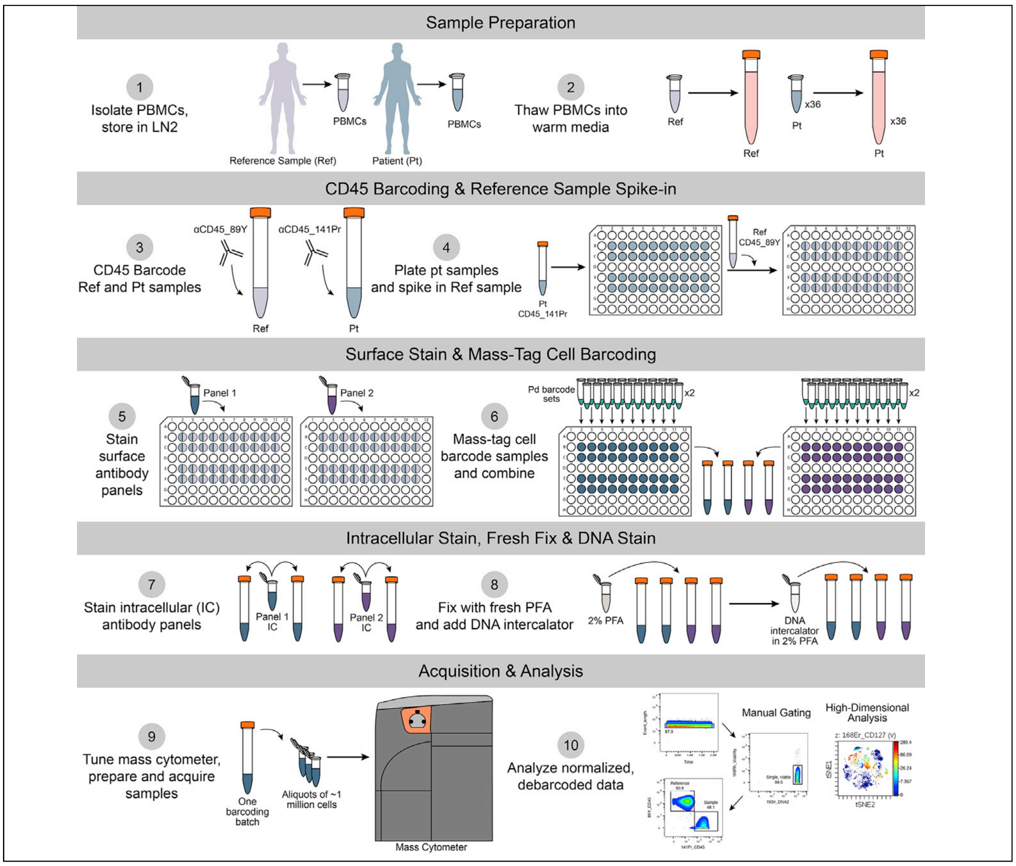
STAR Protocols

Protocol

High-Throughput Mass Cytometry Staining for Immunophenotyping Clinical Samples



As mass cytometry (MC) is implemented in clinical settings, the need for robust, validated protocols that reduce batch effects between samples becomes increasingly important. Here, we present a streamlined MC workflow for high-throughput staining that generates reproducible data for up to 80 samples in a single experiment by combining reference sample spike-in and palladium-based mass-tag cell barcoding. Although labor intensive, this workflow decreases experimental variables and thus reduces technical error and mitigates batch effects.

Emily M. Thrash, Katja Kleinsteuber, Emma S. Hathaway, Matthew Nazzaro, Eric Haas, F. Stephen Hodi, Mariano Severgnini

emily_thrash@dfci.harvard.edu (E.M.T.)
mariano_severgnini@dfci.harvard.edu (M.S.)

HIGHLIGHTS

Design and development of custom mass cytometry 35+ antibody panels

Sample barcoding, reference sample spike-in strategy controls for batch effects

Achieve stability and reproducibility for large-scale mass cytometry experiments

Thrash et al., STAR Protocols *1*, 100055
September 18, 2020
https://doi.org/10.1016/j.xpro.2020.100055

STAR Protocols

CellPress
OPEN ACCESS

Protocol

High-Throughput Mass Cytometry Staining for Immunophenotyping Clinical Samples

Emily M. Thrash,[1,3,*] Katja Kleinsteuber,[1] Emma S. Hathaway,[1] Matthew Nazzaro,[1] Eric Haas,[2] F. Stephen Hodi,[1] and Mariano Severgnini[1,4,*]

[1]Center for Immuno-Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, USA

[2]Longwood Medical Area CyTOF Core, Dana-Farber Cancer Institute, Boston, MA 02215, USA

[3]Technical Contact

[4]Lead Contact

*Correspondence: emily_thrash@dfci.harvard.edu (E.M.T.), mariano_severgnini@dfci.harvard.edu (M.S.)
https://doi.org/10.1016/j.xpro.2020.100055

SUMMARY

As mass cytometry (MC) is implemented in clinical settings, the need for robust, validated protocols that reduce batch effects between samples becomes increasingly important. Here, we present a streamlined MC workflow for high-throughput staining that generates reproducible data for up to 80 samples in a single experiment by combining reference sample spike-in and palladium-based mass-tag cell barcoding. Although labor intensive, this workflow decreases experimental variables and thus reduces technical error and mitigates batch effects.

BEFORE YOU BEGIN

Conjugate Antibodies to Metal Isotopes

Timing: 4 h

Many antibodies are available commercially, though custom conjugations allow for a researcher to be more flexible in their panel design by implementing specific clones and/or antibody-metal combinations to fit with the rest of the panel. This protocol is adapted from the Maxpar® Antibody Labeling User Guide with major changes listed below:

1. Pre-Load the polymer with Lanthanide
   a. Perform a quick spin of the Lanthanide vial using a tabletop mini-centrifuge
   b. Perform the polymer + Lanthanide incubation in an incubator rather than a warm water bath
2. Purify Lanthanide-loaded polymer
   a. After discarding the column flow-through from centrifugation add 300 μL of C-Buffer to the filter, in contrast to 400 μL
   b. Centrifuge the filter containing the C-Buffer at 12,000 × *g* for 30 min at 4°C, in contrast to 23°C
   c. Only one wash with C-Buffer is necessary
3. Buffer exchange and partially reduce the antibody
   a. Add 300 μL of R-Buffer to a 30 kDa filter, in contrast to bringing up to 400 μL of R-Buffer and using a 50 kDa filter
   b. Centrifugation during this step is performed at 4°C
4. Purify the partially reduced antibody
   a. All centrifugations in this step are performed at 12,000 × *g* for 30 min at 4°C, in contrast to 23°C
5. Retrieve the partially reduced antibody and Lanthanide-loaded polymer
6. Conjugate antibody with Lanthanide-loaded polymer

   a. Resuspend the Lanthanide loaded polymer with C-Buffer, bringing the total volume up to 60 μL
      i. Measure the residual volume before adding any additional C-Buffer since it may already be at 60 μL
   b. Incubate the Lanthanide-loaded polymer + C-Buffer for 60 min at 37°C, in contrast to 90 min
7. Wash metal conjugated antibody
   a. Add 300 μL of W-Buffer to the antibody conjugation mixture
8. Perform a buffer exchange for long-term storage of metal conjugated antibodies
   a. Supplement antibody stabilization buffer with 0.05% sodium azide
   b. Add 350 μL of antibody stabilization buffer + 0.05% sodium azide to each conjugated antibody
   c. Centrifuge 12,000 × *g* for 10 min at 4°C
   d. Label the top and side of a new collection tube
   e. Add antibody stabilization buffer to bring up filter volume to 75 μL
      i. Measure residual volume before adding any additional buffer
      ii. Pipette to mix and rinse the walls of the filter
   f. Carefully, invert the 30 kDa filter containing antibody stabilization buffer over into a new collection tube such that the contents fall into the new collection tube
   g. Centrifuge the inverted filter/collection tube assembly at 1,000 × *g*, 2 min, at 4°C
   h. Invert the filter within the same collection tube so that it is right-side up.
      i. Add another 75 μL antibody stabilization buffer + 0.05% sodium azide
      ii. Use a pipette to mix and rinse the walls of the filter
   j. Invert the filter over the same collection tube and repeat centrifugation (1000 × *g*, 2 min, at 4°C)
   k. Store at 4°C until ready to titrate (see "Titrate the Antibody Panel" in step 14, below)

⚠ CRITICAL: This protocol is specific for the X8 polymer, not the MCP9 polymer.

Note: Expected recovery of antibody after conjugation is 60%.

Pause Point: Conjugated antibodies can be stored for up to 6 months. This protocol is routinely used to conjugate and titrate antibodies and stain samples within 6 months of conjugation without degraded signal. Using this protocol, a reduction in an antibody's signal intensity has been observed when stored beyond 6 months after conjugation.

Prepare Reference Sample

Timing: 4 days, 3 h

Reference sample spike-in with CD45 barcoding serves as an essential quality control for analyzing batch effects (Kleinsteuber et al., 2016). A healthy donor leukoreduction apheresis collar was processed for PBMCs (Patel et al., 2018) and stimulated with CD3/CD28 Dynabeads to activate both adaptive and innate immune responses. If these conditions do not produce positive controls for each marker in the panel, please refer to Troubleshooting Problem 1.

9. Isolate PBMCs from a leukoreduction apheresis collar and cryopreserve half, labeled as "ex vivo" (Patel et al 2018)
10. Plate the remaining processed PBMCs in a tissue culture treated flask with supplemented RPMI (recipe in Materials and Equipment section) at a concentration of 2 million cells/mL
11. Add CD3/CD28 Dynabeads at a 1:10 number of beads to number of cells ratio
12. Incubate the cells for 48 h at 37°C in a 5% CO_2, humid environment
13. After incubation, cryopreserve and store in liquid nitrogen as above, labeled as "stim"

Titrate the Antibody Panel

Timing: 4 h for sample preparation, ≈1 h of acquiring samples per antibody being titrated on a mass cytometer

Titrating antibodies, both commercially purchased and conjugated by the researcher, is necessary to achieve optimal staining and minimize nonspecific binding. Titrations should be performed on samples resembling the sample type for which the MC panel will be applied on. In our clinical laboratory, this is cryopreserved human PBMCs isolated from whole blood. The protocol listed below is for titrating a single antibody.

