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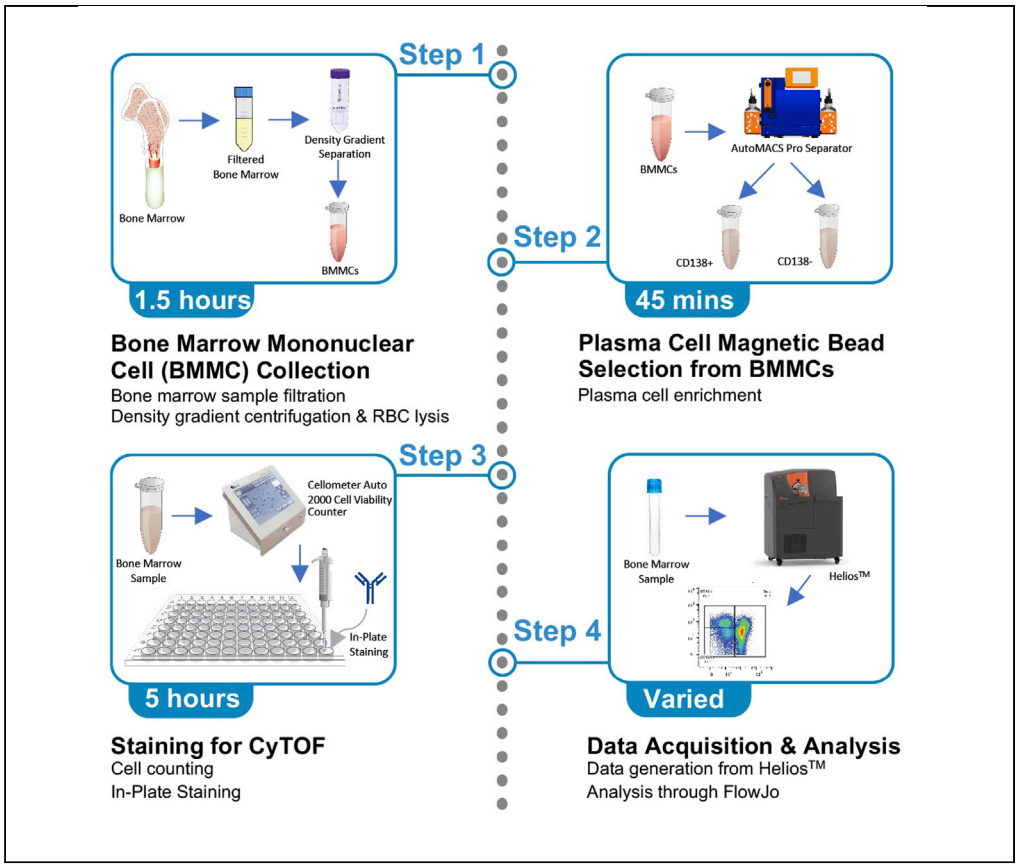
Protocol

Mass cytometry staining for human bone marrow clinical samples



This protocol details a staining technique optimized for immunophenotyping of human bone marrow immune populations using mass cytometry. The protocol accounts for the limitations of working with human bone marrow, such as reduced viability, low cell counts, and fragile cell pellets, to successfully acquire single viable cells ready for downstream analysis. This assay can be used to characterize the activation, exhaustion, and cytotoxicity of immune populations and ensure comprehensive immunophenotyping of human bone marrow clinical samples.

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Highlights

Staining protocol for assessment of immune cells in human bone marrow clinical samples

Validated mass cytometry panel for distinction of major immune populations

Insights on gating strategy for multiparameter mass cytometry data analysis

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Mass cytometry staining for human bone marrow clinical samples

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SUMMARY

This protocol details a staining technique optimized for immunophenotyping of human bone marrow immune populations using mass cytometry. The protocol accounts for the limitations of working with human bone marrow, such as reduced viability, low cell counts, and fragile cell pellets, to successfully acquire single viable cells ready for downstream analysis. This assay can be used to characterize the activation, exhaustion, and cytotoxicity of immune populations and ensure comprehensive immunophenotyping of human bone marrow clinical samples.

BEFORE YOU BEGIN

Preparation for bone marrow mononuclear cell (BMMC) collection

Timing: 10 min

Ethics approval was obtained from Dana-Farber/Harvard Cancer Center Institutional Review Board (IRB 14-174) and all patients provided informed consent

1. Bring Ficoll-Paque PLUS, 1× PBS, and 10× RBC Lysis Buffer to room temperature (19°C–22°C)
2. Prepare a working solution of 20 mL 1× RBC Lysis Buffer by diluting 2 mL 10× RBC Lysis Buffer with 18 mL Distilled Ultrapure Water
3. Prepare Freezing Media containing 90% FBS and 10% DMSO by volume and keep at 4°C until use

Note: Each sample requires approximately 1 mL freezing media for the CD138+ cell fraction (expected cell count of 1 × 10^6 CD138+ cells) and 4 mL of freezing media for the CD138- cell fraction (expected cell count of 4 × 10^6 CD138+ cells).

Human bone marrow mononuclear cell (BMMC) collection

Timing: 90 min

To isolate bone marrow mononuclear cells (BMMC), BMMCs are first enriched from aspirates by density gradient centrifugation using Ficoll-Paque within SepMate™ PBMC Isolation Tubes. Any remaining erythrocytes are then briefly lysed using 1× RBC Lysis Buffer, and samples are washed with 1× PBS to remove residual lysis buffer and any cellular debris.

