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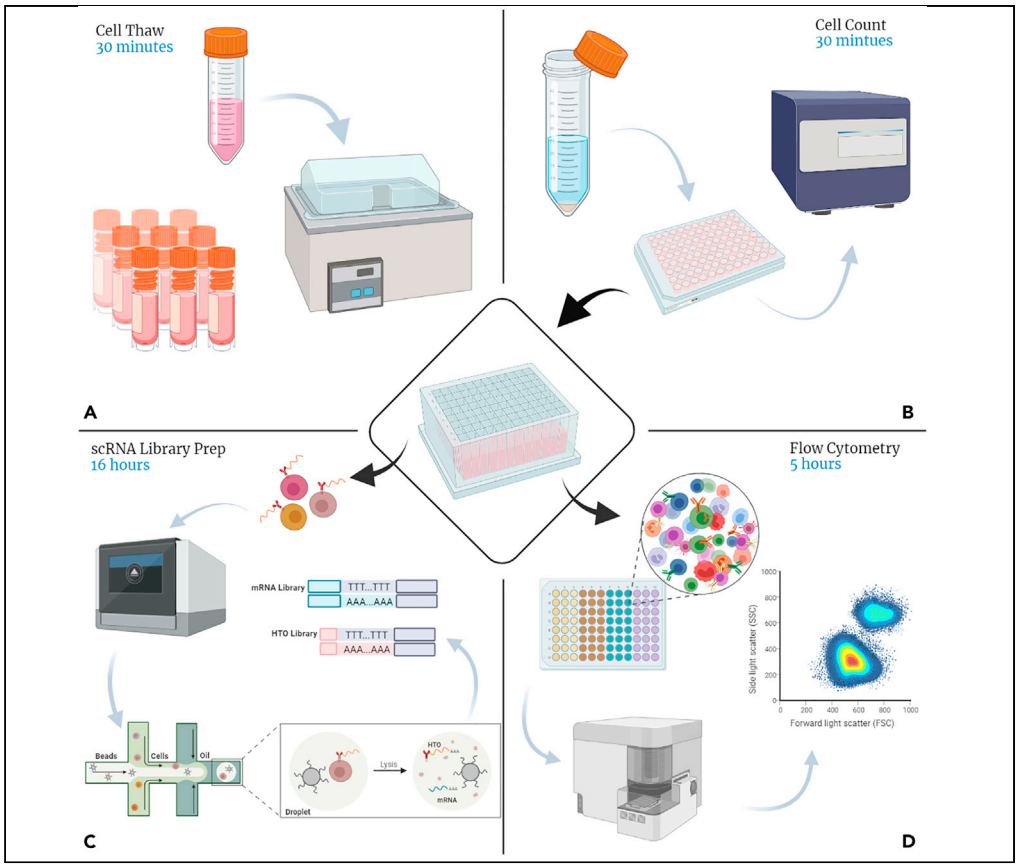
Optimized workflow for human PBMC multiomic immunosurveillance studies



Deep immune profiling is essential for understanding the human immune system in health and disease. Successful biological interpretation of this data requires consistent laboratory processing with minimal batch-to-batch variation. Here, we detail a robust pipeline for the profiling of human peripheral blood mononuclear cells by both high dimensional flow cytometry and single-cell RNA-seq. These protocols reduce batch effects, generate reproducible data, and increase throughput.

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Highlights

Details a robust pipeline for the profiling of human PBMC

Outline a cell thaw protocol compatible with flow cytometry and single cell RNA-seq

Focus on batch effect reduction with a bridging cell control and commercial buffers

Increased throughput with automated liquid handling and cell counting

Genge et al., STAR Protocols 2, 100900
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Optimized workflow for human PBMC multiomic immunosurveillance studies

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SUMMARY

Deep immune profiling is essential for understanding the human immune system in health and disease. Successful biological interpretation of this data requires consistent laboratory processing with minimal batch-to-batch variation. Here, we detail a robust pipeline for the profiling of human peripheral blood mononuclear cells by both high-dimensional flow cytometry and single-cell RNA-seq. These protocols reduce batch effects, generate reproducible data, and increase throughput.

For complete details on the use and execution of this protocol, please refer to Savage et al. (2021).

BEFORE YOU BEGIN

Prepare workspace

This protocol should be performed in a Biosafety level 2 laboratory. All cell processing including 10x Genomics GEM Generation should be performed in a Class II Biological Safety Cabinet (BSC) with a vacuum connection for aspirating media. The vacuum line must be connected to a vacuum trap containing bleach. Ensure that the BSC, pipettes, and additional equipment to be used in the BSC such as the Integra VIAFLO 96 and 10× Chromium Controllers are either sterile or have been cleaned with 70% Reagent Alcohol prior to use. Turn on centrifuges and chill to 4C. After the 10x GEM-generation step has been completed, the protocol can continue in a PCR-Free working area to avoid potential contamination from unrelated amplicons. After the cDNA amplification step has been completed, libraries can be prepared in a ''post-amplification'' working area.

Regulatory compliance for use of primary human material

Peripheral blood mononuclear cells (PBMC) used in this study were collected with written informed consent under the supervision of an appropriate Institutional Review Board (IRB) Protocol. Human samples were de-identified and assigned bar code labels linking to metadata such as subject number, blood draw type etc.

Batch control PBMC for process quality control and batch correction

For longitudinal studies where multiple assay batches will be run and compared, a consistent PBMC batch control sample should be included to assess the quality of each assay run and to provide a consistent control for batch-to-batch correction of data. In our case, a single donor leukopak,

comprising 400 vials of 25 million cryopreserved PBMC per vial was used as a process quality control reference sample and for batch correction of flow cytometry and 10× scRNA-seq data for the duration of the longitudinal study. The batch control PBMC viable cell count and viability (%) were consistent over one year with a standard error less than 1% .

Batch size and design principles

The following protocol is primarily designed to run up to 24 total samples, comprising 23 on-study samples and 1 batch control. Where possible, longitudinal samples from a single subject are processed within the same batch. However, that is not always logistically feasible. A batch will contain PBMC from both healthy subjects and those from disease cohorts. Cells from each vial of 5 million PBMC are processed for flow cytometry (4 panels) and scRNA-seq. The batch control PBMC serves as a process QC control and is used for batch correction. To this end, the high rigor of this protocol minimizes batch variance. For example, cell hashing in scRNA-seq mitigates well and chip variance, resulting in a low 0.1% batch variance based on measurements of technical reproducibility of the batch control (Talla et al., 2021).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TotalSeq™-A0251 anti-human Hashtag 1 Antibody	BioLegend	Cat # 394601, RRID:AB_2750015
TotalSeq™-A0252 anti-human Hashtag 2 Antibody	BioLegend	Cat # 394603, RRID:AB_2750016
TotalSeq™-A0253 anti-human Hashtag 3 Antibody	BioLegend	Cat # 394605, RRID:AB_2750017
TotalSeq™-A0254 anti-human Hashtag 4 Antibody	BioLegend	Cat # 394607, RRID:AB_2750018
TotalSeq™-A0255 anti-human Hashtag 5 Antibody	BioLegend	Cat # 394609, RRID:AB_2750019
TotalSeq™-A0256 anti-human Hashtag 6 Antibody	BioLegend	Cat # 394611, RRID:AB_2750020
TotalSeq™-A0257 anti-human Hashtag 7 Antibody	BioLegend	Cat # 394613, RRID:AB_2750021
TotalSeq™-A0258 anti-human Hashtag 8 Antibody	BioLegend	Cat # 394615, RRID:AB_2750022
TotalSeq™-A0259 anti-human Hashtag 9 Antibody	BioLegend	Cat # 394617, RRID:AB_2750023
TotalSeq™-A0260 anti-human Hashtag 10 Antibody	BioLegend	Cat # 394619, RRID:AB_2750024
TotalSeq™-A0262 anti-human Hashtag 12 Antibody	BioLegend	Cat # 394623, RRID:AB_2750025
TotalSeq™-A0263 anti-human Hashtag 13 Antibody	BioLegend	Cat # 394625, RRID:AB_2750026
TotalSeq™-A0264 anti-human Hashtag 14 Antibody	BioLegend	Cat # 394627, RRID:AB_2750027
TotalSeq™-A0265 anti-human Hashtag 15 Antibody	BioLegend	Cat # 394629, RRID:AB_2750028
Mouse anti-human CD3/BUV395 (UCHT1)	BD Biosciences	Cat # 563546, RRID:AB_2744387
Mouse anti-human CD45/BUV496 (HI30)	BD Biosciences	Cat # 624283, RRID:AB_2868405
Mouse anti-human CD15/BUV563 (W6D3)	BD Biosciences	Cat # 624284, RRID:AB_2868406
Mouse anti-human CD45RA/BUV615 (HI100)	BD Biosciences	Cat # 624297, RRID:AB_2875550
Mouse anti-human CD14/BUV661 (MφP9)	BD Biosciences	Cat # 741684, RRID:AB_2868407
Mouse anti-human CD8/BUV737 (RPA-T8)	BD Biosciences	Cat # 624286, RRID:AB_2868408
Mouse anti-human CD11c/BUV805 (B-ly6)	BD Biosciences	Cat # 624287, RRID:AB_2868409
Mouse anti-human CD25/BV421 (M-A251)	BD Biosciences	Cat # 562442, RRID:AB_11154578
Mouse anti-human CD4/BV480 (SK3)	BD Biosciences	Cat # 566104, RRID:AB_2739506
Mouse anti-human CD16/BV605 (3G8)	BD Biosciences	Cat # 563172, RRID:AB_2744297
Mouse anti-human CD123/BV650 (6H6)	BioLegend	Cat # 306020, RRID:AB_2563827
Mouse anti-human CD127/BV711 (A019D5)	BioLegend	Cat # 351328, RRID:AB_2562908
Mouse anti-human IgD/BV750 (IA6-2)	BD Biosciences	Cat # 747484, RRID:AB_2868411
Mouse anti-human CD304/BV786 (U21-1283)	BD Biosciences	Cat # 743132, RRID:AB_27412
Mouse anti-human CD141/BB515 (1A4)	BD Biosciences	Cat # 565084, RRID:AB_2739058
Mouse anti-human CD11b/PerCP-Cy5.5 (M1/70)	BD Biosciences	Cat # 561114, RRID:AB_2033995
Mouse anti-human CD19/BB790 (HIB19)	BD Biosciences	Cat # 624296
Mouse anti-human CD27/PE (O323)	BioLegend	Cat # 302808, RRID:AB_314300
Mouse anti-human TCRαß/PE-Dazzle594 (IP26)	BioLegend	Cat # 306726, RRID:AB_2566599
Mouse anti-human CD34/PE-Cy5 (581)	BD Biosciences	Cat # 555823, RRID:AB_396152
Mouse anti-human CD197/PE-Cy7 (G043H7)	BioLegend	Cat # 353226, RRID:AB_11126145

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse anti-human CD38/APC (HB-7)	BioLegend	Cat # 356606, RRID:AB_2561902
Mouse anti-human CD56/APC-R700 (NCAM16.2)	BD Biosciences	Cat # 565139, RRID:AB_2744429
Mouse anti-human HLA-DR/APC-Cy7 (L243)	BioLegend	Cat # 307618, RRID:AB_493586
Biological samples		
Cryopreserved human PBMC (longitudinal batch control)	BIOIVT	Cat # HUMAN-PBMC-U-190715
Cryopreserved human PBMC	Clinical partners	N/A
Chemicals, peptides, and recombinant proteins		
AIM V medium	GIBCO	Cat # 12055-091
DPBS w/o Ca/Mg (DPBS)	COSTAR	Cat # 21-031-CM
Clorox germicidal bleach	VWR	Cat # 8901-620
Reagent alcohol 70% (v/v)	Ricca	Cat # 3546.70–5
Molecular Biology Grade Water (MBGW)	Hyclone	Cat # SH3053802
SPRISelect	Beckman Coulter	Cat # B23319
Dynabeads™ MyOne™ SILANE	10x Genomics	Cat # PN-2000048
DNA Suspension Buffer ("Low TE")	TEKnova	Cat # T0220
10% Tween 20 solution	Bio-Rad	Cat # 1610781
50% Glycerol	TEKnova	Cat # G1798
Buffer EB	QIAGEN	Cat # 19086
Ethanol, 200 proof	Pharmco	Cat # 111000200CSPP
Bovine Serum Albumin (BSA)	Sigma-Aldrich	Cat # A2934-100G
NxGen RNAse Inhibitor (40U/ul)	Lucigen	Cat # 30281-2
KAPA HiFi HotStart ReadyMix	Roche	Cat # 7958935001
PhiX control library	Illumina	Cat # FC-110-3001
Human TruStain FcX	BioLegend	Cat # 422302
DMSO (anhydrous)	Invitrogen	Cat # D12345
Cell Staining Buffer	BioLegend	Cat # 420201
Brilliant Stain Buffer Plus	BD Horizon	Cat # 566385
FluoroFix Buffer (contains 4% p-formaldehyde)	BioLegend	Cat # 15713
Critical commercial assays		
ViaStain AOPI Staining Solution	Nexcelom Bioscience	Cat # C52-0106-25mL
BD FVS510 Live Dead Stain	BD Horizon	Cat # 564406
Chromium Next GEM Single Cell 3′ GEM, Library & Gel Bead Kit v3.1	10X Genomics	Cat # PN-1000121
Chromium Next GEM Chip G Single Cell Kit	10X Genomics	Cat # PN-1000120
HS NGS Fragment Kit (1 -6000bp)	Agilent	Cat # DNF-474-0500
Quant-iT™ PicoGreen™ dsDNA Assay Kit	Invitrogen	Cat # P7589
Oligonucleotides		
HTO-cDNA Custom Primer 5′ GTG ACT GGA GTT CAG ACG TGT GCT C	Integrated DNA Technologies	N/A
SI PCR Custom Primer 5′ AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT C	Integrated DNA Technologies	N/A
Single Index Kit T Set A	10X Genomics	Cat # PN-1000213
HTO i7 Sample Index Plate, custom	Integrated DNA Technologies	N/A
Software and algorithms		
FlowJo	BD Biosciences	v 10.7
SpectroFlo	Cytek	v 2.2.0.4
Other		
50 mL polypropylene Falcon conical tube	COSTAR	Cat # 352098
Serological pipettes (5, 25, 50mL)	VWR	Cat # 89130-896, 900, 902
Aspirating pipettes (2 mL)	VWR	Cat # 414004-265
Pipette Tips RT LTS 1000µL wide-orifice, filter	Mettler-Toledo Rainin	Cat # 30389218
Pipette Tips RT LTS 1000µL, filter	Mettler-Toledo Rainin	Cat # 30389213
Pipette Tips RT LTS 200µL, filter	Mettler-Toledo Rainin	Cat # 30389240
Pipette Tips RT LTS 20µL, filter	Mettler-Toledo Rainin	Cat # 30389226