14. Prepare 5 million cells of stimulated and ex vivo samples in 15 mL conical tubes
    a. Thaw cryopreserved human PBMC samples (details listed below in major steps 5–13, under PBMC Sample Thawing and Staining Preparation)
    b. Count cells and record viability and total cells/mL
    c. Determine the volume needed to resuspend samples to 5 million cells/mL
    d. Put cells on ice
15. Prepare ^{103}Rh viability staining media
    a. Thaw Rhodium-103 (^{103}Rh) and dilute ^{103}Rh to final concentration 1:500 in supplemented RPMI (recipe in Materials and Equipment, below)
16. Centrifuge cells (757 × *g*, 3 min, 4°C) and decant supernatant
17. Add ^{103}Rh viability staining media to samples for cells to be at 5 million cells/mL, and pipette to mix
18. Stain the two different stimulation condition samples with different metal isotopes of CD45 (CD45 Barcoding, as first described by (Kleinsteuber et al., 2016))
    a. Add CD45_89Y (1:200) to ex vivo sample
    a. Add CD45_141Pr (1:1000) to stimulated cells
    b. Incubate for 15 min at 37°C in a water bath
    c. Bring up volumes to 15 mL with CyFACS (recipe in Materials and Equipment, below) and centrifuge (757 × *g*, 3 min, 4°C)
    d. Decant supernatant and wash with another 15 mL of CyFACS (757 × *g*, 3 min, 4°C)
    e. Mix ex vivo and stimulated samples 1:1 in one 15 mL conical
    f. Count cells and concentrate to 10 million cells/mL in CyFACS
19. Plate 1:1 mixed sample into a 96-well plate, 200 μL per well (2 million cells/well)
    a. Plate 8 wells per antibody being titrated
20. Centrifuge plate (757 × *g*, 3 min, 4°C) and decant supernatant
21. Fc Block samples
    a. Dilute FcR blocking reagent 1:10 with CyFACS
    b. Add 15 μL of diluted FcBlock to each sample, mix by pipetting, incubate for 10 min on ice
22. Add 10 μL of CyFACS to all wells
23. Prepare serial dilutions of the antibody being titrated in a 1.7 mL Microcentrifuge Tube (see Table 1 below):

Table 1. Serial Dilution Preparation for Titrating Mass Cytometry Antibodies

Tube #	μL of antibody	μL CyFACS	Final dilution
1	4.8 μL	55.2	1:25
2	30 μL from tube 1	30	1:50
3	30 μL from tube 2	30	1:100
4	30 μL from tube 3	30	1:200
5	30 μL from tube 4	30	1:400
6	30 μL from tube 5	30	1:800
7	30 μL from tube 6	30	1:1600

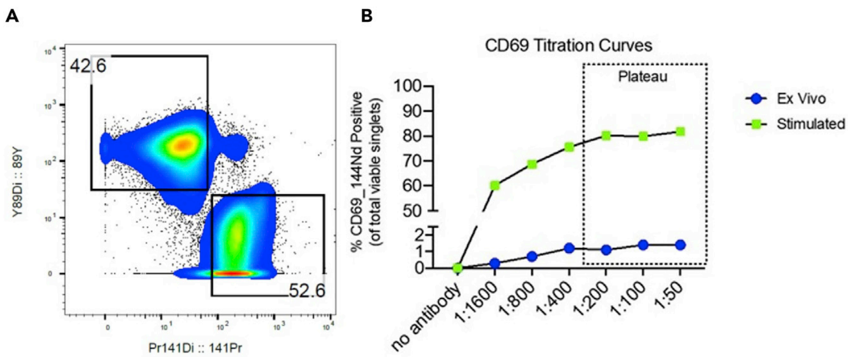


Figure 1. Single Antibody Titration Data Generated by Mass Cytometry

(A) Ex vivo samples stained with 89Y_CD45 and stimulated samples stained with 141Pr_CD45 are separated in downstream manual gating analysis by plotting the two channels on a biaxial plot.

(B) CD69_144Nd single antibody titration data plotted as percent of total viable singlets.

   a. Antibody serial dilutions are prepared at concentrations of 1:1600, 1:800, 1:400, 1:200, 1:100, 1:50, 1:25
   b. If there is a previous lot available for the antibody being titrated, test the previous lot at its predetermined concentration
   c. Add 25 μL of the diluted antibody to the appropriate well

24. Continue with MC Staining and sample acquisition protocol at "Palladium Mass-tag Cell Barcoding Strategy and Fixing Samples"
25. Select titration based on plateau of detection for that marker (Figure 1)

Note: Conjugation protocols may not be successful. If no concentration response is observed between the titration dilutions, then the conjugation will need to be repeated, or the titration experiment may be performed with additional antibodies to test for positive expression on different cell types, as described as optional below (Figure 2). See Troubleshooting Problem 2 for additional details.

Optional: The mass cytometry titration protocol can be expanded to add antibodies that have been previously titrated, to aid in the gating of the antibody currently being titrated. Prepare a master mix (MM) of these antibodies in a 1.7 mL Microcentrifuge Tube so that each sample will be stained with 10 μL of MM. This 10 μL of MM will replace the 10 μL of CyFACS in step 22 above. We recommend running a sample stained with the MM only. In a MM-only sample, 25 μL CyFACS will replace the 25 μL of diluted antibody. A typical MM we use for titration is shown below (Table 2). Lineage markers are included in the MM which allows for the marker being titrated to be analyzed on major immune cell types as shown (Figure 2).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Human CD45 (HI30)-Y89	Fluidigm	Cat#3089003B
Anti-Human CD45 (HI30)-141Pr	Fluidigm	Cat#3141009B
Anti-Human CD19 (HIB19)-142Nd	Fluidigm	Cat#3142001B

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-Human CD69 (FN50)-144Nd	Fluidigm	Cat#3144018B
Anti-Human CD15/SSEA-1 (W6D3)-144Nd	Fluidigm	Cat#3144019B
Anti-Human CD4 (RPA-T4)-145Nd	Fluidigm	Cat#3145001B
Anti-Human CD8a (RPA-T8)-146Nd	Fluidigm	Cat#3146001B
Anti-Human CD11c (Bu15)-147Sm	Fluidigm	Cat#3147008B
Anti-Human CD274/PD-L1 (29E.2A3)-148Nd	Fluidigm	Cat#3148017B
Anti-Human CD25 (2A3)-149Sm	Fluidigm	Cat#3149010B
Anti-Human CD134/OX40 (ACT35)-150Nd	Fluidigm	Cat#3150023B
Anti-Human CD86/B7.2 (IT2.2)-150Nd	Fluidigm	Cat#3150020B
Anti-CD278/ICOS (C398.4A)-151Eu	Fluidigm	Cat#3151020B
Anti-Human CD95/Fas (DX2)-152Sm	Fluidigm	Cat#3152017B
Anti-Human TCRgd (11F2)-152Sm	Fluidigm	Cat#3152008B
Anti-Human CD45RA (HI100)-153Eu	Fluidigm	Cat#3153001B
Anti-Human TCR Va7.2 (3C10)-153Eu	Fluidigm	Cat#3153024B
Anti-Human TIM-3 (F38-2E2)-154Sm	Fluidigm	Cat#3154010B
Anti-Human CD163 (GHI/61)-154Sm	Fluidigm	Cat#3154007B
Anti-Human CD279/PD-1 (EH12.2H7)-155Gd	Fluidigm	Cat#3155009B
Anti-Human CD183/CXCR3 (G025H7)-156Gd	Fluidigm	Cat#3156004B
Anti-Human CD137/4-1BB (4B4-1)-158Gd	Fluidigm	Cat#3158013B
Anti-Human CD33 (WM53)-158Gd	Fluidigm	Cat#3158001B
Anti-Human CD337/NKp30 (Z25)-159Tb	Fluidigm	Cat#3159017B
Anti-Human/Mouse Tbet (4B10)-160Gd	Fluidigm	Cat#3160010B
Anti-Human CD294/CRTH2 (BM16)-163Dy	Fluidigm	Cat#3163003B
Anti-Human Galectin-9 (9M1-3)-163Dy	Fluidigm	Cat#3163002B
Anti-Human CD223/LAG-3 (11C3C65)-165Ho	Fluidigm	Cat#3165037B
Anti-Human IL-10 (JES3-9D7)-166Er	Fluidigm	Cat#3166008B
Anti-Human CD197/CCR7 (G043H7)-167Er	Fluidigm	Cat#3167009A
Anti-Human CD11b/Mac-1 (ICRF44)-167Er	Fluidigm	Cat#3167011B
Anti-Human CD127/IL-7Ra (A019D5)-168Er	Fluidigm	Cat#3168017B
Anti-Ki-67 (B56)-168Er	Fluidigm	Cat#3168007B
Anti-Human CD33 (WM53)-169Tm	Fluidigm	Cat#3169010B
Anti-Human CD152/CTLA-4 (14D3)-170Er	Fluidigm	Cat#3170005B
Anti-Human CD185/CXCR5 (RF8B2)-171Yb	Fluidigm	Cat#3171014B
Anti-Human CD68 (Y1/82A)-171Yb	Fluidigm	Cat#3171011B
Anti-Human CD273/PDL2 (24F.10C12)-172Yb	Fluidigm	Cat#3172014B
Anti-Human/Mouse Granzyme B (GB11)-173Yb	Fluidigm	Cat#3173006B
Anti-Human CD56 (NCAM16.2)-176Yb	Fluidigm	Cat#3176008B
Anti-Human CD16 (3G8)-209Bi	Fluidigm	Cat#3209002B

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
LEAF™ Purified anti-human CD154 Antibody (CD40L)	BioLegend	Cat#310812; Clone 24-31
Purified anti-human CD123	BioLegend	Cat#306002; Clone 6H6
Purified anti-human CD28 (Maxpar® Ready)	BioLegend	Cat#302937; Clone CD28.2
Purified anti-human CD3 (Maxpar® Ready)	BioLegend	Cat#300443; Clone UCHT1
Purified anti-human CD357 (GITR)	BioLegend	Cat#371202; Clone 108-17
Ultra-LEAF™ Purified anti-human CD14	BioLegend	Cat#301862; Clone M5E2
Ultra-LEAF™ Purified anti-human CD314 (NKG2D)	BioLegend	Cat#320814; Clone 1D11
Ultra-LEAF™ Purified anti-human HLA-DR	BioLegend	Cat#307648; Clone L243
Ultra-LEAF™ Purified anti-human IFN-γ Antibody	BioLegend	Cat#506512; Clone B27
TIGIT Monoclonal Antibody (MBSA43), Functional Grade	eBioscience	Cat#16-9500-82
Biological Samples		
Leukoreduction apheresis collar	Brigham and Women's Hospital	N/A
Chemicals, Peptides, and Recombinant Proteins		
BD Cytofix/Cytoperm™ Fixation/ Permeabilization Solution Kit (Fixation/ Permeabilization solution and BD Perm/ Wash™ Buffer)	BD Biosciences	Cat#554714
Antibody Stabilizer (PBS)	Candor Bioscience	Cat#131050
Dimethyl Sulfoxide, Fisher BioReagents™ (DMSO)	Fisher Scientific	Cat# BP231-100
Cell-ID™ Intercalator-Ir—125 μM	Fluidigm	Cat#201192A
Cell-ID™ Intercalator-Rh—500 μM	Fluidigm	Cat#201103A
EQ™ Four Element Calibration Beads	Fluidigm	Cat#201078
Maxpar® Cell Acquisition Solution	Fluidigm	Cat#201240
MaxPar® Cell Staining Buffer	Fluidigm	Cat#201068
Antibiotic-Antimycotic (100×)	Gibco	Cat#15240062
Dynabeads™ Human T-Activator CD3/CD28	Gibco	Cat#11131D
Fetal Bovine Serum, certified, heat inactivated (FBS)	Gibco	Cat#10082147
PBS, pH 7.4	Gibco	Cat#10010023
RPMI 1640 Medium	Gibco	Cat#11875093
UltraPure™ DNase/RNase-Free Distilled Water	Invitrogen	Cat#10977015
FcR Blocking Reagent, Human	Miltenyi Biotec	Cat#130-059-901
AOPI Staining Solution in PBS	Nexcelom Biosciences	Cat#CS2-01060-5ML
Bovine Serum Albumin (BSA), 30% in 0.85% NaCl	Sigma-Aldrich	Cat#A7284
Sodium azide, 10% (w/v) solution in DI water	Teknova	Cat#S0209
Bond-Breaker™ TCEP Solution, Neutral pH	Thermo Scientific	Cat#77720
Pierce™ 16% Formaldehyde (w/v), Methanol-free	Thermo Scientific	Cat#28906