4. Pre-wet a 100 μm MACS® SmartStrainer with 1× PBS within a 50 mL conical tube. Discard 1× PBS used to pre-wet MACS® SmartStrainer from your 50 mL conical tube
5. Slowly filter bone marrow sample through the pre-wet MACS® SmartStrainer into 50 mL conical tube, using the end of a syringe plunger to break apart any clots above the filter and to filter out any bone debris
6. Make note of the volume of bone marrow aspirate post-filtering, then dilute bone marrow sample with 1× PBS three times its volume (for example, dilute 10 mL of bone marrow with 30 mL PBS)
7. Mix the 1× PBS and bone marrow by gently inverting the tube 8–10 times
8. Add 15 mL of Ficoll-Paque to a 50 mL SepMate™ tube by carefully pipetting it through the central hole of the plastic insert. The top of the density gradient medium should be just above the insert

   Note: Any large bubbles present within the density gradient medium should be removed by spinning at 300 g for 1 min prior to loading your sample.

9. Gently layer the diluted bone marrow onto the Ficoll-Paque by pipetting it down the side of the SepMate™ tube. The bone marrow should overlay on top of the Ficoll-Paque.

   Note: If the volume of diluted bone marrow is larger than 30 mL, prepare multiple SepMate™ tubes and evenly distribute the sample

   ⚠ CRITICAL: Do not let the Ficoll-Paque and bone marrow aspirate mix under the plastic insert within the SepMate™ tube, as this will affect the yield, purity and 127I content of the isolated BMMCs.

10. Centrifuge the SepMate™ tube(s) containing the diluted bone marrow aspirate and Ficoll-Paque at 1200 g for 20 min at room temperature, with the brake on
11. Carefully pour the top layer above the plastic insert from the SepMate™ tube(s) (which contains enriched BMMCs) into a new 50 mL conical tube

    ⚠ CRITICAL: Do not hold SepMate™ tube in the inverted position for longer than 2 seconds, as this will cause separated RBCs to pass through the plastic insert into the new 50 mL conical tube.

12. Discard the SepMate™ tube containing RBCs and granulocytes in biohazard waste
13. Centrifuge the new at 300 g for 8 min, with the brake on
14. Pour off the supernatant and resuspend the cell pellet in 20 mL of 1× RBC Lysis Buffer, and gently invert the tube 8–10 times
15. Immediately after centrifuge the 50 mL conical tube containing sample at 300 g for 8 min with the brake on
16. Pour off supernatant and resuspend the pellet in 40 mL PBS. Mix by gently inverting the tube 8–10 times
17. Centrifuge at 300 g for 8 min with the brake on
18. Pour off supernatant and resuspend pellet in 10 mL PBS
19. Count cells using 0.4% Trypan Blue (1:1 dilution, 10 μL sample in 10 μL Trypan Blue) with the Invitrogen™ Countess II Automated Cell Counter (or an alternative cell counter)
    a. Remove 10 μL of resuspended sample and mix well with 10 μL 0.4% Trypan Blue
    b. Load 10 μL of diluted of the Trypan Blue diluted sample to a Countess Cell Chamber
20. The resuspended sample now contains BMMCs. Proceed to plasma cell magnetic bead enrichment using this cell count

Plasma cell magnetic bead selection from BMMCs

Timing: 45 min

Once BMMCs have been isolated from the marrow aspirate, the sample is then enriched for plasma cells, using Miltenyi CD138 Microbeads* (Human) and the autoMACS® Pro Separator. This process will produce both CD138+ and CD138- fractions

21. Centrifuge resuspended BMMCs at 300 g for 8 min, with the brake on
22. Aspirate supernatant and resuspend BMMCs in 80 μL of autoMACS Running Buffer and 20 uL CD138 Microbeads per 2.0×10^7 BMMCs**

Note: *Other separation kits are acceptable yet were not validated when developing this protocol. **Change volume of both beads and running buffer in proportion to BMMC cell count obtained in Step 19 of BMMC Collection.

23. Mix by gently pipetting without introducing bubbles, and incubate at 4°C for 15 min
24. After 15 min, wash the BMMC and magnetic bead mixture by pipetting very gently with 2 mL autoMACS Running Buffer
25. Centrifuge samples at 300 g for 10 min, with the brake on
26. Aspirate the supernatant and resuspend the bead pellet in 500 μL autoMACS Running Buffer
27. Transfer the resuspended sample to a new 15 mL conical tube
28. Label 2 new additional 15 mL conical tubes as "CD138+" and "CD138-", followed by the sample ID
29. Place the 15 mL tube containing the re-suspended sample in the autoMACS® Pro Separator tube rack in the designated sample slot, and place the empty pre-labeled 15 mL conical tubes in each of the appropriate locations for the positive and negative sample fractions to deposit into
30. Ensure there are adequate volumes of autoMACS Washing Buffer, autoMACS Running Buffer, 70% EtOH, and sufficient space in the waste in reagent reservoirs attached to the autoMACS® Pro Separator
31. Select the protocol named "Possel" on the autoMACS® Pro Separator to enable automated bead selection to begin
32. Once autoMACS® Pro Separator is done with enriching CD138+ and CD138- cells fractions, centrifuge samples at 300 g for 8 min, with the brake on
33. Remove supernatant and resuspend each sample in 1 mL of PBS.
34. Count each cell fraction ($CD138^+$/$CD138^-$) using 0.4% Trypan Blue (1:1 dilution, 10 μL sample in 10 μL Trypan Blue) with the Invitrogen™ Countess II Automated Cell Counter (or an alternative cell counter)
    a. Remove 10 μL of resuspended sample and mix well with 10 μL 0.4% Trypan Blue
    b. Load 10 μL of diluted of the Trypan Blue diluted sample to a Countess Cell Chamber
35. Dilute both $CD138^+$ and $CD138^-$ fractions to 10 mL total by adding 9 mL PBS to wash away remaining autoMACS running buffer
36. Centrifuge samples at 300 g for 8 min, with the brake on.
37. Carefully aspirate and discard supernatant.