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
PCR 8 -tube strips (0.2mL)	USA Scientific	Cat # 1402–4700
Eppendorf Safe-Lock tubes (1.5 mL)	Eppendorf	Cat # 022363204
Eppendorf snap cap tubes (5 mL)	Eppendorf	Cat # 0030119487
96 well 0.8 mL polypropylene deep well storage plate	Thermo Fisher Scientific	Cat # AB0765
Cellaca MX cell counter plates	Nexcelom Bioscience	Cat # CHM24-A100-020
Cellometer SD100 Cell-Counting slides	Nexcelom Bioscience	Cat # CHT4-SD100-002
Microseal B plate seal	Bio-Rad	Cat # AB0765
96-well skirted Eppendorf plate	Eppendorf	Cat # 951020401
96-well semi skirted Eppendorf plate	Eppendorf	Cat # 0030129326
5 mL polystyrene tubes	Falcon/VWR	Cat # 60819-820
300 mL Clear Advantage™ Reservoirs (Polypropylene)	Integra	Cat # 6348
Heavy Duty Aluminum 18in by 500ft	Foilman	Cat # B07BR29XV7
Griptips, Low Retention Tips in Racks, 300 µL, Green	Integra Biosciences	Cat # 6535
Pipet-Lite™ LTS Pipette L-1000XLS+	Mettler-Toledo Rainin	Cat # 17014382
Pipet-Lite™ LTS Pipette L-200XLS+	Mettler-Toledo Rainin	Cat # 17014391
Pipet-Lite™ LTS Pipette L-20XLS+	Mettler-Toledo Rainin	Cat # 17014392
Pipet-Lite™ LTS Pipette L-2XLS+	Mettler-Toledo Rainin	Cat # 17014393
Pipet-Lite™ Multi Pipette L8-200XLS+	Mettler-Toledo Rainin	Cat # 17013805
Pipet-Lite™ Multi Pipette L8-20XLS+	Mettler-Toledo Rainin	Cat # 17013803
Pipet-Lite™ Multi Pipette L8-10XLS+	Mettler-Toledo Rainin	Cat # 17013802
Beckman Avanti J-15R Centrifuge	Beckman Coulter	Cat # B99517
Beckman JS-4.750 Swinging-Bucket Rotor & Buckets	Beckman Coulter	Cat # B77580
Beckman JS-4.750 Swinging-Bucket Rotor Microplate Carriers	Beckman Coulter	Cat # B83980
Beckman centrifuge tube bucket covers	Beckman Coulter	Cat # 392805
Beckman centrifuge microplate carrier covers	Beckman Coulter	Cat # 393070
Beckman 30 mm conical tube adapter	Beckman Coulter	Cat # 393267
Eppendorf Centrifuge 5430	Eppendorf	Cat # 5430
Serological Pipette Controller	Thermo Scientific	Cat # 9511
Mini-Centrifuge	Southwest Science	Cat # SC1012
Vortex Mixer	Southwest Science	Cat # SBV1000
Cold Block for 1.5 mL tubes	Corning	Cat # 432038, 432036
Cold Block for 96-well skirted/semi-skirted plates	Integra	Cat # 6250
DynaMag-96 Side Skirted magnetic plate stand	Invitrogen	Cat # 12027
Plate Heat Sealer	4titude	Cat # 4ti-0665
Chromium Controller	10x Genomics	Cat # 1000202, 1000204
Fragment Analyzer	Agilent	Cat # M5311AA
Spectramax iD3	Molecular Devices	ID3-STD
Thermal Cycler	Bio-Rad	Cat # 1851196
Cellaca MX high-throughput automated cell counter	Nexcelom Bioscience	Cat # Cellaca MX
Cellometer Spectrum Image Cytometry System	Nexcelom Bioscience	Cat # Cellometer Spectrum
Integra VIAFLO 96 handheld electronic 96 channel pipette	Integra Biosciences	Cat # VIAFLO 96
PCR 96 well Cooling block	Integra Biosciences	Cat # 6250
Milli-Q IQ 7000	Milli-Q	Cat # ZIQ7000T0
Cytek Aurora spectral flow cytometer	Cytek Biosciences	Cat # Aurora

MATERIALS AND EQUIPMENT

Preparation of materials

RNA-seq FcX Staining Buffer: 10% BSA

Reagent	Final concentration	Amount
MBGW	N/A	50 mL
BSA	10%	5 g
Total	**N/A**	**50 mL**

Store at 2°C–8°C for up to 1 week.

RNA-seq Human TruStain FcX Master Mix (FcX MM)		
Reagent	Final concentration	Amount
Human TruStain FcX	26.7%	5 µL
10% BSA	73.3%	13.7 µL
Total	**N/A**	**18.7 µL**
Store at 2°C–8°C for up to 1 week.		

RNA-seq Staining Buffer: DPBS 1× + 2% BSA		
Reagent	Final concentration	Amount
DPBS (1×)	0.9 X	500 mL
BSA	2%	10 g
Total	**N/A**	**500 mL**
Store at 2°C–8°C for up to 1 week.		

GEM-Reverse Transcription Master Mix (GEM-RT MM)		
Reagent	Final concentration	Amount
RT Reagent B	58.2%	18.8 µL
Template Switch Oligo	7.4%	2.4 µL
Reducing Agent B	6.2%	2.0 µL
RT Enzyme C	26.9%	8.7 µL
NxGen RNAse Inhibitor (40U/µL)	16U, 1.2%	0.4 µL
Total	**N/A**	**32.3 µL**
Store on ice for up to 1 h.		

⚠ CRITICAL: Reducing Agent B – irritant and toxicity. Dispose of any residual material in a defined hazardous liquid waste stream.

Dynabeads Cleanup Mix		
Reagent	Final concentration	Amount
Cleanup Buffer	91%	182 µL
Dynabeads MyOne SILANE	4%	8 µL
Reducing Agent B	2.5%	5 µL
Nuclease-free Water	N/A	5 µL
Total	**N/A**	**200 µL**
Store at 20°C–25°C for up to 1 day.		

⚠ CRITICAL: Cleanup Buffer – acute toxicity. Dispose of any residual material in a defined hazardous liquid waste stream.

Elution Solution 1		
Reagent	Final concentration	Amount
Qiagen Buffer EB	98%	98 µL
10% Tween 20	1%	1 µL
Reducing Agent B	1%	1 µL
Total	**N/A**	**100 µL**
Store at 20°C–25°C for up to 1 day.		

80% ethanol solution		
Reagent	Final concentration	Amount
Nuclease-free Water	N/A	8 mL
200 proof Ethanol	80%	32 mL
Total	**N/A**	**40 mL**
Store at 20°C–25°C for up to 1 week		

cDNA amplification reaction mix		
Reagent	Final concentration	Amount
Amp Mix	75.8%	50 µL
cDNA Primers	22.7%	15 µL
HTO cDNA Additive Primer (0.2 µM)	3 nM, 1.5%	1 µL
Total	**N/A**	**66 µL**
Store on ice for up to 1 h.		

⚠ CRITICAL: Amp Mix – irritant. Dispose of any residual material in a defined hazardous liquid waste stream.

HTO Amp Master Mix (MM)		
Reagent	Final concentration	Amount
2× KAPA HiFi PCR MM	95.2%	50 µL
SI PCR Oligo (10 µM)	480 nM, 4.8%	2.5 µL
Total	**N/A**	**52.5 µL**
Store on ice for up to 1 h.		

⚠ CRITICAL: 2× KAPA HiFi PCR MM – acute toxicity. Dispose of any residual material in a defined hazardous liquid waste stream.

HTO index plate		
Reagent	Final concentration	Amount
HTO Index Plate	10 µM	40 µL
Store at −20C for up to 3 months.		

Fragmentation mix		
Reagent	Final concentration	Amount
Fragmentation Buffer	33.3%	5 µL
Fragmentation Enzyme	66.7%	10 µL
Total	**N/A**	**15 µL**
Store on ice for up to 1 h.		

⚠ CRITICAL: Fragmentation Buffer – acute toxicity and skin sensitization. Dispose of any residual material in a defined hazardous liquid waste stream.

Ligation mix		
Reagent	Final concentration	Amount
Ligation Buffer	40%	20 μL
DNA Ligase	20%	10 μL
Adapter Oligos	40%	20 μL
Total	**N/A**	**50 μL**
Store on ice for up to 1 h.		

Index PCR mix		
Reagent	Final concentration	Amount
Amp Mix	83.3%	50 μL
SI Primer (0.2 μM)	0.1 μM, 17.7%	10 μL
Total	**N/A**	**60 μL**
Store on ice for up to 1 h.		

Single Index Kit T Set A Plate		
Reagent	Final concentration	Amount
Single Index Kit T Set A	N/A	10 μL
Store at −20C for up to 3 months.		

Fixable viability dye stock		
Reagent	Final concentration	Amount
FVS510 dye powder	385 μg/mL	100 μg
DMSO	N/A	260 μL
Total	**N/A**	**260 μL**
Store at −20C for up to 3 months.		

STEP-BY-STEP METHOD DETAILS

Cell thaw

Timing: 30 min

In this section, the procedure for thawing human PBMC for subsequent High Dimensional Flow Cytometry and 10x Genomics v3.1 scRNA-seq (single-cell RNA-sequencing) with antibody-based cell hashing workflows is described. Typically, a batch of 23 PBMC samples and one bridging control leukopak sample are processed for downstream assays. Adding thawed PBMC directly to pre-warmed media streamlines the first step of the thaw, and the use of AIM V media eliminates serum (a potential source of activating compounds as well as lot-to-lot variability).

Note: If working with fresh or cultured cells, proceed to "cell count and normalization".

1. Prepare cell thawing media
   a. Transfer 30 mL of AIM V media into one 50 mL conical tube for each sample to be processed. Place on ice until ready to thaw cells.
   b. Transfer 40 mL of AIM V media into one 50 mL conical tube for each sample to be processed. 30 min prior to cell thawing, place the 40 mL AIM V tubes into a 37°C water bath.

Note: Other serum-free media may be used in place of AIM V.

2. Thaw Cells
   a. Thaw PBMC vials rapidly by swirling in the water of a 37°C water bath.
   b. Observe the thawing contents and remove from the water bath when only a small pellet of ice is visible (approximately two minutes).
3. Transfer Cells
   a. Using a wide-bore P-1000 tip, transfer the contents of the PBMC vial to a 50 mL conical tube containing 40 mL of pre-warmed (37°C) AIM V.
   b. Take up 1 mL of AIM V from the conical tube and use it to wash out any remaining cells in the PBMC vial.
   c. Transfer the 1 mL back to the conical tube.
   d. Repeat steps 2a -2c for each PBMC sample.
4. Centrifuge Cells
   a. Transfer the conical tubes to the centrifuge.
   b. Attach the aerosol containment caps to the buckets.
   c. Centrifuge the cells for 400 *g* for 10 min at 4°C.
5. Resuspend Cells
   a. Use a 2 mL aspirating pipette connected to the vacuum line of the BSC to carefully aspirate the supernatant.
   b. Finger vortex or flick the tubes to disrupt the cell pellet.
   c. Using a 5 mL serological pipette, transfer 5 mL of AIM V from a 50 mL conical tube containing 30 mL ice cold AIM V to the conical tube containing the cells.
   d. Pipette mix to resuspend the cells.

