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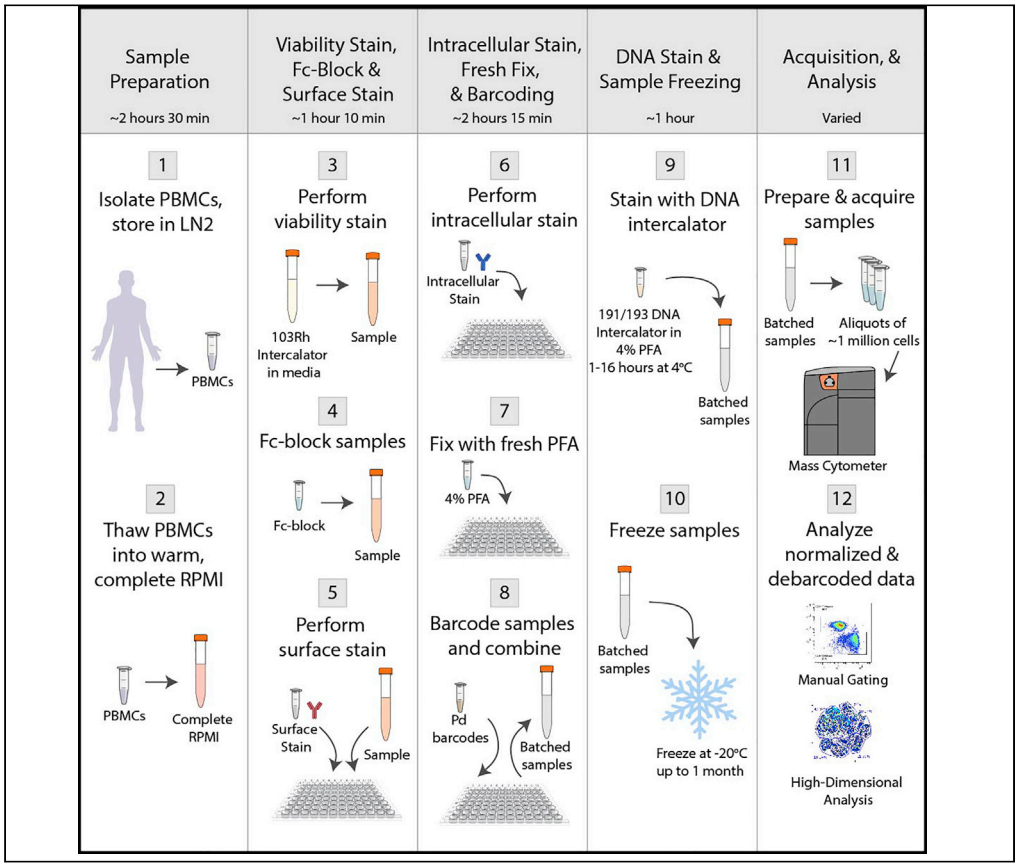
Simplified mass cytometry protocol for in-plate staining, barcoding, and cryopreservation of human PBMC samples in clinical trials



With the increasing use of mass cytometry in clinical research, a simplified and standardized protocol for immunophenotyping human peripheral blood mononuclear cells (PBMCs) in clinical trials is needed. We present a simplified in-plate staining protocol for up to 80 samples, for laboratories of all mass cytometry expertise levels, aimed to generate reproducible datasets for large clinical cohorts. In this protocol, we provide details on the requirements to obtain meaningful results, spanning from sample quality, barcoding, and batch-freezing of stained samples.

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Highlights

Mass cytometry protocol to stain, barcode, and freeze up to 80 ready-to-acquire samples

Freezing stained, barcoded samples allows for better management of machine acquisition

Insights on gating strategy and multiparameter data analysis

Useful for all expertise levels to generate datasets with reduced batch effects

Abrecht et al., STAR Protocols 3, 101362
June 17, 2022
https://doi.org/10.1016/j.xpro.2022.101362

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Simplified mass cytometry protocol for in-plate staining, barcoding, and cryopreservation of human PBMC samples in clinical trials

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https://doi.org/10.1016/j.xpro.2022.101362

SUMMARY

With the increasing use of mass cytometry in clinical research, a simplified and standardized protocol for immunophenotyping human peripheral blood mononuclear cells (PBMCs) in clinical trials is needed. We present a simplified in-plate staining protocol for up to 80 samples, for laboratories of all mass cytometry expertise levels, aimed to generate reproducible datasets for large clinical cohorts. In this protocol, we provide details on the requirements to obtain meaningful results, spanning from sample quality, barcoding, and batch-freezing of stained samples.

BEFORE YOU BEGIN

Generation of peripheral blood mononuclear cells (PBMCs) from whole blood clinical samples

Timing: 30 min

Whole blood samples were obtained per the blood collection protocol approved by the Institutional Review Board of Brigham and Women's Hospital. All participants gave written informed consent prior to the blood draw. To isolate PBMCs from whole blood, standard density gradient centrifugation was performed using Ficoll-Paque™ Plus.

1. Add 12 mL of Ficoll-Paque™ Plus per 50 mL conical tube.
2. Evenly distribute 25 mL of whole blood into necessary number of 50 mL conical tubes.
3. Dilute each 50 mL conical containing $\leq$ 25 mL of whole blood with 5 mL of PBS.
4. Slowly and gently layer the diluted whole blood sample on top of the Ficoll-Paque™ with a maximum volume of 35 mL per conical tube.