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
Maxpar® X8 Antibody Labeling Kit, 143Nd	Fluidigm	Cat#201143A
Maxpar® X8 Antibody Labeling Kit, 156Gd	Fluidigm	Cat#201156A
Maxpar® X8 Antibody Labeling Kit, 159Tb	Fluidigm	Cat#201159A
Maxpar® X8 Antibody Labeling Kit, 161Dy	Fluidigm	Cat#201161A
Maxpar® X8 Antibody Labeling Kit, 162Dy	Fluidigm	Cat#201162A
Maxpar® X8 Antibody Labeling Kit, 164Dy	Fluidigm	Cat#201164A
Maxpar® X8 Antibody Labeling Kit, 166Er	Fluidigm	Cat#201166A
Maxpar® X8 Antibody Labeling Kit, 174Yb	Fluidigm	Cat#201174A
Maxpar® X8 Antibody Labeling Kit, 175Lu	Fluidigm	Cat#201175A
Cell-ID™ 20-Plex Pd Barcoding Kit	Fluidigm	Cat#201060
Software and Algorithms		
FlowJo	Becton, Dickinson & Company	https://www.flowjo.com/solutions/flowjo/downloads
Cytobank	Cytobank Inc.	https://www.cytobank.org/
CyTOF Software Version 6.7.1014	Fluidigm	https://www.fluidigm.com/software
Other		
1.7 mL Polypropylene Microcentrifuge Tubes	Corning Inc.	Cat#MCT-175-C-S
150 cm^2 Cell Culture Flask, treated	Corning Inc.	Cat#3291
15 mL Polypropylene Centrifuge Tubes	Corning Inc.	Cat#430052
50 mL Polypropylene Centrifuge Tubes	Corning Inc.	Cat#430829
96-well V-bottom plate, untreated	Corning Inc.	Cat#3896
Falcon® 5 mL Round Bottom Polypropylene Tubes (FACS Tubes)	Corning Inc.	Cat#352063
Falcon® 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap 35 µm Cell Strainer Snap Cap	Corning Inc.	Cat#352235
Amicon Ultra-0.5 Centrifugal Filter Unit, 3 kDa	Millipore	Cat#UFC500396
Amicon Ultra-0.5 Centrifugal Filter Unit, 30 kDa	Millipore	Cat#UFC503024
Ultrafree-MC Centrifugal Filter, 0.1 µm	Millipore	Cat#UFC30VV00
ThermoFisher Scientific™ Sorvall™ Legend™ XTR Centrifuge TX-1000 rotor	ThermoFisher Scientific™	Cat#75004521
ThermoFisher Scientific Sorvall™ Legend™ Micro 21R	ThermoFisher Scientific™	Cat#75002446
FisherBrand Mini-Centrifuge	ThermoFisher Scientific™	Cat#12-006-901
Fisher Scientific Digital Vortex Mixer	ThermoFisher Scientific™	Cat#0215370
ThermoFisher Scientific Precision™ Water Bath	ThermoFisher Scientific™	Cat#TSCIR19
ThermoFisher Scientific Forma™ Steri-Cycle™ CO_2 Incubator	ThermoFisher Scientific™	Model 370
Nexcelom Biosciences Cellometer Auto 2000 Cell Counter	Nexcelom Biosciences	Auto 2000
SD100 Slides	Nexcelom Biosciences	CHT4-SD100-002
Fluidigm Helios™ Mass Cytometer	Fluidigm	PN#101-0723
WB Injector for Helios™ Mass Cytometer	Fluidigm	Cat#107950

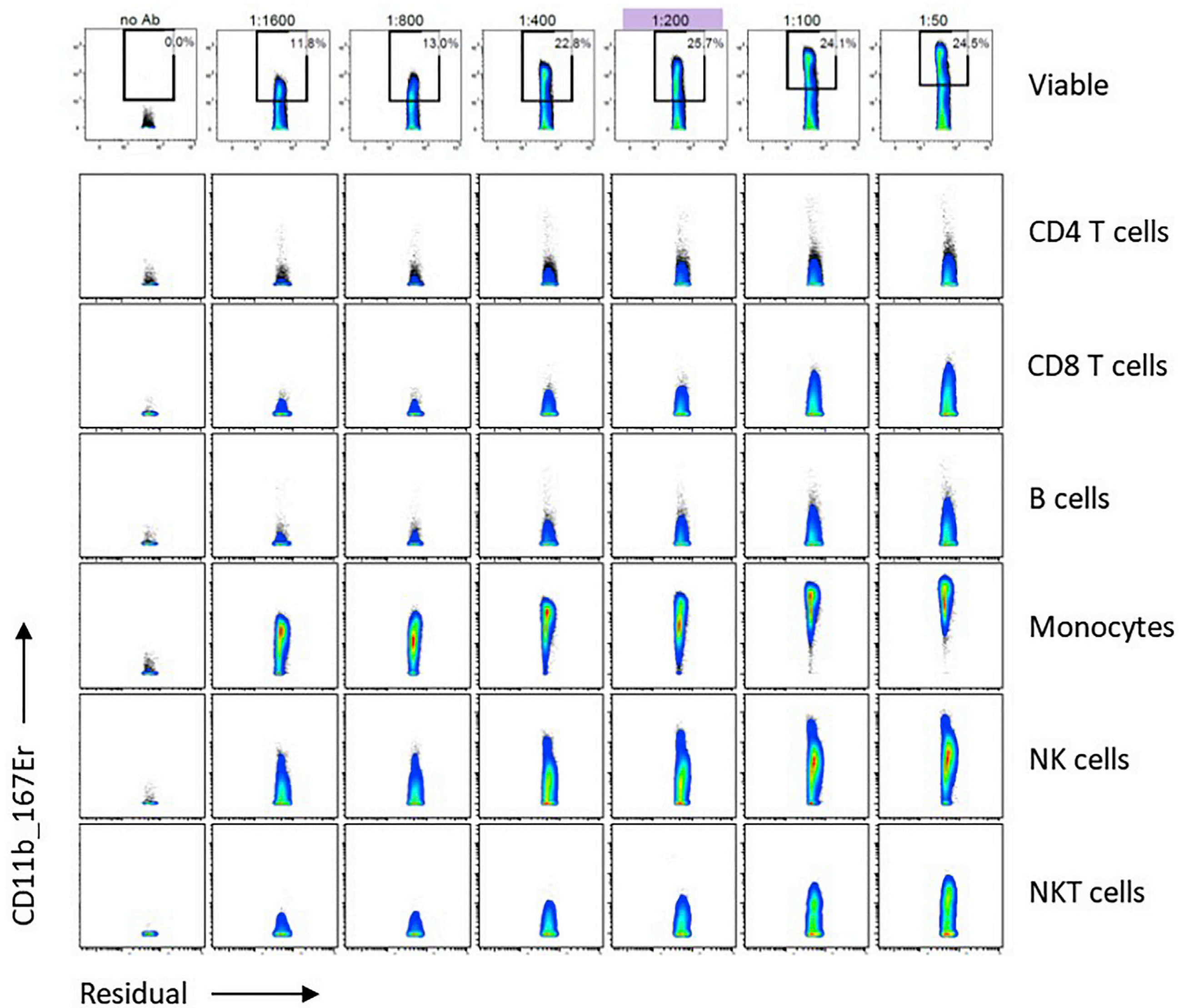


Figure 2. Using Surface Antibody Master Mix to Analyze Mass Cytometry Titration Data
CD11b titration data is used as an example for analyzing a marker with differential expression on cell types. This assists in placing the manually set gate on the viable cells. CD11b expression is expected to be positive on monocytes, with lower to no expression on B, T, and NKT cells. Increased background is observed as the antibody increases to 1:50 dilution in these cell types.

⚠ CRITICAL: Only the 35 μm cell strainer snap cap included with the Falcon® 5 mL Round Bottom Polystyrene Test Tube will be used. Polypropylene tubes are substituted for the polystyrene tubes to minimize cell adhesion to the tubes.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Mariano Severgnini (Mariano_Severgnini@DFCI.Harvard.edu).

Materials Availability

This study did not generate new unique reagents.

Table 2. Surface Antibody Master Mix for Titrating Mass Cytometry Antibodies

Surface Staining				
Marker	Metal	Clone	Dilution Factor	μL Added to MM
CD19	^{142}Nd	HIB19	800	12.50
CD3	^{143}Nd	UCHT1	200	5.00
CD4	^{145}Nd	RPA-T4	200	5.00
CD8a	^{146}Nd	RPA-T8	100	10.00
CD11c	^{147}Sm	Bu15	1600	6.25
CD14	^{162}Dy	M5E2	400	2.50
CD56	^{176}Yb	NCAM16.2	800	12.50
CD16	^{209}Bi	3G8	200	5.00
CyFACS				141.25
Total MM Volume				200.00
Volume MM/sample (μL)	10			
# of samples (+10%–20%)	20			

Data and Code Availability

There is no dataset/code associated with the paper.

MATERIALS AND EQUIPMENT

Human Samples

For human samples, healthy donor samples were obtained per the blood collection protocol approved by the Institutional Review Board (IRB) of Brigham and Women's Hospital. All participants gave written informed consent prior to blood draw. Healthy donor whole blood was collected in apheresis leukoreduction collar that contain Anticoagulant Citrate Dextrose Solution USP(ACD) Solution A. The age/developmental stage, sex, and gender identity is unknown per the IRB between Brigham and Women's Hospital and Dana-Farber Cancer Institute.

Equipment

- Centrifugation × *g* are calculated using the maximum radius
- All centrifugations are set at 4°C
- In this protocol the following equipment refers to:
  - Refrigerated benchtop centrifuge: Thermo Scientific Sorvall™ Legend™ XTR Centrifuge with TX-1000 rotor
    - Acceleration and deceleration set to 9 for all centrifugations
  - Refrigerated microcentrifuge: ThermoFisher Scientific Sorvall™ Legend™ Micro 21R
  - Tabletop mini-centrifuge: FisherBrand Mini-Centrifuge
  - Vortex: Fisher Scientific Digital Vortex Mixer
  - Automated cell counter: Nexcelom Biosciences Cellometer Auto 2000 Cell Counter with disposable counting slides (SD100 slides)
    - Program for cell counting: "Immune cells, low RBC assay (AOPI viability)"

Alternatives: A hemocytometer may be used if an automated cell counter is not available. However, this will add a substantial amount of time to the experiment, especially if the experiment includes 80 samples.

Recipes

Supplemented RPMI

Reagent	Volume (mL)	Final Concentration
RPMI 1640 Medium	500	n/a
FBS	50	10%
100× antibiotic-antimycotic	5	1×

Store at 4°C for up to 2 weeks

CyFACS

Reagent	Volume (mL)	Final Concentration
PBS	500	n/a
30% BSA	8.3	0.5%
5% sodium azide	2	0.02%

Store at 4°C for up to 6 weeks

Freezing Media

Reagent	Volume (mL)	Final Concentration
FBS	25	85%
DMSO	4.4	15%

Store at 4°C for up to 2 weeks

⚠ CRITICAL: Sodium azide is toxic if swallowed or inhaled and fatal in contact with skin. It should always be handled in a fume hood while wearing protective gloves and lab coat.

⚠ CRITICAL: Glass or anything that could have been exposed to soap including autoclaved materials cannot be used.

STEP-BY-STEP METHOD DETAILS

Prepare Surface Antibody Master Mix and Labeling

Timing: ≈2 h (for 80 samples)

We recommend performing these experiment setup steps the day before staining due to the length of the protocol, the longest step being antibody master mix preparation. Here, we stain 36 experimental samples with two mass cytometry panels.

1. Locate and set aside sample vials in liquid nitrogen, for quick retrieval on the day of staining
2. Make surface antibody master mixes, diluting in CyFACS (Tables 3 and 4), and store at 4°C
3. Label cell-counting slides and 15 mL conical tubes with sample identifier, one each per sample
4. Fill 15 mL conical tubes with 12 mL of supplemented RPMI and put in 4°C

Optional: The surface master mix preparation and labeling can be performed prior to PBMC Sample Thawing and Staining Preparation, though the workflow on the thawing and staining day will become more laborious and time consuming. Therefore, it is recommended to perform this section of the protocol the day before staining.