Cell count and normalization

Timing: 30 min

The purpose of this step is to quantify the cell concentration of each sample, to resuspend each sample at the appropriate concentration for downstream assays. The Nexcelom Cellaca MX high-throughput cell counter performs 24 cell counts in three minutes. The fluorescent AOPI (Acridine orange/Propidium iodide) method quantifies viable nucleated cells (AO-positive green cells) and dead nucleated cells (PI-positive red cells). As PI quenches AO, a viable cell count and dead cell count are generated. As only nucleated cells are counted, contaminating red blood cells are excluded from the count, eliminating the need for red blood cell lysis. The thawed PBMC samples, normalized to 10 million viable cells per mL in ice cold DPBS, are compatible with both our 10x Genomics scRNA-seq and high dimensional flow cytometry workflows.

Note: Alternative cell counting methods such as Trypan Blue dye exclusion plus red blood cell lysis may be used in place of high-throughput AOPI cell counting. For small batches of samples, a manual hemacytometer may also be suitable.

6. Load Cellaca MX cell counter plate (Figure 1)
   a. Using a P-200 pipette, transfer 30 µL of each cell suspension to a PCR strip tube containing 30 µL of ViaStain AOPI solution. Mix thoroughly.
   b. Using a multi-channel P-200 pipette, mix and transfer 50 µL of the AOPI-stained cells to the counting wells of a Nexcelom cell counting plate. Use Row C for samples 1–12 and Row G for samples 13–24, if applicable.
   c. Go to the Setup menu on the Cellaca MX. Name the plate and select the assay type.

Note: The default assay "MX04.0_AOPI_LiveDead" (Fluorescence exposure settings F1-100msec and F2-300 msec) is used for standard cell counts.

   d. Go to the Load Plate menu. Import or enter the sample names for each well.

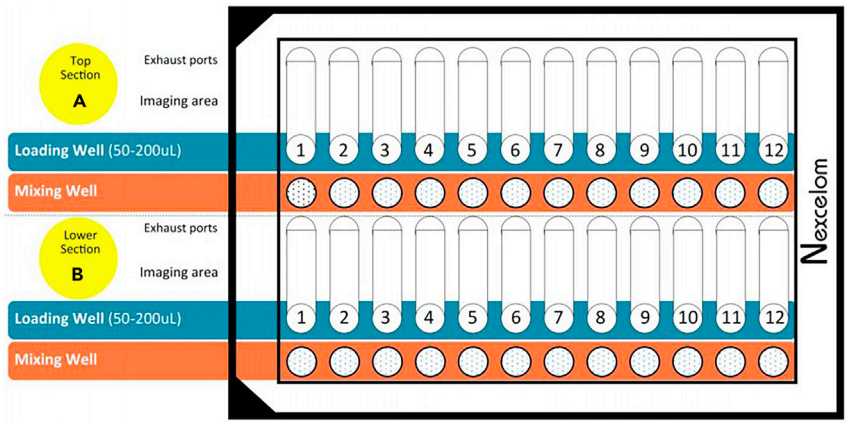

Figure 1. Nexcelom cell counting plate

e. Select Eject Plate: the plate loader will open.
f. Place the plate in the loader and select Load Plate. The plate will be drawn into the counter.
g. Go to the Preview & Count menu. Preview BR1 and FL1 to confirm that the cells are present with bright green fluorescence. See troubleshooting section problem 1 for focusing errors.

7. Count Cells (Figure 2)
   a. Select Count to initiate cell counting. Counting 24 wells takes approximately three minutes.
   b. Once counting is finished, a results sheet will be generated listing the viable cell count, dead cell count, and viability as a percentage. Use the total viable cell counts to calculate resuspension volumes for a target concentration of 10 million viable cells/mL for RNA-seq processing.
   c. Go to the Load Plate menu and select Eject Plate. Dispose of the plate in a proper biohazardous waste container.
   d. Select Load Plate to return the empty plate loader into the Cellaca.
8. Centrifuge Cells
   a. Pour the remaining 25 mL of cold AIM V into each 50 mL conical tube, for a total volume of 30 mL in each.
   b. Place the tubes in the centrifuge.
   c. Attach the aerosol containment caps to the buckets.
   d. Centrifuge the cells for 400 *g* for 10 min at 2°C–8°C.
9. Resuspend Cells
   a. Use a 2 mL aspirating pipette connected to the vacuum line of the BSC to carefully aspirate the supernatant.
   b. Finger vortex or flick the tubes to disrupt the cell pellet.
   c. Add cold (2°C–8°C) DPBS to adjust the cell concentration to 10 million viable cells per mL (based on the Nexcelom Cellaca MX viable cell count).
   d. Transfer normalized samples to a deep well Masterplate on ice and use a Microseal B plate seal to cover.

High-throughput 10x single-cell RNA-Seq with cell hashing, GEM generation, and reverse transcription

Timing: 4 h

In this section, samples undergo "Cell Hashing" as described by Stoeckius et al. (2018) via staining with TotalSeq™-A anti-human Hashtag Oligo (HTO) conjugated antibodies on the 10x Chromium Next GEM Single Cell 3′ assay platform (v3.1). Excess, unbound antibody is removed via robotic

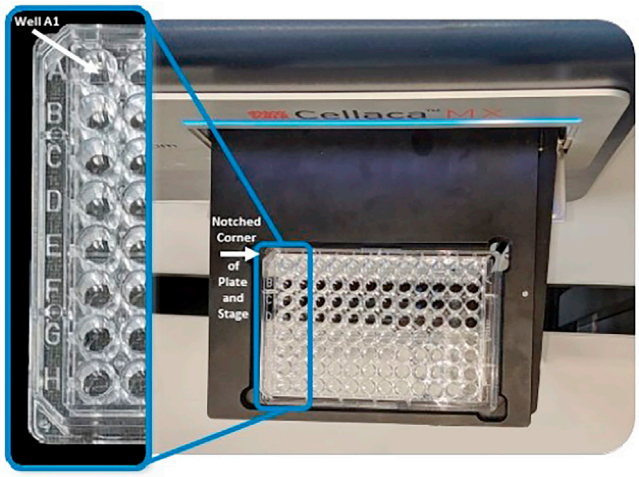

Figure 2. Load plate in the Nexcelom plate loader

washes and samples are then normalized and pooled with a limit of 12 samples per pool. Pooled samples are loaded onto the 10x Chromium Controller Chip G in replicate to achieve ~20K singlets per sample at a concentration of 64,000 cells per well. This generates Gel Bead-In Emulsions (GEMs). GEMs then undergo reverse transcription (GEM-RT) after which can be stored at 2°C–8°C for up to 72 h or −20°C for up to a week.

10. Cell Hashing
    a. Make Human TruStain FcX and 10% BSA Master Mix for the appropriate number of reactions.
    b. Transfer 500,000 Human PBMCs in 50 µL DPBS from the deep well masterplate (see Cell count and normalization section) to sample plate.
    c. Add 18.7 µL of the Human TruStain FcX and 10% BSA Master Mix to each sample.
    d. Set multi-channel pipette to 60 µL and slowly pipette-mix 5 times.
    e. Apply Microseal 'B' adhesive seal and incubate on ice for 10 min.
    f. Prepare a master mix of 1 µL (0.5 µg) of each unique TotalSeq™-A anti-human Hashtag Antibody and 31.3 µL 2% BSA/DPBS (RNA-seq Staining Buffer) for each sample. Include a 20% overage or slop volume.
    g. Set multi-channel pipette to 80 µL and slowly pipette-mix 5 times.
    h. Apply adhesive seal and incubate plate on ice for 20 min.
    i. During the incubation step turn on Integra VIAFLO 96 and click "RUN" to home instrument. Follow prompt on screen for further set-up.
    j. Prepare Integra platform, pipette tips (7 per sample), reservoirs, reservoir tray, and Integra cold block (Figure 3).

⚠ CRITICAL: Set up Integra platform and save operation programs in VIAFLO 96 before starting.

⚠ CRITICAL: When running the Integra VIAFLO 96 programs ensure that the platform is set such that the plate in "B" aligns with the "B" position on the instrument and the black adjustment lever is in the middle/straight position to ensure that the tips, reservoir, and plate align during the automated wash steps.

⚠ CRITICAL: Use new pipette tips for every removal and resuspension step (7 tips per sample).

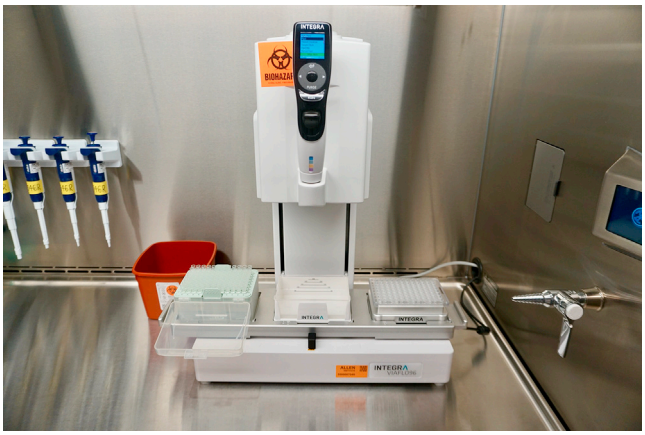

Figure 3. Integra VIAFLO 96 initial set up

Integra VIAFLO 96 in a BSC with tips seated in the left position ("A"), a reservoir seated in the middle position ("AB"), and a cold block with a 96-well plate seated in the right position ("B").

⚠ CRITICAL: During the following "Wash" thaw 10x GEM Generation reagents as described in step 3a.

k. Add 60 mL of 2% BSA/DPBS to an Integra reservoir and hold on ice when not in use.

l. Set the reservoir containing 60 mL of 2% BSA/DPBS tray and place it in the "AB" position on the Integra VIAFLO 96 stage.

m. Remove the seal from the sample plate and place the sample plate in an Integra cold block on the instrument in the "B" position.

n. Run the 'RNA-Seq Wash 1' program to add 150 µL 2% BSA/DPBS solution to each sample and mix 5 times.

o. Transfer the plate in a cold-block to a stable surface. Seal plate with a Micro-Optical qPCR adhesive seal, and centrifuge at 750 *g* for 5 min at 2°C–8°C (ensure centrifuge is pre-cooled). During each centrifugation step, hold the cold block on ice.

p. Carefully transfer the pelleted sample plate to the cold block and back to the "B" position on the Integra VIAFLO 96. Remove the seal.

q. Replace the buffer reservoir with a new empty reservoir and label it "Waste."

r. Run the 'RNA-Seq Remove' program on Integra to remove 200 µL of the clear supernatant from each well, leaving 50 µL above the pellet.

s. With the buffer reservoir in place, run the 'RNA-Seq Wash 2' program on the Integra.

t. Transfer plate in cold block to a stable surface, seal plate with a Micro-Optical qPCR adhesive seal, and centrifuge at 750 *g* for 5 min at 2°C–8°C (ensure centrifuge is pre-cooled).

u. Carefully transfer the pelleted sample plate to the cold block and back to the "B" position on the Integra VIAFLO 96. Remove the seal.

v. With the waste reservoir in place, run the 'RNA-Seq Remove' program on Integra.

w. With the buffer reservoir in place, run the 'RNA-Seq Wash 2' program.

x. Transfer plate to a stable surface, seal plate with a Micro-Optical qPCR adhesive seal, and centrifuge at 750 *g* for 5 min at 2°C–8°C (ensure centrifuge is pre-cooled).

y. With the waste reservoir in place, run the 'RNA-Seq Remove' program on Integra.

z. With the buffer reservoir in place, run the 'RNA-Seq Final' program on the Integra to resuspend the cell pellet. Total volume is now 200 µL.

aa. Transfer the plate to ice and proceed immediately to the next step.

11. Cell Count and Pooling

a. Refer to "Cell count and normalization" section to count samples on the Cellaca MX cell counter. Use 27.5 µL of sample and 27.5 µL of AOPI dye to conserve sample volume.

⚠ CRITICAL: Due to high background noise from oligo-conjugated, cell hashing antibodies use the MX04.0_AOPI_scRNA-Pipeline Assay custom program (Fluorescence exposure settings F1, 300 msec and F2, 350 msec).

b. Use the live cell concentration to normalize pools such that each sample is pooled at an equal fraction and will be sufficient to overload at up to 64K cells per well during 10x GEM generation.