Note: Given bone marrow research aspirates low volume and the low prevalence of plasma cells within the bone marrow niche, the CD138+ cell pellet is often less than 1 million cells and difficult to visualize after pelleting. Therefore, it is best to aspirate and discard all but the last ~50 uL of supernatant.

38. Resuspend the $CD138^+$ fraction in 1 mL of Freezing Media (90% FBS + 10% DMSO) and aliquot into a single cryovial

39. Resuspend the CD138$^-$ fraction in 4 mL of Freezing Media and aliquot into 4 individual cryovials
40. Place vials in CoolCell FTS30 (or alternative slow-freeze container) and into a -80°C Freezer for 24 h before moving to long-term storage in liquid nitrogen.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Human CD45 (HI30)-89Y—100 Tests (1:100)	Fluidigm	Cat#3089003B
Anti-Human CD196/CCR6 (G034E3)-141Pr—50 Tests (1:100)	Fluidigm	Cat#3141003A
Anti-Human CD19 (HIB19)-142Nd—100 Tests (1:100)	Fluidigm	Cat#3142001B
Anti-Human CD45RA (HI100)-143Nd—100 Tests (1:100)	Fluidigm	Cat#3143006B
Anti-Human CD69 (FN50)-144Nd—100 Tests (1:100)	Fluidigm	Cat#3144018B
Anti-Human CD4 (RPA-T4)-145Nd—100 Tests (1:100)	Fluidigm	Cat#3145001B
Anti-Human IgD (IA6-2)-146Nd—100 Tests (1:100)	Fluidigm	Cat#3146005B
Anti-Human CD20 (2H7)-147Sm—100 Tests (1:100)	Fluidigm	Cat#3147001B
Anti-Human CD14 (RMO52)-148Nd—100 Tests (1:100)	Fluidigm	Cat#3148010B
Anti-Human CD56/NCAM (NCAM16.2)-149Sm—100 Tests (1:100)	Fluidigm	Cat#3149021B
Anti-Human CD138/Syndecan-1 (DL-101)-150Nd—25 tests (1:100)	Fluidigm	Cat#3150012C
Anti-Human TNFa (mab11)-152Sm—100 Tests (1:100)	Fluidigm	Cat#3152002B
Anti-Human TIGIT (MBSA43)-153Eu—100 Tests (1:100)	Fluidigm	Cat#3153019B
Anti-Human CD3 (UCHT1)-154Sm—100 Tests (1:100)	Fluidigm	Cat#3154003B
Anti-Human CD27 (L128)-155Gd—100 Tests (1:100)	Fluidigm	Cat#3155001B
Anti-Human IFNg (B27)-158Gd—100 Tests (1:100)	Fluidigm	Cat#3158017B
Anti-Human CD161 (HP-3G10)-159Tb—100 Tests (1:100)	Fluidigm	Cat#3159004B
Anti-Human/Mouse MIP1beta (D21-1351)-160Gd—100 Tests (1:100)	Fluidigm	Cat#3160013B
Anti-Human/Mouse Tbet (4B10)-161Dy—100 Tests (1:100)	Fluidigm	Cat#3161014B
Anti-Human FoxP3 (259D/C7)-162Dy—50 Tests (1:100)	Fluidigm	Cat#3162024A
Anti-Human CD183/CXCR3 (G025H7)-163Dy—100 Tests (1:100)	Fluidigm	Cat#3163004B
Anti-Human IL-17A (N49-653)-164Dy—100 Tests (1:100)	Fluidigm	Cat#3164002B
Anti-Human CD279/PD-1 (EH12.2H7)-165Ho—100 Tests (1:100)	Fluidigm	Cat#3165042B
Anti-Human CD197/CCR7 (G043H7)-167Er—50 Tests (1:100)	Fluidigm	Cat#3167009A
Anti-Human CD8 (SK1)-168Er—100 Tests (1:100)	Fluidigm	Cat#3168002B
Anti-Human CD25 (2A3)-169Tm—100 Tests (1:100)	Fluidigm	Cat#3169003B
Anti-Human Granzyme B (GB11)-171Yb—100 Tests (1:100)	Fluidigm	Cat#3171002B
Anti-Human CD57 (HCD57)-172Yb—100 Tests (1:200)	Fluidigm	Cat#3172009B
Anti-Human HLA-DR (L243)-173Yb—100 Tests (1:200)	Fluidigm	Cat#3173005B
Anti-Human CD94 (HP-3D9)-174Yb—100 Tests (1:100)	Fluidigm	Cat#3174015B
Anti-Human Perforin (B-D48)-175Lu—100 Tests (1:100)	Fluidigm	Cat#3175004B
Anti-Human CD127/IL-7Ra (A019D5)-176Yb—100 Tests (1:100)	Fluidigm	Cat#3176004B
Anti-Human CD16 (3G8)-209Bi—100 Tests (1:100)	Fluidigm	Cat#3209002B
Anti-Granzyme K antibody [GM-24C3] (ab3771) (1:100)	Abcam	Cat#ab3771
Chemicals, peptides, and recombinant proteins		
Ficoll-Paque Plus	Global Life Sciences	Cat#45001740
1× Phosphate-Buffered Saline without Calcium & Magnesium	Mediatech	Cat #21040CV
10× RBC Lysis Buffer	BioLegend	Cat #420301
0.4% Trypan Blue Solution	Life Technologies	Cat #15250061
autoMACS Running Buffer – MACS Separation Buffer	Miltenyi Biotec	Cat #130091221

