated sample (B) was treated independently. Panel C shows the expression of two cytokines IFNγ and TNFα for CD4 and CD8 T cells.

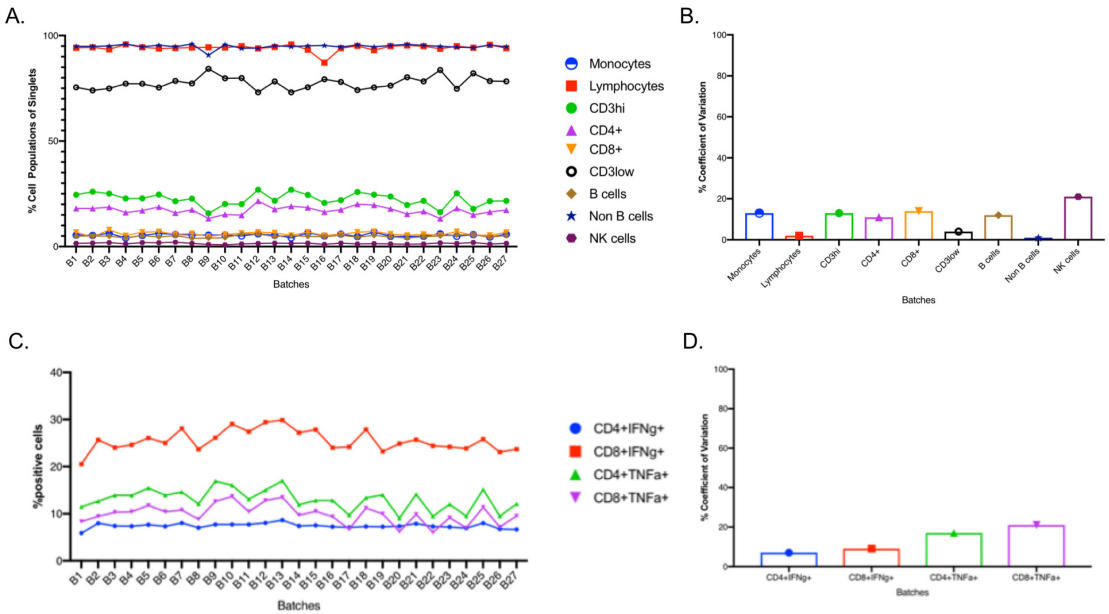

Figure 3. Expression of cell populations and cytokines across batches. A and C show the frequencies of cell lineages and functional subsets, respectively, across 27 batches of healthy control sample replicates. Frequencies are expressed as a percentage of singlets (3A) or of $CD4^+$ or $CD8^+$ T cells (3C). B and D show the percent coefficient of variation calculated across the 27 batches for the cell lineages (3B) and functional subsets (3D).

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Recipes

1. CyPBS
   1x PBS without heavy metal contaminants, such as 10x PBS
   Made in MilliQ water
   Sterile filter before use
2. CyFACS buffer
   1x CyPBS with 0.1% BSA
   2 mM EDTA (from 0.5 M EDTA stock)
   0.05% sodium azide (from 10% stock)
   Made in MilliQ water
   Sterile filter before use

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Competing interests

The authors declare that they have no conflicts or competing interests.

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