⚠ CRITICAL: A cell viability cut-off of 70% is recommended to ensure the quality of the data, however differences in samples due to a patient's disease profile should be considered. In past preparations, cell viabilities as low as 50% have been processed due to patient disease profile. See troubleshooting section problem 2 for potential solution if cell viability is low.

c. Based on the number of 10× wells to be run, calculate the amount of batch control to spike into each pool (should be 1/5th of the amount of a sample i.e., for 12 samples pooled at 100K cells/sample, pool 20K cells of the bridging control).
d. Pass the pool through a labeled 35 μM cell strainer cap securely held in place on top of a 1.5 mL screw-top centrifuge tube.
e. Take a cell count of the pool using the Nexcelom Cellometer. Mix 12.5 μL of AOPI + 12.5 μL sample pool, pipette-mix, remove plastic from Cellometer slide and place on a clean surface, load 20 μL of AOPI stained sample pool to the slide chamber. Record the total cells/mL and viability.
f. Dilute the sample pool in DPBS for 10× GEM Generation. Use the following equation to determine volume (μL) of the sample pool must be diluted in DPBS for 10× GEM Generation:
Volume of Cells = [Number of Cells per Well ×Number of 10x Wells ×1.2] ÷ Number of Cells per μL
g. Use the following equation to calculate how much (μL) DPBS to add for the sample pool dilution calculated above:
Volume of DPBS = [43.3 μL of Sample Pool Diln ×Number of 10x Wells ×1.2] -Volume of Cells

12. GEM Generation & RT
    a. Allow RT reagent, resuspended Template Switch Oligo (see following step), Reducing Agent B and Gel beads to equilibrate to 20°C–25°C for at least 30 min.

⚠ CRITICAL: If opening a new kit, add 80 μL Low TE to the TSO, vortex 15 seconds and incubate at 20°C–25°C for at least 30 min.

    b. Make up the GEM Reverse Transcription Master Mix (GEM RT MM).
    c. Add 31.8 μL of the GEM RT MM to each strip tube well equal to the number of 10x-wells to be loaded.
    d. Add 43.3 μL of cells from each sample dilution to each well corresponding to the number of 10x Chip G wells.
    e. Keep strip tubes on ice while preparing each chip.

13. Chip Preparation (Figure 4)
    a. Load a chip into the holder without touching the top of the wells.
    b. Dispense 50% glycerol into any unused chip wells if processing less than 8 samples on a chip:

⚠ CRITICAL: DO NOT add glycerol solution to the bottom, 'NO FILL' row.

    c. Set pipet to 70 μL and mix RT Master Mix + Sample by slowly pipetting up and down 5 times then dispense 70 μL of the sample + Master Mix into row 1 without introducing bubbles.

d. Vortex the Gel Beads for 30 s, remove strip tube from holder and briefly centrifuge, and return to holder.
e. Slowly aspirate 50 μL Gel Beads. Dispense into row 2 without introducing bubbles. Wait 30 s before moving to the next step.
f. Dispense 45 μL Partitioning Oil into row 3 wells.
g. Attach the 10x Gasket and ensure the gasket holes are aligned with the wells. Avoid touching the smooth surface (Figure 5).
h. Load the chip into the 10x Chromium controller and press play. Avoid tilting the chip in the process. (~18 min run time) (Figure 6).
i. IMMEDIATELY unload the chip, discard the gasket, and fold the lid back until the holder clicks into place so the chip is sitting at a 45 degree angle (Figure 7).
j. Slowly aspirate 100 μL GEMs from the lowest point of row 3 slowly rotating the pipette away to ensure that the tips stay at the lowest point (Don't allow a seal to form between the tips and the well) (Figure 8).
k. Taking ~20 s, dispense GEMs into a semi-skirted 96-well PCR plate on ice, keeping pipette tips against the sidewalls of the wells at a 45 degree angle.
l. Repeat for each additional chip as needed. Keep GEMs on ice no longer than an hour.
m. Load the plate onto a Thermal Cycler and run the 'GEM RT' program with the following cycling conditions

Steps	Temperature	Time	Cycles
GEM-RT	53°C	45 min	1
Enzyme Degradation	85°C	5 min	1
Hold	4°C	Forever	

Pause point: store at 2°C–8°C for up to 72 h or −20°C for up to one week.

GEM recovery, cDNA amplification, HTO library preparation

Timing: 5 h

In this section, GEMs are broken, recovered cDNA and HTO fragments are amplified. Gene expression cDNA libraries and HTO libraries are separated and size-selected via SPRI-Select bead-based cleanup. HTO libraries are amplified and recovered before storing both sets of libraries at 2°C–8°C for up to 72 h or −20°C for up to one week.

14. Post GEM-RT Cleanup and Gene Expression/HTO Amplification
    a. Allow Reducing Agent B, cDNA Primers, & Dynabeads to come to 20°C–25°C 30 min.
    b. Place Amp Mix and Additive HTO Primer (0.2 μM) on ice.
    c. Thaw Cleanup Buffer for 10 min (or more) at 65°C ensuring there is no precipitate.
    d. Make the Dynabead Cleanup Mix and Elution Solution 1, mix and store at 20°C–25°C until use.
    e. Add 125 μL Recovery Agent to each sample. Do not mix. Incubate 2 min at 20°C–25°C and wait for the biphasic mixture to fully separate.
        i. If biphasic separation is incomplete, securely seal the plate with a Micro-Optical qPCR adhesive seal, invert 5 times and briefly centrifuge.
    f. Pipetting from the bottom of the well, slowly remove and discard 125 μL of recovery agent (pink) without removing any clear or opaque sample (Figure 9).
    g. Vortex the Cleanup Mix well and add 200 μL to each sample. Heat-seal the plate and invert 10× to mix.
    h. Incubate for 10 min at 20°C–25°C.
    i. At the 5-min mark, invert 10× to mix again.

Figure 4. 10x chip G well loading

j. Place the plate on a DynaMag-96 magnsetic plate stand for 3+ minutes, remove and discard only the clear supernatant.

⚠ CRITICAL: Take care to avoid removing beads not bound to the magnet. Beads may bind to the walls of the well. If beads are aspirated, they can be pipetted back into the well to rebind the magnet.

k. Add 300 µL 80% EtOH to each well avoiding the pellet, wait 30 s.
l. Remove and discard EtOH.
m. Add 200 µL 80% EtOH to each well avoiding the pellet, wait 30 s.
n. Remove and discard EtOH.
o. Centrifuge briefly, place plate back on magnetic plate stand and remove the remaining EtOH; let air dry on magnetic plate stand for 30 s.
p. Resuspend beads in 35.5 µL Elution Solution 1. Pipette-mix without introducing bubbles. Incubate at 20°C–25°C for 2 min.

Optional: Seal and briefly centrifuge when incubation is complete.

q. Place the plate on a magnetic plate stand, once the solution clears, transfer 35 µL of sample to a new plate.
r. Make up the cDNA Amplification Reaction Mix.
s. Add 65 µL cDNA Amplification Reaction Mix to each 35 µL sample well.
t. Heat seal, cool immediately, invert 5x and centrifuge.
u. Run the following "cDNA Amplification" program with 11 cycles of amplification

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	15 s	11 cycles
Annealing	63°C	20 s	
Extension	72°C	1 min	
Final extension	72°C	1 min	1
Hold	4°C	Forever	

⏸ **Pause point:** store at 4°C for up to 72 h or −20°C for up to one week.

v. Add 60 µL SPRI beads to each sample well, incubate for 5 min at 20°C–25°C.
w. Place on a magnetic plate stand to pellet beads for 10 min. Transfer 155 µL of the clear supernatant to a new semi-skirted 96-well PCR plate.
x. Heat seal the "HTO" plate and store it on ice while you complete the following steps on the gene expression libraries.

Figure 5. 10x chip G with gasket

y. Add 200 μL 80% EtOH to each well avoiding the pellet, wait 30 s.
z. Remove and discard EtOH.
aa. Repeat steps z and aa for a total of 2 washes.
bb. Centrifuge plate briefly and remove remaining EtOH. Air dry on a magnetic plate stand for 30 s. Resuspend in 40.5 μL Elution Buffer and incubate for 2 min.
cc. Place on a magnetic plate stand until the solution clears and transfer 40 μL of clear supernatant to a new plate.
dd. Make a 1:10 dilution using 45 μL Elution Buffer + 5 μL of Gene Expression Library.

15. HTO Library Amplification and Cleanup.
    a. Remove the semi-skirted plate that was stored on ice and bring it to 20°C–25°C.
    b. Add 140 μL SPRI-Select beads, heat seal, and vortex to mix. Incubate for 10 min at 20°C–25°C.
    c. Place on a magnetic plate stand and discard the clear supernatant.
    d. Add 200 μL 80% EtOH to each well avoiding the pellet, wait 30 s.
    e. Remove and discard EtOH.
    f. Repeat steps d and e for a total of 2 washes.
    g. Centrifuge plate briefly, place on magnet and remove remaining EtOH. Air dry on magnet for 30 s.
    h. Resuspend the beads in 50 μL MBGW.
    i. Add 100 μL SPRI beads directly to the resuspended beads. Incubate for 10 min at 20°C–25°C.
    j. Place on a magnetic plate stand for 10 min and discard the clear supernatant.
    k. Add 200 μL 80% EtOH to the pellet. Wait 30 s.
    l. Remove and discard EtOH, being careful to not touch the beads.
    m. Repeat steps k and l for a total of 2 washes.

Figure 6. Load the 10x Chromium controller

Figure 7. Unload the 10x chip

n. Centrifuge briefly and remove remaining EtOH. Air dry on a magnetic plate stand for 30 s.
o. Resuspend the beads in 45 µL Elution Buffer, incubate for 5 min at 20°C–25°C.
p. Place on magnetic plate stand and transfer the clear supernatant into a new labeled 96-well semi-skirted plate.
q. Make HTO Amplification Master Mix.
r. Add 52.5 µL HTO Amplification Master Mix to each sample well.
s. Add 2.5 µL of a unique HTO Index to each sample from the HTO index plate.
t. Run the following "HTO Index Amplification" PCR program, with 10 cycles of amplification

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	20 s	10 cycles
Annealing	64°C	30 s	
Extension	72°C	20 s	
Final extension	72°C	5 min	1
Hold	4°C	Forever	

Pause point: store at 4°C for up to 72 h or −20°C for up to one week.

u. Add 160 µL SPRI beads, heat seal, vortex to mix. Incubate at 20°C–25°C for 5 min.
v. Place on magnetic plate stand and discard the clear supernatant
w. Add 200 µL 80% EtOH to each well avoiding the pellet, wait 30 s.
x. Remove and discard EtOH.
y. Repeat for a total of 2 washes.
z. Centrifuge briefly and remove remaining EtOH. Air dry on a magnetic plate stand for 30 s.
aa. Resuspend the beads in 30 µL Elution Buffer, incubate for 5 min at 20°C–25°C.
bb. Place on a magnetic plate stand and transfer 30 µL of sample to a new plate.
cc. Make 1:10 dilution (45 µL Elution Buffer + 5 µL HTO Final Libraries) and record the barcode and well location.
dd. QC HTO Libraries alongside the RNA once library prep is complete.

16. Gene Expression Library Intermediate QC
    a. Run 1 µL of the 1:10 diluted Gene Expression Library prior to continuing library preparation on an Agilent Bioanalyzer or Fragment Analyzer to report cDNA quality and yield.

Gene expression library preparation

Timing: 3.5 h

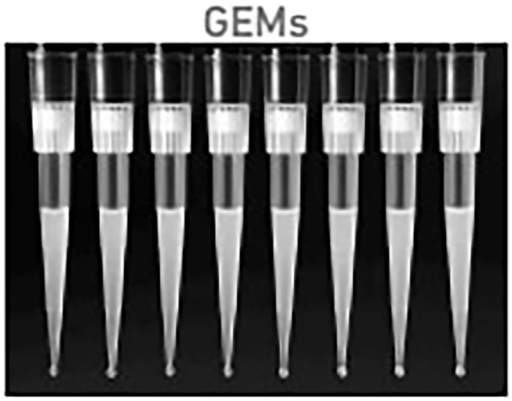

Figure 8. Slowly aspirate GEMs

This section outlines preparation of 25% of the recovered gene expression cDNA libraries for sequencing. The remaining 75% may be stored at −20C for up to 3 months for future use if needed. Fragmented and A-tailed recovered libraries are size-selected for via SPRI-select, bead-based cleanup. Adapters are ligated, recovered libraries are then indexed and amplified before proceeding to QC.

17. Gene Expression Library Prep
    a. Thaw Fragmentation Buffer, Adapter Oligos, Ligation Buffer, SI Primer, and Chromium i7 Index plate (PN-220103).
    b. Remove Fragmentation Enzyme, DNA Ligase, and Amp Mix from -20°C storage only when needed.
    c. Make the Fragmentation Master Mix.
    d. Transfer 10 µL of purified cDNA sample to a new library prep plate, retaining the well position.
    e. Add 25 µL Elution Buffer and 15 µL Fragmentation Mix to each sample and mix.
    f. Transfer to a pre-cooled thermal cycler and run the following "Fragmentation and A-Tail" program.