⚠ CRITICAL: Do not let the Ficoll-Paque™ Plus and blood mix together, as this will affect the yield and purity of isolated PBMCs.

5. Centrifuge conical tubes at room temperature (RT), (800 g, 20 min), with the brake off.

6. Carefully collect PBMC 'buffy' layer and transfer to a new 50 mL conical tube and fill it up to 50 mL with room temperature (17°C–21°C) PBS, mixing well.

   Optional: If cell viability loss is observed, FBS can be added to the room temperature PBS in step 6 (PBS with 1% FBS).

7. Count cells using AO/PI (1:1 dilution; 20 μL sample in 20 μL AO/PI) with the Cellometer® Auto 2000 Cell Viability Counter (or alternative method).
   a. Gently invert the conical tubes 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.
8. Centrifuge conical tubes from step 6 containing PBMCs in PBS (400 g, 5 min, RT).

   ⚠ CRITICAL: Be careful to not touch the Ficoll-Paque™ layer with the pipette tip when collecting the PBMCs clouds, as this contributes to iodine contamination of the samples and can negatively affect quality and analysis.

Freezing of PBMCs

Timing: 10 min

The PBMCs are frozen in freezing media to preserve the cells until needed for mass cytometry staining.

9. After centrifugation, carefully aspirate and discard supernatant.
10. According to cell count, resuspend PBMC pellet at a concentration of 1×10^7 in freezing media (85% FBS, 15% DMSO).
11. Aliquot 500 μL of resuspended pellet into cryovials for a final concentration 5×10^6 cells per cryovial.
12. Place vials in controlled-rate freezing container (for example CoolCell™) and transfer to −80°C overnight.
13. Transfer vials the following day to liquid nitrogen for storage until mass cytometry staining.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
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 CD8 (RPA-T8)-146Nd—100 Tests (1:100)	Fluidigm	Cat#3146001B
Anti-Human CD11c (Bu15)-147Sm—100 Tests (1:100)	Fluidigm	Cat#3147008B
Anti-CD278/ICOS (C398.4A)-148Nd—100 Tests (1:100)	Fluidigm	Cat#3148019B
Anti-Human CD56/NCAM (NCAM16.2)-149Sm—100 Tests (1:100)	Fluidigm	Cat#3149021B
Anti-Human CD223/LAG-3 (11C3C65)-150Nd—100 Tests (1:100)	Fluidigm	Cat#3150030B
Anti-Human CD123/IL-3R (6H6)-151Eu—100 Tests (1:100)	Fluidigm	Cat#3151001B
Anti-Human TCRgd (11F2)-152Sm—100 Tests (1:100)	Fluidigm	Cat#3152008B
Anti-Human CD185/CXCR5 (RF8B2)-153Eu—100 Tests (1:100)	Fluidigm	Cat#3153020B
Anti-Human CD3 (UCHT1)-154Sm—100 Tests (1:200)	Fluidigm	Cat#3154003B
Anti-Human CD27 (L128)-155Gd—100 Tests (1:100)	Fluidigm	Cat#3155001B
Anti-Human CD14 (HCD14)-156Gd—100 Tests (1:100)	Fluidigm	Cat#3156019B

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-Human CD33 (WM53)-158Gd—100 Tests (1:100)	Fluidigm	Cat#3158001B
Anti-Human TIGIT (MBSA43)-159Tb—100 Tests (1:100)	Fluidigm	Cat#3159038B
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 CD57 (HCD57)-163Dy—100 Tests (1:200)	Fluidigm	Cat#3163022B
Anti-Human IL-17A (N49-653)-164Dy—100 Tests (1:100)	Fluidigm	Cat#3164002B
Anti-Human IFNg (B27)-165Ho—100 Tests (1:100)	Fluidigm	Cat#3165002B
Anti-Human CD314/NKG2D (ON72)-166Er—100 Tests (1:100)	Fluidigm	Cat#3166016B
Anti-Human CD197/CCR7 (G043H7)-167Er—50 Tests (1:100)	Fluidigm	Cat#3167009A
Anti-Ki-67 (B56)-168Er—100 Tests (1:100)	Fluidigm	Cat#3168007B
Anti-Human CD25 (2A3)-169Tm—100 Tests (1:100)	Fluidigm	Cat#3169003B
Anti-Human CD152/CTLA-4 (14D3)-170Er—100 Tests (1:100)	Fluidigm	Cat#3170005B
Anti-Human CD195/CCR5 (NP-6G4)-171Yb—50 Tests (1:50)	Fluidigm	Cat#3171017A
Anti-Human CD38 (HIT2)-172Yb—100 Tests (1:100)	Fluidigm	Cat#3172007B
Anti-Human HLA-DR (L243)-173Yb—100 Tests (1:200)	Fluidigm	Cat#3173005B
Anti-Human CD279/PD-1 (EH12.2H7)-174Yb—100 Tests (1:100)	Fluidigm	Cat#3174020B
Anti-Human CD194/CCR4 (L291H4)-175Lu—50 Tests (1:50)	Fluidigm	Cat#3175035A
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
Chemicals, peptides, and recombinant proteins		
Ficoll-Paque™ Plus	Sigma-Aldrich	Cat#17144003
Dimethyl Sulfoxide (DMSO)	Fisher BioReagents	Cat#BP231-100
Gibco™ Phosphate-Buffered Saline (PBS)	Thermo Fisher Scientific	Cat#10010023
Antibiotic-Antimycotic (100×)	Gibco	Cat#15240096
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 Medium 1640 [+] L-glutamine	Thermo Fisher Scientific	Cat#11875093
Gibco™ Premium Plus Fetal Bovine Serum, heat inactivated, qualified (FBS)	Thermo Fisher Scientific	Cat#10438026
UltraPure™ DNAse/RNAse-Free Distilled Water	Invitrogen	Cat#10977023
ViaStain™ AOPI Staining Solution	Nexcelom Biosciences	Cat#CS2-0106-5mL
Cell-ID™ Intercalator-103Rh—500 μM	Fluidigm	Cat#201103A
Cell-ID™ Intercalator-191/193Ir—125 μM	Fluidigm	Cat#201192B
Maxpar® Cell Acquisition Solution	Fluidigm	Cat#201237
Maxpar® Cell Staining Buffer	Fluidigm	Cat#201068
Maxpar® 10× Barcode Perm Buffer	Fluidigm	Cat#201057
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 Fisher Scientific	Cat#28906
CyTOF® Tuning Solution, 250 mL	Fluidigm	Cat#201072
Critical commercial assays		
Cell-ID™ 20-Plex Pd Barcoding Kit	Fluidigm	Cat#201060
Biological samples		
Human PBMCs isolated from whole blood clinical samples (sex, age as required par study)	Any Supplier	N/A
Software and algorithms		
FlowJo v.10	Becton Dickinson & Company	https://www.flowjo.com/solutions/flowjo/downloads/
GraphPad Prism v9.2	GraphPad Software, LLC	https://www.graphpad.com/scientific-software/prism/
CyTOF Software v7.0.8493	Fluidigm	https://www.fluidigm.com/software