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CD138 MicroBeads, Human	Miltenyi Biotec	Cat #130051301
DMSO	Sigma-Aldrich	Cat #D2650
PBS, pH 7.4 (1×)	Gibco	Cat#10010023
Bovine Serum Albumin (BSA), 30% ± 2% in 0.85% NaCl	Sigma-Aldrich	Cat#A7284
Sodium Azide, 10% (w/v) solution in Ultra-Pure H_2O	Teknova	Cat#S0209
RPMI 1640 (1×) Medium	Gibco	Cat#11875093
Fetal Bovine Serum, heat inactivated, qualified (FBS)	Gibco	Cat#10438026
Antibiotic-Antimycotic (100×)	Gibco	Cat#15240096
UltraPure™ DNAse/RNAse-Free Distilled Water	Invitrogen	Cat#10977023
ViaStain™ AOPI Staining Solution	Nexcelom Bioscience	Cat#CS2-0106-5mL
Cell-ID™ Intercalator-103Rh—500 μM	Fluidigm	Cat#201103A
Cell-ID™ Intercalator-191/193Ir—125 μM	Fluidigm	Cat#201192A
Maxpar® Cell Acquisition Solution	Fluidigm	Cat#201237
Maxpar® Cell Staining Buffer	Fluidigm	Cat#201068
EQ™ Four Element Calibration Beads	Fluidigm	Cat#201078
eBioscience™ Permeabilization Buffer (10×)	Invitrogen	Cat#00833356
eBioscience™ Fixation/Permeabilization Concentrate	Invitrogen	Cat#00512343
eBioscience™ Fixation/Permeabilization Diluent	Invitrogen	Cat#00522356
Human TruStain FcX™ (FcR Blocking Solution)	BioLegend	Cat#422302
Pierce™ 16% Formaldehyde Solution (w/v), Methanol-free	Thermo Scientific	Cat#28906
Critical commercial assays		
Maxpar® X8 Antibody Labeling Kit, 166Er—4 Rxn	Fluidigm	Cat#201166A
Biological samples		
Human bone marrow samples (sex, age as required par study)	Any supplier	N/A
Software and algorithms		
FlowJo	Becton Dickinson & Company	https://www.flowjo.com/solutions/flowjo/downloads/
CyTOF Software v7.0.8493	Fluidigm	https://www.fluidigm.com/software
Other		
SepMate-50 Tubes	Stemcell Technologies	Cat #85460
10 mL Serological Pipet	Corning Inc.	Cat #357530
25 mL Serological Pipet	Corning Inc.	Cat #1367530
25 mL Serological Pipet	Corning Inc.	Cat #1367530
MACS SmartStrainer 100 μm	Miltenyi Biotec	Cat #130098463
autoMACS Pro	Miltenyi Biotec	Cat #130092545
CoolCell FTS30	Thermo Fisher Scientific™	Cat #07210008
Countess II Automated Cell Counter	Thermo Fisher Scientific™	Cat #AMQAX1000
Countess Cell Counting Chamber Slides	Thermo Fisher Scientific™	Cat #C10228
Falcon 96-Well Polystyrene Microplate, TC Treated	Corning Inc.	Cat #353296
15 mL Polypropylene Centrifuge Tubes	Corning Inc.	Cat#430052
50 mL Polypropylene Centrifuge Tubes	Corning Inc.	Cat#430829
Cellometer® SD100 Cell Counting Chamber Slides	Nexcelom Bioscience	CHT4-SD100-014
70 μm Cell Strainer, Polypropylene Frame	Biologix	Cat#15-1070
Eppendorf® 1.5 mL Safe-Lock Microcentrifuge Tubes, natural	Eppendorf	Cat#022363204
96-well V-Bottom Plate, untreated	Corning Inc.	Cat#3896
Polystyrene 96-well Microplate Corner Notch Lid	Corning Inc.	Cat#3930
Falcon® 5 mL Round Bottom Polypropylene Tubes (FACS tubes)	Corning Inc.	Cat#352063
Falcon® 5 mL Round Bottom Polystyrene Tubes with 35 μm Cell Strainer Snap Caps	Corning Inc.	Cat#352235

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
ThermoFisher Scientific™ Sorvall™ Legend™ XTR Refrigerated Centrifuge, 120VAC	Thermo Fisher Scientific™	Cat#75004521
Fisherbrand™ Mini-Centrifuge 100–240V, 50/6-Hz Universal Plug, Grey	Thermo Fisher Scientific™	Cat#12-006-901
Fisher Scientific Digital Vortex Mixer	Thermo Fisher Scientific™	Cat#0215370
Thermo Scientific™ Precision™ Circulating Water Bath	Thermo Fisher Scientific™	Cat#TSCIR19
Cellometer® Auto 2000 Cell Viability Counter	Nexcelom Bioscience	Auto 2000
Fluidigm Helios™ Mass Cytometer	Fluidigm	N/A
Nebulizer for Helios™ Mass Cytometer	Fluidigm	Cat#107144
WB Injector for Helios™ Mass Cytometer	Fluidigm	Cat#107950

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

MATERIALS AND EQUIPMENT

Complete RPMI		
Reagent	Final concentration	Amount
RPMI Medium 1640 (1×)	n/a	500 mL
FBS	10%	50 mL
Antibiotic-Antimycotic (100×)	1%	5 mL
Total	**n/a**	**555 mL**
Store at 4°C for up to 2 weeks		

CyFACs		
Reagent	Final concentration	Amount
PBS	n/a	500 mL
30% BSA	0.5%	8.3 mL
5% Sodium Azide	0.02%	2 mL
Total	**n/a**	**510.3 mL**
Store at 4°C for up to 6 weeks		

FoxP3 fixation/permeabilization buffer		
Reagent	Final concentration	Amount
Fixation/Permeabilization Concentrate	25%	4 mL
Fixation/Permeabilization Diluent	75%	16 mL
Total	**n/a**	**20 mL**
Store at 4°C during the day of experiment, prepare fresh every time		

⚠ CRITICAL: Sodium Azide is carcinogenic and toxic if swallowed, inhaled, or if in contact with skin. Handle in a fume hood using personal protective equipment.