Steps	Temperature	Time	Cycles
Pre-cool block	4°C	Forever	
Fragmentation	32°C	5 min	1
End Repair and A-tailing	65°C	30 min	1
Hold	4°C	Forever	

    g. Add 30 µL SPRI beads to each sample well, heat seal, cool, and vortex. Incubate for 5 min at 20°C–25°C.
    h. Place the plate on a magnetic plate stand for at least 3 min and transfer 75 µL of clear supernatant to a new set of columns in the plate.
    i. Add 10 µL SPRI beads to each sample heat seal, cool, and vortex. Incubate for 5 min at 20°C–25°C.
    j. Place on a magnetic plate stand and discard the clear supernatant.
    k. Add 125 µL 80% EtOH to each well avoiding the pellet, wait 30 s.
    l. Remove and discard EtOH.
    m. Repeat steps k and l for a total of 2 washes.
    n. Centrifuge briefly, place plate back on the magnet and remove remaining EtOH. Air dry on a magnetic plate stand for 30 s.

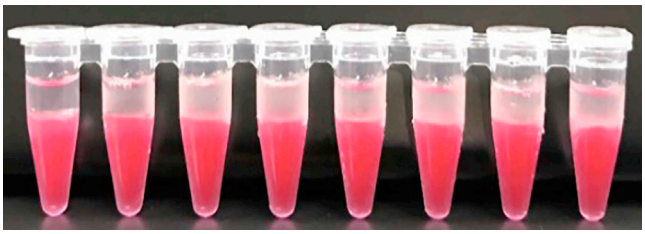

Figure 9. Remove recovery agent

o. Resuspend the beads in 50.5 μL Elution Buffer, heat seal, cool, and vortex. Incubate for 5 min at 20°C–25°C.
p. Place on a magnetic plate stand and transfer 50 μL of sample to a new 96-well skirted PCR plate.
q. Make the Ligation Master Mix.
r. Add 50 μL Adaptor Ligation Mix to 50 μL of sample.
s. Place the plate on the pre-warmed thermal cycler and run the following "Ligation" program:

Steps	Temperature	Time	Cycles
Pre-warm block	20°C	Forever (skip to next step to run)	
Ligation	20°C	15 min	1
Hold	4°C	Forever	

t. Add 80 μL SPRI beads to each sample well and incubate for 5 min at 20°C–25°C.
u. Place on a magnetic plate stand and discard the clear supernatant.
v. Add 200 μL 80% EtOH to each well avoiding the pellet, wait 30 s.
w. Remove and discard EtOH.
x. Repeat for a total of 2 washes.
y. Centrifuge briefly and remove remaining EtOH. Air dry on a magnetic plate stand for 30 s.
z. Resuspend the beads in 30.5 μL Elution Buffer, incubate for 5 min at 20°C–25°C.
aa. Place on a magnetic plate stand and transfer 30 μL of sample to a new plate
bb. Make Index PCR Master Mix.
cc. Add 60 μL Index PCR Mix to each 30 μL sample.
dd. Add 10 μL of an individual i7 sample index from the Single Index Kit T Set A to each well and record the well ID.
ee. Run the "Library Index Amplification" PCR program on a thermal cycler:

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	45 s	1
Denaturation	95°C	20 s	13 cycles
Annealing	54°C	30 s	
Extension	72°C	20 s	
Final extension	72°C	1 min	1
Hold	4°C	Forever	

Pause point: store 2°C–8°C up to 72 h or −20°C for one week.

ff. Add 60 μL SPRI beads to each sample well, heat seal, cool, and vortex. Incubate for 5 min at 20°C–25°C.

gg. Place the plate on a magnetic plate stand and wait until the solution has cleared, transfer 150 μL of the clear supernatant to a new set of columns in the plate.
hh. Add 20 μL SPRI beads to each sample and incubate for 5 min at 20°C–25°C.
ii. Place on a magnetic plate stand and discard 165 μL of the clear supernatant.
jj. Add 180 μL 80% EtOH to the pellet. Wait 30 s
kk. Remove and discard EtOH.
ll. Repeat steps kk and ll for a total of 2 washes
mm. Centrifuge briefly and remove remaining EtOH. Air dry on a magnetic plate stand for 30 s.
nn. Resuspend the beads in 35.5 μL Elution Buffer, incubate for 5 min at 20°C–25°C.
oo. Place on a magnetic plate stand and transfer 35 μL of sample to a new plate once the solution has cleared.
pp. Make 1:10 dilution (45 μL Elution Buffer + 5 μL HTO Final Libraries) and record the barcode and well location.

Library QC and sequencing

Timing: 3 h

This section details fragment size analysis via Agilent Fragment Analyzer and quantification of libraries via PicoGreen DNA Quantitation and pooling libraries for Illumina Next Generation Sequencing. High quality libraries were generated .

18. Library QC and Sequencing
    a. Determine each library's average fragment size with a Fragment Analyzer.
    b. Determine each library's concentration using the Quant-iT™ PicoGreen™ dsDNA Assay or Kapa SYBR Fast qPCR Complete Assay for BioRad® iCycler. See troubleshooting section problem 3 for potential solution to low library concentration.
    c. Based on the recommendations of the quantification assay used, calculate size-adjusted molarity (nM) based on the average base pair size obtained via QC for each library.
    d. Determine the targeted number of reads per each library, we recommend:
        i. 45K reads per cell for each Gene Expression Library.
        ii. 3K reads per cell for each HTO library.
    e. Using each well's size-adjusted molarity (nM) and targeted number of reads, pool a normalized concentration of each Gene Expression and HTO Library into a 1.5 mL low-retention, screw-cap tube.
    f. Dilute pool to desired concentration and volume for sequencing with Qiagen Buffer EB or similar Tris low EDTA buffer.
        i. Typically, 10 nM with a total volume of 150 μL.
    g. Determine the appropriate Illumina sequencing platform and flow cell size to sequence the pooled RNA and HTO library pools together with the following sequencing parameters:
        i. Paired-end, Single-Index
        ii. Read lengths:
            R1: 28
            R2: 91
            i7 Index: 8
        iii. Include 1% PhiX control spike-in.
19. Storage and Freezing
    a. Using reagent guidelines provided on kit boxes and containers, place all reagents in the appropriate freezers. Store custom oligo stocks at 2°C–8°C for up to 1 month. Store the custom HTO i7 stock plate at −80°C for up to 3 months. Store the custom oligo working dilutions and working HTO i7 index plate at −20°C for up to 3 months.

b. In the event the operator is no longer able to continue this protocol at any step where a safe stopping point and storage of libraries is not explicitly referenced, refer to 10x Genomics support for sample storage guidance.
c. Store all the sequencing-ready libraries at −20°C for up to 1 year.

High-dimensional flow cytometry sample preparation

This section describes the flow cytometry procedure for staining and data acquisition of human peripheral blood mononuclear cells (PBMCs) with high dimensional immunophenotyping panels. This protocol has been optimized for longitudinal studies by incorporating features such as a bridging control sample in each batch for normalization, and commercial reagents for staining, washing, and fixation to reduce batch variation. Automated pipetting with the Integra VIAFLO 96 electronic pipette reduced processing time while maintaining high cell viability and retention. The Integra mixing operation programs were optimized to ensure sample pellets were fully resuspended to avoid variable staining due to clumping outlined in troubleshooting problem 4. The data displayed in this protocol is from a 25-color immune survey panel acquired on the Cytek Aurora five laser spectral cytometer but this method can be adopted for any flow cytometry experiment and instrumentation.

⚠ CRITICAL: All incubations prior to sample fixation are at 2°C–8°C protected from light and sealed with a Microseal B adhesive seal.

⚠ CRITICAL: All sample plate centrifuge steps are at 750g for 5 min at 2°C–8°C with swinging centrifuge buckets and aerosol containment covers.

⚠ CRITICAL: DPBS and Cell Staining Buffer should be chilled to 2°C–8°C and kept on ice when not in use.

⚠ CRITICAL: Store the Integra cold block on ice when not in use.

⚠ CRITICAL: Set up Integra platform and save custom Flow Cytometry operation programs in VIAFLO 96 before starting.

⚠ CRITICAL: Use new pipette tips for every removal and resuspension step (18 tips per sample).

Note: The Integra VIAFLO 96 steps may be substituted with manual washing/aspiration using the same volumes noted in the protocol.

Flow cytometry cell staining

Timing: 3.5 h

This section details how to aliquot and stain samples to allow robust longitudinal flow cytometry analysis.

20. Aliquot Samples and Controls to Staining Plate
    a. Adjust the volume of each sample to 10 million viable per mL with DPBS. If the final volume is less than 120 µL, add DPBS so that there is at least 120 µL in the well.

Note: Less than 120 µL can be used, as the samples will be centrifuged, and the supernatant aspirated before the first staining step. Ensure that each well receives the desired number of cells.

b. Add 100 μL (equivalent to 1 million viable cells) of each sample to the corresponding labeled wells of the 96-well semi-skirted Eppendorf sample plate. Insert the sample plate into a pre-cooled Integra PCR 96 well cooling block (2°C–8°C).
c. Add 50 μL (equivalent to 0.5 million cells) of extra batch control cells to empty wells of the sample plate, or an additional plate, for staining single color controls. Make sure to include a negative control.

Note: If single color controls have previously been recorded as Library Reference Controls in the SpectroFlo software, they do not need to be recorded again. The normalized reference controls will work for future batches of the same panel.

Note: If single color controls are on a separate plate, repeat all steps for both the sample and control plates.

Alternatives: If extra batch control samples are not available, other PBMC samples can be used for single color controls. If cells are not available, compensation beads can also be used, but must be optimized before running the experiment.

d. Move the plate and cold block into the right most Integra position. Place a reservoir with 50 mL of cold DPBS into the middle Integra position.
e. Run the Integra program "FLOW_DILUTE" to add 150 μL of DPBS to each sample well.
f. Seal the plate with an adhesive seal and centrifuge at 750 *g* for 5 min at 2°C–8°C.

21. Prepare and apply Viability and Fc Blocking Solution
    a. Prepare the Viability and Fc Blocking solution in a 5 mL Eppendorf tube and store on ice protected from light for up to 15 min.

Reagent	Volume per sample (μL)[a]
FVS510 Fixable Viability Dye	0.3
FcX	2.4
DPBS	117.3

[a] Includes 20% overage

b. Aliquot 100 μL of Viability and Fc Blocking solution to the wells corresponding to samples and Viability single color control in an Eppendorf 96-well full skirt plate. Store in the dark at 2°C–8°C.

Note: Do not add the Viability and Fc Blocking Solution to all control wells, only the single color control for the Viability stain.

c. Remove the sample plate from the centrifuge. Insert the plate into the cold block, and transfer to the right most Integra position. Remove the adhesive seal.
d. Label a new reagent reservoir as biohazardous waste and place it in the middle position of the Integra deck. Run the Integra program "FLOW_REMOVE" to remove 250 μL of supernatant from sample wells.

Note: Once the Integra aspiration step completes, immediately remove the waste reservoir from the middle integra position and discard the fluid in a waste bottle with 10% bleach solution.

e. Place the 96-well full-skirt plate containing the Viability and Fc Blocking solution in the middle position on the Integra.

Table 1. Flow cytometry antibody master mix solution

Conjugate	Target	Clone	Volume per sample (µL)[a]
Cell Staining Buffer	N/A	N/A	57.6
Brilliant Stain Buffer Plus	N/A	N/A	12
BUV395	CD3	UCHT1	2.4
BUV496	CD45	HI30	2.4
BUV563	CD15	W6D3	1.2
BUV615	CD45RA	HI100	0.6
BUV661	CD14	MφP9	2.4
BUV737	CD8	RPA-T8	0.6
BUV805	CD11c	B-ly6	3.6
BV421	CD25 (IL2Rα)	M-A251	2.4
BV480	CD4	RPA-T4	0.6
BV605	CD16	3G8	2.4
BV650	CD123 (IL3Rα)	6H6	2.4
BV711	CD127 (IL7Rα)	A0195D5	2.4
BV750	IgD	IA6-2	1.2
BV786	CD304	U21-1283	2.4
BB515	CD141	1A4	3.6
PerCp-Cy5.5	CD11b	M1/70	0.6
BB790	CD19	HIB19	1.2
PE	CD27	O323	1.2
PE/Dazzle594	TCRαß	IP26	3.6
PE-Cy5	CD34	581	6
PE/Cy7	CD197 (CCR7)	G043H7	3.6
APC	CD38	HB-7	1.2
APC-R700	CD56	NCAM16.2	1.2
APC/Cy7	HLA-DR	L243	1.2

[a]Includes 20% overage.

f. Run the Integra program "FLOW_RSP_MM" to add 90 µL of Viability and Fc Blocking solution to each sample and mix ten times.
g. Incubate for 30 min at 2°C–8°C protected from light.
h. Label a new reagent reservoir as Cell Staining Buffer and place it in the middle position of the Integra deck. Add 50 mL of Cell Staining Buffer to the reservoir. Insert the sample plate on the cold block, remove the seal and place on the right of the stage on the Integra.
i. Run the Integra program "FLOW_DILUTE" to add 150 µL of Cell Staining Buffer to each sample well.
j. Seal the plate and centrifuge at 750 *g* for 5 min at 2°C–8°C.