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
CoolCell™ LX Cell Freezing Vial Containers	Corning Inc.	Cat#07210001
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 Biosciences	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 Polystyrene 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
ThermoFisher Scientific™ Sorvall™ Legend™ XTR Refrigerated Centrifuge, 120VAC	Thermo Fisher Scientific	Cat#75004521
Fisherbrand™ Mini-Centrifuge 100–240 V, 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 Biosciences	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. Falcon® 5 mL Round Bottom 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%	8 mL
Fixation/Permeabilization Diluent	75%	32 mL
Total	**n/a**	**40 mL**

Store at 4°C during the day of experiment, prepare fresh every time.

Table 1. Surface antibody master mix, titrated for staining of up to 3 M cells per condition

Marker	Metal	Clone	Volume per sample (µL)
CD19	142Nd	HIB19	1.00
CD45RA	143Nd	HI100	1.00
CD69	144Nd	FN50	1.00
CD4	145Nd	RPA-T4	1.00
CD8	146Nd	RPA-T8	1.00
CD11c	147Sm	Bu15	1.00
CD278/ICOS	148Nd	C398.4A	1.00
CD56/NCAM	149Sm	NCAM16.2	1.00
CD223/LAG3	150Nd	11C3C65	1.00
CD123/IL-3R	151Eu	6H6	1.00
TCRgd	152Sm	11F2	1.00
CD185/CXCR5	153Eu	RF8B2	1.00
CD3	154Sm	UCHT1	0.50
CD27	155Gd	L128	1.00
CD14	156Gd	HCD14	1.00
CD33	158Gd	WM53	1.00
TIGIT	159Tb	MBSA43	1.00
CD57	163Dy	HCD57	0.50
CD314/NKG2D	166Er	ON72	1.00
CD197/CCR7	167Er	G043H7	1.00
CD25	169Tm	2A3	1.00
CD195/CCR5	171Yb	NP6G4	2.00
CD38	172Yb	HIT2	1.00
HLA-DR	173Yb	L243	0.50
CD279/PD-1	174Yb	EH12.2H7	1.00
CD194/CCR4	175Lu	L291H4	2.00
CD127/IL-7Ra	176Yb	A019D5	1.00
CD16	209Bi	3G8	1.00
CyFACS	n/a	n/a	21.50 µL
Total	n/a	n/a	50.00 µL

⚠ 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 80 samples.

STEP-BY-STEP METHOD DETAILS

PBMC thawing, cell counting, and viability staining

Timing: 2 h

To prepare the cryopreserved PBMC samples for mass cytometry staining, the samples must first be thawed and counted.

⚠ CRITICAL: This step assumes the availability of two technicians to facilitate the quick and efficient thawing and counting of samples.

1. Before staining, annotate and fill the appropriate number of 15-mL conical tubes (one per sample) with 9 mL of warmed to 37°C RPMI +10% FBS +1% Antibiotic-Antimycotic (named Complete RPMI from now on), and prepare necessary counting slides.
2. Prepare the cell surface antibody master mix in CyFACS (Table 1).

