Reagents and Materials

- Cells to be stained
- FACS buffer – can be cell dependent
    - 1x Ca/Mg2+ free PBS
    - Either FBS (1-10%) or BSA (0.1-1%)
    - *Optional:* EDTA (0.5-5mM) if you have very sticky cells
    - Commonly used: 1% FBS in 1x PBS (consider filtering FBS to remove debris)
- Antibodies
- Live/dead stain (this protocol is for unfixable stains such as DAPI, PI, and Sytox dyes)
- 5 mL FACS tubes: Falcon #352008

FMO = Fluorescence Minus One

Procedure

1. Prepare single cell suspension
2. Lyse RBCs (optional)
3. Count cells
4. Adjust cell concentration to 0.1-5 million cells per 50 µL FACS buffer
    a. Number of cells needed depends on your experiment.

    Note: General starting point is often 1 million cells per tube.
5. Pipette 50 µL of cells into Eppendorf or FACS tubes for staining
    a. Prepare one tube per sample
6. Pool remaining cells for unstained and FMO controls
    a. If you have 10 colors, make sure you have 10 x 50 = 500 µL pooled cells
    b. Ideally keep concentration the same as sample concentration, but you could use less cells in the FMOs if you really can't avoid it. You can also eliminate some FMOs that have clear positive populations.
7. Add Fc block
    a. 1-5 µl per sample following manufacturer's protocol

    Note: Anytime you need to pipette 1 µL, it's easier to make a 1:10 dilution in FACS buffer and add 10 µL to each sample

    b. Don't forget to add Fc block to pooled sample!
8. Make sure samples are mixed by gently vortexing or pipetting up and down
9. Incubate at 4°C for 10-15 minutes
10. Add antibody cocktail – 50 µL total volume per tube
    a. Example for a single color staining: if 2 µL of antibody are needed per tube, first combine 2 µL of antibody with 48 µL of FACS buffer, and then add the 50 µL of antibody cocktail to the 50 µL of cells for a total staining volume of 100 µL
    b. Example for a multi-color staining: prepare master mix with all antibodies (example below for the preparation of a master mix to stain 10 samples) so that appropriate amount of each antibody is in 50 µL (when added to cells, final staining volume is 100-110 µL, calculate the dilution factor for 100µL final volume)

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i.

	1X	10X
FITC	1	10
APC	5	50
PE	2	20
FACS Buffer	42	420
TOTAL	50	500

ii. Always add an extra 1-2 to your master mix to account for pipetting error, so a 10x master mix will be good for 8-9 samples

c. For FMOs: combine 50 µL FMO antibody mix (all antibodies except one) with 50 µL pooled cells

11. Incubate in the dark at 4°C for 30 minutes (cover tubes with alu foil if needed)
12. Wash cells with FACS buffer
    a. 1 mL if staining in Eppendorf tubes
    b. 2 mL if staining in flow tubes
13. Centrifuge
14. Aspirate, pipette, or dump tubes to remove supernatant
    a. Be careful not to disturb the pellet!
15. Resuspend in FACS buffer
    a. Volume depends on the number of cells
    b. At least 350 µL for acquisition on Fortessa or Symphony
16. If cells were stained in Eppendorf tubes, transfer to 5 mL FACS tubes
17. A few minutes before running samples on the cytometer, add live/dead stain (see manufacturers protocol)

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