Note: The timing and reagent consumption in this protocol is relative to the handling of 20 samples.

Table 1. Surface antibody master mix

Marker	Metal	Clone	Volume per sample (μL)
CD45	89Y	HI30	1.00
CCR6	141Pr	G034E3	1.00
CD19	142Nd	HIB19	1.00
CD45RA	143Nd	HI100	1.00
CD69	144Nd	FN50	1.00
CD4	145Nd	RPA-T4	1.00
IgD	146Nd	IA6-2	1.00
CD20	147Sm	2H7	1.00
CD14	148Nd	RM052	1.00
CD56	149Sm	NCAM16.2	1.00
CD138	150Nd	DL-101	1.00
TNF-α	152Sm	Mab11	1.00
TIGIT	153Eu	MBSA43	1.00
CD3	154Sm	UCHT1	1.00
CD27	155Gd	L128	1.00
IFN-γ	158Gd	B27	1.00
CD161	159Tb	HP-3G10	1.00
CXCR3	163Dy	G025H7	1.00
IL-17a	164Dy	N49-653	1.00
PD-1	165Ho	EH12.2H7	1.00
CCR7	167Er	G043H7	1.00
CD8a	168Er	SK1	1.00
CD25	169Tm	2A3	1.00
CD57	172Yb	HCD57	0.50
HLA-DR	173Yb	L243	0.50
NKG2C	174Yb	HP-3D9	1.00
CD127	176Yb	A019D5	1.00
CD16	209Bi	3G8	1.00
CyFACs	n/a	n/a	24 μL
Total	n/a	n/a	50 μL

STEP-BY-STEP METHOD DETAILS

Bone marrow thawing and cell counting

Timing: 1 h

To prepare the cryopreserved bone marrow samples for mass cytometry staining, the samples must first be thawed, counted, and viability assessed.

1. Before staining, fill the appropriate number of 15-mL conical tubes (one per sample) with 9 mL of RPMI +10% FBS +1% Antibiotic-Antimycotic (named Complete RPMI from now on) and place at 37°C
2. Prepare the surface antibody master mix in CyFACs (Table 1)
3. Thaw the samples by placing the frozen vial directly into the 37°C water bath until content is thawed, approximately 45 s, few small ice crystals might remain in the vial.
4. Transfer the totality of thawed cell suspension into the 15 mL conical containing 9 mL of 37°C complete RPMI
5. Count cells using AO/PI (1:1 dilution; 20 μL sample in 20 μL AO/PI) with the Cellometer® Auto 2000 Cell Viability Counter
   a. Gently invert the conical containing the freshly thawed cells to ensure accurate cell counts
   b. Add 20 μL of the AO/PI-diluted sample to a Cellometer® SD100 Cell Counting Chamber
   c. Cells should be >90% viable

6. Adjust concentration of cell suspension to 3 × 10^6 cells/sample in complete RPMI
7. Centrifuge at 400 g for 10 min
   a. While the sample is spinning down, prepare the 103Rh Viability Stain (1:500 dilution of 103Rh Cell-ID intercalator in 37°C warm complete RPMI)
8. After the sample is spun down, carefully aspirate and discard the supernatant
9. Resuspend the sample in 1 mL of 103Rh Viability Stain
10. Incubate for 15 min at room temperature
11. Once incubation is complete, fill conical up to 5 mL with complete RPMI and spin down (400 g, 10 min)
12. Carefully aspirate and discard supernatant without disturbing the cell pellet
13. Resuspend each cell pellet in 50 μL CyFACs and proceed to Fc-block and surface staining

⚠ CRITICAL: If cell viability is below 90%, filter the samples using 70 μM cell pre-wetted strainers to obtain a single cell suspension.

Optional: The surface antibody master mix can be prepared during the viability stain incubation, but we recommend preparing the cocktail prior to the thawing of clinical samples to avoid any critical errors.

Fc-block and surface staining

Timing: 1 h

This portion of the protocol stains the sample with pre-selected metal-tagged antibodies to be acquired and used in downstream analysis. Prior to antibody staining, the samples are Fc-blocked to prevent non-specific staining of antibodies.

14. Fc-block procedure:
    a. Add 5 μL undiluted Fc-block to each sample
    b. Incubate on ice for 10 min
       i. During the incubation, aliquot the prepared surface antibody cocktail and distribute 50 μL/well according to 96 well plate map
15. Surface staining:
    a. Once the incubation is complete, distribute the totality of Fc-blocked samples into the appropriate wells (already containing the antibody cocktail) according to the plate map
    b. Incubate for 30 min at room temperature
       i. During incubation, prepare the eBioScience™ FoxP3 Fixation/Permeabilization Buffer as per manufacturer protocol (https://tinyurl.com/foxp3TF) (briefly, mix 1 part fix/perm concentrate (00-5123-43) + 3 parts fix/perm diluent (00-5223-56))
       ii. During ^{incubation}, prepare the eBioScience™ Perm/Wash solution by diluting 2 mL of 10× Permeabilization Buffer stock into 18 mL of Ultra-Pure H_20
    c. Pellet cells by plate centrifugation (400 g, 5 min)
    d. Carefully aspirate supernatant (~ 100 μL), wash once with 200 μL CyFACs, spin plate down (400 g, 5 min) and proceed to intracellular staining

Optional: The surface antibody master mix can be added individually to each sample well after the Fc-blocked cells have been plated, but this increases risk of technical error and experimental time.