Table 3. Adaptive Mass Cytometry Panel

Marker	Metal	Clone	Vendor	Dilution Factor
Surface Master Mix				
CD19	^{142}Nd	HIB19	FDM	800
CD3	^{143}Nd	UCHT1	CIO	100
CD69	^{144}Nd	FN50	FDM	200
CD4	^{145}Nd	RPA-T4	FDM	200
CD8a	^{146}Nd	RPA-T8	FDM	100
CD11c	^{147}Sm	Bu15	FDM	1600
PD-L1	^{148}Nd	29E.2A3	FDM	100
CD25	^{149}Sm	2A3	FDM	400
OX40	^{150}Nd	ACT35	FDM	50
ICOS	^{151}Eu	C398.4A	FDM	100
CD95	^{152}Sm	DX2	FDM	100
CD45RA	^{153}Eu	HI100	FDM	50
TIM-3	^{154}Sm	F38-2E2	FDM	50
PD-1	^{155}Gd	EH12.2H7	FDM	100
CXCR3	^{156}Gd	G025H7	FDM	200
4-1BB	^{158}Gd	4B4-1	FDM	100
GITR	^{159}Tb	108-17	CIO	100
CD40L	^{161}Dy	24-31	CIO	100
CD14	^{162}Dy	M5E2	CIO	200
CRTH2	^{163}Dy	BM16	CIO	50
TIGIT	^{164}Dy	MBSA43	CIO	100
LAG3	^{165}Ho	11C3C65	CIO	100
CD28	^{166}Er	CD28.8	CIO	100
CCR7	^{167}Er	G043H7	FDM	100
CD127	^{168}Er	A019D5	FDM	100
CD33	^{169}Tm	WM53	FDM	200
CXCR5	^{171}Yb	51505	FDM	100
PD-L2	^{172}Yb	24F.10C12	FDM	50
CD123	^{174}Yb	6H6	CIO	100
HLA-DR	^{175}Lu	L243	CIO	100
CD56	^{176}Yb	NCAM16.2	FDM	800
CD16	^{209}Bi	3G8	FDM	200
Intracellular Master Mix				
T-bet	^{160}Gd	4B10	FDM	100
CTLA-4	^{170}Er	14D3	FDM	200
Granzyme B	^{173}Yb	GB11	FDM	800

Titrated antibodies of the adaptive immune response focused mass cytometry panel and dilution factors to make master mixes. Vendor listed as "CIO" refers to Center for Immuno-Oncology and indicates the antibody metal conjugation was performed in our lab.

Optional: Antibody surface master mixes can be stored at -80°C without observable differences (Figure 3). Other groups have shown that a large batch of premixed antibody master mix can be made and stored at -80°C for at least 9 months (Schulz et al., 2019).

Table 4. Innate Mass Cytometry Panel

Marker	Metal	Clone	Vendor	Dilution Factor
Surface Master Mix				
CD19	^{142}Nd	HIB19	FDM	800
CD3	^{143}Nd	UCHT1	CIO	100
CD15	^{144}Nd	W6D3	FDM	100
CD4	^{145}Nd	RPA-T4	FDM	200
CD8a	^{146}Nd	RPA-T8	FDM	100
CD11c	^{147}Sm	Bu15	FDM	1600
PD-L1	^{148}Nd	29E.2A3	FDM	100
CD25	^{149}Sm	2A3	FDM	400
CD86	^{150}Nd	IT2.2	FDM	100
ICOS	^{151}Eu	C398.4A	FDM	100
TCRgd	^{152}Sm	11F2	FDM	50
TCR Va7.2	^{153}Eu	3C10	FDM	100
CD163	^{154}Sm	GHI/61	FDM	50
PD-1	^{155}Gd	EH12.2H7	FDM	100
NKG2D	^{156}Gd	1D11	CIO	50
CD33	^{158}Gd	WM53	FDM	400
NKp30	^{159}Tb	Z25	FDM	100
CD14	^{162}Dy	M5E2	CIO	200
Galectin-9	^{163}Dy	9M1-3	FDM	100
TIGIT	^{164}Dy	MBSA43	CIO	100
LAG3	^{165}Ho	11C3C65	FDM	100
CD11b	^{167}Er	ICRF44	FDM	200
CD68	^{171}Yb	Y1/82A	FDM	100
PD-L2	^{172}Yb	24F.10C12	FDM	50
CD123	^{174}Yb	6H6	CIO	100
HLA-DR	^{175}Lu	L243	CIO	100
CD56	^{176}Yb	NCAM16.2	FDM	800
CD16	^{209}Bi	3G8	FDM	200
Intracellular Master Mix				
IFNγ	^{161}Dy	B27	CIO	100
IL-10	^{166}Er	JES3-9D7	FDM	100
Ki-67	^{168}Er	B56	FDM	200
CTLA-4	^{170}Er	14D3	FDM	200
Granzyme B	^{173}Yb	GB11	FDM	800

Titrated antibodies of the innate immune response focused mass cytometry panel and dilution factors to make master mixes. Vendor listed as "CIO" refers to Center for Immuno-Oncology and indicates the antibody metal conjugation was performed in our lab.

Note: Dilution Factors listed in Tables 3 and 4 are specific to human cryopreserved PBMCs. All researchers using this protocol should titrate antibodies on their sample type of interest and can use these dilution values as guides. Antibody dilutions are estimated at 6 μg/mL for a 1:100 dilution factor

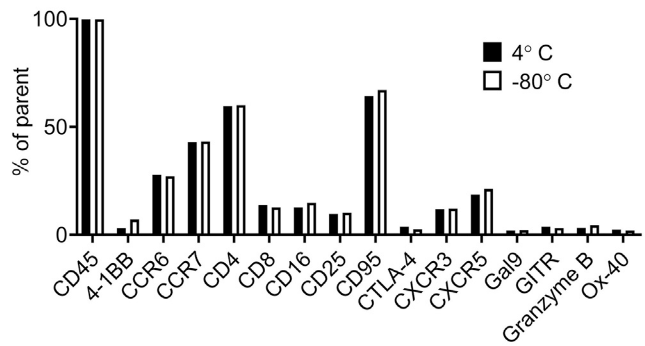


Figure 3. Comparing Storage Conditions of Surface Master Mix

Metal-conjugated antibody surface master mixes were prepared and stored at -80°C or 4°C for 16 h and healthy donor PBMCs were stained. Clones and metals are the same as used in the adaptive and innate panels, as listed in Tables 3 and 4.

Note: Commercially purchased antibodies listed here do not need to be re-titrated between lots.

⚠ CRITICAL: In-house conjugated antibodies must be titrated for every new conjugation lot.

PBMC Sample Thawing and Staining Preparation

Timing: ≈2 h (for 80 samples)

This step details an optimized thawing protocol for PBMC samples from liquid nitrogen storage to achieve high (>95%) viability and concentrates the samples appropriately for the next steps (Holland et al., 2018).

5. Place 15 mL conical tubes (filled with supplemented RPMI) in the water bath
6. Prepare a liquid waste container with ≈5 mL of 10% bleach
7. Fill two large ice buckets with ice
8. Thaw each sample vial one at a time in a water bath set at 37°C for ≈45 s (for a sample volume of 1 mL) until just melted
9. Quickly pipette contents of the vial into the appropriate, prelabeled 15 mL conical tube
    a. If multiple vials of reference sample are used, these can be combined into a single 15 mL conical tube, taking care to not overfill it
10. Count cells and record viability and total cell/mL
    a. Mix 15 μL of cell suspension with 15 μL of trypan blue for a 1:1 mixture
    b. Load 20 μL of 1:1 mixture onto cell counting slide
    c. Insert cell counting slide into Cellometer and count
11. Centrifuge all samples (757 × *g*, 3 min, 4°C)
12. Keep samples on ice after they are finished spinning
13. Calculate how much volume is needed to resuspend all samples to 5 million cells/mL

⚠ CRITICAL: Ensure that there is enough reference sample after counting: approximately 20 million of each ex vivo and stimulated reference sample are needed for an 80-sample experiment. 2 million cells are needed from each of the reference sample stimulation conditions (ex vivo and stimulated) to run as separate solo wells (1 solo well for each stimulation condition per barcode set; 4 barcode sets are needed for 80 samples). 0.5 million of 1:1 ex vivo and stimulated mixed reference sample is spiked into each experimental sample (80 experimental samples = 40 million cells of 1:1 mixed reference sample).

Viability Stain and CD45 Barcoded Reference Sample Spike-in

Timing: ≈1 h (for 80 samples)

At the end of this step the experimental samples and reference sample will be stained for viability, using Rhodium-103 (^{103}Rh), barcoded with CD45, and plated in a 96-well v-bottom plate. The experimental sample and reference sample are stained with CD45 conjugated to different metal isotopes: experimental samples with CD45 conjugated to ^{141}Pr (CD45_141Pr) and reference sample stained with CD45 conjugated to ^{89}Y (CD45_89Y). This CD45 barcoding step allows the reference sample to be spiked-in to the experimental samples and provides a quality control. Experimental sample in our lab is typically a cryopreserved PBMC clinical trial patient sample. Reference sample is a 1:1 mixed sample from a single donor of resting PBMCs ("ex vivo") and stimulated PBMCs ("stim"). As an additional quality control, the ex vivo and stim reference samples are also stained as independent samples on the plate.

14. Make a master mix of ^{103}Rh and RPMI with ^{103}Rh diluted to a final concentration of 1:500
    a. Use calculated volume (from step 13) to determine how much ^{103}Rh + RPMI master mix is needed and round up ≈20% for overhead
15. Decant supernatant from reference sample conical tubes
16. Add the appropriate volume of ^{103}Rh-RPMI master mix to the reference sample for a final cell concentration of 5 million cells/mL
17. Pipette up and down to disrupt the cell pellet
18. Add CD45_89Y at a 1:200 dilution to reference sample suspended in ^{103}Rh-RPMI
19. Add CD45_141Pr at a dilution of 1:1000 to remaining ^{103}Rh-Media
20. Decant supernatant from experimental samples and add appropriate volume of CD45_141Pr-103Rh-Media to experimental samples to resuspend each to 5 million cells/mL
    a. See step 13 for calculated volumes
21. Incubate samples for 15 min at 37°C in the water bath
22. After CD45-barcoding and viability incubation is finished, immediately bring volume in each conical tube up to 10 mL with CyFACS and centrifuge: 757 × *g*, 3 min, 4°C
23. Decant supernatant, resuspend cell pellet in 10 mL CyFACS by gently pipetting up and down, and centrifuge samples: 757 × *g*, 3 min, 4°C.
    a. If residual volume remains after decanting, pipette out the remaining volume, leaving the cell pellet as dry as possible
24. Calculate volume needed to resuspend cells to a concentration of 15 million cells/mL
    a. Calculate without factoring in any cell loss, use cell counts from step 10
25. Resuspend samples to 15 million cells/mL with CyFACS
26. Plate experimental and reference samples in a 96-well v-bottom plate (Figure 4A):
    a. Add 1.5 million cells (100 μL) of experimental sample to appropriate wells (yellow)
    b. Add 2 million cells (133 μL) of ex vivo sample to ex vivo alone wells (blue, row 10)
    c. Add 2 million cells (133 μL) of stimulated sample to stimulated alone wells (blue, row 11)
27. Combine the remaining ex vivo and stimulated reference sample 1:1
28. Spike-in the reference sample to experimental sample wells (Figure 4B):
    a. Add 0.5 million cells of 1:1 mixed reference sample (33 μL) to each experimental well (green)
    b. If an experimental sample's cell number is insufficient, then add more 1:1 mixed reference sample to reach a final cell number of 2 million total cells and a total volume of 133 μL in the well. See Troubleshooting Problem 3 for more details.
29. Add 67 μL of CyFACS to each well containing sample (total 200 μL/well) and centrifuge (757 × *g*, 3 min, 4°C)