3. Thaw the samples by placing the rack with 10 frozen vials directly into the 37°C circulating water bath until content is thawed, approximately 45 s, few small ice crystals might remain in the vial.
4. Immediately transfer the totality of thawed cell suspensions into the 15 mL conical tubes containing 9 mL of 37°C complete RPMI.
5. Repeat step 3 and 4 for remaining batches of frozen vials.
6. Filter the samples using 70 μM cell pre-wetted strainers to obtain a single cell suspension.
7. Count cells using AO/PI (1:1 dilution; 20 μL sample in 20 μL AO/PI) with the Cellometer® Auto 2000 Cell Viability Counter (or alternative cell counter).
   a. Gently invert the conical tube containing the freshly thawed cells to ensure cells are fully resuspended.
   b. Add 20 μL of the AO/PI-diluted sample to a Cellometer® SD100 Cell Counting Chamber.
   c. Cells should be >90% viable.
8. According to the cell count, adjust volume of cell suspension to a final concentration of 3×10^6 cells per condition in complete RPMI.

   Note: To prepare samples for staining, prepare a total of 3×10^6 cells for each condition. If more cells will be stained, this will require careful titration of antibodies.

9. Centrifuge samples (400 g, 5 min, RT).
   a. While the samples are spinning down, prepare the 103Rh Viability Stain (1:500 dilution of 103Rh Cell-ID™ Intercalator-Rh in 37°C warm complete RPMI).
10. After the sample is spun down, carefully aspirate and discard the supernatant.
11. Resuspend the sample in 1 mL of warm, Complete RPMI+103Rh Viability Stain.
12. Incubate the samples for 15 min at room temperature (17°C–25°C).
    a. During incubation, aliquot 50 μL of surface stain antibody cocktail per well into designated v-bottomed wells of the 96-well polystyrene plate.
13. Once incubation is complete, fill conical tube up to 10 mL with complete RPMI and spin down (400 g, 5 min, RT).
14. Carefully aspirate and discard supernatant without disturbing the cell pellet.
15. Resuspend each cell pellet in 50 μL CyFACS and proceed to Fc-block and surface staining.

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

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.

16. Fc-block:
    a. Add 5 μL undiluted Fc-block to each sample.
    b. Incubate the samples on ice for 10 min.
17. 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.
18. Incubate the staining plate for 30 min at room temperature.
    a. During incubation, prepare the eBioScience™ FoxP3 Fixation/Permeabilization Buffer as per manufacturer protocol (www.thermofisher.com) (briefly, mix 1 part fix/perm concentrate (00-5123-43) + 3 parts fix/perm diluent (00-5223-56)).
    b. During incubation, prepare the eBioScience™ Perm/Wash solution by diluting 5 mL of 10× Permeabilization Buffer stock into 45 mL of Ultra-Pure H_20.

Table 2. Intracellular antibody master mix

Marker	Metal	Clone	Volume per sample (µL)
MIP1beta	160Gd	D21-1351	1.00
Tbet	161Dy	4B10	1.00
FoxP3	162Dy	259D/C7	1.00
IL-17a	164Dy	N49-653	1.00
IFNg	165Ho	B27	1.00
Ki-67	168Er	B56	1.00
CD152/CTLA-4	170Er	14D3	1.00
Perm/Wash	n/a	n/a	43.00 µL
Total	n/a	n/a	50.00 µL

19. After incubation is complete, centrifuge the plate to pellet cells (400 g, 5 min, RT).
20. Carefully aspirate and discard supernatant from each staining well using the multi-channel pipette, set for approximately 20 µL less than the volume in the well to avoid aspirating the pellet. For best results, tilt the plate at a 45° angle and rest the pipette tip on the edge of the well, right before the well funnels down into the v-bottom. This applies for every aspiration step in this protocol.
21. Add 200 µL CyFACs/well using multichannel pipette and centrifuge the plate (400 g, 5 min, RT).

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.

Permeabilization and intracellular staining

Timing: 1.5 h

This portion of the protocol describes staining the samples with pre-selected metal-tagged antibodies that target intracellular markers.

22. Carefully aspirate and discard supernatant from each staining well using multichannel pipette (as in step 20).
23. Resuspend samples thoroughly in 200 µL of FoxP3 Fixation/Permeabilization Buffer per well, using multichannel pipette.
24. Incubate the plate for 30 min at room temperature.
    a. During incubation, prepare the intracellular antibody master mix (Table 2).

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

25. Centrifuge plate (800 g, 5 min, RT) and wash samples once with 200 µL of Perm/Wash per well using multichannel pipette.
26. Carefully aspirate and discard supernatant (as in step 20).
27. Resuspend each sample in 50 µL of Perm/Wash per well using multichannel pipette.
28. Add 50 µL of intracellular master mix to each well.
29. Incubate the plate for 30 min at room temperature.
30. After incubation is complete, add 100 µL of Perm/Wash solution per well using multichannel pipette and centrifuge plate (800 g, 5 min, RT).

31. Wash samples with 200 μL of Perm/Wash solution per well using multichannel pipette, centrifuge the plate (800 g, 5 min, RT), and carefully aspirate the supernatant as in step 20.
    a. During spin, prepare fresh 4% PFA solution from 16% stock PFA in Ultra-Pure H_2O.
32. Add 200 μL of freshly prepared 4% PFA per well using multichannel pipette.
33. Centrifuge the plate (800 g, **10 min**, RT), aspirate the supernatant (as in step 20), and proceed to barcoding.
    a. During spin, prepare the 191/193 DNA Intercalator at a 1:5,000 dilution of 191/193 Cell-ID™ Intercalator-Ir to FoxP3 Fixation/Permeabilization Buffer.
    b. During spin, prepare Barcode Perm Buffer according to manufacturer's protocol (www.fluidigm.com) by diluting 1 part 10× Barcode Perm Buffer in 9 parts PBS.