Note: The FoxP3 Fixation/Permeabilization Buffer is prepared in excess and will be needed for the DNA stain at the end of the protocol.

Table 2. Intracellular antibody master mix

Marker	Metal	Clone	Volume per sample (μL)
MIP1-β	160Gd	D21-1351	1.00
T-Bet	161Dy	4B10	1.00
FoxP3	162Dy	259D/C7	1.00
Granzyme K	166Er	2471A	0.50
Granzyme B	171Yb	GB11	0.50
Perforin	175Lu	B-D48	0.50
Perm/wash	n/a	n/a	45.5 μL
Total	n/a	n/a	50 μL

Permeabilization and intracellular staining

Timing: 1.5 h

This portion of the protocol stains the sample with pre-selected metal-tagged antibodies that target intracellular markers. Prior to intracellular staining, the samples are incubated with eBiosciences™ FoxP3 Fixation/Permeabilization Buffer to preserve cellular morphology while allowing the intracellular antibodies across the plasma membrane to stain intracellularly.

16. Discard the supernatant and resuspend samples thoroughly in 200 μL of FoxP3 Fixation/Permeabilization Buffer
17. Incubate for 30 min at room temperature
    a. During incubation, prepare the intracellular antibody master mix

⚠ CRITICAL: Intracellular antibody master mix is made in Perm/Wash.

Note: Granzyme K was conjugated to the 166Er metal isotope, prior to this protocol via the Fluidigm Maxpar® X8 Antibody Labeling Kit according to the manufacturer protocol (https://tinyurl.com/maxpAbLabeling).

18. Spin plate down (**800 g**, 5 min) and wash once with 200 μL of Perm/Wash
19. Carefully aspirate and discard supernatant
20. Resuspend sample in 50 μL of Perm/Wash
21. Add 50 μL of intracellular master mix (Table 2) to each well containing sample
22. Incubate for 30 min at room temperature
23. After incubation is complete, add 100 μL of Perm/Wash and spin plate down (800 g, 5 min)
24. Wash once with 200 μL of Perm/Wash
25. During spin, prepare fresh 4% PFA in Ultra-Pure H_2O
26. Add 200 μL of fresh 4% PFA to each sample
27. Spin down at 800 g for 10 min and proceed to DNA stain and Pre-Acquisition Wash
    a. During spin, prepare the 191/193 DNA Intercalator at a 1:5000 dilution of 191/193 Cell-ID Intercalator to FoxP3 Fixation/Permeabilization Buffer

⚠ CRITICAL: Spin time was increased to 10 min to improve cell recovery post-fixation of cells.

DNA stain and pre-acquisition wash

Timing: 1.5 h

The final steps of the protocol stain the cell nuclei to allow for single-cell detection and discrimination by the Helios™ mass cytometer. After completing the DNA stain, the samples undergo a series of washes to prepare for acquisition.

28. Add 200 μL of 191/193 DNA Intercalator to each sample
29. Incubate for 1 h at 4°C

Pause Point: Samples can be incubated in intercalator solution overnight (or 8–16 h) at 4°C and protocol can be resumed the following morning

Optional: If timing of the protocol needs to allow the acquisition within the same day – intercalator staining *can* be combined with intracellular staining, if extended to 45 min.

30. After the incubation is complete, pellet cells by plate centrifugation (800×*g*, 5 min)
31. Carefully aspirate and discard supernatant
32. Resuspend the samples in 200 μL of MaxPar Cell Staining Buffer
33. Transfer the sample into a labeled, polypropylene tube with a 35 μm cell pre-wetted strainer cap
34. Add 300 μL of MaxPar Cell Staining Buffer per tube, bringing the total volume to 500 μL
35. Spin the tubes down (800 g, 10 min)
36. Carefully aspirate and discard supernatant
37. Resuspend the sample in 500 μL of MaxPar Cell Acquisition Solution (CAS)
    a. Load 20 μL of sample directly into Cellometer® SD100 Cell Counting Chamber
38. Spin the tube down (800 g, 5 min)
    a. Count the cells using the Cellometer® Auto 2000 Cell Viability Counter during spin and record final cell count
39. Prepare a 1:5 dilution of MaxPar EQ Calibration beads in CAS (CAS/EQ)

⚠ CRITICAL: Vortex the EQ Calibration beads for 1 min before preparing CAS solution.

40. Carefully aspirate and discard supernatant
41. Resuspend samples at a concentration of 1 x 10^6 cells per 1 mL of CAS/EQ solution.
42. Filter resuspended sample through 35 μm cell pre-wetted strainer cap and proceed to mass cytometer acquisition.

EXPECTED OUTCOMES

At the end of this protocol, FCS files will be generated for every sample acquired on Helios™ and normalized using the FCS Processing tab of the Fluidigm CyTOF Software 7.0.8493. Prior to immunophenotyping, the FCS files must be cleaned as described in Figure 1 (Bagwell et al., 2020) (Thrash et al., 2020). Briefly, cell events are gated by event length versus time, beads (to remove EQ calibration beads), and Gaussian parameters (Residual, Width, Offset). Single cells are determined by a bi-axial plot of both DNA Intercalator channels. Specific to bone marrow, we identified live, CD45+ cells, and then confirmed the depletion of CD138 before continuing analysis. As noted in Figure 2, CD45 bone marrow populations are donor-dependent and can vary from a CD45 smear to distinct $CD45^{hi}$ and $CD45^{dim}$ populations. Further analysis focused on the $CD45^{hi}$ lymphocyte population, excluding the non-viable and $CD45^{dim}$ erythroid populations (Cloos et al., 2018).