22. Generate Master Mix and Stain Cells

⚠ CRITICAL: Centrifuge antibody storage vials at 10,000g for 10 min prior to adding to mastermix to reduce antibody aggregates outlined in troubleshooting problem 5 .

a. Record the lot number of each antibody vial and note lot changes between batches.
b. Keep antibodies protected from light at 2°C–8°C until needed.
c. Prepare the antibody master mix solution in a 5 mL Eppendorf tube (Table 1) and place on ice, protected from light for up to 1 h.
d. Vortex the antibody master mix tubes for 10 s. Centrifuge the tubes at 3000 *g*, 2°C–8°C for 2 min.
e. Transfer 90 µL master mix into corresponding wells of a 96-well full-skirt plate. Keep the master mix solution plate at 2°C–8°C protected from light for up to 1 h until staining.

f. Insert the sample plate on the cold block and remove the adhesive seal. Place the biohazardous waste reservoir in the middle position of the Integra deck.
g. Run the Integra program "FLOW_REMOVE" to remove 250 μL of supernatant from sample wells.

Note: Once the Integra aspiration step completes, immediately remove the waste reservoir from the middle integra position and discard the fluid in a waste bottle with 10% bleach solution.

h. Place the 96-well full-skirt plate containing 100 μL of antibody master mix in each well needed in the middle position on the Integra.
i. Run the Integra program "FLOW_RSP_MM" to add 90 μL of antibody master mix to the sample wells and mix ten times.
j. Add the corresponding antibody volume to each single color control well.

Note: The volume of each antibody added to single color controls should be the same as the volume for a single sample, including the 20% overage.

k. Seal the plate and incubate for 30 min at 2°C–8°C protected from light.
l. Insert the sample plate on the cold block and remove the adhesive seal. Place the reagent reservoir containing Cell Staining Buffer in the middle position of the Integra deck.
m. Run the Integra program "FLOW_DILUTE" to add 150 μL of Cell Staining Buffer to each sample well.
n. Seal the plate and centrifuge at 750 *g* for 5 min at 2°C–8°C.
o. Insert the plate into the cold block and remove the adhesive seal. Place the waste reservoir in the middle position of the Integra deck.
p. Run the Integra program "FLOW_REMOVE" to remove 250 μL of supernatant from sample wells. Remove the waste reservoir from the middle integra position and discard the supernatant in a waste bottle with 10% bleach solution.
q. Place the reagent reservoir containing 50 mL Cell Staining Buffer in the middle position of the Integra deck.
r. Run the Integra Program "FLOW_WASH" to add 250 μL of Cell Staining Buffer to each sample well and mix ten times.
s. Seal the plate and centrifuge at 750 *g* for 5 min at 2°C–8°C.
t. Place the sample plate on the cold block, remove the seal and place it on the right of the stage on the Integra. Place the waste reservoir in the middle position of the Integra deck.
u. Run the Integra program "FLOW_REMOVE" to remove 250 μL of supernatant from sample wells.

Note: Once the Integra aspiration step completes, immediately remove the waste reservoir from the middle integra position and discard the fluid in a waste bottle with 10% bleach solution.

v. Place the reagent reservoir containing 50 mL Cell Staining Buffer in the middle position of the Integra deck.
w. Run the Integra Program "FLOW_WASH" to add 250 μL of Cell Staining Buffer to each sample well and mix ten times.
x. Seal the plate and centrifuge at 750 *g* for 5 min at 2°C–8°C.

Note: Two full washes are needed after antibody master mix staining to ensure no excess antibody is in the solution when the cells are fixed as this could lead to non-specific attachment of antibody to the cell surface.

y. Insert the plate on the cold block and remove the adhesive seal. Place the waste reservoir in the middle position of the Integra deck.
z. Run the Integra program "FLOW_REMOVE" to remove 250 μL of the supernatant from sample wells.

Note: Once the Integra aspiration step completes, immediately remove the waste reservoir from the middle integra position and discard the fluid in a waste bottle with 10% bleach solution.

23. Fix Stained Cells
    a. Remove the waste reservoir from Integra stage and place a 96-well full-skirt plate containing 110 μL of 20°C–25°C FluoroFix Buffer in each well in the middle position on the Integra.

⚠ CRITICAL: FluoroFix – BioLegend: 4% p-formaldehyde - acute toxicity.

Note: Other 4% p-formaldehyde fixation buffers may be used in place of FluoroFix Buffer.

b. Run the Integra program "FLOW_FIX" transfer 100 μL of FluoroFix Buffer from the fixation plate to the sample plate and mix ten times.
c. Incubate for 30 min protected from light at 20°C–25°C.
d. Insert the sample plate on the cold block and remove the adhesive seal. Place the reagent reservoir containing Cell Staining Buffer in the middle position of the Integra deck.
e. Run the Integra program "FLOW_DILUTE" to add 150 μL of Cell Staining Buffer to each sample well.
f. Seal the plate with an adhesive seal and centrifuge at 750 *g* for 5 min at 2°C–8°C.
g. Place the plate on the cold block and remove the adhesive seal. Place the waste reservoir in the middle position of the Integra deck.
h. Run the Integra program "FLOW_REMOVE" to remove 250 μL of supernatant from sample wells.

Note: Once the Integra aspiration step completes, immediately remove the waste reservoir from the middle integra position and discard the fluid in a waste bottle with 10% bleach solution.

i. Place the reagent reservoir containing 50 mL Cell Staining Buffer in the middle position of the Integra deck.
j. Perform a post-fixation wash by running the Integra Program "FLOW_WASH" to add 250 μL of Cell Staining Buffer to each sample well and mix ten times.
k. Seal the plate with a Microseal B adhesive seal and centrifuge at 750 *g* for 5 min at 2°C–8°C.

Note: Only one wash is needed because the fixative has been diluted beyond efficacy.

l. Place the sample plate on the cold block, remove the seal and place on the right of the stage on the Integra. Place the waste reservoir in the middle position of the Integra deck.
m. Run the Integra program "FLOW_REMOVE" to remove 250 μL of supernatant from sample wells.

Note: Once the Integra aspiration step completes, immediately remove the waste reservoir from the middle integra position and discard the fluid in a waste bottle with 10% bleach solution.

n. Place the reagent reservoir containing 50 mL Cell Staining Buffer in the middle position of the Integra deck. Run the Integra program "FLOW_FINAL" to add 100 μL of Cell Staining Buffer and mix ten times to resuspend the cells.
o. Seal the plate with an adhesive seal and cover with aluminum foil and store at 2°C–8°C.

Pause point: Cells are fixed at this stage and may be stored at 2°C–8°C for up to 18–24 h before data acquisition.

Flow cytometry data acquisition

Timing: 4 h (for a full 96 well plate)

This section includes information on settings used to acquire processed samples on a five laser Cytek Aurora.

⚠ CRITICAL: Acquire data within 24 h of staining and fixing cells.

24. Prepare sample plate
    a. Remove the seal from the sample staining plate. Mix the wells 10 times to resuspend the samples.
    b. Transfer the 100 μL of sample volume from each well to a 96 well U bottom plate.
    c. Add 160 μL of Cell Stain Buffer to the original sample staining plate and mix 10 times. Transfer 160 μL of the wash volume to the 96 well U bottom plate for acquisition.

Note: The total volume in the 96 well U bottom plate should be 260 μL per well.

25. Set up Cytek Aurora cytometer
    a. Startup and QC instrument according to manufacturer's recommendations in plate mode.

Note: Using the same QC bead lot throughout the course of a longitudinal study will improve consistency. It is recommended that you purchase enough of the same bead lot for the duration of your study.

    b. Set the instrument to acquire 200 μL of sample from each well, mix each well before acquisition, and backflush the sample probe 2 times after every well.

Note: These settings will allow for ~50 μL of dead volume per well and acquire samples at a rate of 5000 events/sec or less. This should prevent any bubbles from entering the stream and causing artifacts in the data.

    c. Load the 96 well U bottom sample plate onto the instrument.

26. Record Reference Controls

Alternatives: You can skip this step if reference controls have been previously recorded for this panel.

    a. In the QC & Set-up tab of the SpectroFlo software, select Reference Controls.
    b. Add the single stain control fluorophores for the panel to be recorded. Label the controls with the corresponding marker and lot number information.
    c. Record 50000 events of the stained controls as Library Reference Controls.

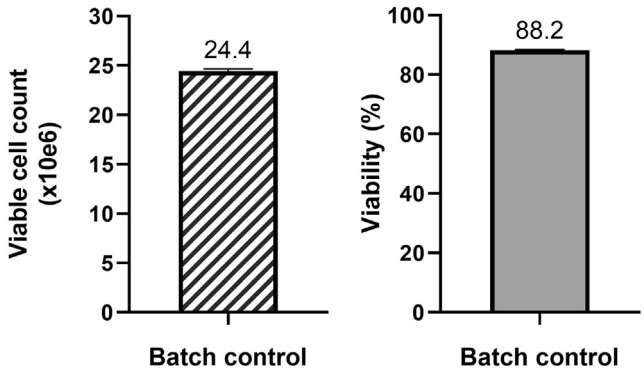

Figure 10. Batch control PBMC

Viable cell count and viability (%) were consistent over one year Between May 2020 and May 2021, the batch control PBMC sample was run in 42 batches. The total cell count mean was 24,436,905 (SEM ± 238,032 or 1%) while the viability (%) mean was 88.2% (SEM ± 0.2%).

27. Acquire data and controls on Cytek Aurora cytometer
    a. Set up a new experiment and label the appropriate experiment details.
    b. If acquiring PBMCs for the first time, adjust the FSC-A and SSC-A voltages so that the cells can be discriminated from debris in the lower left portion of the FSC vs. SSC plot. Otherwise, use the same acquisition settings as previous batches.
    c. Acquire and record samples from the plate.

Note: Data collection should take approximately 4 h for a full 96 well plate.

    d. Unmix collected data with corresponding Reference Controls saved in the SpectroFlo Library.
    e. Export FCS data files as FCS 3.1 format
    f. The acquired data can be analyzed with FlowJo or other commonly used software such as Cytobank, FlowCore, CATALYST etc., using linear scales for FSC-A, SSC-A and FCS-H, and biexponential scales for the other markers.

EXPECTED OUTCOMES

Use of the outlined cell thawing and counting protocol showed consistent cell recovery and viability values for the batch control leukopak PBMC sample over a period of 1 year composed of 42 batches (Figure 10).

For typical fragment analyzer traces showing expected intermediate gene expression cDNA library, final gene expression library and final hashtag oligo (HTO) library see Figures 11, 12, and 13.

The expected output of this protocol is high quality longitudinal flow cytometry data over multiple batches. With a starting input of one million cells, the typical recorded number of cells from the cytometer is ~400,000 cells. Recording a high number of cells improves the counts and statistical significance of rare cell types (Figures 14 and 15).

QUANTIFICATION AND STATISTICAL ANALYSIS

Flow cytometry data was gated manually with FlowJo 10.7 using the gating strategy shown in Figure 14, based on prior knowledge of flow cytometry gating for immune populations.

Data analysis for the scRNA-seq and integration of data sets are not encompassed within this protocol but are described in Savage et al. (2021), Swanson et al. (2021) and Talla et al. (2021).

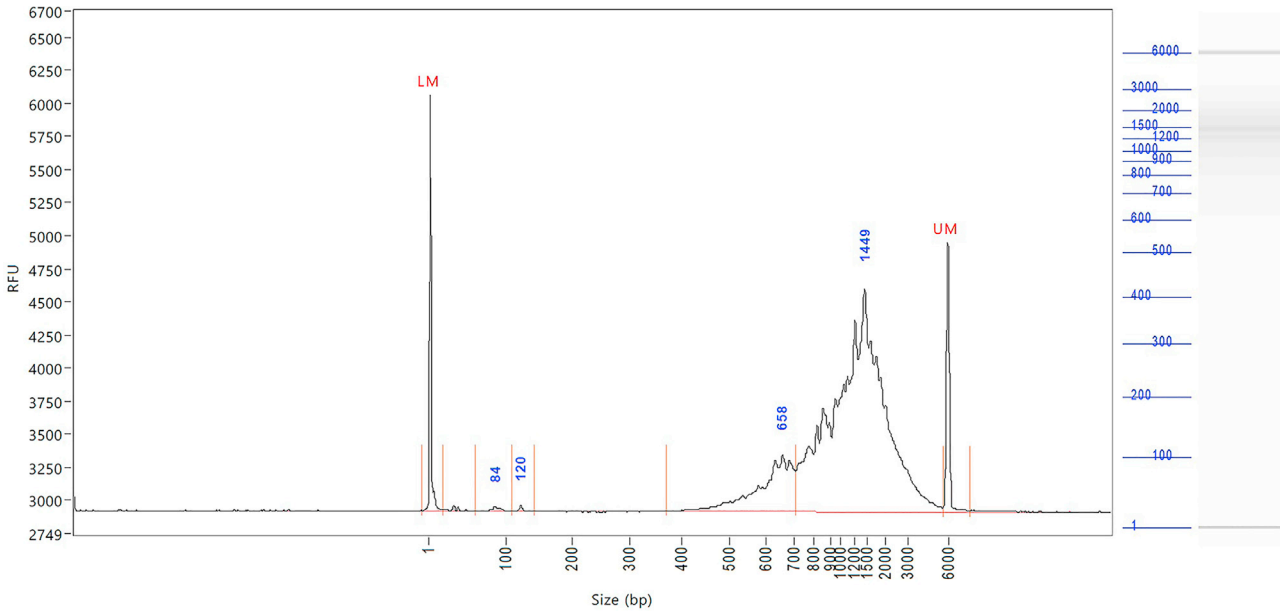

Figure 11. Intermediate gene expression cDNA library

A 1:10 dilution of the intermediate gene Expression library is run as a quality control step on Fragment Analyzer or Bioanalyzer. Above is an example of an expected trace from the Fragment Analyzer. LM and UM indicate a 35 bp Lower Marker reference and a 10,380 bp Upper Marker reference, respectively.

