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Cell sorting for common and rare immune population enrichment and single cell omics

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Tarran S. Rupall[1], Carla Jones[1], Gosia Trynka[1]

[1]Wellcome Sanger Institute

Human Cell Atlas Metho...

Carla Jones
Wellcome Sanger Institute

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We use this protocol and it's working

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Abstract

AIM: This protocol aims to enrich common and rare immune cells from cryopreserved PBMC from healthy donors from JAGUAR sites for multimodal single cell analysis

Materials

Thawing PBMCs:

- 15mL Falcon tubes
- 5mL FACS tubes
- Complete RPMI-1640 (Gibco, RPMI, 10% FBS, 1% Penicillin/Streptomycin, 1% L-Glutamine)
- Cell Staining Buffer (Biolegend, Cat #420201)
- AO/PI staining solution (Revvity, cat #CS2-0106-5ML)
- High-Throughput Counting Plates (Revvity, cat #CHM24-A100-001)

FACS staining:

- SpectraComp Unmixing Controls (Cambridge bioscience cat #SSB-05-B)
- Human TruStain FcXTM blocker (Biolegend, Cat #422302)
- True-Stain Monocyte Blocker (Biolegend, Cat #426102)
- Brilliant Stain buffer plus (Biolegend, Cat #566385)
- CD20-SUV387 (BD Bioscience, cat #375528)
- CD19-SBUV605 (Bio-Rad, cat #MCA1940SBUV605)
- CD25-BUV737 (BD Bioscience, cat #741832)
- CD11c-BV421 (BioLegend, cat #337226)
- CD14-SBV475 (BD Bioscience, cat #MCA1568SBV475)
- CD8-SBV570 (BioLegend, cat #MCA1226SBV570)
- CD56-BV650 (BioLegend, cat #362532)
- CD117-BV750 (BD Bioscience, cat #747514)
- CD34-BV750 (BD Bioscience, cat #746987)
- CD127-FITC (BioLegend, cat #11-1278-42)
- CD16-RB613 (BD Bioscience, cat #759229)
- CD4-RB744 (BD Bioscience, cat #570466)
- HLA-DR-RY586 (BD Bioscience, cat #568153)
- CD3-AF594 (BioLegend, cat #300446)
- CD45-SBY800 (Bio-Rad, cat #MCA87SBY800)
- TCRgd-AF647 (BioLegend, cat #331214)
- LiveDead-Zombie NIR (BioLegend, cat #423105)

CITEseq staining:

- 1.5mL LoBind protein binding Eppendorf's
- 5mL FACS tube mesh filter
- Total-seq C Human Universal Cocktail v1.0 (Biolegend, cat #399905)
- TotalSeqTM-C0148 anti-human CD197 (CCR7) (Biolegend, cat #353251)
- Heat Inactivated Fetal Bovine Serum (HI FBS)

10X:

- GEM-X Universal 5' Gene Expression v3, 16 samples (10X Genomics, cat #1000699)
- Chromium GEM-X Single Cell 5' Chip Kit v3, 4 chips (10X Genomics, cat #1000698)
- 50% Glycerol
- 0.2mL PCR 8-strip tubes (Starlab, cat #I1409-3700)