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

Palladium-based barcoding

Timing: 45 min

This portion of the protocol applies a post stain 6-choose-3 palladium based barcoding system to mitigate potential procedural variations while retaining staining efficiency. We applied a post-stain barcoding scheme to preserve fixation sensitive staining while maintaining the acquisition consistency of running pooled samples. This Palladium-based Barcoding Strategy is adapted from the protocol "Cell-ID 20-Plex Pd Barcoding Kit" on www.fluidigm.com.

34. Remove barcode tubes from −20°C, thaw, and briefly spin down barcodes in mini centrifuge.
35. Resuspend barcodes in 100 μL Barcode Perm Buffer.
36. Resuspend freshly fixed cells from step 33 in 100 μL Barcode Perm Buffer per well using multichannel pipette.
37. Transfer the totality (100 μL) of resuspended barcodes from step 35 to respective wells containing samples using multichannel pipette.
38. Incubate the plate for 30 min at room temperature.
39. Centrifuge plate (**800 g**, 5 min, RT) and wash samples once with 200 μL of Barcode Perm Buffer using multichannel pipette.
40. Resuspened each well with 200 μL of Barcode Perm Buffer using multichannel pipette and combine all samples from a single barcode batch (up to 20 samples) into a 15 mL conical tube.
41. Wash once with 5 mL Barcode Perm Buffer, pellet the cells by centrifugation (800 g, 5 min, RT), and proceed to DNA staining.

Optional: We recommend including a healthy donor sample in each barcoding batch (i.e., 19 clinical samples, one healthy donor sample). If running multiple full barcodes, the same normal donor should be used throughout batches to assess data quality.

Optional: If running a smaller cohort of patients (less than 20 samples), barcoding *may* be omitted. In this case, proceed from step 33 to step 42 and adjust all the buffer/wash volumes to 200 μL/well. Samples can be frozen directly in plate: using multichannel pipette add 100 μL of freezing medium per well to the spun-down cells from step 47.

DNA staining and plate freezing

Timing: 1 h

The final steps stain the cell nuclei to allow for single-cell detection and discrimination by the Helios™ mass cytometer. After completing the DNA stain, the samples are prepared for short-term

storage until acquisition. Prior to acquisition the samples are thawed and prepared accordingly for optimal Helios™ acquisition.

42. Per full barcode (20 samples) add 5 mL of a 1:5,000 dilution of 191/193 DNA Intercalator in FoxP3 Fixation/Permeabilization Buffer.
43. Incubate samples 1 h at 4°C.

Pause point: Samples can be incubated in 191/193 DNA Intercalator staining solution up to 16 h (or overnight) at 4°C instead of 1 h incubation.

44. After the incubation is complete, pellet cells by plate centrifugation (800 g, 5 min, RT).
45. Carefully aspirate and discard supernatant.
46. Wash once with 2 mL of Cell Staining Buffer.
47. Centrifuge the tube(s) (800 g, 5 min, RT) and carefully aspirate the supernatant.
48. Resuspend the pellet(s) in 500 µL of freezing media (85% FBS, 15% DMSO) and immediately transfer to −20°C for storage.

⚠ CRITICAL: This protocol was optimized and validated for short-term storage of stained and frozen samples (up to 1 month).

Pre-acquisition thawing and wash

Timing: 45 min

49. On the acquisition day, thaw samples with 500 µL of **warm** Complete RPMI per tube(s) (100 µL per well if no barcoding was applied).
50. Centrifuge the thawed samples (800 g, 5 min, RT).
51. Wash once with 2 mL of Cell Acquisition Solution (CAS).
52. Count the total number of cells with Cellometer® Auto 2000 Cell Viability Counter, and pellet cells by centrifugation (800 g, 5 min).
    a. Load 20 µL of sample directly into Cellometer® SD100 Cell Counting Chamber to count total cell concentration (in absence of stain).
    b. Count the cells using the Cellometer® Auto 2000 Cell Viability Counter during spin and record final cell count.
53. 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 EQ beads/CAS solution.

54. Resuspend the samples at a concentration of 1×10^6 cells per 1 mL in EQ beads/CAS solution.
55. Filter resuspended sample through a 35 µm pre-wetted cell strainer cap into a labeled, polypropylene tube in 2 mL aliquots.
56. Proceed to Helios™ mass cytometer acquisition (Fluidigm).

EXPECTED OUTCOMES

Our panel presents a comprehensive scope of the innate and adaptive immune system within one mass cytometry panel, consisting of 35 markers (Figure 1A) with the ability to define multiple immune phenotypes (Figure 1B).

At the end of this protocol, FCS files are generated for every sample acquired on Helios™ and normalized using the FCS Processing tab of the Fluidigm CyTOF Software 7.0.8493.