LIMITATIONS

This protocol provides a method for generating high quality longitudinal flow cytometry data from fixed human PBMCs. This method may be adapted for other tissue types, but development is needed to ensure the same quality of data over time.

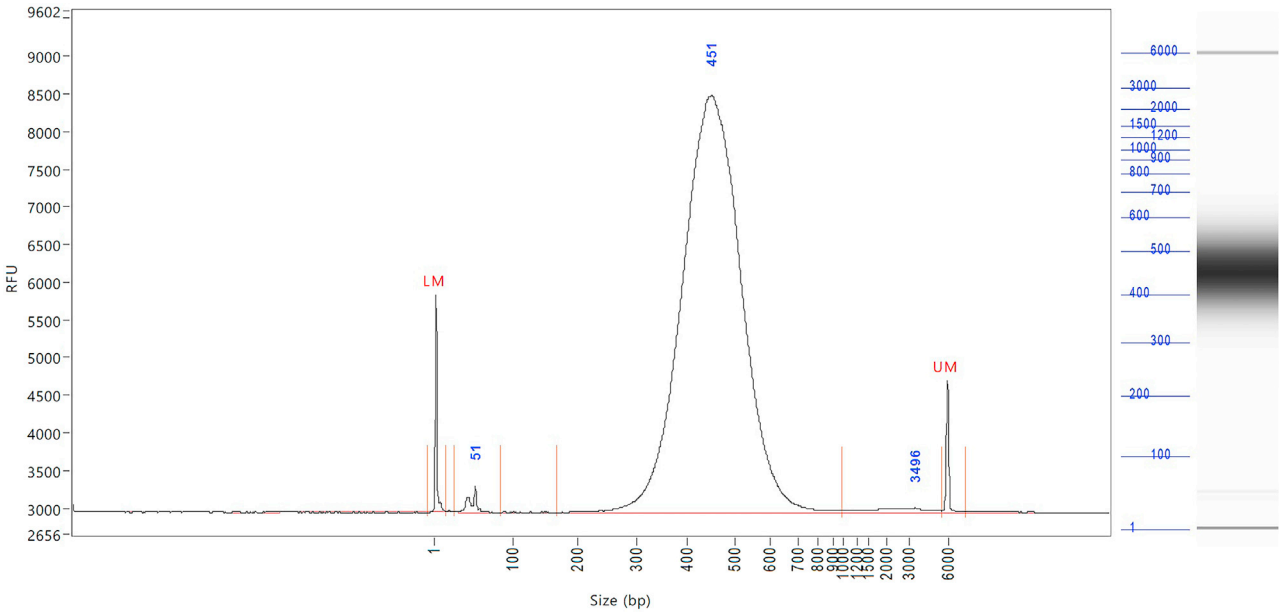

Figure 12. Final gene expression library, ready for sequencing

A 1:10 dilution of the final, sequencing ready gene expression library is analyzed to determine the average size of the library. Above is an example of an expected, 440–460bp, trace from the Fragment Analyzer. LM and UM indicate a 35 bp Lower Marker reference and a 10,380 bp Upper Marker reference, respectively.

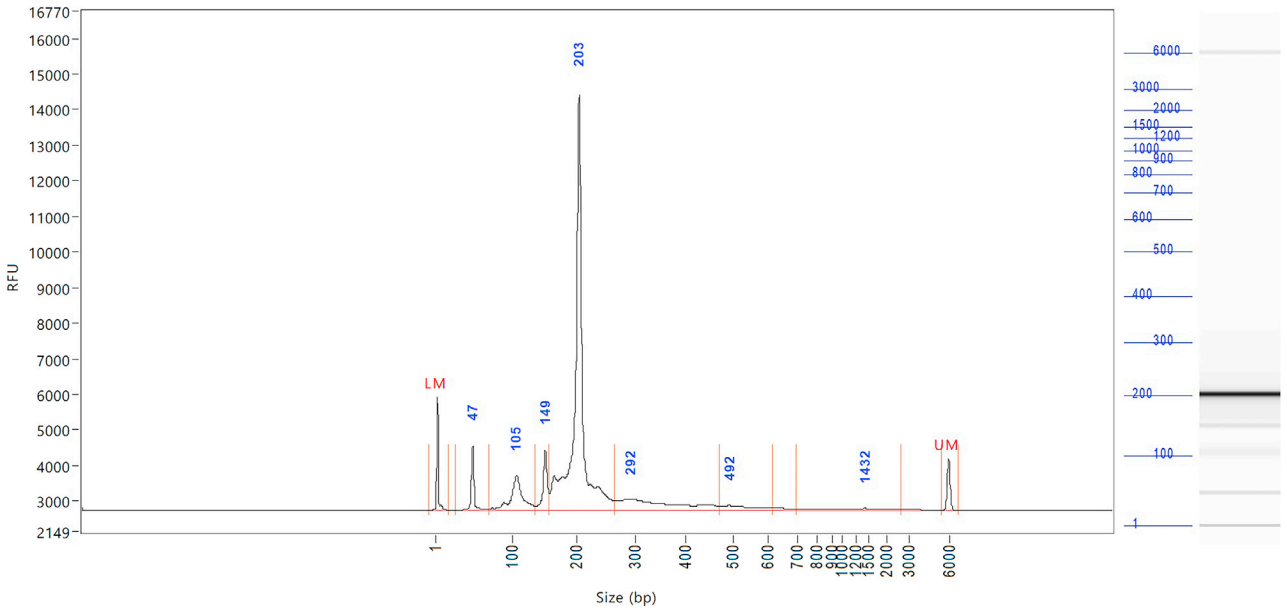

Figure 13. Final hashtag oligo (HTO) library, ready for sequencing

A 1:10 dilution of the final HTO library is run on the Fragment Analyzer. Above is an example of an expected, 200-210 bp, trace from the Fragment Analyzer. This is the corresponding HTO library to both intermediate and final gene expression libraries shown above. LM and UM indicate a 35 bp Lower Marker reference and a 10,380 bp Upper Marker reference, respectively.

This protocol has been optimized for scRNAseq data generation from human PBMC with cell viability greater than 70%.

The number of samples for each pool is limited to the number of unique HTOs and HTO i7 indexes available.

The number of samples per each pool is also limited by sequencing capacity on a NovaSeq S4 flow-cell which is only able to sequence 12 GEX libraries and 12 corresponding HTO libraries at the appropriate depth.

TROUBLESHOOTING

Problem 1

Cellaca MX cell counter.

Cells appear out of focus, or the Green Fluorescence signal FS1 is dull when viewing loaded cell counter plate (cell count and normalization step 6g), shown in Figure 16.

Potential solution

Stop the preview and select Auto-Focus. Preview BR1 and FL1 again to confirm the cells are in focus. If necessary, adjust the focus further using the fine and coarse focus adjustments.

> *Note:* The focus can only be adjusted when the preview has been stopped. A new preview must be done each time the focus is adjusted.

Problem 2

scRNA-seq low cell viability.

If a sample's cell viability is too low (usually below 50%) when reviewing cell viability results (cell hashing, gem generation, and reverse transcription step 11b) it is recommended not including that

Example Population Gating from Survey Panel

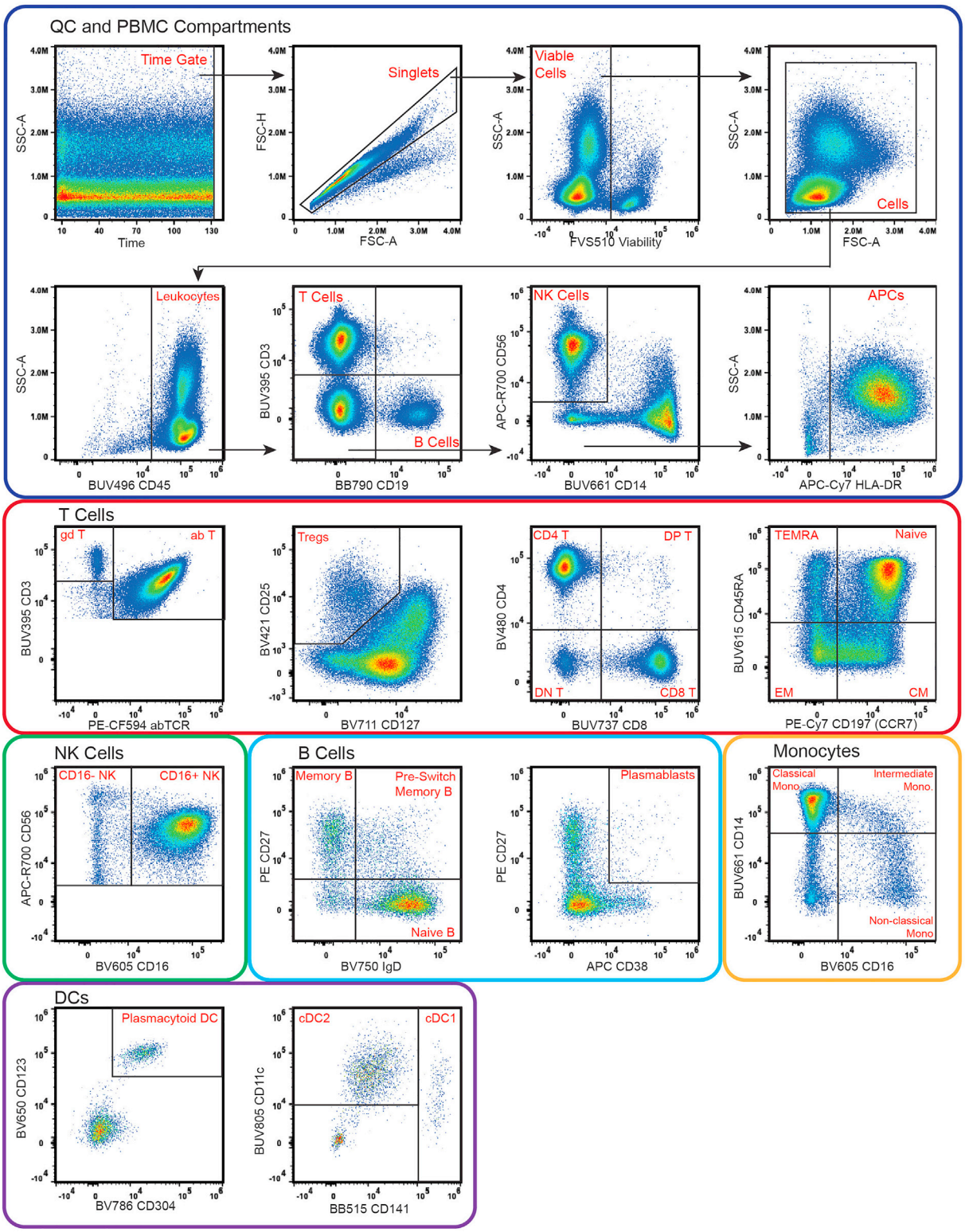

Figure 14. Hierarchical Gating of 25 Marker immunophenotyping panel

Example gating from the 25 marker immune survey panel demonstrated in this protocol. This panel provides protein expression for classifying markers and identifies over 30 different immune subsets including T cell, B cells, NK cells, dendritic cells, and monocytes. APCs = Antigen Presenting Cells, CM = Central Memory T cells, DC = Dendritic Cells, DN T = Double Negative T cells, DP T = Double Positive T cells, EM = Effector Memory T cells, gd T = Gamma Delta T cells, NK = Natural Killer, TEMRA = T Effector Memory cells Re-expressing CD45RA, Treg = Regulatory T cells.

sample in the pool for GEM generation. Adjust the pooling calculation for the batch control accordingly. Dropping a sample with low viability ensures the correct number of cells are loaded per sample and maintaining high quality single-cell data.