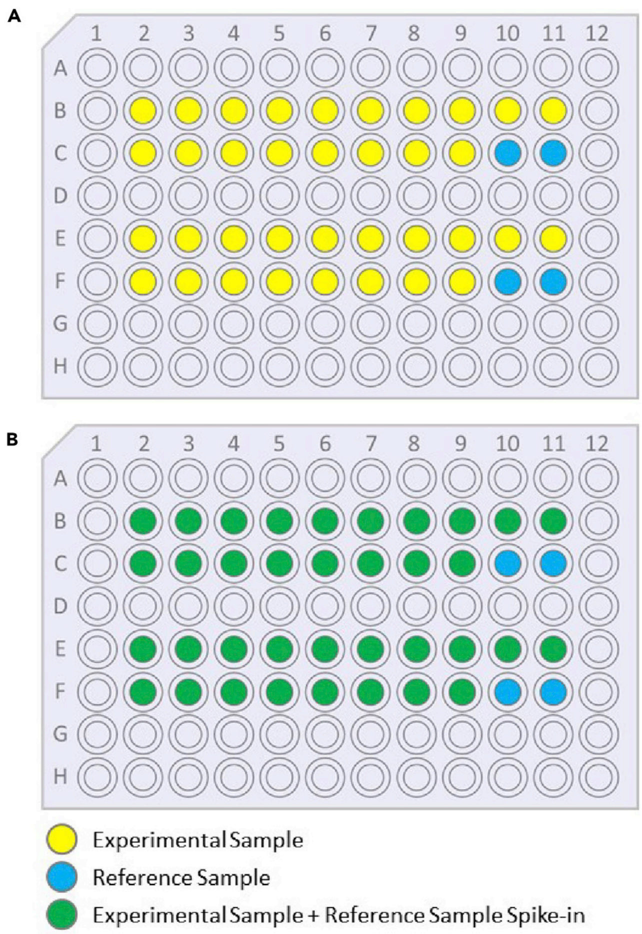


Figure 4. Plate Layout for Experimental Samples, Reference Samples, and CD45-Barcoded Reference Sample Spike-in

(A) Experimental samples (yellow wells) and reference samples (blue) are plated. Wells C10 and F10 contain the ex vivo reference sample, and wells C11 and F11 contain the stimulated reference sample. Keeping the two stimulated conditions separate guides downstream analysis (Figure 8).

(B) 1:1 mixed reference sample is "spike-in" to experimental sample (green wells). Each well now contains 2 million cells.

> *Note:* Each experimental sample is decanted just before being resuspended with CD45_141Pr-103Rh-Media. If multiple antibody panels are going to be used, we recommend having a separate plate for each panel.

FcR Block and Surface Stain

Timing: ≈1 h

Here, the Fc receptors on the cells are blocked and the cells are stained with the surface master mix.

30. Prepare FcR blocking reagent by diluting 1:10 with CyFACS
31. Add 15 μL of diluted FcR blocking reagent to each well and mix gently by pipetting
32. Incubate for 10 min on ice
33. Remove surface master mix from 4°C (prepared the day before)
34. Filter the surface master mix with 0.1 μM spinfilter in the microcentrifuge: 14,000 × *g*, 5 min, 4°C

   a. Measure the master mix volume after filtering and adjust to the original volume with CyFACS
   b. Keep at 4°C until ready to add to samples
35. After the FcR blocking incubation is up, add 25 µL of filtered surface master mix per sample
   a. If staining with multiple antibody panels, add that surface master mix to the appropriate samples, not to all samples.
36. Incubate for 45 min on ice
37. Add 150 µL of CyFACS and centrifuge: 757 × *g*, 3 min, 4°C
38. Wash 1× with 200 µL PBS

Note: Make 5 mL of FcR blocking reagent and use a multichannel pipette to speed up this step. After samples are plated, we recommend performing all steps with a multichannel pipette, except for adding the surface master mix.

Palladium Mass-Tag Cell Barcoding Strategy and Fixing Samples

Timing: 45 min

Fluidigm's Cell-ID™ 20-Plex Pd Barcoding Kit stains 20 individual samples using different combinations of 6 palladium (Pd) isotopes. Here, we provide additional logistics to stain 80 samples with 4 Pd barcode sets, with ex vivo and stimulated reference samples run as separate samples in each barcode set for quality control purposes (sample barcoding strategy is outlined in Figure 5). The ex vivo and stimulated reference samples are run separately, not as the 1:1 mixed reference sample, to guide manual gating (described in Expected Outcomes, Figure 8). At the end of this section, 20 samples will be combined into one 15 mL conical tube, and there will be four 15 mL conical tubes.

39. Prepare reagents:
   a. 1× Fix I Buffer
      - Dilute 5× Fix I Buffer with PBS to make 1× working stock of Fix I Buffer
      - ≈200 µL are needed per sample
   b. 1× Barcode Perm Buffer
      - Dilute 10× Barcode Perm Buffer with PBS
      - ≈600 µL are needed per sample
   c. 1× Perm/Wash
      - Dilute 10× Perm/Wash with UltraPure Water
      - ≈20 mL of 1× Perm/Wash per barcode batch
40. Add 200 µL of 1× Fix I to each sample and incubate for 10 min at 23°C
41. Thaw 4 full sets of 20 Pd-isotope barcodes for 10 min at 23°C until fully thawed
42. Quickly centrifuge barcodes using the PCR tube adapter in a mini-centrifuge (≈5 s)
43. Centrifuge samples: 935 × *g*, 3 min and decant supernatant
44. Resuspend cells with 200 µL of 1× Barcode Perm Buffer
45. Centrifuge samples at 935 × *g* and decant supernatant
46. Repeat wash (steps 44–45)
47. Resuspend cells in 100 µL of 1× Barcode Perm Buffer
48. Resuspend barcodes in 100 µL 1× Barcode Perm Buffer
49. Transfer 100 µL of resuspended barcodes to samples
50. Gently mix by pipetting
51. Incubate for 30 min at 23°C
52. Centrifuge samples (935 x *g*, 3 min) and wash samples twice with 200 µL of CyFACS
   a. Spin samples at 935 x *g* for 3 min between washes
53. Add 200 µL of CyFACS to each sample
54. Combine samples from each barcode set to an appropriately labeled 15 mL conical tube

55. Repeat steps 53 and 54, to ensure transfer of every cell
56. Bring volume in the 15 mL conical tubes up to 10 mL with CyFACS
57. Centrifuge samples at 935 x *g* for 3 min and decant supernatant
58. Add 1 mL of BD Cytofix/Cytoperm to each 15 mL conical tube containing the barcoded samples
59. Incubate for 20 min at 23°C
60. Add 10 mL 1× Perm Wash to each sample and centrifuge (935 x *g*, 3 min)
61. Wash 1× with 10 mL of 1× Perm Wash
62. Decant and use a pipette to aspirate after the last Perm Wash to leave pellet as dry as possible

Alternatives: MilliQ water can be used in place of UltraPure Water

Note: Palladium Mass-Tag Cell Barcoding Strategy protocol is adapted from the protocol "Cell-ID 20-Plex Pd Barcoding Kit" on www.fluidigm.com. The barcodes can be centrifuged (in step 42) in the orange plate they come in.

⚠ CRITICAL: Do not thaw and refreeze barcodes.

⚠ CRITICAL: After samples are fixed in step 40 perform all centrifugations are performed at 935 x *g*.

Intracellular Stain

⏲ Timing: 45 min

This step details the protocol for preparing the intracellular antibody master mix and performing the intracellular staining. If intracellular markers are not a part of the panel this entire section can be skipped.

63. Prepare intracellular antibody master mixes using 1× Perm/Wash (see Tables 3 and 4)

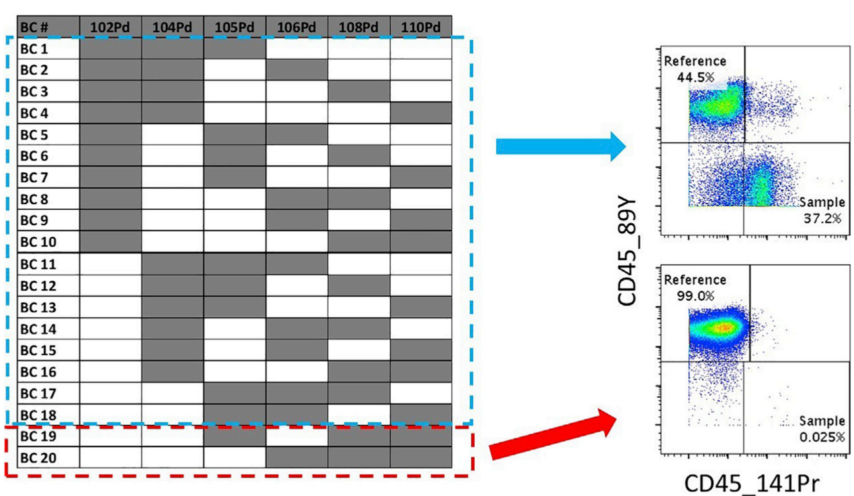


Figure 5. 20-Plex Palladium Isotope Barcode Strategy

A Barcode ID # (BC #) is assigned to each sample. Each BC # is positive for 3 of the six different Palladium isotopes (102, 104, 105, 106, 108, 110 Pd). In this schema, grayed out boxes indicate that this BC # is positive for that isotope. BC 1–18 are experimental samples with reference sample spike-in, and BC 19 and 20 are reserved for reference sample controls. Downstream gating of CD45 barcoding is shown on the right to indicate what samples are in which BC #.

64. Filter intracellular antibody master mixes with a 0.1 μM spinfilter in a microcentrifuge: 14,000 × *g*, 5 min, 4°C
    a. Measure the master mix volume after filtering and adjust to the original volume with 1× Perm/Wash
    b. Keep at 4°C until ready to add to samples
65. Add 100 μL of intracellular master mix to each barcode batch
66. Incubate for 30 min on ice
67. Add 10 mL of CyFACS and centrifuge (935 × *g*, 3 min)
68. Decant supernatants, resuspend pellets with 10 mL PBS and centrifuge (935 × *g*, 3 min)
69. Decant supernatants and store samples on ice

Optional: Intracellular antibody master mix can be made at any point of the protocol, or the day prior to staining. Store the unfiltered antibody master mix at 4°C. Filter just prior to adding the master mix to samples.

Fresh Fix and DNA Intercalator Staining

Timing: 10 min

At the end of this major step, samples are fixed and stained with a DNA intercalator (Cell-ID™ Intercalator-Ir).

70. Prepare a fresh batch of 2% formaldehyde fix (FA) solution
    a. 6 mL of 2% FA is needed per barcode batch
    b. Dilute fresh 16% Paraformaldehyde (PFA) with PBS
71. Vortex the cell pellet for 15 s
72. Add 5 mL of 2% FA solution to each barcode set of samples
73. Vortex for 30 s, thoroughly disrupting the pellet
74. Incubate for 10 min at 23°C
75. During the fresh fix incubation, thaw the DNA intercalator at RT for about 5 min, until total thawed
76. After the 10 min Fresh Fix incubation, centrifuge samples at 935 x g for 3 min
77. Add the DNA intercalator to 2% PFA at 1:5000
78. Add 1 mL of 2% PFA + DNA intercalator to each barcode batch and resuspend cells
79. Count samples for cell concentration of each barcode batch
    a. Pipette 20 μL of cell suspension (in 2% PFA + DNA intercalator) into a cell counting slide, labeled with the respective barcoding batch
    b. Count (using "total concentration" assay) and record cells/mL

Pause Point: Store at 4°C until acquisition

Instrument Tuning and Sample Acquisition

Timing: sample acquisition: 12–24 h (across two instruments)

Below, we outline the sample acquisition procedure for Helios™ Mass Cytometers that were adapted for superior data quality. The startup procedure follows the instrument manufacturer's guidelines with a few modifications. It is important to properly tune the Helios™ prior to sample acquisition to ensure the highest possible data quality can be collected. The washing and sample acquisition procedure is a modified version of the manufacturer's guidelines, tailored to large sample numbers with long-duration sample acquisitions.

