CIMAC CyTOF Harmonized SOP/Approach

Introduction

This protocol is a version of the harmonized CIMAC CyTOF cell surface staining protocol adapted specifically for the initial multi-site comparison pilot

Application

Staining cryopreserved PBMC and lyophilized reference samples and using lyophilized cell surface panel in all centers and comparing the data for cross-site validation. Note that both channels Ta and Nb have to be on for the acquisition.

Reagents

1. Thawing Media: RPMI 1640 (+L-Glutamine) + 10% FBS, stored at 4°C
2. Benzonase (Pierce™ Universal Nuclease for Cell Lysis), ThermoFisher Cat#88701; Keep at -20°C
3. Rh103 Intercalator, 500 µM (Fluidigm #201103A)
4. CSM: PBS+0.2%BSA+0.02%NaN3
5. Cell-ID™ Intercalator-Ir, 500 µM, Fluidigm Cat# 201192B, Aliquots kept at -20°C.
6. Pierce™ 16% Formaldehyde (w/v), Methanol-free; Catalogue number 28906
7. 10 X CyPBS (Rockland, catalog number: MB-008)
8. 10X PBS (Gibco, catalog number: 70011-044)
9. BSA (Sigma, A1933)
10. MilliQ water
11. EQ four element calibration beads, Fluidigm Cat#201078, lot#P17A0502
12. Human TruStainFcX™ (FcReceptor Blocking solution); Biolegend
13. Lyophilized Heavy Metal Vericells (Biolegend)
14. Vericell reconstitution buffer (Biolegend)
15. MaxPar CAS (Fluidigm Cat. No. 201237) for WB **or MaxPar Water for NB users**
16. Tuning solution (Fluidigm Cat. No 201072)
17. 5mL tube with cell strainer cap (Corning 352235)

1. Healthy Donor and Unstim/Stim PBMC thawing:

a. Keep the frozen PBMC vial on dry-ice until time to thaw; Prepare 10 ml of RPMI-1640 media with 1:10,000 benzonase, per vial to be thawed. Warm this media to 37°C in a water bath.
b. Thaw the vial at 37°C until 2/3 of the PBMC are thawed and transfer slowly to 10 ml RPMI with benzonase.
c. Centrifuge @ 300 g for 5 min.
d. Remove supernatant and gently resuspend the cell pellet in 5ml of RPMI+10%FBS.
e. Count cells and record post thaw count and viability.
f. Centrifuge cells again @ 300g for 5mins
g. Resuspend cells in 1ml of RPMI+10%FBS and prepare aliquots of 2 million cells from each sample for staining and keep on ice.
For the Unstim/Stim samples, combine 1:1 for a total of 2 million cells.

2. Viability staining:

a. Prepare 1x Rh103 staining media by diluting the stock to 1 μM staining solution in pre-warmed cell culture medium (RPMI+10% FBS)
b. Add 1ml of 1x Rh103 staining medium to the cells and mix well.
c. Incubate at 37°C for 20 min.
d. Spin down cells @ 300g for 5mins and aspirate supernatant (confirm pellet before aspirating the supernatant)
e. Wash the cells by adding 1ml CSM (PBS + 0.2% BSA + 0.02% NaN3), spin @ 300g for 5 min

3. Reconstitution of lyophilized Vericells

a. Retrieve reconstitution buffer and Vericells and warm to room temp for 5mins
b. Add 1.3 mL of reconstitution buffer to glass vial and incubate at RT for 10mins
c. Centrifuge Vericells @ 1500g for 1 min.
d. Remove supernatant and gently resuspend the cell pellet in 1 ml of CSM and centrifuge @ 1500g for 1 min and aspirate
e. Resuspend cells in 1ml of CSM and count cells (count should be ~6 million cells)
f. Spike in 200,000 reconstituted vericells into each sample of 2 million PBMCs

4. Reconstitution of 2x antibody cocktail from lyo panel in CSM

a. Reconstitute the lyo panel in a volume of 50 μl CSM (per one sample) and incubate for 5mins at RT (avoid touching the lyosphere with the pipette tip)
b. Transfer antibody cocktail to 0.1μm PVDF filter tubes (Millipore UFC30VV00)
c. Pipette 1μl of each additional liquid antibody (per sample) to the antibody cocktail (CD45-89Y, CD45RA-143Nd, PDL1-156Gd, CD69-164Dy) and 2 μl of PD1-153Eu.
d. Centrifuge antibody cocktail in filter tube @ 12,000g for 1 min

5. Cell Surface staining:

a. Prepare 2X TruStain Fcblock (FcX) – 1μl of FcX+50μl CSM per sample
b. Spin down aliquoted cells with the spiked in reconstituted Vericells @ 300g for 5mins and aspirate supernatant
c. Resuspend cells in 50 μl of CSM+FcX
d. Add 50 μl of filtered antibody cocktail to the cells and mix well
e. Incubate for 30 min on ice
f. Wash the cells by addition of 1 ml CSM and spin down @ 300g for 5 min.

