Multi-color cell surface staining protocol

This is a general protocol for multi-color staining involving live/dead discrimination by Zombie dye and a stain with multiple fluorophore-conjugated antibodies.

Make sure to keep cells on ice during every step, unless otherwise noted!

1. Adjust cells to 10^7 cells/mL using RPMI 1640 + 1% FBS
2. Add 100 µL of cell solution to appropriate wells of a 96 well, round-bottom plate
3. Dilute cells to 250 µL with FACS buffer (PBS + 2% FBS)
4. Pellet cells by centrifugation (300 x g, 3 minutes, 4 C)
5. Decant cells by flicking supernatant into sink
6. **Resuspend cell pellet with 50 µL 1:200 Zombie stain in FACS buffer** and incubate at room temperature for 15 minutes, protected from light
7. Dilute cells to 250 µL with FACS buffer, then pellet and decant
8. FC block: add 25 uL to prevent off target staining (5 min incubation) resuspend cell pellet
9. **Resuspend cell pellet with 50 µL of multi-color staining panel** and incubate for 30 minutes on ice, protected from light
10. Wash cells 3X by dilution to 250 µL with FACS buffer, pelleting, and decanting
11. **Add 100 µL of 1% PFA in PBS to each well, using a solvent reservoir and multi-channel pipette**, mixing well immediately after addition of fixative
12. Incubate for at least 30 minutes on ice, protected from light
13. Wash cells twice by dilution to 250 µL with FACS buffer, pelleting, and decanting
    a. PFA-fixed cells are more durable and can be pelleted at 500xg
    b. If performing ethanol fixation after PFA fixation, see below
14. Resuspend cells in 150 µL FACS buffer, keep at 4 C protected from light until time of acquisition

If performing PFA + ethanol fixation (useful for certain DNA stains):

- After step 11, dilute to 250 µL with FACS buffer, pellet, and decant
- Dilute to 250 µL with PBS, pellet, and decant
- Resuspend cell pellets in 50 µL PBS
- Transfer cells to 450 µL **ice-cold** 70% ethanol in cluster tubes, using a multi-channel pipette to mix well immediately after addition of cells
- Transfer cluster tubes, in rack and wrapped in foil, to -20 C freezer

Before downstream use of cells, removing ethanol is generally advisable. Pellet ethanol-fixed cells at 1000xg for 5 minutes in cluster tubes and decant.

Compensation (beads, can be done in tandem with cells):

1. Vortex the bead bottle vigorously to resuspend
2. Add half as many drops of beads to a 1.7 mL tube as the number of compensation needed
3. Dilute beads 4X with FACS buffer (each drop is 50 µL), plus an extra 50 µL of void volume
4. Distribute 200 µL of bead solution to individual wells of a 96 well plate
5. Pellet beads by centrifugation (300 x g, 3 minutes, 4 C) and decant into sink
6. Resuspend beads in 100 µL of compensation staining solution
    a. In most cases, a 1:100 stain is sufficient, but in certain cases this may be too bright for the detector setting used for the corresponding cell stain
7. Incubate beads for 30 minutes on ice, protected from light
8. Wash beads twice by dilution to 250 µL with FACS buffer, pelleting, and decanting
9. Resuspend beads in 150 µL of FACS buffer and keep at 4 C and protected from light until acquisition

Compensation (live/dead):

1. Transfer 2 x 10^6 splenocytes or comparable cells to a 1.7 mL tube
2. Incubate cells for 20 minutes at 56 C in a heating block to induce necrosis

3. Allow solution to cool on ice for 5 minutes, then add 2 x 10^6 live cells
4. Dilute cells to 1 mL with FACS buffer, pellet, and decant
5. **Resuspend cells in 200 µL 1:400 Zombie stain in FACS buffer** and incubate at room temperature for 15 minutes, protected from light
6. Repeat step 4 twice
7. Resuspend cells in 400 µL 1% PFA in PBS and incubate for 30 minutes on ice, protected from light
8. Repeat step 4
9. Resuspend cells in 400 µL FACS buffer and keep at 4 C, protected from light until acquisition

Reagents:
UltraComp eBeads Plus -- https://www.thermofisher.com/order/catalog/product/01-3333-42#/01-3333-42
Zombie red fixable viability dye (one of many colors available) -- https://www.biolegend.com/en-us/products/zombie-red-fixable-viability-kit-9338
Cluster tubes -- https://www.fishersci.com/shop/products/costar-cluster-tube-system-6/p-195364