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Immune profiling using 16-color panel for PD patient PBMCs

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We use this protocol and it's working

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Abstract

This is the protocol for immune profiling using 16-color panel for PD patient PBMCs.

Materials

Materials:

1. 1x PBS
2. Flow cytometry staining buffer (Stain Buffer) – 1x PBS + 2% FBS, filtered with 0.2um filter
3. Brilliant buffer plus
4. Staining Kit: eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (00-5523-00)
5. PBMC isolated from buffy coat for no stain control and Ghost dye only control
6. 15ml tubes, frozen vials, V-bottom 96-well plates, FACS tubes with cell strainer snap cap

Samples: PD patient PBMCs; 1M cells per sample

Troubleshooting

Master tubes summary:

- 1
 1. Ghost dye stain: Day 1
 2. Fc blocker: Day 1
 3. Surface marker: Day 1
 4. Intracellular markers: Day 1
 5. Foxp3 fix/perm buffer: Day 2
 6. FoxP3 wash buffer: Day 1

Day 1 (the day before staining) for 16 colors staining:

- 2 Compo-Beads staining:
 - 2.1 1. Label 1.5ml tubes with Compo-beads control IDs
 - 2.2 2. Add 1 drop of Compo-bead into corresponding tube (1 drop for each tube, totally 15 drops)
 - 2.3 3. Add antibody into corresponding tubes
 - 2.4 4. Vortex well, and incubate at 4°C for 30 min, avoid light
 - 2.5 5. Wash with 1 mL Stain Buffer
 - 2.6 6. Spin at 1500 rpm for 5 min
 - 2.7 7. Dump the supernatant, and directly tap on kim-wiper once gently
 - 2.8 8. Resuspend into 500 µl Stain Buffer
 - 2.9 9. Wrap all the tubes with plastic wrap to prevent evaporation, and cover with foil



2.10 10. Store at 4°C

3 Ghost dye bead staining

3.1 1. Add 2x drops of ArC positive beads (green top) into the FACS tube, labeled as **GB-16 for Ghost dye bead staining**

3.2 2. Add 0.5 µl of Ghost Violet 510 into the tube

3.3 3. Vortex well, and incubate at 4°C for 30 min

3.4 4. Wash with 1 mL Stain Buffer

3.5 5. Spin at 1500 rpm for 5 min

3.6 6. Dump the supernatant, and directly tap on kim-wiper once gently

3.7 7. Resuspend into 500 µl Stain Buffer

3.8 8. Wrap all the tubes with plastic wrap to prevent evaporation, and cover with foil

3.9 9. Store at 4°C
- *Note: Add 2x drops of ArC negative beads (white top) into the tube right before running FACS.*

4 Prepare the surface antibody cocktails using brilliant buffer

4.1

1. Prepare full cocktails: Mix antibodies together by the volume calculated.



	A	B	C	D	E	F
	Sur face	Marke rs	Volum e (μ l)	*N	Brilliant buffer (μ l)	Aliquot
	Y	CD19	5		10	Total XXX μ l; 64ul for each sample
	Y	CD3	5			
	Y	CD11c	5			
	Y	CXCR 5(CD1 85)	5			
	Y	CD103	5			
	Y	PD-1 (CD27 9)	5			
	Y	CD45	5			
	Y	CD4	5			
	Y	CD8	5			
	Y	CD11b	1			
	Y	CD56	5			
	Y	CD25	3			
	Tot al	54		10*N=		

4.2 2. Vortex well, and store at 4°C

5 Prepare the intracellular staining cocktails using Foxp3 wash buffer



5.1 Full Intracellular panel for 82 samples

IC Antibody	Volume/ Sample (μl)	* N	Foxp3 wash buffer
FoxP3 eFluor 450	5		35*N μl
Ki67 APC	5		
CD107a PE	5		
Total	XXX μl; 50ul / sample		

Vortex well, and store at 4°C

6 Prepare the Fc blocker: 1:100 dilution with stain buffer

6.1

Antibody	Volume/ Sample (μl)	*N	For each sample
CD16/CD32	1		100 μl
Stain buffer	99		

Vortex well and store at 4°C

7 Ghost dye master tube: 0.5ul Ghost dye + 100ul PBS for each sample

7.1 1. Remove Ghost Dye vial from freezer and allow to equilibrate to room temperature.

7.2 2. Quick spin Ghost Dye vial before opening.

7.3 3. Make Ghost Dye staining solution, and vortex well



- Note: Ghost Dyes are formulated in DMSO, pipet carefully and slowly.

		Volume/ Sample (μl)	*N	For each sample
	Ghost dye Volume/ Sample (μl)	0.5		100 μl
	PBS	100		

- 8 FoxP3 Fix/Perm buffer: 1:4 dilution; 100ul for each sample; prepare it on day 2
- 9 FoxP3 wash buffer: 1:10 dilution by water
- 10 Prepare 15ml columns, FACS tubes with cell strainer snap cap and 78 frozen vials; label them accordingly.