Before start

Warm media is required for optimal recovery during cell thawing

Thawing PBMCs

21m

1 Take PBMC vials and heat in water bath (37°C) for 45 seconds to form ice pellet. 1m

2 Add 1mL of warmed media to ice pellet. 1m

3 Add all volume to the 15mL of warm RPMI media. 1m

4 Immediately spin down, for 5 minutes, 400g at room temperature (RT). 5m

5 Quickly remove supernatant (SN) and gently resuspend in 1mL of cell staining buffer (CSB). 1m

6 Transfer 1mL of samples to 5mL FACS tubes on ice. 1m

7 Top up with 3mL of CSB.

8 Centrifuge samples for 5 minutes, 400g at room temperature (RT). 5m

9 Remove supernatant & gently resuspend in total volume of 100uL of CSB. 1m

10 Take 5uL of sample and dilute 1:10 and count using dead/live stain. 5m

Prepare single colour controls

47m

11 Vortex spectra comp beads for 4 seconds.

12 Add 50uL of beads into a 96 deep well plate (2mL). 5m

13 Briefly spin down each antibody. 1m

14 Stain each well with the correct amount of antibody as denoted in the tables below. 10m

	Antibody marker	Volume per 50uL of beads
	CD20	1
	CD19	1
	CD25	0.5
	CD11c	1
	CD14	1
	CD8	1
	CD56	0.5
	CD117	0.5
	CD127	0.5
	CD16	0.5
	CD4	1
	HLA-DR	0.5
	CD3	0.5
	CD45	1
	TCRgd	0.1

15 Pipette mix 5 times. 1m

16 Incubate for 30 minutes on ice in the dark. 30m

Washing single colour controls 34m

17 Add 950uL of cell staining buffer to each well and pipette mix well x5. 2m

18 Spin down plate, for 5 minutes, 400g at 4oC. 5m

19 Remove supernatant from plate by aspirating 950uL. 2m

20 Add 950uL cell staining buffer to beads and resuspend. 2m

21 Repeat this wash for a total of 3 times. 20m

22 On final resuspension, add 300uL of cell staining buffer to wells and pipette mix x5 times. 2m

23 Label FACS tubes and transfer all volume per well to correct labelled FACS tubes. 1m

24 Keep FACS tubes in fridge at 4oC in the dark until ready for FACS.

Prepare samples for Antibody staining 29m

25 For each donor sample aliquot 5 million cells into 5mL FACS tubes and dilute with CSB for a total volume of 120uL (25 million cells per mL). 5m

26 Add 95uL of True-stain Monocyte Blocker to 95uL of Human TruStain FcX (biolegend) to create a master mix blocker. 1m

27 Add 30uL of master mix blocker to each sample and pipette mix well. 5m

28 Incubate on ice for 10 minutes. 10m

29 Aliquot 1 million cells of a control sample to 2 FACS tubes labelled 'dead' and 'unstained'. 1m

30 Transfer 1mL of CSB to the 'unstained' FACS tube. 1m

31 Add 0.5uL of Zombie Near Infrared (Zombie NIR) to the 'dead' FACS tube and and top up volume to 100uL with CSB 1m

32 Prepare FACS Antibody cocktail in a 1.5mL Lobind eppendorf. 5m
Add 110.6uL of CSB
Add 190uL of Brilliant stain buffer plus
Add 9uL of Zombie NIR
Add FACS antibodies (follow table below).

	Antibody marker	Volume per 18M cells (uL)
	CD117	30.6

	Antibody marker	Volume per 18M cells (uL)
	CD11c	30.6
	CD127	30.6
	CD14	30.6
	CD16	30.6
	CD19	30.6
	CD20	59.4
	CD25	30.6
	CD3	30.6
	CD34	30.6
	CD4	30.6
	CD45	30.6
	CD56	90
	CD8	30.6
	HLA-DR	14.4
	TCRgd	59.4

FACS antibody staining

33m

33 Pipette mix antibody cocktail well and transfer 150uL of antibody cocktail into each sample.

3m

34 Pipette mix each sample well and incubate on ice for 30 minutes in the dark.

30m

Prepare CITEseq antibody cocktail

27m

35 Equilibrate 5 lyophilised Total-seq C Human Universal Cocktail vials to room temperature for 5 minutes.

5m

36 Place vials in a micro-centrifuge and spin down at 10,000g for 30 seconds at RT.

30s

37 Rehydrate lyophilised vials by adding 27.5uL of CSB to each vial - vortex for 10 seconds.

1m

38 Incubate at RT for 5 minutes.

5m

39 After cocktail incubation, vortex it again and spin down at 10,000g for 30 seconds at RT. 30s

40 Transfer 27.5uL of reconstituted cocktail to 1.5mL Lobind protein binding eppendorf tube. 1m

41 Centrifuge reconstituted cocktails and CCR7 totalseq antibody in a microcentrifuge at 14,000g for 10 minutes at 4°C. 10m

