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1. Transfer cell suspension (~10^6 cells) from each well to a microcentrifuge tube and spin at 800 rcf for 3 min. Aspirate media.
   - *Combine replicate samples as needed to achieve this amount of starting material*
2. Resuspend cells in 1 ml PBS and spin at 800 rcf for 3 min. Aspirate supernatant.
   - *Throughout the protocol, the supernatant will be discarded*
3. Resuspend cells in 160 µl PBS.
   - *There is typically ~20 µl residual volume before adding the PBS, bringing the total volume to ~180 µl*
4. Spike in 20 µl 37% PFA, vortex, and incubate at room temperature for 15 min.
   - *The final concentration of PFA is 3.7%*
5. Spin at 800 rcf for 3 min and aspirate the PFA solution.
6. Resuspend cells in 1 ml PBS and spin at 800 rcf for 3 min. Aspirate supernatant.
7. Repeat Step 6.
8. Permeabilize cells with 0.3% Triton X-100 in PBS.
   - *Add 15 µl of a 10% Triton X-100 solution to 485 µl of PBS per tube*
   - *Cells can be stored at 4°C for days to weeks at this point*
9. Change to a fresh microcentrifuge tube and spin at 800 rcf for 3 min.
10. Wash pellet with 500 µl of PBS + 0.1% Tween-20 and spin at 800 rcf for 3 min.
11. Block cells in 1× Western Blocking Reagent (Roche #11921673001) diluted in PBS + 0.1% Tween-20 for 1 hour at room temperature.
    - *100 µl of blocking solution is sufficient for each tube*
    - *Tubes should be placed on Eppendorf thermomixer set at 700 rpm with the temperature control off*
12. Spin at 800 rcf for 3 min and aspirate the blocking solution.
13. Add primary antibody at the appropriate dilution in blocking solution overnight at room temperature.
    - *100 µl of primary solution is sufficient for each tube*
    - *Tubes should be placed on Eppendorf thermomixer set at 700 rpm with the temperature control off*
14. Spin at 800 rcf for 3 min and aspirate the primary solution.
15. Wash pellet with 500 µl of PBS + 0.1% Tween-20 and spin at 800 rcf for 3 min. Aspirate supernatant.
16. Repeat Step 15.
17. Add secondary antibody at the appropriate dilution in blocking solution for 1 hr at room temperature.
    - *100 µl of secondary-antibody solution is sufficient for each coverslip*
    - *1:200 dilution of species-specific, highly-crosslinked Alexa-labeled antibodies from Molecular Probes; these fluorochromes do not need to be protected from ambient light during the staining process*
    - *Tubes should be placed on Eppendorf thermomixer set at 700 rpm with temperature control off*
18. Spin at 800 rcf for 3 min and aspirate the secondary solution.
19. Wash pellet with 500 µl of PBS + 0.1% Tween-20 and spin at 800 rcf for 3 min. Aspirate supernatant.
20. Repeat Step 19.
21. Counterstain cells with 0.5 µg/ml DAPI in PBS for 5 min.
    - *Apply 100-300 µl of DAPI in PBS per tube*
    - *Tubes may rest in tube rack at this time*
22. Spin at 800 rcf for 3 min and aspirate the DAPI solution.
23. Wash pellet with 500 µl of PBS + 0.1% Tween-20 and spin at 800 rcf for 3 min. Aspirate supernatant.
24. Repeat Step 23.
25. Incubate cells with 10 mM $CuSO_4$ in 50 mM NH_4Ac (pH 5.0; acidify with acetic acid) for 10 min.
    - *$CuSO_4$ treatment dramatically reduces autofluorescence of cells, while only slightly diminishing the fluorescence of Cy- and Alexa-family dyes (Schnell et al. J. Histochem. Cytochem. 47:719 (1999))*

- *Higher concentrations of $CuSO_4$ reduced signal, whereas lower concentrations are less effective at quenching autofluorescence*
- *Apply 100-300 μl of $CuSO_4$ treatment per tube*
- *Tubes may rest in tube rack at this time*

26. Spin at 800 rcf for 3 min and aspirate the $CuSO_4$ quenching solution.
27. Wash pellet with 500 □l of PBS + 0.1% Tween-20 and spin at 800 rcf for 3 min.
28. Carefully aspirate remaining liquid and resuspend pellet in 12 μl of antifade mounting medium.
    - *We use 0.5% N-propyl gallate in 90% glycerol + 1x phosphate buffer (pH 8.0)*
    - *Cut 20 µl pipette tips at the first gradation to avoid breaking up cell aggregates when handling the pellet.*
29. Add the resuspended cells to the center of a 22 x 22 mm coverslip and then use a clean microscope slide to pick up the coverslip after the microscope slide has touched the mounting medium
    - *To reduce spherical aberration, use a minimal amount of cells suspended in mounting medium on each coverslip. Allow the embedding medium to spread slowly across the entire surface of the coverslip once coverslip is mounted on slide.*
    - *Carefully push out excess liquid or air bubbles as needed*
30. Seal the edges of the coverslip in clear nail polish and allow to air dry.
31. Store slides at 4°C until imaging.