A

Mass Cytometry Panel for Immunophenotyping PBMCs

Lineage	T Cell Specific	Checkpoint	Activation Markers	Memory Markers	Maturation Markers	Exhaustion Markers
CD11c	CCR4	CTLA-4	CD69	CCR7	CD57	CXCR5
CD123	CCR5	LAG-3	HLA-DR	CD27		
CD14	CD127	PD-1	ICOS	CD38		
CD16	CD125	TIGIT	Ki-67	CD45RA		
CD19	FoxP3		MIP1b			
CD3	IL-17a		NKG2D			
CD33	T-bet					
CD4						
CD56						
CD8						
TCRgd						

B

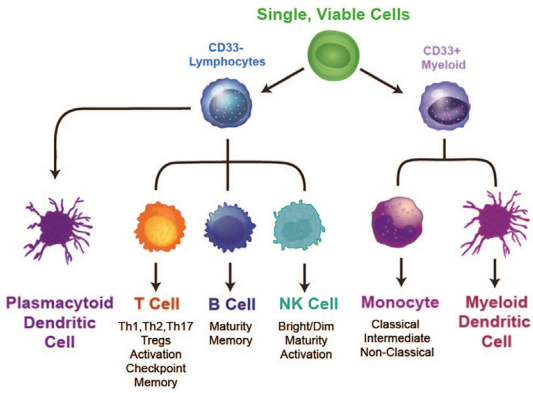

Figure 1. Immunophenotyping of innate and adaptive immune cell subsets

(A) The mass cytometry panel of 35 markers to immunophenotype PBMCs.

(B) Immune phenotypes of single, viable PBMCs.

This panel covers the major immune cell subsets, including activation, checkpoint, maturity, and memory markers. The data was cleaned following Gaussian parameters (Figure 2A). The myeloid subset determines monocyte subpopulations: classical ($CD33^{+}CD16^{-}CD14^{+}$), intermediate ($CD33^{+}CD16^{+}CD14^{+}$), and nonclassical ($CD33^{+}CD16^{+}CD14^{-}$) (Figure 2B). The lymphocyte subset distinguishes Natural Killer cells ($CD33^{-}CD19^{-}CD3^{-}CD56^{+}$), Natural Killer T cells ($CD33^{-}CD19^{-}CD3^{+}CD56^{+}$) amongst other CD56+ T cells, and B cells (Figure 2B). The lymphocyte subset also distinguishes CD4 T cells and T regulatory cells (Figure 2C), and CD8 T cells (Figure 2D).

LIMITATIONS

Low quality sample (for example sample with low viability) can jeopardize debarcoding efficiency and analysis of all samples in a barcoding batch. Therefore, we suggest that low-quality samples are in a separate barcode batch from high quality samples.

TROUBLESHOOTING

Problem 1

High level of 127-Iodine contamination in samples (step 4 and data analysis).

Potential solution

127 Iodine contamination most frequently comes from Ficoll-Paque, as it contains sodium diatrizoate. If the Ficoll-Paque layer was disturbed while collecting the immune cell layer during PBMC isolation, the isolated cells can have trace amounts of Iodine contamination. This can potentially be resolved by adding supplementary washes after thawing.

Problem 2

Shift in lower mass channels expression level in barcoded samples (step 42 & data analysis).

Although sample phenotypes were not significantly affected by barcoding it is important to note that there is potential for signal shift in the lower mass channels for barcoded samples. As shown in Figure 3, the signal shift is absent in individually acquired samples (non-barcoded and single barcode) and does not occur in the full barcode sample until approximately 5.0 million acquired events. The signal shift appears during acquisition of full barcode samples due to the high number of cells when multiple samples are combined, and therefore requiring extended acquisition time. For example, the signal continually degraded over acquisition time, causing the 145Nd CD4 positive

