1. PRINCIPLE

Carboxyfluorescein succinimidyl ester (CFSE) is a dye that binds to intracellular structures and is divided equally among the daughter cells if proliferation takes place. Thus, measuring the fluorescence intensity of CFSE in the cells will reveal how many cells have undergone how many rounds of cellular division as each division should halve the fluorescence intensity of the parent cell.

2. SPECIMEN REQUIREMENT

2.1. Primary Specimen: EDTA anti-coagulated peripheral blood. Blood samples should be received in the lab within 24 hours of draw.

2.2. Cells: PBMCs isolated by HANC PBMC processing SOP. Not validated on frozen/thawed PBMCs.

3. REAGENTS:

3.1. CFSE Stock Solution (Life Technologies Cat # C1157)

3.1.1. 25mg dissolved in 8.98 mL of DMSO (.01mM concentration) and divided into 15µL aliquots. Stored at -20°C.

3.2. Dulbecco's phosphate buffered saline (DPBS, e.g. Gibco Cat # 14190-144). Store at room temperature.

3.3. RPMI 1640 (e.g. Gibco Cat # 21870-076). Store at 4°C.

3.4. Heat Inactivated Fetal Bovine Serum (Gemini Bio-Products Cat # 100-500). Store at -20°C.

3.4.1. Needed for complete medium, but should also freeze some in 2.5mL aliquots for quenching of CFSE (stored at -20°C).

3.5. Penicillin/Streptomycin (Life Technologies Cat # 15140-122). Store at -20°C.

3.6. L-Glutamine (200mM) (Life Technologies Cat # 25030-024). Store at -20°C.

3.7. 2x Complete Medium

3.7.1. RPMI 1640 with 20% FBS containing 400 U/mL penicillin and 400 µg/mL streptomycin and 4mM L-glutamine.

3.8. 0.2% BSA in D-PBS

3.8.1. 200mg BSA (Bovine Serum Albumin, Sigma Cat # A7906) dissolved in 100mL Dulbecco's PBS (DPBS) and sterile filtered. Stored at 4°C (stable for 3 months).

3.9. Frozen stock of 2x stimulants in 2x Complete Medium

3.9.1. Stimulants will be defined by the protocol

3.9.2. Prepare batch of 2x stimulants to use over the course of the protocol and store at -80°C

3.10. Bleach

3.11. Staining buffer

3.11.1. DPBS containing 0.1% NaN3 + 1% BSA. Stored at 4°C for up to three months.

3.12. Fixation Buffer after antibody staining:

3.12.1. DPBS containing 1% paraformaldehyde (PFS, Sigma Cat # P-6148). Stored at 4°C (stable for 6 months).

3.13. Live/Dead Cell Viability Stain

3.13.1. Invitrogen Live/Dead stains (panel will be determined by the protocol)

3.14. Conjugated Monoclonal Antibodies

3.14.1. Moconclonal antibody panel will be determined by the protocol. Store reagents at 4°C. Avoid exposure to light.

4. MATERIALS

4.1. Class 2 Biosafety cabinet

4.2. Centrifuge

4.3. Refrigerator 4°C

4.4. Freezer -20°C

4.5. Freezer -80°C

4.6. Incubator 37°C

4.7. Ice
4.8. Falcon 12mm x 75mm tubes with snap cap (Cat#352063)
4.9. 24-well plate
4.10. Micropipettes capable of dispensing 20µL, 100µL, 1000µL
4.11. Disposable pipette tips
4.12. Vortex
4.13. Interval timers
4.14. Disposable Gloves
4.15. Lab coat
4.16. Safety Glasses
4.17. Flow cytometer capable of at least 7-color detection

5. CFSE STAINING AND CELL CULTURE

5.1. Isolate roughly $4x10^6$ PBMCs for each stimulant/control and place in a 15mL conical tube
5.2. Add enough DPBS to fill to 15mL and spin the isolated PBMCs at 400xg for 10 minutes and discard supernatant into bleach.
5.3. Thaw a 2.5mL aliquot of FBS and place in a bucket of ice.
5.4. Loosen the pellet by finger flicking and re-suspend the cells in 5mL DPBS/0.2% BSA
5.5. Spin at 400g for 10 minutes and discard supernatant.
5.6. Loosen the pellet by finger flicking and re-suspend the cells in 250µL DPBS/0.2% BSA.
5.7. Dilute 4µL of CFSE stock in 1mL DPBS/0.2% BSA (mix well) and IMMEDIATELY (do not mix in advance) add 250µL of this to the PBMCs. Vortex for one second. (Protect CFSE solution and CFSE labeled cells from light).
5.8. Incubate the cells for 10 min at 37°C (with the 15mL cap somewhat loose).
5.9. Add 2.5mL of cold FBS to the CFSE labeled cells and vortex for one second.
5.10. Incubate on ice for 5 minutes.
5.11. Centrifuge cells at 400xg for 10 minutes.
5.12. Remove one each of the frozen 2x stock solutions and let thaw.
5.13. Re-suspend cells in 10mL of RPMI 1640.
5.14. Repeat steps 5.12 and 5.13 for a total of two washes
5.15. Re-suspend cells in 1.5mL of RPMI 1640.
5.16. Add 0.5mL of stock solutions and 0.5mL of cells to wells of a 24-well plate.
5.17. Incubate the plate at 37°C for 7 days.
5.18. After 7 days, harvest cells from 24-well plate and place into labeled 12mm x 75mm flow tubes
5.19. Wash twice with 2 mL of PBS and proceed to Live/Dead staining, followed by surface antibody staining as per your usual flow staining protocol

6. Harvesting Cells and Antibody Staining
6.1. Harvest the cells and place each well into a labeled 12x75mm test tube.
6.2. Wash each well once with 1mL DPBS and add to harvested cells.
6.3. Spin the cells for 3 minutes in the Clay Adams Sero-Fuge.
6.4. Discard supernatant and blot tubes on a paper towel.
6.5. Wash, again, with 2mL of PBS
6.6. Re-suspend in 1mL of DPBS and add 1µL of Live/Dead Violet.
6.7. Incubate in the dark at room temperature for 30 minutes.
6.8. Add 1mL of wash buffer.
6.9. Spin the cells for 3 minutes in the Clay Adams Sero-Fuge.
6.10. Discard supernatant and blot tubes on a paper towel.

6.11. Re-suspend with 2mL wash buffer and repeat steps 4.9 and 4.10
6.12. Create an antibody “cocktail” of:
    6.12.1. 15µL CD3 PerCP
    6.12.2. 15µL CD4 PE-Cy7
    6.12.3. 15µL CD27 APC-H7
    6.12.4. 30µL CD8 PE
    6.12.5. 30µL CD45RA APC
    6.12.6. 210 µL wash buffer
6.13. Add 100µL of antibody/wash buffer and stain for 10 minutes in the dark.
6.14. Repeat step 4.11
6.15. Add 100µL of flow wash buffer and 200µL of 1% PFA to the flow tubes.
6.16. Run tubes on the LSRII flow cytometer.