CFSE Cell Labeling Protocol - Mouse Splenocytes – In Vitro Studies

* Notes:
1. All the procedures must be performed in a tissue culture hood – ALL STERILE
2. See additional protocols for CFSE Cell Culture Set Up for Activation of T cells using plate-bound antibodies and basic immunophenotyping/flow cytometry, prior to beginning

Protocol Overview:
CFSE - Carboxyfluorescin succinimidyl ester is a fluorescent cell dye used to stably label cells, track their migration, and monitor cell division, both in vitro and in vivo. CFSE is covalently linked to intracellular proteins and is progressively halved within daughter cells following each division. Cell division (i.e., proliferation) is monitored by flow cytometry using the FITC channel.

Materials:
1. CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes/Invitrogen #C34554)
   a. CellTrace™ CFSE (Component A), 10 vials, each containing 50 ug of lyophilized powder
   b. DMSO (Component B), 1 vial containing 0.5 mL of high-quality dimethylsulfoxide
   c. *Note: Components should be stored desiccated at ≤-20°C until use.
   d. AVOID REPEATED FREEZING AND THAWING. Allow the products to warm to room temperature before opening the vials. Both DMSO and solid CFSE should be stable for at least 6 months.
   e. A 5 mM CellTrace™ CFSE Stock Solution can be prepared immediately prior to use by dissolving the contents of one vial (Component A) in 18 uL of the DMSO provided (Component B)
2. Freshly isolated splenocytes from BALB/c mice
3. Supplies for tissue harvest:
   a. Sterile Surgical instruments (scissors, forceps, limb holders, dissecting board)
   b. Sterile 60x15mm Tissue Culture Dishes from BD Falcon (Cat# 353002)
   c. Sterile 70μM Nylon Cell Strainers from BD Falcon (Cat# 352350)
   d. Sterile plungers from 1 mL Syringes for teasing the tissues from BD Falcon (Cat# 309602)
   e. Beaker with 95% Ethanol Solution
   f. CO₂ euthanasia chamber
4. Sterile 6-well Tissue Culture Plates from BD Falcon (Cat# 353046)
5. Sterile RPMI 1640 + 5 % FBS (GIBCO Cat# 11875-093; Sigma Cat# F4135)
6. Sterile Complete Mouse Culture Medium:
   a. RPMI 1640 + L Glutamine + 10 % FBS (GIBCO Cat# 11875-093; Sigma Cat# F4135)
   b. 0.5 mL Cell culture 2β Mercaptoethanol from GIBCO (Cat# 21985-023)
      Stock: 1,000X, 55 mM in D-PBS. Store at 4°C
   c. 0.1 mL Gentamicin from GIBCO (Cat# 15750-060)
      Stock: 50 mg/mL. Store at room temp.
   d. Filter Sterilize Medium through 0.2um 500 mL bottle filter
7. 1x ACK (ammonium chloride) RBC lysing buffer at room temperature.
   a. To prepare a 1X working solution (to be used at room temperature), dilute 10X STCOK 1:10 with distilled water. Keep tightly closed.
   b. 10X ACK Solution:
Ammonium Chloride, ACS 82.9 g
Potassium Bicarbonate, USP 10.0 g
Ethylenediamine tetraacetic acid (EDTA) disodium salt 0.37 g
Water, distilled QS to 1.0 liter final

8. Pre-warmed Sterile 1X PBS/0.1 % BSA from GIBCO (Cat# 15260-037)
9. Sterile 15 mL and 50 mL conical tubes from BD Falcon (Cat# 352099; Cat# 352098)
10. 12x75 polystyrene flow tubes from BD Falcon (Cat# 8562)
11. Flow Cytometer w/488 nm laser (FITC detection filter set – usually FL1)
12. Tissue Culture Hood, centrifuges, pipets

Procedures:

Mouse Splenocyte Isolation
1. Mouse Euthanasia: 10min in sealed pre-charged CO₂ chamber. Dunk into 95% Ethanol solution.
2. Ascetically remove spleen, place in cell strainer within the tissue culture dish, add 3 mL of medium (RPMI 1640 + 5 % FBS)
3. Tease spleen tissue through 70 µm strainer w/ the syringe plunger until only stromal tissue remains in strainer.
4. Add 5 mL of medium through 70 µm cell strainer, leave stromal tissue in strainer.
5. Aspirate fluid (now ~8 mL) with serological pipette and transfer to 15cc conical.
6. Centrifuge for 5 min at 1,500 rpm, 4°C.
7. Aspirate supernatant from cell pellet at the bottom of the conical.
8. Resuspend pellet in 5 mL of Room Temp. “1X ACK” solution to lyse RBCs, let sit 5 min at Room Temp.
9. Neutralize ACK with ~10 mL medium.
10. Centrifuge for 5min at 1,500 rpm, 4°C.
11. Discard supernatant, resuspend pellet in 5 mL medium & mix thoroughly.
12. Count cells on Coulter Counter, count three times and average (Program B).
13. Store splenocyte suspension on ice throughout the rest of procedure.

CFSE Labeling
*Note: (See reference for CFSE concentration calculations, cell culture set up, and cell harvest for flow analysis to refer to steps 14-22)*
14. CFSE-label appropriate cell number based on experiment design. Resuspend cells of interest in prewarmed (37°C) 1x PBS/0.1% BSA at a final concentration of 1 x 10⁶ cells/mL.
15. Incubate cells with 5 uM CFSE final (Determined to be optimal for in vitro studies) at 37°C water bath for 10 min. in a tube that can accept 5 volumes more buffer
16. Quench labeling reaction with 5 volumes of ice-cold complete mouse culture medium.
17. Incubate 5 min. on ice.
18. Centrifuge for 5 min at 1,500 rpm, 4°C.
19. Wash cells by resuspending pellet in Complete Mouse Medium.
20. Pellet and resuspend cells in Complete Mouse Medium and repeat.
21. CFSE-labeled cells are now ready for in vitro culture.

In vitro Assay/Detection
22. Set up *in vitro* cell cultures per experimental design.
23. Harvest cells as desired for experimental purposes at specific time points. Resuspend culture and pull ~1x10^6 cells for analysis.

**Support Protocols**

**Con A Stimulation to Activate Splenocytes:**
- Concanavalin A type IV, Sigma Cat# 2010, aliquoted stock vials of 10,000 ug/mL
- Suggested final concentration for in vitro activation is 2.5 ug/mL

**Anti-CD3/CD28 (Plate-Bound) Stimulation to Activate Splenocytes:**

**Materials:**
1. Anti-mouse CD3 from BD (Cat# 553057; stock = 1mg/mL) – Sterile, endotoxin free and azide free
2. Anti-mouse CD28 from BD (Cat# 553294; stock = 1mg/mL) – Sterile, endotoxin free and azide free
3. Sterile 1X PBS from GIBCO (Cat# 14040-133)
4. Sterile 6-well tissue culture plates from BD Falcon (Cat# 353046)

**Procedures:**
1. Coat the wells of a 6-well plate with 2mL of 2ug/mL final of each antibody
2. Cover the plate and gently tap to coat entire well.
3. Incubate plate 90 min at 37°C.
   - a. NOTE: Plates can also be prepared the night before and stored at 4°C
4. When ready to use, wash wells (3x) with 200–500 uL sterile PBS.
   - a. Invert and flick plate to remove wash buffer.