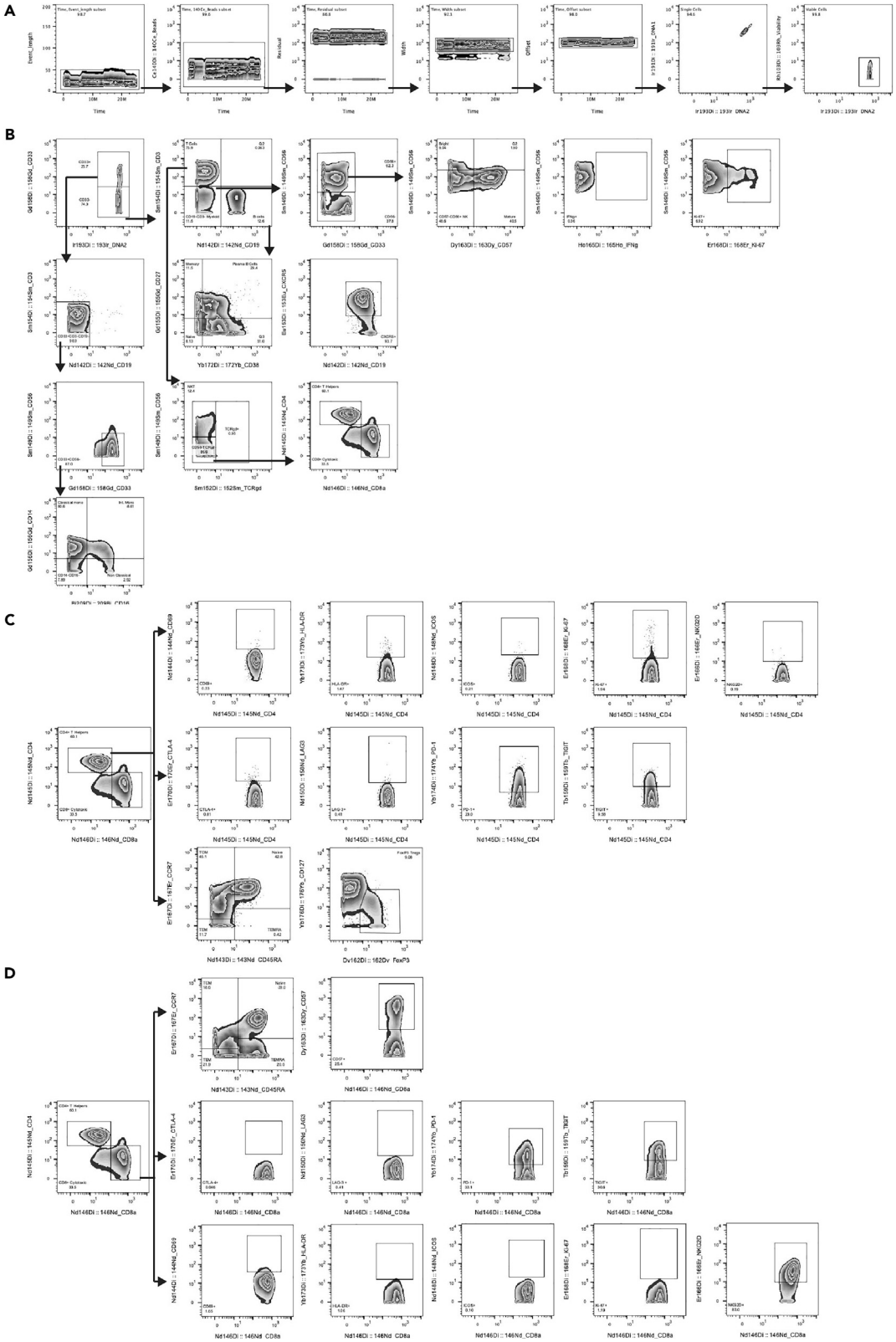
A
B
C
D

Figure 2. Gating strategy of innate and adaptive immune cell subsets

(A) Clean-up gating strategy used for identifying of viable single cell population, using gaussian parameters and 191Ir/193Ir biaxial plots.

(B) Cleaned gating strategy to identify monocyte, B cell, NKT cell, and NK cell populations.

(C) Cleaned CD4+ T cell populations and marker expression.

(D) Cleaned CD8+ T cell populations and marker expression.

and negative populations to eventually merge, leaving no clear separation. Interestingly in our experience change of fixation method did not improve the outcomes.

Potential solution

To mitigate signal shift, samples should be acquired on the machine in aliquots of 1×10^6 cells per 1 mL in EQ beads/CAS solution and that newly developed panels be tested with a fully barcoded set of donor samples to ensure vital populations remain discernable before applying the panel to clinical samples. Before a clinical experiment, the barcode condition should be determined and applied to all samples in the same cohort.

Problem 3

Significant machine time use required to acquire all cellular events of 80 samples (step 56).

Although barcoding samples aids in the reduction of batch effects by allowing for combined acquisition, the length of acquisition time might become problematic due to high cell volume (Sumatoh et al., 2017). With an optimal acquisition rate of 500 events per second, the mass cytometer can only effectively analyze 2 million cells per hour. Assuming an eight-hour workday for one operator on a single machine, the threshold is around 20 million cells per day of acquisition. In addition to the limitation of machine availability, we found lengthy acquisition time to lead to signal shifts in the lower mass channels, as described above (Figure 3). This forces multiple acquisition days for a single cohort.

Potential solution

This protocol was developed to allow for successful staining of 80 samples during one workday, and subsequent acquisition of 1 batch of 20 barcoded samples per machine day. We recommend

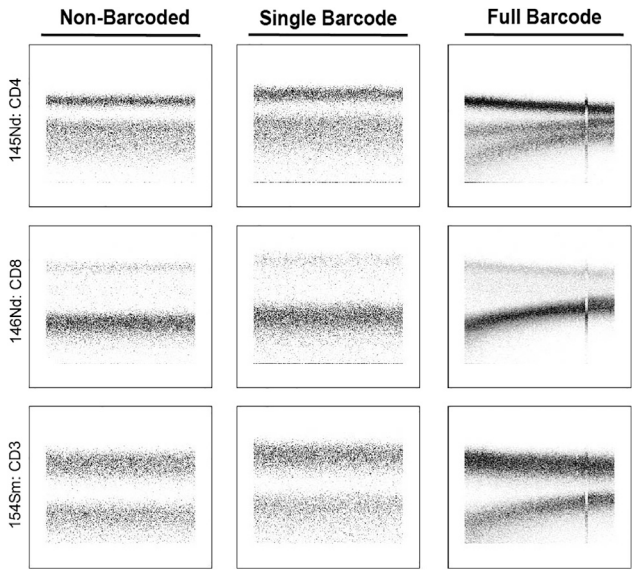

Figure 3. Signal shift in individual sample acquisition compared to combined sample acquisition

Biaxial time plots of CD4, CD8, and CD3 marker expression in individually acquired samples (non-barcoded and single barcode) compared to combined sample acquisition (full barcode).

