Fluorescence Microscopy:

Intracellular IF staining protocol with PFA / Triton (The Donnelly Method):

1. Wash specimen with pre-warmed (37°C) 1x PBS.
2. Add pre-warmed (37°C) fixative buffer (4% PFA in 1x PBS) and incubate for 15 minutes at 37°C.
3. Remove the fixative and wash with 1x PBS. Store the specimen at 4°C in 1x PBS or proceed with permeabilization.
4. Permeabilize cells (0.1% Triton X-100 in 1x PBS) for 10 minutes at room temperature.
5. Block specimen for 1 hour at room temperature in blocking buffer (1% BSA in 1xPBS-filtered with 0.22 um filter).

   Perform the below steps in the dark if using fluorescence:
6. Dilute the antibody in the blocking buffer (1:200) and incubate samples overnight at 4°C in a humidified chamber (Note: 2h RT incubation will not yield a signal).
7. Wash 4x 5 minutes with 1x PBS.
8. Dilute Hoechst (DNA stain) 1:2000 in 1x PBS and incubate at room temperature for 20 minutes.
9. Wash 2x 5 minutes with 1x PBS.
10. Aspirate the remaining PBS and mount coverslips on a glass slide. Let the slides cure overnight, then seal the edges of the coverslips with clear nail polish.

Intracellular IF staining protocol with PFA / Methanol (The Apostol Method):

1. Wash specimen once with 0.05% TritonX-100 in 1X PBS for 2-5 minutes.
2. Add 1 ml fixative buffer (50% methanol / 4% paraformaldehyde in 1x PBS).
3. Incubate 4 hours at 4°C.
4. Remove fixative buffer and wash with PBST (the fixed samples can now be stored at 4°C for up to 2 weeks).
5. Optional: Incubate slides with 1% SDS for 5 minutes
6. Wash 3x with PBST for 5 min each.
7. **Perform the below steps in the dark if using fluorescence:**
8. Apply Antibody (1:200 in PBS), for 1-2 hours at room temperature.
9. Wash 1x with PBST.
10. Cover with a cover slip and seal the edges with clear nail polish. Allow to dry in the dark for 5-10 min.

Flow Cytometry:

Intracellular FC staining with PFA / Triton protocol:

1. Harvest, wash the cells and adjust cell suspension to a concentration of $1x10^6$ cells per tube.
2. Spin cells and remove supernatant. Wash cells once with 1X PBS.
3. Spin cells and remove supernatant. Add 1 ml fixative buffer (4% PFA in 1x PBS). Incubate 15 minutes at 37°C.
4. Spin cells and remove supernatant. Wash cells once with 1xPBS (the fixed cells can now be stored at 4°C for up to 2 weeks).
5. Optional: Spin cells and remove supernatant, add 1ml 1% SDS, incubate for 5 minutes.
6. Spin cells and remove supernatant. Permeabilize cells with 1ml permeabilisation buffer (0.1% Triton X-100 in 1x PBS) for 10 minutes at room temperature.
7. Block specimen for 1 hour at room temperature in blocking buffer (1% BSA in 1xPBS-filtered with 0.22 um filter).
   Perform the below steps in the dark if using fluorescence:
8. Aliquot 200ul for each test into a new tube ($2x10^5$ cells).
9. Add 1ul Antibody (1:200 final conc.), quick vortex and incubate overnight at 4°C (Note: 2h RT incubation will not yield a signal).
10. Wash 4x with 500ul PBS by spinning cells and removing supernatant each time.
11. Resuspend to a final 500ul PBS and analyse with Flow cytometer.

Intracellular FC staining with PFA / Methanol protocol:

12. Harvest, wash the cells and adjust cell suspension to a concentration of $1x10^6$ cells per tube.
13. Spin cells and remove supernatant. Wash cells once with 0.05% TritonX-100 in 1X PBS.
14. Spin cells and remove supernatant. Add 1 ml fixative buffer (50% methanol / 4% paraformaldehyde in 1x PBS). Incubate 4 hours at 4°C.
15. Spin cells and remove supernatant. Wash cells once with PBST (the fixed cells can now be stored at 4°C for up to 2 weeks).
16. Optional: Spin cells and remove supernatant, add 1ml 1% SDS, incubate for 5 minutes.
17. Wash 3x with PBST for 5 min each by spinning cells and removing supernatant each time.
18. Spin cells and remove supernatant, resuspend in 1ml PBS and aliquot 200ul for each test into a new tube ($2x10^5$ cells).
    Perform the below steps in the dark if using fluorescence:
19. Add 1ul Antibody (1:200 final conc.), quick vortex and incubate for 1-2 hours at room temperature.
20. Wash 3x with 500ul PBS by spinning cells and removing supernatant each time.
21. Resuspend to a final 500ul PBS and analyse with Flow cytometer.

Western Blot:

1- Load 10ug denatured cell lysate and transfer to membrane.
2- Block membrane with 3% BSA in a clean/new Falcon tube or tray for 1 hour (**Important: 1 - Do not block with milk or serum; 2- Do not use re-usable trays/containers (milk stuck on the plastic from previous WBs will deactivate the antibodies**).
3- Wash membrane 3 x 5 min in TBS (no tween) - this step is to remove unbound BSA which will mask antibodies.
4- Incubate with fresh antibody in TBS overnight in a cold room or 2 hours at room temperature (**Important: Do not include BSA or tween in the antibody dilution mixture; 2- Do not reuse diluted antibodies**).
5- Wash membrane 3 x 5 min in TBST
6- Apply ECL reagent and proceed to detection