After isolating the $CD45^{hi}$ lymphocyte population, we manually gated our desired lineage populations. This included monocytes ($CD45^+CD138^-CD19^-CD14^+$), B cells ($CD45^+CD138^-CD14^-CD19^+$), T cells ($CD45^+CD138^-CD14^-CD19^-CD56^-CD3^+$), and NK cells ($CD45^+CD138^-CD14^-CD19^-CD3^-CD56^+$). The lineage gating strategy is shown in Figure 3. The remaining markers in the panel were used to further characterize the phenotype and function of each cell type.

LIMITATIONS

This protocol was established for staining extracellular proteins and secreted proteins, including cytokines, and other intracellular antigens. It is not applicable for staining of activation-induced phosphorylated antigens.

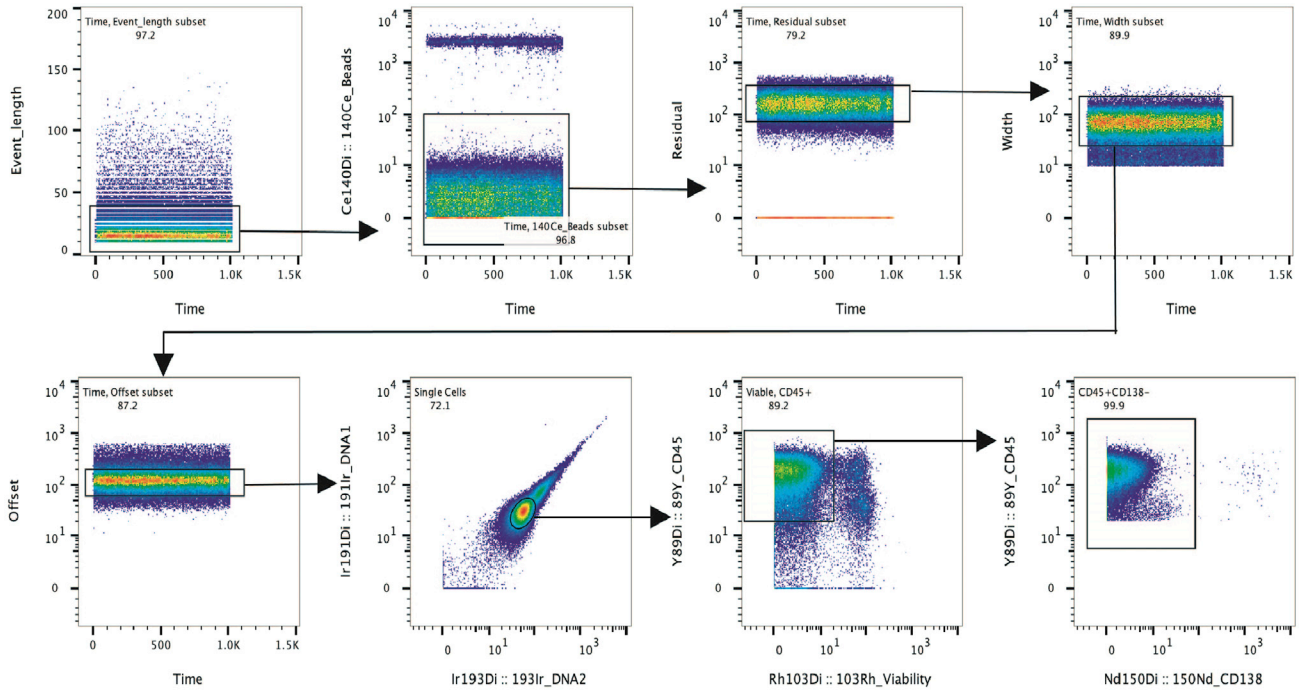

Figure 1. Clean up gating strategy used for identifying of viable, CD45^{+}CD138^{-} single cell population using gaussian parameters and 191Ir/193Ir biaxial plots

The single cells were gated against bi-axial plots versus CD45 and CD138 to confirm the population of interest for downstream analysis.

TROUBLESHOOTING

Problem 1

Low cell recovery at the end of staining protocol (step 41)

Potential solution

Do not use microcentrifuge 1.5 mL or 2 mL tubes for this protocol, always use 96 well, V-bottom plate. Do not discard supernatant through plate flicking, but instead aspirate the volume to discard using a multichannel pipette.

Problem 2

High level of 127-iodine contamination in your samples (step 9 and data analysis)

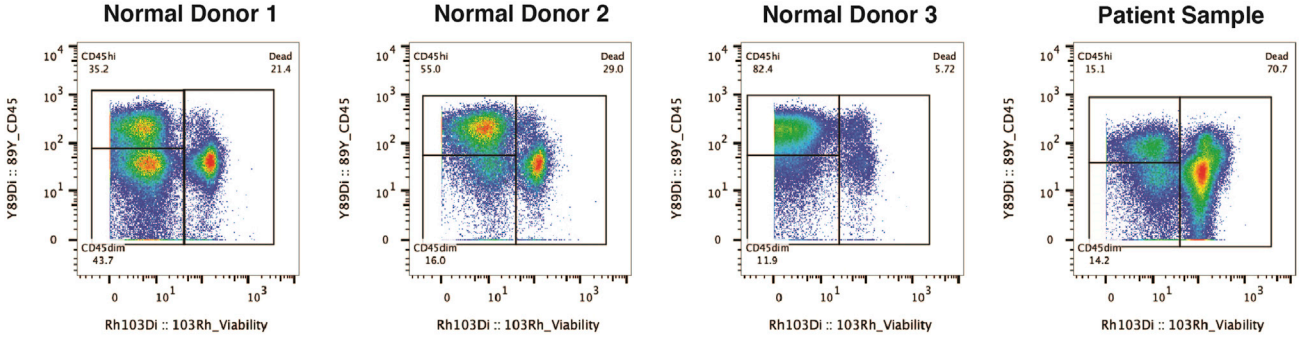

Figure 2. CD45 expression level, as it is a phosphatase, is expressed in different levels on progenitor/stem cells and is donor dependent