Day 2

- 11 Control cells:
 - 11.1 1. Add 9ml RPMI medium into each 15mL tube.
 - 11.2 2. Thaw one vial of PBMCs from -80°C at 37°C until it has a small piece of ice at a time. Add PBMCs into the 15mL tube.
 - 11.3 3. Count cell numbers, and record the volume, viability and concentration.
 - 11.4 4. Spin down PBMCs for 5min at 1500rpm, resuspend in RPMI medium to get the final concentration of **10×10⁶/ml**.
 - 11.5 5. Add 100ul PBMCs into the V-bottom plates; Will need two wells, one is for **no stain control (A)**, and one for **Ghost dye only control (B)**



12 PBMC Samples:

- 12.1 1. Take all the PBMCs from LN2 to -80°C.
- 12.2 2. Add 9ml RPMI medium into each 15mL tube.
- 12.3 3. Thaw maximum 5 vials of PBMCs from -80°C at 37°C until it has a small piece of ice at a time. Add PBMCs into the 15mL tube.
- 12.4 4. Count cell numbers, and record the volume, viability and concentration.
- 12.5 5. Spin down PBMCs for 5min at 1500rpm, resuspend in RPMI medium to get the final concentration of $10 \times 10^6/\text{ml}$.
- 12.6 6. Add 100ul PBMCs into the V-bottom plates; freeze the rest cells back using Mr. Frosty™ Freezing Container for future use.
- 12.7 7. Spin down PBMCs in V-bottom 96-well plate (26 samples for each group), discard the supernatant, add 200ul Stain buffer into the each well to wash the cells.
- 12.8 8. Spin down PBMCs for 5min at 1500rpm, discard the supernatant.

13 Ghost dye staining: Include Ghost dye only control

- 13.1 9. Add 100 μl of the Ghost Dye staining solution into cells (**Include Ghost dye only control**).
- 14 10. Incubate cells for 30 minutes at 4°C protected from light.
- 14.1 11. Wash cells with 100ul Stain buffer.
- 14.2 12. Spin down at 1500 rpm for 5 min



- 14.3 13. Aspirate the supernatant.
- 14.4 The samples are ready for Fc blocking except **Ghost dye only control**. For **Ghost dye only control**, store cells at 4°C for future permeabilization.
- 15 FC Blocking: For PD patient PBMC samples
 - 15.1 14. Add 100uL of FC blocker into cells
 - 15.2 15. Incubate cells at RT for 10 min
 - 15.3 16. Wash the samples with 100ul Stain buffer
 - 15.4 17. Spin down at 1500 rpm for 5 min (with Eppendorf tube centrifuge)
 - 15.5 18. Aspirate the supernatant (the pellets are ready for surface staining)
- 16 Surface staining: For PD patient PBMC samples
 - 16.1 19. Add 64 µl of surface staining cocktail from yesterday into samples, and mix them well
 - 16.2 20. Incubate at 4°C for 30 min, protected from the light
 - 16.3 21. Wash with 100ul Stain Buffer, spin down at 1500 rpm for 5 min



- 16.4 22. Aspirate the supernatant (the pellets are ready for the cell permeabilization)
- 17 Cell Permeabilization: Include no stain control and Ghost dye only control
- 17.1 23. Prepare Foxp3 perm buffer (1:4 dilution)
- 17.2 24. Add 100uL of Foxp3 perm buffer into each tube, and mix them well
- 17.3 25. Incubate cells at RT for 60 min
- 17.4 26. Wash with 100ul Foxp3 wash buffer
- 17.5 27. Spin down at 1500 rpm for 5 min
- 17.6 28. Aspirate the supernatant (the pellets are ready for the intracellular staining).
- 18 Intracellular staining:
- 18.1 29. Add 50 µl of the intracellular antibody cocktails from yesterday, and mix them well
- 18.2 30. Incubate at 4 °C for 30 min
- 18.3 31. Wash with 100ul Foxp3 wash Buffer
- 18.4 32. Spin down at 1500 rpm for 5 min
- 18.5 33. Aspirate the supernatant



18.6 34. Resuspend the pellets into 200ul stain Buffer

18.7 35. Vortex, and store at 4°C

Analyze the samples using The BD LSRFortessa™ Cell Analyzer.

19 Gating strategy

T cell: CD45+CD3+CD19-/CD45+

CD4 T cell: CD45+CD3+CD19-CD4+ CD8-/ T cell

Treg cell: CD45+CD3+CD19-CD4+ CD8- CD25+ Foxp3+/CD4 T

P-CD4 cell: CD45+CD3+CD19-CD4+ CD8-Ki67+ /CD4 T

CD8 T cell: CD45+CD3+CD19-CD4- CD8+/T cell

P-CD8 T cell: CD45+CD3+CD19-CD4- CD8+Ki67+/CD8 T cell

CD8-CD103 cell: CD45+CD3+CD19-CD4- CD8+CD103+/CD8 T cell

CD8-CD107 cell: CD45+CD3+CD19-CD4- CD8+CD107+ (?) /CD8 T cell

CD8-PD1 cell: CD45+CD3+CD19-CD4- CD8+PD-1+ (?) /CD8 T cell

B cell: CD45+CD3-CD19+/CD45

P-B cell: CD45+CD3-CD19+Ki67+/B cell

NK cell: CD45+CD3-CD19-CD56+CD11C-/non T and non B

P-NK cell: CD45+CD3-CD19-CD56+CD11C-Ki67+/NK

DC cell: CD45+CD3-CD19-CD56-CD11C+/non T and non B

P-DC cell: CD45+CD3-CD19-CD56-CD11C+/NK

Monocyte: CD45+CD3-CD19-CD11b+CD11C-/non T, B, DC, NK

ASAP 77 PBMCs – Columbia cohort:

1. #UPL222C is missing (Well D4, no tube received from Columbia)

2. Wells A5, B5, C5, D5, E5, F5, G5, H5 have poor results (removed Treg, CD103, CD107, Ki67,

Monocytes)