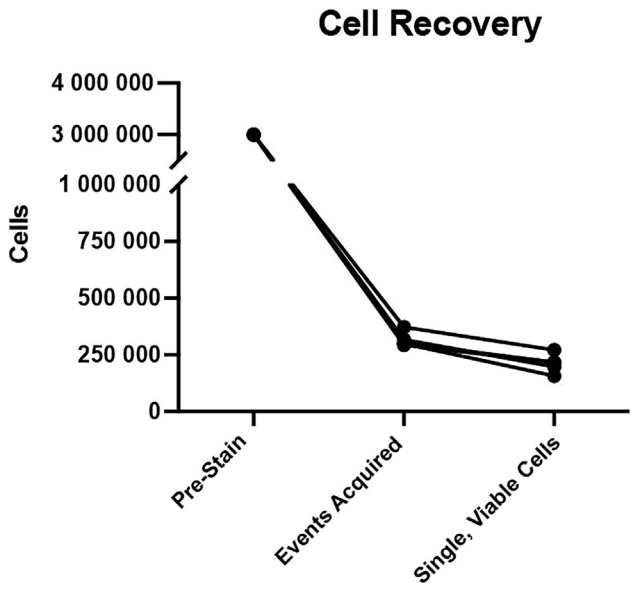

Figure 4. Cell recovery at different timepoints during CyTOF preparation and protocol
Cell recovery data for number of cells per sample before CyTOF staining, after machine acquisition, and after routine clean-up gating to obtain single, viable cells.

thawing every barcoded sample batch on its day of acquisition. For best results, the stained clinical samples should be thawed and acquired within a one-week time frame to achieve best instrument consistency (Gonder et al., 2020). We recommend adding the healthy donor sample to each barcode batch, to allow for batch normalization if needed.

Problem 4
Low Cell Count for specific populations.

Our streamlined protocol takes into consideration the importance of obtaining a high cell count. Initial poor cell viability will result in unacceptably low yield of single, live cells (Olsen et al., 2019). Through experimental exploration, we established a threshold for an acceptable cell count to confidently produce accurate cell population quantifications. Cell loss is a result of improper sample handling, poor cryopreservation methods, inadequate fixation, and prolonged staining protocols, specifically the washing steps (Maecker and Harari, 2015). Using our described protocol, we obtained an average staining cell recovery rate of 50%, or approximately 1–1.5 million cells per sample at the end of staining protocol. In addition to cell loss while staining, mass cytometers have a typical machine cell recovery rate of 30%–50% (Maecker and Harari, 2015). After routine clean up gating using Gaussian parameters, we had an end point cell count of at least 200,000 $CD45^{+}103Rh^{-}$ alive single cells per patient available to analyze (Figure 4.). For an in-depth immune phenotyping encompassing small cellular populations, samples need to have a minimum of 100,000 alive single cells analyzed per condition (Marsh-Wakefield et al., 2021) (Maecker and Harari, 2015).

Potential solution
Do not increase the number of washes, as those were optimized for best cell recovery. Do not use Eppendorf tubes at any point of staining, as there is a huge loss of cells during centrifugation in this type of tubes. The use of 96-well plates was optimal for the best cell recovery, thus always use plate or 15 mL conical tubes at precise steps. Do not decrease time of or modify fixation methods recommended in this protocol.

Problem 5

103Rh Viability weak staining intensity (step 11 and data analysis).

Staining 3 × 10^6 cells with a concentration of 1:500 103Rh viability stain should produce two distinct populations of 103Rh negative live cells and 103Rh positive dead cells. If there is no 103Rh positive populations or the positive populations forms a smear rather than a distinct population, downstream analysis could be negatively affected.

Potential solution

We recommend thawing a frozen aliquot of 103Rh no earlier than the morning of staining. Additionally, we do not recommend using frozen aliquots that are over one year old, as we have noticed decreased staining intensity after one year, even with proper storage and preparation. Additionally, be careful when modifying fixation reagents, as 103Rh is not compatible with all fixation methods, such as methanol fixation.

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.

ACKNOWLEDGMENTS

We are thankful to Suzan Lazo, lead of DFCI CyToF core, for technical support.

AUTHOR CONTRIBUTIONS

Conceptualization, J.B.; methodology, C.A., M.H., M.N., M.B., E.H., J.D., and J.B.; investigation, C.A., M.H., and J.B.; resources, C.A., M.H., J.B., F.S.H., and M.S.; writing – original draft, C.A., M.H., and J.B.; visualization, M.H. and J.D.; funding acquisition, F.S.H. and M.S.; supervision, J.B.

DECLARATION OF INTERESTS

Dr. Hodi reports grants and/or personal fees from Bristol-Myers Squibb, Merck, EMD Serono, Novartis, Surface, Compass Therapeutics, Apricity, Sanofi, Pionyr, 7 Hills Pharma, Torque, Bicara, Pieris Pharmaceutical, Checkpoint Therapeutics, Genentech/Roche, Bioentre, Gossamer, Iovance, Trillium, Catalym, Immunocore, Amgen, and Zumutor outside the submitted work. In addition, Dr. Hodi has a patent (#20100111973) with royalties paid and patents (#7250291, #9402905, #10279021, #10106611, #20170248603, #20160046716, #20140004112, #20170022275, #20170008962, and #20170343552) pending.

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