80. Retrieve samples from 4°C storage
81. Resuspend and transfer contents from 15 mL conical tubes into labeled 1.7 mL Microcentrifuge Tubes for washing

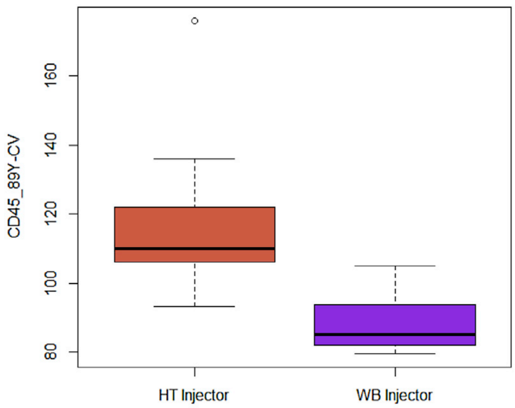


Figure 6. Comparison of Injector Type on Signal Variability as Accessed by CD45_89Y Coefficient of Variable

Identical samples were acquired in replicate 100,000 events each for two different acquisition modes, Wide Bore (WB) and High Throughput (HT) injectors. The WB injector significantly decreased the variability of the CD45_89Y signal. p-value=0.0001, Welch Two Sample T-test.

82. Centrifuge samples for 5 min at 800 × *g* at 23°C
83. Aspirate supernatant by pipette
84. Add 1 mL Cell Staining Buffer (CSB) to each sample and pipette or vortex to resuspend
85. Centrifuge for 5 min at 800 × *g* at 23°C
86. Aspirate supernatant by pipette
87. Add 1 mL Cell Acquisition Solution (CAS) to each sample and pipette or vortex to resuspend
88. Label additional 1.7 mL Microcentrifuge Tubes for each sample
89. Aliquot approximately 750,000 - 1 million cells from each sample into the respective additional 1.7 mL Microcentrifuge Tubes (use cell counts from step 79)
90. Bring total volume of each aliquot up to 1 mL in CAS
91. Centrifuge for 5 min at 800 × *g* at 23°C
92. Aspirate supernatant from all Eppendorf tubes and leave pelleted at 4°C until ready to acquire
93. Prepare stock of 1:5 EQ Bead/CAS mixture by combining 4 mL EQ Beads with 16 mL CAS in a 50 mL conical tube and vortex to mix
94. Resuspend one sample aliquot in 1 mL of EQ Bead/CAS mixture and filter through 35 μm blue filter cap into a polypropylene FACS Tube (per instrument)
95. Acquire each sample for 7200 s, with each aliquot being acquired one at a time for approximately 30 min each
96. Adjust concentration with 1:5 EQ Bead/CAS mixture if necessary, to achieve an acquisition rate of 300–350 events/s
97. Once samples have been acquired, the files are normalized, concatenated (if necessary), and de-barcoded in CyTOF software

⚠ CRITICAL: Cell pellet must be aspirated to be as dry as possible in step 86. Samples sitting in CAS can lead to degraded signal in some channels as seen in Figure 7. This is circumvented by reducing exposure to CAS, as mentioned, and by aliquoting samples that run for only 30–45 min at a time.

Note: This protocol has successfully been tested on PBMC samples processed from leukoreduction apheresis product or PMBCs isolated from whole blood using Ficoll density-gradient centrifugation (Holland et al., 2018).

Note: Instrument start-up and tuning can be performed simultaneously with sample washing. For longer acquisitions, it is helpful to only wash two of the four groups of samples at first, then wash the other two groups of samples when 45 min remain of the initial sample acquisitions. Leaving the samples in the DNA-Ir step is the most stable method for those samples that will be run later.

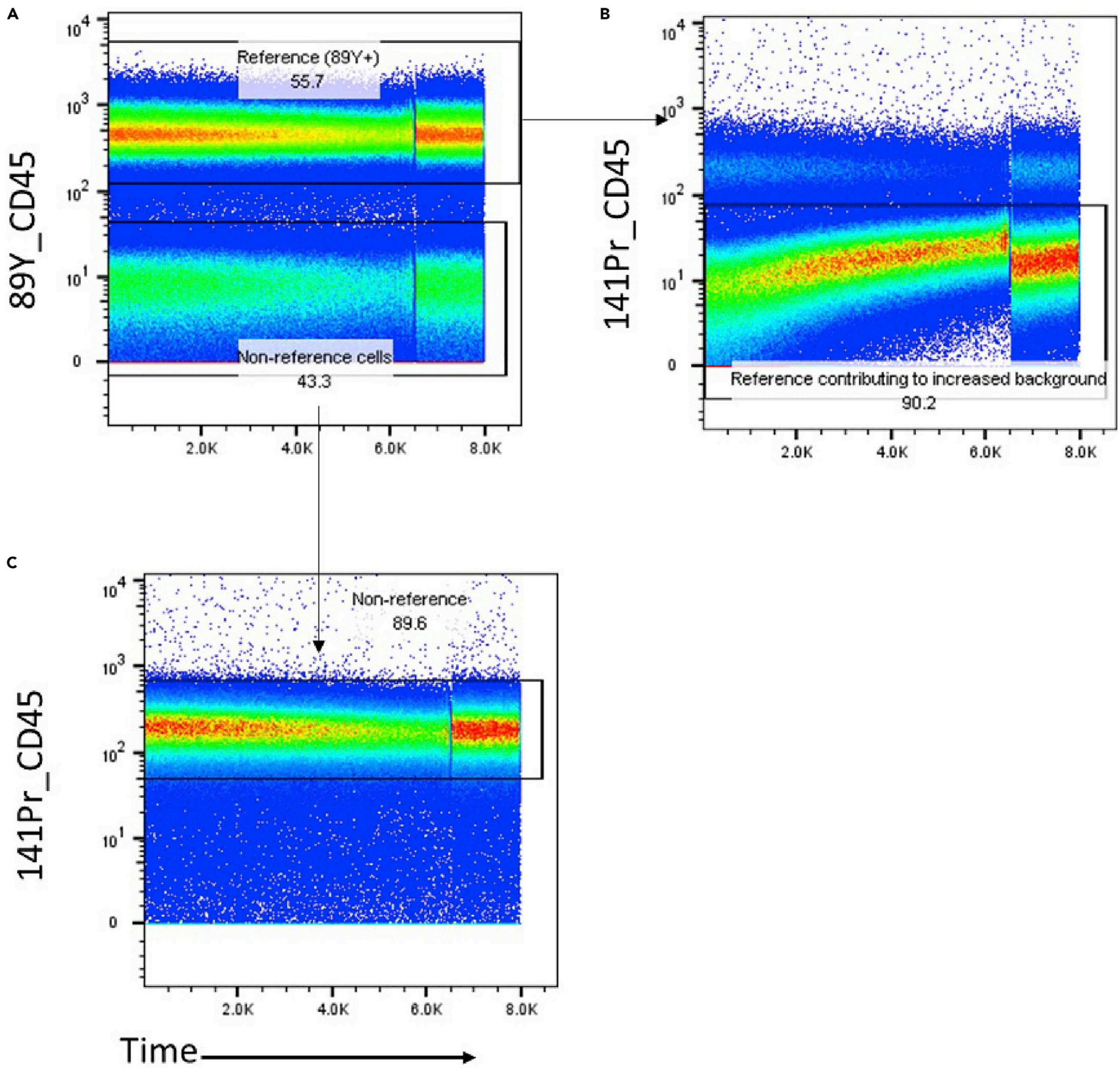


Figure 7. Tracking Increased Signal Drift Over Time during Long Sample Acquisitions

(A) 89Y_CD45 positive reference cells contribute to an increase in background signal when resuspended over the course of an acquisition of at least 2 h.

(B) Reference cells start as 141Pr_CD45 negative but contribute to increased background signal over time.

(C) 89Y_CD45 negative cells are shown to be 141Pr_CD45 positive cells. These analyses were performed on "live, intact, single cells", thus the increase in background signal in the ^{141}Pr channel cannot be attributed to cell doublets.

Note: This protocol is specific for WB injector and sample acquisition in CAS. Using this protocol, the WB injector with CAS showed superior data quality on both instruments at the Longwood Medical Area CyTOF Core compared to Fluidigm's High Throughput (HT) injector (as shown in Figure 6). We have published more comprehensive results in collaboration with a multi-site comparison study to compare WB vs. HT injectors (Lee et al., 2019).

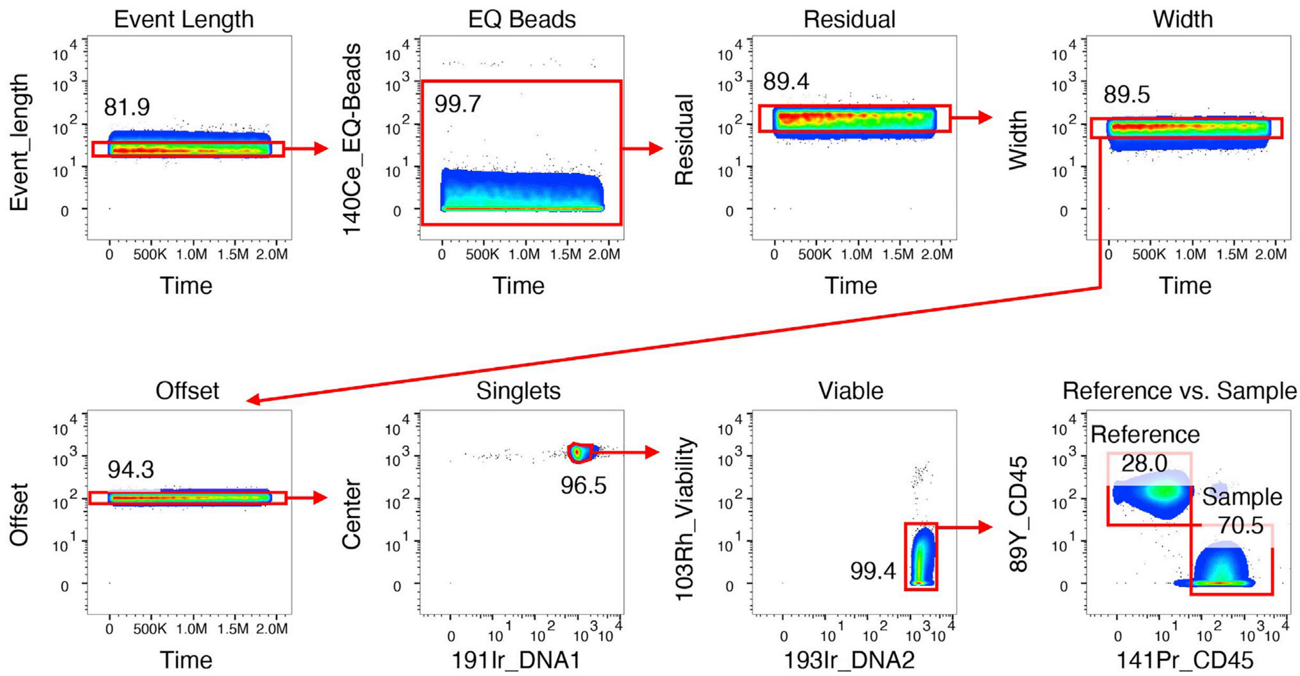


Figure 8. Clean-up Gating with CD45 Barcoded Reference Sample Spike-in

A representative single fcs file is cleaned-up to identify viable single cells with reference sample and experimental sample separated by manually gating on the CD45 channels, ^{89}Y and ^{141}Pr.

