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1. Dissociate ~10^6 cells extensively with 250–500 μl trypsin
   - The protocol is compatible with a variety of plating schemes and densities
   - *Fewer cells will not make it through the entire procedure*
   - *Complete trypsinization (as long as 30–45 min for some cells) is important to obtained a fully dissociated single-cell suspension*
2. Break up cell clumps by adding 1 ml PBS to each well and pipetting up and down several times.
3. Transfer cells from each well to a microcentrifuge tube and spin at 800 rcf for 3 min.
4. Resuspend cells in 1 ml PBS and spin at 800 rcf for 3 min.
5. For standard flow cytometry without live-dead discrimination, skip to Step #9.
   - *Live-dead discrimination is helpful for identifying non-apoptotic forms of cell death and for artifactual staining that could be observed in nonviable cells*
   - *The live-dead stain should not be used if a DAPI counterstained is planned or the FL1 channel is going to be used without compensation*
6. Resuspend cells in 1 ml PBS and add 1 μl violet-fluorescent reactive dye (Invitrogen #L34955; dissolve each vial in 55 μl DMSO and use within 1–2 weeks) to each sample.
   - *This dye can be used to exclude dead cells on flow cytometers with a violet laser line*
7. Incubate on ice for 30 min and spin at 800 rcf for 3 min.
8. Resuspend cells in 1 ml PBS and spin at 800 rcf for 3 min.
9. Resuspend cells in 160 μl PBS.
   - *There is typically ~20 μl residual volume before adding the PBS, bringing the total volume to ~180 μl*
10. Spike in 20 μl 37% PFA, vortex, and incubate at room temperature for 15 min.
11. Spin at 800 rcf for 3 min, and place samples on ice.
12. Aspirate the PFA solution and immediately permeabilize cells with 200 μl ice-cold MeOH.
    - *Cells can be stored at –20°C for days to weeks at this point.*
13. Change cells to a fresh tube
    - *Moving the cell suspensions to a fresh tube allows a better pellet to be formed at the first centrifugation, when a large fraction of cells can be lost*
14. Spin at 800 rcf for 3 min.
15. Wash cells with 500 μl PBS + 0.1% Tween-20 (PBS-T), and spin at 800 rcf for 3 min.
16. Resuspend cells in 100 μl 1× Western blocking reagent (Roche #11921673001; aliquot 10× stock and freeze at –20°C) in PBS-T and incubate for 30 min at room temperature.
    - *Western blocking reagent provides a more stringent block than goat serum or BSA and gives greater specificity to the staining; however, this block may be too stringent for some antibodies*
    - *An alternative low-stringency blocking solution is 1% BSA (use throughout all the subsequent staining steps)*
17. Spin at 800 rcf for 3 min.
18. Resuspend cells in 100 μl of primary antibody solution diluted in 1× Western blocking reagent in PBS-T and incubate for 1 hr at room temperature.
    - *Antibody dilutions must be determined empirically for each primary (1:50, 1:100, and 1:200 or 0.1 – 1 μg is a good starting range of dilutions, but some will require up to ten-fold less antibody)*
19. Spin at 800 rcf for 3 min.
20. Wash cells with 500 μl PBS-T, and spin at 800 rcf for 3 min.
21. Repeat Step #20.
22. Resuspend cells in 100 μl of secondary antibody solution diluted in 1× Western blocking reagent in PBS-T and incubate for 1 hr at room temperature.
    - *Antibody dilutions must be determined empirically for each secondary*

- *We have optimized the following secondaries:*
    - *Alexa 488-conjugated goat anti-mouse/rabbit (Invitrogen) for FL1 channel — 1:1000 dilution (0.2 μg)*
    - *PE-conjugated goat anti-mouse/rabbit (Jackson ImmunoResearch) for FL2 channel — 1:200 dilution (0.25 μg) PE is light sensitive and will fade over time with storage*
    - *PerCP-conjugated goat anti-mouse/rabbit (Jackson ImmunoResearch) for FL3 channel — 1:100 dilution (0.5 μg) PerCP is very light sensitive… keep protected at all times!*
    - *Alexa 647-conjugated goat anti-mouse/rabbit (Invitrogen) for FL4 channel — 1:5000 dilution (0.04 μg)*
- *PE is the brightest as for a single-channel experiment; Alexa 488 pairs with Alexa 647 with essentially zero compensation required, but prevents doublet discrimination on standard FACSCalibur instruments; Alexa 488 paired with PerCP allows doublet discrimination but will require slight compensation to account for bleedthrough*

23. Wash cells with 500 μl PBS-T, and spin at 800 rcf for 3 min.
24. Repeat Step #23.
25. Resuspend cells in 200 μl PBS-T and transfer to flow cytometry tubes (Falcon #352008).