42 Label a new 1.5mL Lobind eppendorf 'CITEseq master mix'
Add 25uL from each of the reconstituted cocktail to the CITEseq master mix eppendorf
Add 16uL of CCR7 totalseq antibody to the CITEseq master mix eppendorf
Add 1359uL of CSB to the CITEseq master mix eppendorf 3m

43 Pipette mix well x15. 1m

CITEseq staining samples 48m

44 After FACS antibody staining incubation add 1mL of cell staining buffer to each sample. 1m

45 Spin down samples for 5 minutes, 4°C at 400g. 5m

46 For dead control remove supernatant and resuspend in 1mL cell staining buffer.

47 Repeat washing for these samples for a total of 3 times. 10m

48 For the other samples remove supernatant and resuspend in 300uL of CITEseq cocktail. 1m

49 Pipette mix samples well x10 times. 1m

50 Incubate on ice for 30 minutes at 4°C in the dark. 30m

Washing samples after CITEseq staining

24m

51 After incubation add 1mL of CSB and mix. 1m

52 Centrifuge samples for 5 minutes at 400g at 4°C. 5m

53 Remove supernatant from FACS tubes and resuspend pellet in 1mL of CSB. 1m

54 Repeat washing steps for a total of 3 times. 15m

55 On final resuspension, resuspend in 1mL of cell staining buffer. 1m

56 Label 6 FACS tubes, 'CD4T', 'CD8T', 'NK', 'B', 'Myeloid', 'Rare' and add 1mL of 100% FBS into each one. 1m

57 Take all samples to FACS on ice for sorting.

Sorting Samples

2h

58 Filter samples with 20uM filter before sorting.

59 Sort targeted immune cell types for each donor into buckets and multiplex donors during sort (shown in table below). 2h

	Bucket	Cell type	Targeted events per cell type	Number of donors to sort	Targeted number of events per bucket
	CD4	CD4T	55	5	275
	CD8	CD8T	55	5	275
	B	B	55	5	275
	NK	NK	40	5	200
	Myeloid	Monocyte	50	5	275
	Myeloid	DC	5	5	
	Rare	Treg	10	5	250

	Bucket	Cell type	Targeted events per cell type	Number of donors to sort	Targeted number of events per bucket
	Rare	NKT	10	5	
	Rare	gdT	10	5	
	Rare	CD4-CD8-/CD4+CD8+	10	5	
	Rare	ILC/Plasmablast	10	5	

60 Gating strategy for enriching common and rare immune cell type:

Gating Strategy.pdf 466.7KB

Prepare samples for 10X

40m

61 Centrifuge cells after sorting for 5 minutes at 4°C at 400g. 5m

62 Remove supernatant and add 50uL of CSB, resuspend pellet. 2m

63 Take 15uL of sample and stain with live/dead dye and count each bucket 10m

64 Combine CD4T, CD8T and NK buckets into one 5mL FACS tube at equal proportions. 2m

65 Combine B, Myeloid and Rare buckets into one 5mL FACS tube at equal proportions. 2m

66 Centrifuge pools for 5 minutes at 4°C at 400g. 5m

67 Afters spin, remove supernatant and resuspend in the residual volume 1m

68 Transfer samples to a PCR strip tube and top up volume with CSB to 40uL if necessary. 3m

69 Take 5uL of each pool and dilute 1:5, stain with live/dead dye and count 10m

Performing GEM generation

33m

70 Take 90,000 cells per sample and dilute with CSB for a total volume of 37.5uL

3m

71 Proceed to follow 10X GEM X protocol for GEM generation.

30m