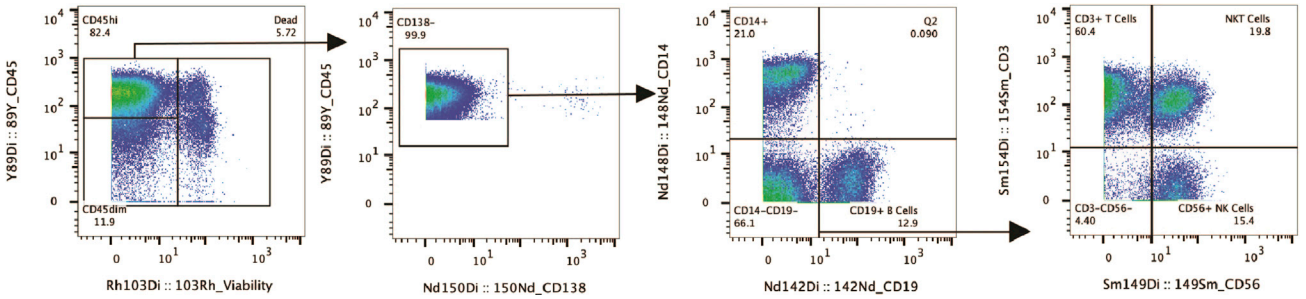

Figure 3. Cleaned, CD45^{hi} population manually gated to define lineage populations

Potential solution

The most popular source of 127-Iodine contamination is Ficoll – Paque, as it contains sodium diatrizoate. It is crucial to carefully remove the immune cells layer without touching the Ficoll - Paque layer or use tubes with a separation membrane. If contamination still occurs, it might be reduced by 3 supplementary washing steps after cell thawing.

Problem 3

Staining seems to "fade" or "streak" over time on certain markers (step 25 and data analysis)

Potential solution

Fixation using freshly prepared PFA is crucial. The fixative agent in the Fix/Perm buffers may degrade over time after repeated exposure to oxygen; freshly prepared PFA assures proper fixation and maintenance of staining signal. We suggest freshly fixing stained cells using 4% PFA. The concentration can be adjusted depending on specific needs of the assay, but not decreased more than 1.6% PFA.

Problem 4

Significantly more than 20 samples to stain and to acquire on mass cytometer (steps 1 and 42)

Potential solution

Up to 80 samples can be stained within the same plate, and the plate can be frozen after the 191-Ir DNA Intercalator incubation in freezing medium containing 90% of FBS and 10% of DMSO (Sumatoh et al., 2017). For long acquisitions, the best solution is to freeze all plates and book the mass cytometer for the number of consecutive days needed. To prepare samples for acquisition, thaw each plate the morning of acquisition, and continue with washes, treating the samples as if they were incubated overnight in a DNA-intercalator.

Problem 5

103Rh Viability Staining seems not to perform when analyzing data (step 9 and data analysis)

Potential solution

Be careful when modifying fixation and permeabilization buffers, as for example 103Rh viability staining is not compatible with methanol fixation. If other fix/perm reagents are being used, staining with Cell-ID™ Cisplatin, Fluidigm #SKU 201064 might replace 103Rh Viability staining. Cisplatin binds covalently to protein and labeling remains strong through subsequent steps of the protocol.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Joanna Baginska, joanna_baginska@dfci.harvard.edu.

Materials availability

There were no new reagents generated.

Data and code availability

FCS files from healthy donors will be available upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.B.; Methodology, M.H., J.D., C.A., J.B., R.S.P., A.N.S., M.S., E.D.L., and D.L.M.; Investigation, M.H. and J.B.; Resources, M.H., R.S.P., A.N.S., D.L.M., M.S.; Writing-Original Draft, M.H., J.D., R.S.P.; Visualization, M.H.; Funding Acquisition I.M.G., F.S.; Supervision, J.B.

DECLARATION OF INTERESTS

Hodi FS: Dr. Hodi reports grants and/or personal fees from Bristol-Myers Squibb, Merck, EMD Serono, grants and Novartis, Surface, Compass Therapeutics, Apricity, Sanofi, Pionyr, 7 Hills Pharma, Torque, Bicara, Pieris Pharmaceuticals, Checkpoint Therapeutics, Genentech/Roche, Bioentre, Gossamer, Iovance, Trillium, Catalym, Immunocore, Amgen, Zumutor, outside the submitted work; in addition, Dr. Hodi has a patent (#20100111973) with royalties paid, patents (#7250291) (#9402905) (# 10279021) (#10106611) (#20170248603) (#20160046716) (#20140004112) (#20170022275) (#20170008962) and (#20170343552) pending. Ghobrial IM : Dr Ghobrial has the following potential conflicts of interest to disclose: Honoraria: Celgene, Bristol-Myers Squibb, Takeda, Amgen, Janssen; Consulting or Advisory Role: Bristol-Myers Squibb, Novartis, Amgen, Takeda, Celgene, Cellectar, Sanofi, Janssen, Pfizer, Menarini Silicon Biosystems Oncopeptides, The Binding Site, GlazoSmithKlein, AbbVi Adaptive; Travel, Accommodations, Expenses: Bristol-Myers Squibb, Novartis, Celgene, Takeda, and Janssen Oncology.

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