Note: The number of additional 1.7mL Microcentrifuge Tubes required for each sample is determined by the total cell count for a given sample. The goal is to reach an acquisition concentration of approximately 700,000 cells/mL which equates to a rate of 350 events/s, in a volume of 1 mL. This is to keep the run time per aliquot to ≈30 min. It minimizes the amount of background, which is seen when samples are acquired for 45 min or longer in one resuspension (Figure 7).

EXPECTED OUTCOMES

At the end of this protocol, fcs files will be generated for 80 samples. Clean-up gating will be required to identify viable singlet cells, and to separate the CD45_89Y+ reference sample spike-in from the CD45_141Pr+ experimental sample. Clean-up gating is routinely performed by manual gating in cytometry software, like Flowjo. Cell events are gated through biaxial plots of time vs. event length, bead channel (to remove the EQ Beads), Gaussian-derived parameters (Residual, Width, Offset, Center), then viability (^{103}Rh) and DNA intercalators (DNA 1 or 2) to identify viable, singlet cell events (shown in Figure 8) (Bagwell et al., 2020). The viable singlet cell events with spike-in sample removed can be manually gated further to find population frequencies of certain cell subsets, or analyzed using high-dimensional analysis algorithms and software kits (Kimball et al., 2018).

Approximately 75% of total cells are lost over the course of staining from initial cell counts (2 million total cells per well) and cleanup analysis (Figure 9) (See Troubleshooting Problem 4 if more loss is routinely observed with this protocol). Manual gating can be continued to identify population frequencies for each marker. Starting with 2 million total cells for staining is enough for gating major immune cell populations and activation, checkpoint markers in the Adaptive and Innate mass cytometry panels. For markers that have dynamic expression, such as OX-40, it can be difficult to find a clear separation of positive and negative populations, and thus challenging to determine the positive expression threshold. The independently stained ex vivo and stimulated reference samples are helpful to guide placing these gates by the increase in marker expression after

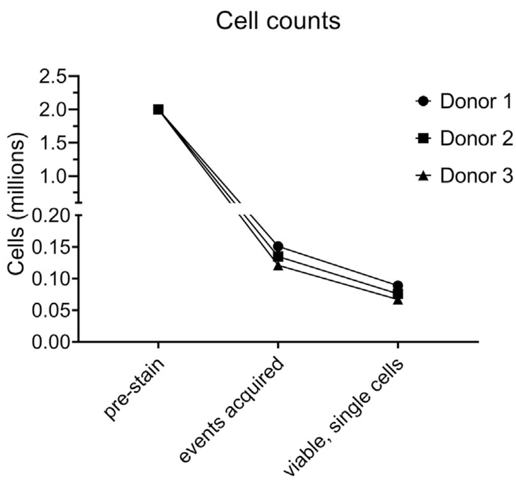


Figure 9. Tracking Cell Loss during Staining Protocol
Cell count changes over the course from post-thawing and plating, to how many events acquired, and after clean-up gating are shown for three independent healthy donor PBMC samples. Three healthy donor PBMC samples were stained for MC using our full protocol. We observed ≈ 75% cell loss (mean values: pre-stain = 2 million, events acquired = 136k, events per sample after clean-up gating (''viable, single cells'') = 77k).

stimulation (Figure 10). Additionally, reference sample spike-in serves as a quality control to compare intra-experiment staining variability within and across barcode sets, as well as to observe staining differences between mass cytometer panels. Analyzing markers that are shared between both the Adaptive and Innate mass cytometry panels (Tables 3 and 4) can reveal batch effects of staining samples independently. In Figure 11, the overlapping markers show consistent staining between both panels and across four sets of 20-Plex Pd Barcoding sets. Importantly, the adaptive and innate mass cytometry panels were prepared independently, thus demonstrating that no batch effects were observed from preparing the antibody surface master mixes (Figures 11A and 11B). The four reference samples that were run independently (not spiked-in) are compared across the two mass cytometry panels (Figure 11C). The population frequencies were comparable across spike-in reference samples, across independent reference barcode samples, across barcode sets and across mass cytometry antibody panels therefore demonstrating that this protocol produces reproducible data in a high-throughput manner for up to 80 samples.

LIMITATIONS

This protocol assumes the use of an automated cell counter, two mass cytometer instruments for acquisition, and at least two technicians for thawing samples. Without these assumptions met, the protocol may take longer than the estimated hands-on time. However, the samples can be acquired over the course of 4 days on one instrument.

Palladium mass-tag cell barcoding, as used in this protocol, partially permeabilizes cells and must be performed after surface staining because it can cause staining issues for certain surface markers. This neglects one of the key benefits of sample barcoding, which is to stain the samples in a single tube and thus mitigating variable of staining samples separately. However, we have shown that this variance of freshly preparing the master mixes is low (% CV < 5 for most markers) (Figure 11B).

This protocol is optimized for immunophenotyping human PBMCs processed from peripheral blood using Ficoll density-gradient centrifugation, and therefore is specialized for analyzing CD45+ cells. This protocol is limited to this sample type. CD45 can be substituted for any marker specific to the researcher's hypothesis. This protocol describes a framework staining a shared marker between experimental and reference sample spike-in with two different metal isotopes.

Preparing the surface and intracellular master mixes fresh for every experiment, as recommended in this protocol, is laborious, time-consuming, and introduces a batch effect between master mix preparation and experiments. In Figure 11, we demonstrate reproducible results from master mixes

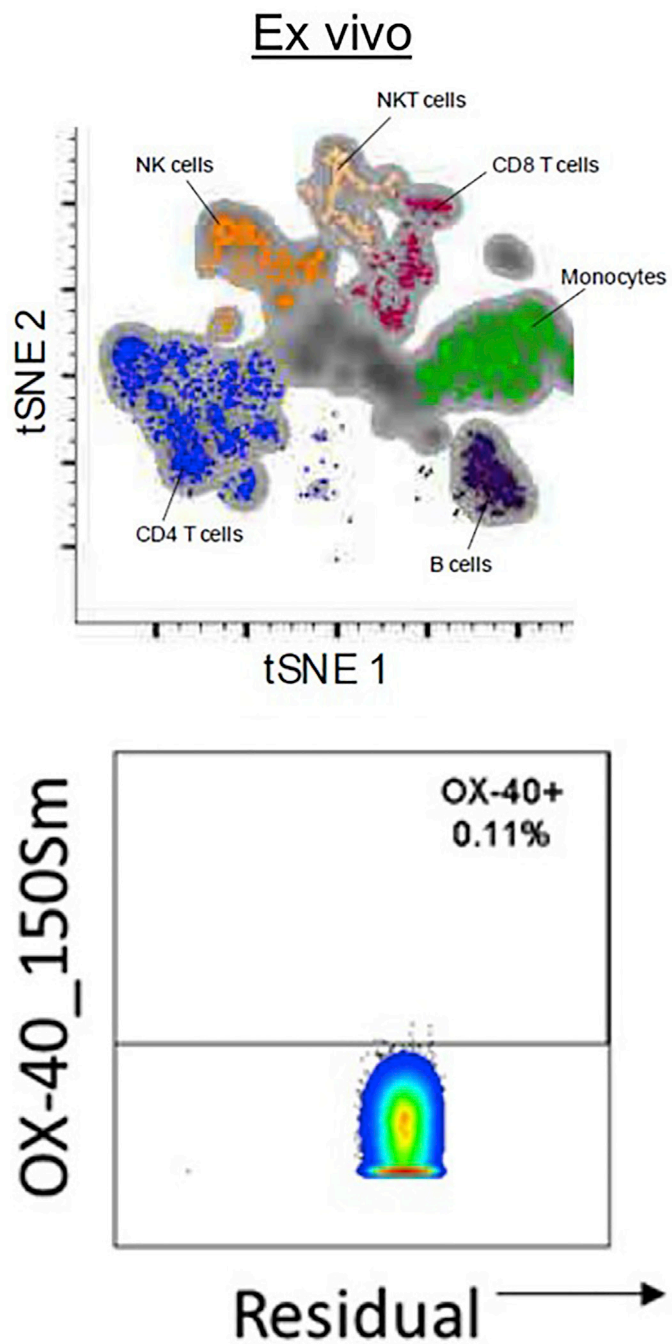


Figure 10. Independent Pd Barcode Ex Vivo and Stimulated Reference Samples Guides Gating

Ex vivo and Stimulated reference sample are run as independent samples of the barcoding strategy.

(A) Samples were analyzed using tSNE algorithm (viSNE maps created using cytobank.com) and show changes of major cell populations. Clusters indicate cell subsets and the major cell lineages are overlaid with colored dot plots. Each dot represents one cell.

(B) Manual gating (Flowjo) of ex vivo and stimulated samples guides where to place positive threshold gate for dynamic markers that are expected to increase with stimulation.

prepared separately. The mass cytometry community has come up with a few options to circumvent the issue of batch effects in preparing master mixes, including making large batches of antibody master mixes and lyophilizing or storing at -80°C. We address storage conditions in Figure 3.

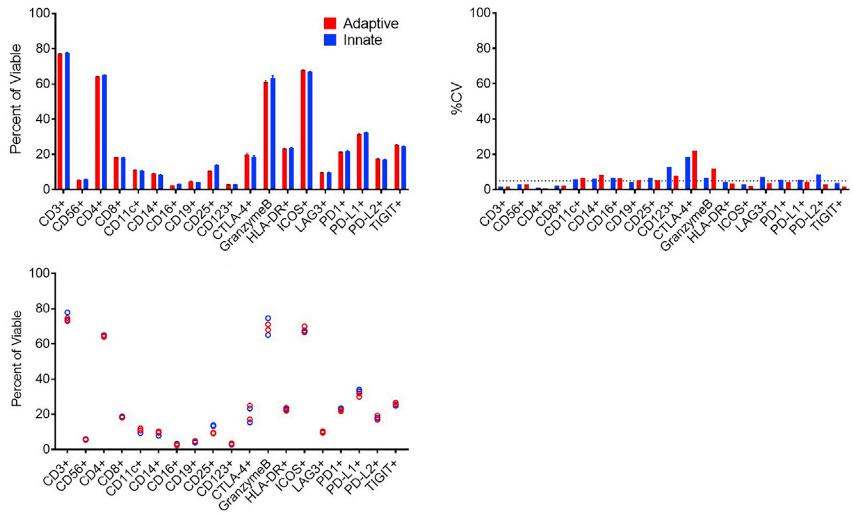


Figure 11. Reference Sample Spike-In and Palladium Sample Barcoding Generates Reproducible Results

Stimulated and ex vivo healthy donor PBMC samples were stained with either the adaptive or innate MC panels. Two sets of palladium sample barcodes were used for each panel. Each barcode set included one independent sample of stimulated and ex vivo and the remaining 18 barcodes were replicate samples of 1:1 mixed stimulated and ex vivo samples.

(A) Manual gating was compared for cellular frequencies as percent of viable for 19 overlapping markers between the adaptive and innate panels for spike-in reference sample (n = 36 replicates spike-in reference samples, bar graph of mean with standard error of mean for error bars).

(B) Percent CV graph of data in shown in (A), dotted line at 5%.

(C) Comparison of 1:1 mixed stimulated and ex vivo samples as independent barcoded samples (n = 2/panel).

Though, preparing large batches of master mixes is typically done by an outside vendor and increases costs. Additional validation experiments would be needed specific to the antibody panels to test if the storage condition impacted each marker's signal intensity. This has not been performed on the Adaptive or Innate panels described in this manuscript.

