PBMC Immunophenotyping (Panels 1-5)

PART 2: Staining Protocol (Panel 5)

REAGENTS AND BUFFER

1. 1x DPBS (Invitrogen, Catalogue number 14190)
2. FACS buffer (1X PBS + 2% FCS + 2mM EDTA)
3. BD Cell fix (BD Biosciences, Catalogue number 340181)
4. BD FACS™ Lysing Solution 10X Concentrate (BD Biosciences, Catalogue 349202)
5. BD Brilliant violet stain buffer (BD Biosciences, 566385)
6. FoxP3 Fixation/Permeabilisation buffer (Invitrogen, Catalogue number 00-5523-00)
7. FoxP3 Permeabilisation wash buffer 10X (Invitrogen, Catalogue number 00-5523-00) diluted 1:10 in dH_20 prior to use
8. Live dead Dye (BD Biosciences, Catalogue number 565388)
9. HoechstDye 33342 (Invitrogen, Catalogue number H3570)

MATERIALS

1. P10, P20, P200 and P1000 filter tips
2. Beaker for waste

EQUIPMENT

1. BSL3 facility
2. BSC2 safety cabinet
3. Centrifuge
4. P10, P20, P200 and P1000 pipettes
5. Multichannel pipette
6. Dispensing troughs for multichannel pipetting
7. BD LSR Fortessa X20

Inside BSL3

Samples are in 96 well V-bottom plates as detailed in '*Sample preparation protocol*'.

Antibody panels can be found in the '*Set up and panels*' document.

1. Centrifuge cells for 5 minutes at 400g at RT.
2. Prepare Live dead (L/D) dye to working concentration (1:4000 dilution of stock prepared according to manufacturer's instructions in 1x PBS)
3. Remove supernatant, and gently resuspend the cell pellets in 100 µl L/D working solution by pipetting up and down. Incubate for 20 minutes at RT, protected from light.
4. After the L/D incubation step is over, centrifuge cells for 5 minutes at 400g at RT.
5. Remove supernatant from cells, and gently resuspend the cell pellet in 200µl of FACS buffer by pipetting up and down.
6. Centrifuge cells for 5 minutes at 400g at RT.
7. Remove supernatant from cells and gently resuspend the cell pellet directly into 100 µl of the master mix made up in BD Brilliant Violet Stain Buffer and incubate for 30 minutes at RT

8. Add 100 µl of FACS buffer to wells and centrifuge cells for 5 minutes at 400g at RT.
9. Remove supernatant from cells, and gently resuspend the cell pellet in 200µl of FACS buffer by pipetting up and down.
10. Centrifuge cells for 5 minutes at 400g at RT.
11. Remove supernatant from cells, and gently resuspend the cell pellet in 200µl of Foxp3 Fixation/Permeabilization Buffer. This buffer is prepared by diluting 1:4 Foxp3 Fixation/Permeabilization Concentrate in Foxp3 Fixation/Permeabilization Diluent
12. Incubate overnight (not more than 18 hours) at 4°C in the dark.

Outside BSL3

The next day, prepare materials and reagents before removal from fridge

- Reservoirs x 2 (3 if RBC lysis needed) for PBS and perm buffer
- Antibody stain – volume dictated by the previous day's sample numbers. 1/50 FoxP3-AF647, 1/50 Ki67-AF700
- Hoechst working concentration = 2µl of Hoechst in 5ml of PBS (Hoechst kept in -80)
- Perm buffer. 1ml of concentrate in 9 ml of dH20 (MilliQ)
- PBS 1x

13. **If samples appear red**: Lyse with 1:10 dilution of RBC Lysis solution in dH_2O (200µl per well for 15 min at RT).
14. Centrifuge cells for 1 minute at 2000rpm at RT. Remove supernatant from cells by flicking out in the fume hood and wash the cells with 200µl of D-PBS. Repeat once more.
15. Spin cells down as before and gently resuspend the cell pellet in 200µl of Foxp3 Permeabilization wash Buffer 1X to wash.
16. Centrifuge again for 1 minute at 2000rpm at RT. Remove supernatant from cells, and gently resuspend the cell pellet in 50µl of Foxp3 Permeabilization Wash Buffer 1X.
17. Add 50µl of Ki67 and FOXP3 antibody diluted in 1x Foxp3 Permeabilization Wash Buffer.
18. Incubate 30 minutes at 4C in the dark.
19. Centrifuge cells for 1 minute at 2000rpm at RT. Remove supernatant from cells, and gently resuspend the cell pellet in 200µl of 1x Foxp3 Permeabilization wash Buffer to wash
20. Centrifuge again for 1 minute at 2000rpm. Remove supernatant from cells, and gently resuspend the cell pellet in 125 µl PBS.
21. Add 125 µl of Hoechst working stock.
22. To prepare this, add 2 µl of Hoechst stock (kept at -80C) in 5ml of PBS.
23. Incubate for 15 minutes at RT in the dark
24. Centrifuge for 1 minute at 2000rpm at RT. Remove supernatant from cells, and gently resuspend the cell pellet in 200 µl PBS to wash.

25. Centrifuge for 1 minute at 2000rpm at RT. Remove supernatant from cells and resuspend cells in 300µl of PBS for acquisition. Transfer to FACS tubes.
26. Acquire on BD LSR Fortessa X20.