Ex vivo cell staining

I – Principle

This SOP provides guide for *ex vivo* fluorescent cell staining of cryopreserved human PBMC/Spleen/Tonsil cells. The cell staining involves staining process for cell surface and intracellular markers labelled with fluorochrome-conjugated antibodies. Cell markers can be used to identify cell subsets based on lineage, differentiation state, and function.

For intracellular staining, cells are normally fixed with fixative agents to stabilize cellular structure, then permeabilised with detergents or organic solvents to allow access for staining antibodies to bind intracellular or intraorganellar marker antigens.

II – Safety overview

- All blood samples should be considered an infection risk and all work be performed in Biosafety Cabinet Class II (BSC-II).
- Standard personal protective equipment (PPE) must be worn when performing laboratory work; this includes long-sleeve lab gown, enclosed footwear, safety glasses and gloves.

III – Required reagents and consumables

MSDS

Chemical Name	CAS. No.
Sodium chloride (NaCl)	7647-14-5

Equipment

- F2 Finnipipette P2, P20, P200, and P1000
- Multichannel pipette 30-300uL
- Eppendorf Centrifuge 5810 R
- Eppendorf Centrifuge 5430
- Class 2 Biosafety Cabinet
- -80 °C Freezer

Reagents/Consumables

- Interpath Aerosol Barrier Tips 10 XL For use with Gilson P10 pipette Cat #24300
- Interpath Aerosol Barrier Tips 20 µL For use with F2 Finnpipette P20 Cat #24500
- Interpath Aerosol Barrier tips 200 µL For use with F2 Finnpipette P200 Cat #24700
- Interpath Aerosol Barrier Tips 1000 µL For use with F2 Finnpipette P1000 Cat #24800
- Sigma-Aldrich Multi-well plate washer/dispenser manifolds 12 position, straight Cat #M2781

- Corning 96-well Clear V-bottom TC-treated Microplate, Individually Wrapped, with Lid, Sterile Cat #3894
- Falcon 50 mL polypropylene tube Cat #227261
- Sarstedt 10 mL conical centrifuge tube Cat #62.9924284
- Copan Interpath sterile 1 mL plastic transfer pipette Cat #201CS01
- Sarstedt 5 mL serological pipette Cat #86.1253.001
- Sarstedt 10 mL serological pipette Cat #86.1253.001
- Costar 25 mL serological pipette Cat #4489
- Falcon 5mL Polystyrene Round-Bottom FACS Tube Cat #352008
- EBioscience Fixation/Permeabilization concentrate Cat #00-5123
- EBioscience Fixation/Permeabilization concentrate Cat #00-5223
- EBioscience Fixation/Permeabilization Buffer (10X) Cat #00-8333
- 1X Filtered Phosphate Buffer Saline (PBS)
- Gibco sterile fetal bovine serum (FBS) Heat-inactivated (HI; 56°C, 45 min) Cat #16000044
- Gibco Trypan blue stain (0.4%) Cat #15250-061
- Sodium Chloride Cat #S5886-10kg

IV - SOP

PBMC Thawing

PROCESS	RECOMMENDED TIME/VOLUME/TEMPERATURE/SPEED/ETC
Thaw PBMC/Spleen/Tonsil cells	• Thaw PBMC/Spleen/Tonsil cells according to the "PBMC Thaw" SOP.
Aliquot PBMC for staining	• After calculating total PBMC/Spleen/Tonsil cells, aliquot $1x10^6$ PBMC/Spleen/Tonsil cells per participant, per timepoint, into a 96-well Clear V-Bottom plate. • Record on plate template.

PBMC Flow Cytometry Staining

PROCESS	RECOMMENDED TIME/VOLUME/TEMPERATURE/SPEED/ETC
Prepare Media Risk 3	• Prepare 2% FBS/PBS solution. Place in fridge. • Prepare a 1X Fixation/Permeabilisation solution by mixing 1 part the 4X Fixation/ Permeabilisation concentrate with 3 parts Fixation/ Permeabilisation diluent. Place in fridge.

	• Prepare a 1X Permeabilisation Buffer, by mixing 1 part 10X concentrate with 9 parts MilliQ H_2O. Place in fridge. • Prepare a 1X solution of Stabilising Fixative by mixing 1 part 3X BD Stabilising Fixative with 2 parts distilled water.
Prepare cell stain master mixes	• Prepare viability, surface stain, and intracellular stain according to experiment-specific flow cytometry panel.
Perform viability staining Risks 1 & 2	• Top wells up to 200 uL/well with PBS. Resuspend 6X by pipetting • Centrifuge plate at 1500rpm for 5 mins. Aspirate supernatant. • Add 200 uL/well of PBS. Resuspend 6X by pipetting. • Centrifuge plate at 1500rpm for 5 mins. Aspirate supernatant. • Add 50 uL/well of Viability stain. Resuspend 6X by pipetting. • Incubate plate in the dark at room temperature for 15 mins. • Add 150 uL/well of 2% FCS/PBS. Resuspend 6X by pipetting. • Centrifuge plate at 1500rpm for 5 mins. Aspirate supernatant.
Perform surface staining Risks 1 & 2	• Add 50 uL/well of Surface stain. Resuspend 6X by pipetting. • Incubate plate in the dark at room temperature for 30 mins. • Add 125 uL/well of 2% FCS/PBS. Resuspend 6X by pipetting. • Centrifuge plate at 1500rpm for 5 mins. Aspirate supernatant. • Add 200 uL/well of 2% FCS/PBS. Resuspend 6X by pipetting. • Centrifuge plate at 1500rpm for 5 mins. Aspirate supernatant.
Perform fixation and permeabilisation Risks 1, 2 & 3	• Add 100 uL/well of 1X Fixation/Permeabilisation solution. Resuspend 6X by pipetting. • Incubate plate in the dark, on ice for 20 mins. • *From this step onwards, set centrifuge at 4 ^oC.* • Add 100 uL/well of 1X Permeabilisation buffer. Resuspend 6X by pipetting. • Centrifuge plate at 1500rpm for 5 mins. Aspirate supernatant. • Add 200 uL/well of 1X Permeabilisation buffer. Resuspend 6X by pipetting. • Centrifuge plate at 1500rpm for 5 mins. Aspirate supernatant.

Perform intracellular staining	• Add 50 uL/well of Intracellular stain. Resuspend 6X by pipetting. • Incubate plate in the dark, on ice for 30 mins. • Add 150 uL/well of 1X Permeabilisation buffer. Resuspend 6X by pipetting. • Centrifuge plate at 1500rpm for 5 mins. Aspirate supernatant. • Add 200 uL/well of 1X Permeabilisation buffer. Resuspend 6X by pipetting. • Centrifuge plate at 1500rpm for 5 mins. Aspirate supernatant.
Fix cells with stabilising fixative	• Add 200 uL/well of 1X Stabilising fixative. Resuspend 6X by pipetting. • Incubate plate in the dark, at room temperature for 20 mins. • Centrifuge plate at 1500rpm for 5 mins. Aspirate supernatant. • Add 200 uL/well of 2% FCS/PBS. Resuspend 6X by pipetting. • Cover plate in foil and store at 4 ^{o}C until acquisition on flow cytometer. • Transfer samples to FACS tubes before acquisition