TROUBLESHOOTING

Problem 1

Ex vivo and stimulated reference sample are not positive for markers in the panel.

Potential Solution

Try other stimulation conditions and combinations to mediate expression of each marker in your panel.

Typically, we employ both the Adaptive and Innate MC panels in one staining experiment, therefore we needed a stimulation condition that could detect each marker in both panels. The panels monitor activation markers with dynamic expression so it is important to use the appropriate stimulation condition that will induce markers for both panels. We tested CD3/CD28 beads for 48 h alone compared to PMA/ionomycin for 2 h, compared to a combination of both beads with PMA/ionomycin (Figure 12). A 48-h CD3/CD28 Dynabead stimulation reduced the total percent of CD11c (top row) compared to the ex vivo condition. However, Lag-3 (middle row) and CD69 (bottom row) expression is virtually undetectable in ex vivo samples. The CD3/CD28 bead stimulation condition is optimal for these panels as it preserved CD11c+ and CD56+ cell populations concomitant with strong induction of activation marker CD69 and checkpoint molecules like Lag-3. This pattern was observed with the CD3/CD28 bead stimulation condition for virtually all activation and checkpoint molecules in the panel, though the ex vivo condition preserved monocyte populations.

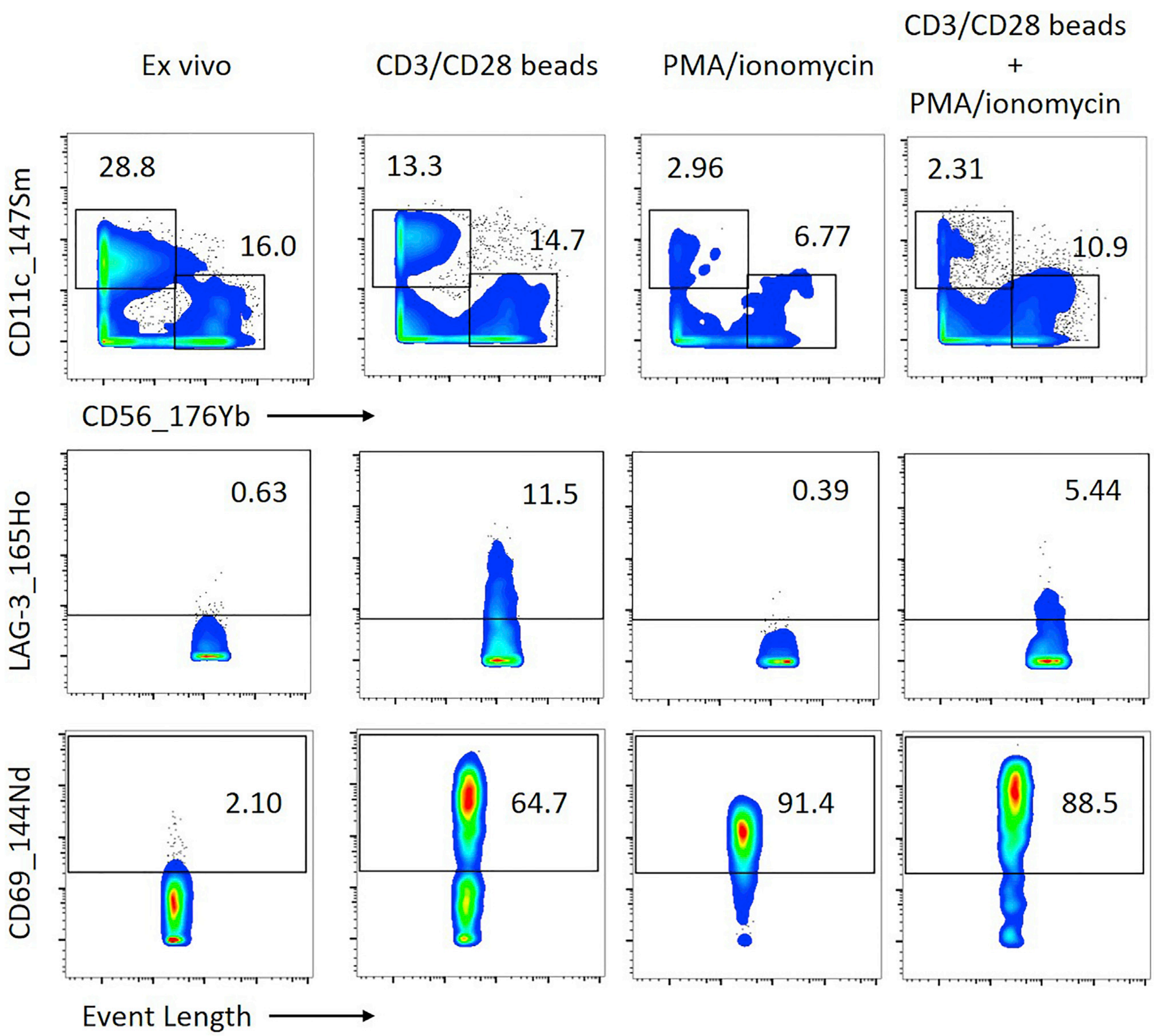


Figure 12. Stimulation Conditions Are Tested to Detect Markers in Mass Cytometry Antibody Panels

Healthy donor PBMC samples were treated with various stimulation conditions at various timepoints: PMA + ionomycin, CD3/CD28 bead stimulation, or a combination of CD3/CD28 beads with PMA + ionomycin. Specific markers are highlighted here to represent the dynamic range of activating these markers. Top row: CD11c. Middle row: Lag-3. Bottom row: CD69.

Problem 2

Antibodies conjugated in the laboratory failed to detect marker appropriately.

Potential Solution

If there is no detection of the marker of interest, then repeat the conjugation. Conjugations can fail. If there is detection, but no plateau indicating saturation, then repeat the titration experiment using major immune lineage markers to characterize the marker staining on specific cell populations (as shown in Figure 2). If other antibodies were used in the titration experiment, try testing for spillover. Run a sample without the titrated antibody. If there is high background signal then consider re-titrating the antibodies that are 16 atomic mass units below the metal channel being titrated, which would indicate oxidation spill-over. Also, try re-titrating antibodies with the same metal (for example ^{146}Nd and ^{145}Nd) to test for isotope impurity.

Problem 3

Antibody signal intensity varies between master mixes and experiments.

Potential Solution

We propose four potential solutions to mitigate antibody staining variance: test for technical error during antibody master mix preparation, check the date of conjugation (if conjugated by the researcher) or contact the vendor (if commercially purchased), check cell counts such that spike-in reference sample brought the total amount of cells in each well to 2 million, re-titrate the antibody in question, and plan out samples to be acquired in the same palladium barcoding set.

It is possible for staining intensities between experiments to vary due to preparing the master mix fresh every time. In Figure 11, we compare markers from separately prepared and filtered master mixes and show that this variance is low. A similar comparison between the reference sample spike-in can be performed to test for technical error introducing batch effects to the master mix preparation. This highlights the value of using the same reference sample spike-in between experiments. In addition, the reference sample ensures that the same number of cells were stained with the antibody master mix. When counting the reference and experimental samples, use total cell counts, since dead cells can be stained with the antibody master mix.

A decrease in marker signal intensity may be observed with the researcher-conjugated antibodies over time. Though each conjugated antibody will have a different shelf-life, we have observed a decreasing signal intensity starting at 6 months. It is important to plan out experiments to ensure that the same conjugated antibody, or the same lot of antibody if commercially purchased, can be used on all samples for the experiment. If timepoint samples are collected, such as a sample at pre-treatment, month 1, month 2, etc. then a normalizing sample should be run with each timepoint sample, such as the pre-treatment sample. In this instance, it is recommended to collect all timepoints and run them in one barcoding batch to decrease intra-patient variance.

Problem 4

More than 75% cell events loss is observed.

Potential Solution

First, check the cell counts step that enough reference sample was spiked-in to the experimental sample for a total count of 2 million cells per well. This is critical since the titration experiments were performed on this set number of cells. Next, compare the cell count steps from the protocol to check where most of the cell loss is occurring. This protocol includes multiple fixation steps that can make the cell pellets fragile. Ensure that fixations adhere to this protocol and do not go over the recommended incubation time. If enough events were acquired, check the clean-up gating for each sample to check for cell populations falling outside the gating and if they can be included in analysis.

ACKNOWLEDGMENTS

We would like to thank our funding sources "The G. Harold & Leila Y. Mathers Foundation." We thank Rachel Cunningham, Martha Holland, and Lake Seymour of the DFCI Center for Immuno-Oncology for their technical assistance with mass cytometry experiments. We also thank Suzan Lazo and John Daly and their team at the Flow Cytometry Cores and Longwood Medical Area CyTOF Core at DFCI for their technical assistance and feedback.

AUTHOR CONTRIBUTIONS

Conceptualization, E.M.T., K.K., F.S.H., and M.S.; Methodology, E.M.T., K.K., E.S.H., M.N., and E.H.; Investigation, E.M.T., K.K., E.S.H., M.N., and E.H.; Resources, F.S.H. and M.S.;

Writing – Original Draft, E.M.T., E.S.H., M.N., and E.H.; Writing – Review & Editing, E.M.T., K.K., E.S.H., M.N., E.H., F.S.H., and M.S.; Visualization, E.M.T., K.K., E.S.H., M.N., and E.H.; Supervision, Project Administration, and Funding Acquisition, F.S.H. and M.S.

DECLARATION OF INTERESTS

K.K. is currently an employee of bluebird bio. F.S.H. serves as a consultant to Genentech, Bristol-Myers Squibb, Merck, Novartis, Amgen, Sanofi, Bayer, Pfizer, EMD Serono, Verastem, Aduro, Celldex, and Incyte.

REFERENCES

Bagwell, C.B., Inokuma, M., Hunsberger, B., Herbert, D., Bray, C., Hill, B., Stelzer, G., LI, S., Kollipara, A., Ornatsky, O., et al. (2020). Automated data cleanup for mass cytometry. Cytometry A *97*, 184–198.

Holland, M., Cunningham, R., Seymour, L., Kleinsteuber, K., Cunningham, A., Patel, T., Manos, M., Brennick, R., Zhou, J., Hodi, F.S., et al. (2018). Separation, banking, and quality control of peripheral blood mononuclear cells from whole blood of melanoma patients. Cell Tissue Bank. *19*, 783–790.

Kimball, A.K., Oko, L.M., Bullock, B.L., Nemenoff, R.A., van Dyk, L.F., and Clambey, E.T. (2018). A beginner's guide to analyzing and visualizing mass cytometry data. J. Immunol. *200*, 3–22.

Kleinsteuber, K., Corleis, B., Rashidi, N., Nchinda, N., Lisanti, A., Cho, J.L., Medoff, B.D., Kwon, D., and Walker, B.D. (2016). Standardization and quality control for high-dimensional mass cytometry studies of human samples. Cytometry A *89*, 903–913.

Lee, B.H., Kelly, G., Bradford, S., Davila, M., Guo, X.V., Amir, E.D., Thrash, E.M., Solga, M.D., Lannigan, J., Sellers, B., et al. (2019). A modified injector and sample acquisition protocol can improve data quality and reduce inter-instrument variability of the helios mass cytometer. Cytometry A *95*, 1019–1030.

Patel, T., Cunningham, A., Holland, M., Daley, J., Lazo, S., Hodi, F.S., and Severgnini, M. (2018). Development of an 8-color antibody panel for functional phenotyping of human CD8+ cytotoxic T cells from peripheral blood mononuclear cells. Cytotechnology *70*, 1–11.

Schulz, A.R., Baumgart, S., Schulze, J., Urbicht, M., Grützkau, A., and Mei, H.E. (2019). Stabilizing antibody cocktails for mass cytometry. Cytometry A *95*, 910–916.