Potential solution

If a second aliquot of this sample is available, thaw, count and replace. If this sample also has low viability, consider sequencing this sample separately through standard 10X 3′ scRNA seq without cell hashing.

Problem 3

scRNA-seq low HTO product, high TSO product.

It can be difficult to separate the amplified HTO libraries from excess TSO if the product concentration is low when reviewing library concentration (library QC and sequencing step 18b).

Potential solution

If an HTO library shows low amplified product and high TSO product, an additional amplification can be run with illumina P7 and P5 adapter primers at a final concentration of 0.1 μM with 3–5 cycles of amplification.

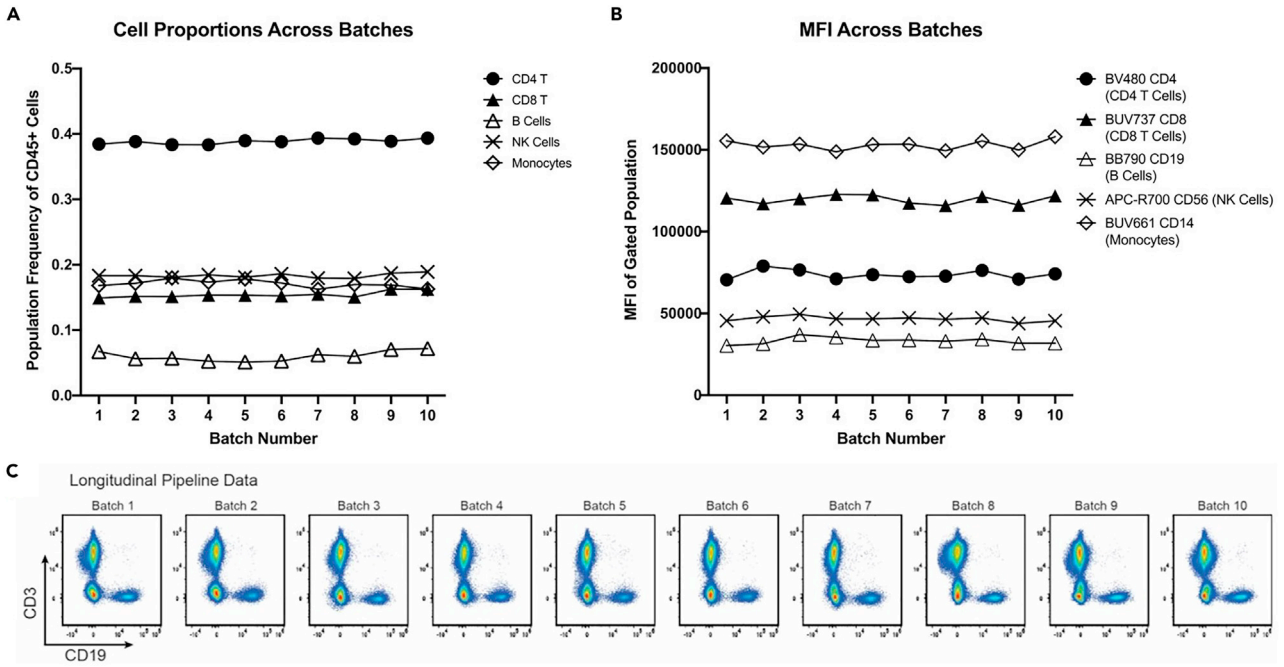

Figure 15. Longitudinal consistency across batches

This protocol provides high quality flow cytometry data over time. The figures above show data from 10 batches spread out across 6 months of pipelines run. Gated events use the same gating strategy shown in Figure 1.

(A) Cell proportions of major cell types from the bridging control sample in each batch.

(B) Median fluorescence intensity (MFI) of the same major cell types across batches.

(C) Bivariate plots of CD45+ cells expressing CD3 versus CD19, demonstrating visual consistency of data over time.

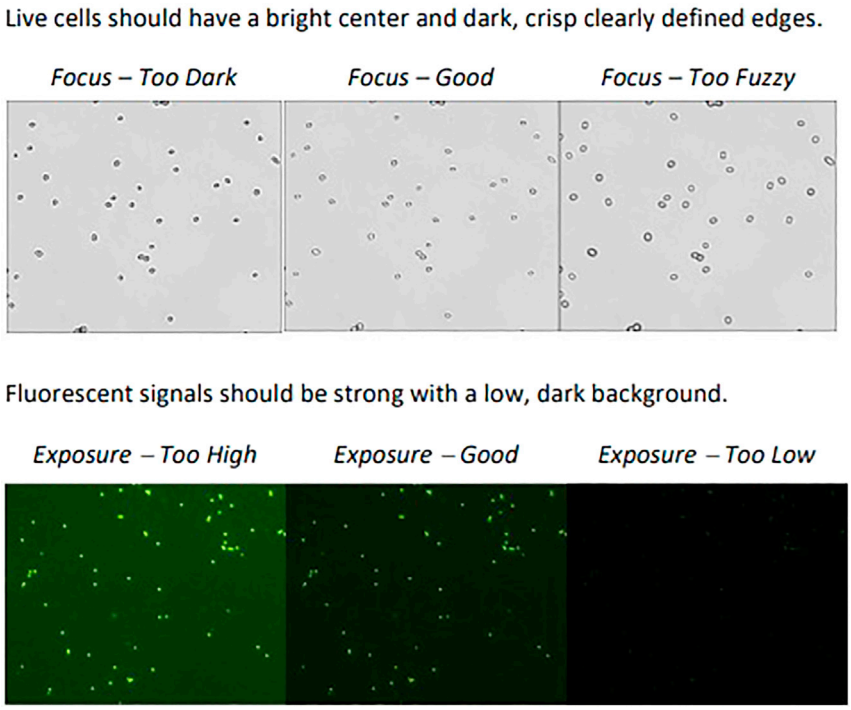

Figure 16. If dark field focus or Fluorescence signal FS1 (Green Acridine Orange nuclear stain) are suboptimal, cell count values will be underestimated

Problem 4

High dimensional flow cytometry, poor quality fluorescence data.

Suboptimal resuspension of cell pellets in the antibody mastermix may lead to streaking of fluorescent signals observed when reviewing acquired flow cytometry data (high dimensional flow cytometry step 27f), shown in Figure 17.

Potential solution

After centrifuging the PBMC samples, cell pellets will not break up completely without proper mixing. If pellets are not well dispersed, some cells are not fully stained, resulting in a signal that streaks towards zero on the axis. This mixing effect is related to how well each pellet is resuspended in the well, so it may appear to occur in random samples. Increasing the number of mixes in each step, particularly during the antibody staining step and using standard width pipette tips instead of Wide Bore tips, will help prevent this mixing effect from impacting data quality. The Integra mixing

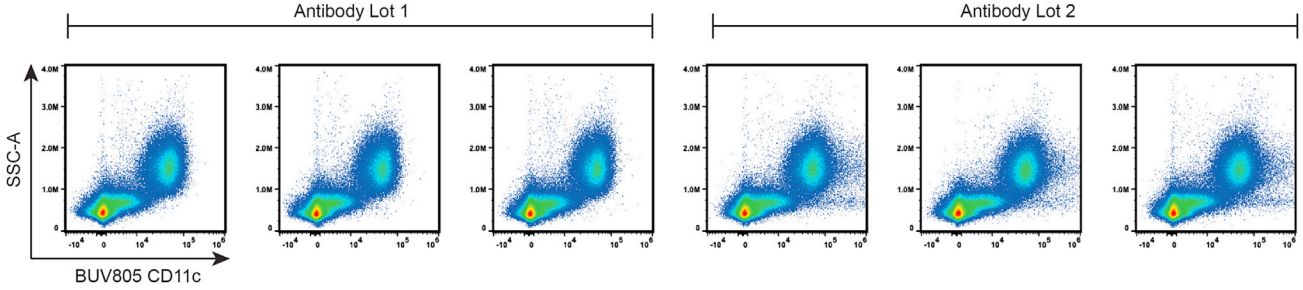

Figure 17. Effect of poor mixing during antibody staining on CD3 and CD45 signals in fixed PBMCs

Some samples are more affected than others.

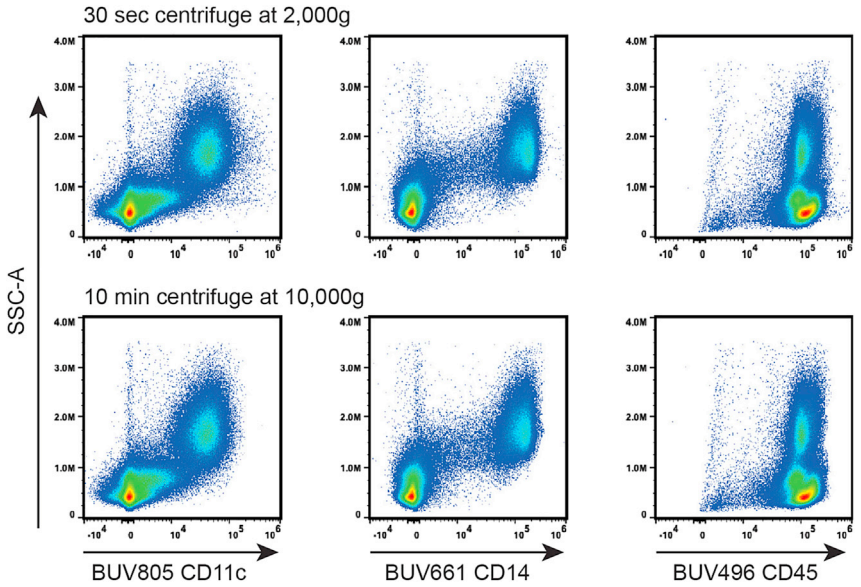

Figure 18. BUV805 CD11c staining across 6 batches over time
After the first 3 batches, a new antibody lot was used that introduced high signal artifacts to the data.

operation programs were optimized (Flow Cytometry Cell Staining steps 1–4) to ensure sample pellets were fully resuspended to avoid variable staining due to clumping.

Problem 5
High dimensional flow cytometry presence of antibody aggregates.

High signal artifacts appear above fully stained populations, sometimes appearing with new antibody lots, when reviewing acquired flow cytometry data (high-dimensional flow cytometry step 27f), shown in Figure 18, resolved in Figure 19. This issue is most likely to happen with BD Brilliant Ultra Violet (BUV) fluorophores.

Potential solution
High signal artifacts are typically from antibody aggregates in the storage vial. Spinning down the vials at 10,000 *g* for 10 min at 2°C–8°C before aliquoting from them can help prevent aggregates

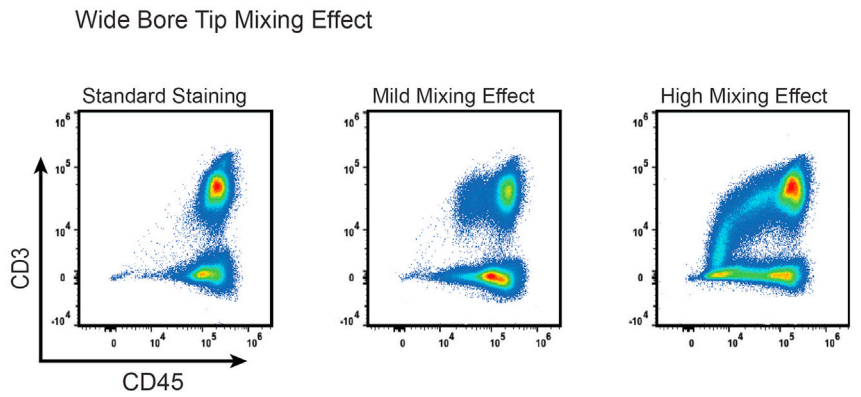

Figure 19. Centrifuging antibody storage vials at 10000 *g* for 10 min prior to preparation of master mixes reduced antibody aggregate artifacts in Brilliant Ultra Violet fluorophores

from staining the sample. The vials were originally centrifuged in a small table top centrifuge at 2,000 *g* for 30 s, but this was insufficient to remove aggregates whereas 10,000 *g* for 10 min removed most of the aggregates as reflected in samples stained with various Brilliant Ultra Violet (BUV) dyes (Figure 19). To ensure that antibody aggregates are minimized, this centrifugation step was included in Flow Cytometry Cell Staining step 22.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Julian Reading (julian.reading@alleninstitute.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all data sets/code generated or analyzed during this study. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

A.T.H., N.K., C.R.R., V.L.H., and J.R. optimized cell thaw, VIAFLO 96, and flow cytometry protocol. P.C.G., M.D.A.W., E.S., C.L., Z.T., and C.G.P. optimized scRNA-seq protocol. T.F.B. led the Allen Institute for Immunology. P.J.S. led the AIFI Molecular Biology group. T.R.T. led the AIFI Experimental Immunology group. J.R., N.K., P.C.G., M.D.A.W., C.R.R., and V.L.H. wrote and reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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