6. Fixation and iridium staining

a. Resuspend cells in 200 μl of 1X PBS
b. Ensure 16% Formaldehyde is opened fresh that day and filtered using a PVDF syringe filter (Millipore Millex, cat# SLVV033RS).
c. Prepare 14.4% isotonic formaldehyde by mixing 1800 μl of 16% formaldehyde with 200ul of 10x PBS.
d. Prepare **Fix Buffer** by mixing:
    i. 2428 μl of PBS
    ii. 320 μl of 2% saponin in PBS solution
    iii. 1332 μl of 14.4% isotonic formaldehyde

e. Prepare a **2X Fix-Ir solution** (200 µl per sample) by:
    i. diluting 2 µl of stock 500 µM Intercalator-Ir in 1 mL Fix Buffer for a 1 µM final conc.
    ii. followed by diluting the 1 µM solution 1:16 (100 µl + 1500 µl) for a final concentration of 0.06 µM.
f. Add 200 µl of 2X Fix-Ir solution to cells in PBS and mix well. The final concentration of Ir is 0.03 µM.
g. Incubate for 30 min at RT
h. Wash once in 1ml CSM and spin down at 1500g for 1 min
i. Store in 200 µl of CSM containing 0.03 µM Intercalator-Ir.
   Dilute stock 500 µM to 1 µM (1 µl + 499 µl)
   Then dilute the 1 µM to 0.03 µM (50 µl + 1600 µl)
j. Store stained cells up to one week @ 4°C

7. Wash

a. Centrifuge in a microcentrifuge at 1500g for 1 min, then rotate the tube 180º and centrifuge at 1500g for an addition 15 seconds so that the pellet slides off the wall and moves to bottom of the tube
b. Carefully aspirate and discard supernatant
c. Gently vortex to disrupt pellet
d. Resuspend cells in 1mL of **MaxPar Water (NB) or MaxPar CAS (WB)**
e. Wash cells **2X in MaxPar Water (NB) or MaxPar CAS (WB)**
    i. Centrifuge in a microcentrifuge at 1500g for 1 min, then rotate the tube 180º and centrifuge at 1500g for an addition 15 seconds so that the pellet slides off the wall and moves to bottom of the tube
    ii. Carefully aspirate and discard supernatant
    iii. Gently vortex to disrupt pellet and store on ice in residual volume until immediately prior to acquisition
    iv. Resuspend cells in 300 µl of **MaxPar Water (NB) or MaxPar CAS (WB)** and filter sample through a cell strainer cap into a FACS tube and count cells.

8. Add a 1/20 dilution of EQ beads.
9. Use narrow bore or wide bore injector.
10. Acquire data using CIMAC_190423.tem acquisition template and name the sample with the following filename format “HD180926_A_MDACC_NB” etc or “NDSTIM-NDUN_MDACC_NB” for the mixed stim/unstim sample.
11. Aim for a target acquisition of a minimum of 300,000 events

Appendix 1

Lyophilized core panel from Mt.Sinai used for cross-site assay harmonization

Isotope	Target	Clone
89 Y		
113 In		
115 In		
139La		
141 Pr		
142 Nd	CD19	HIB19
143 Nd	CD45RA	HI100
144 Nd		
145 Nd	CD4	RPA-T4
146 Nd	CD8a	RPA-T8
147 Sm		
148 Nd	CD16	3G8
149 Sm		
150 Nd	CD1c	L161
151 Eu	CD123	6H6
152 Sm	CD66b	G10F5
153 Eu		
154 Sm		
155 Gd	CD27	O323
156 Gd		
158 Gd		
159 Tb		
160 Gd	CD14	M5E2
161 Dy	CD56	B159
162 Dy		
163 Dy		
164 Dy		
165 Ho		
166 Er		
167 Er		
168 Er	CD3	UCHT1
169 Tm		
170 Er	CD38	HB-7
171 Yb		
172 Yb		
173 Yb		
174 Yb	HLA-DR	L243
175 Lu		
176 Yb		
209 Bi		

Appendix 2

Table for acceptable CVs for various cell types based on ranges obtained from healthy control samples stained at each site and acquired:

	B cells	CD8 T cells	CD4 T cells	CD14 monocytes	CD16 monocytes	NK cells	Basophils	pDCs	CD11c DCs
Range for % of total event	5.3-10.4	18.7-34.4	20.6-34.1	7.9-18.8	0.7-2.7	6.6-16.7	0.33-1.6	0.13-0.29	0.33-1.7
CV	7.3	4.8	3.2	9.4	11.8	9.04	31.5	12.2	12.5