Fluorescence Microscopy:

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

1. Wash specimen with 1x PBS at room temperature.
2. Add cold fixative buffer (4% PFA in 1x PBS) and incubate for 10 minutes at room temperature.
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 (0.5% Triton X-100 in 1x PBS) for 30 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 add to specimen in a humidified chamber (covered to protect from light), shake gently overnight at 4°C (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.

If the above protocol doesn't give a signal, you could try the below changes separately or in combination to increase permeabilisation and allow our antibodies to penetrate the cell.

Step 2: Use cold 2% PFA and fix by incubating for 10min at room temperature
Step 3: Do not store specimens, use immediately
Step 4: Permeabilise with 0.5% Triton X-100 for 30 minutes at 37°C.
Step 6: Incubate with antibody overnight at room temperature instead of 4°C.

If the Donnelly Method does not work, please use the Apostol intracellular staining method in the next page, which permeabilises better than triton-X-100.

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. Resuspend in 500ul PBS.
4. Add 500ul fixative buffer (8% PFA in 1x PBS to achieve final PFA conc 4%). Incubate 15 minutes at 37°C.
5. 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).
6. Optional: Spin cells and remove supernatant, add 1ml 1% SDS, incubate for 5 minutes.
7. 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.
8. 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:
9. Aliquot 200ul for each test into a new tube ($2x10^5$ cells).
10. Add 1ul Antibody (1:200 final conc.), quick vortex and incubate overnight at 4°C (Note: 2h RT incubation will not yield a signal).
11. Wash 4x with 500ul PBS by spinning cells and removing supernatant each time.
12. Resuspend to a final 500ul PBS and analyse with Flow cytometer.

If the above protocol doesn't give a signal, you could try the below changes separately or in combination:

Step 4: Add cold 4% PFA and incubate for 10min at room temperature (Final PFA conc 2%),
Step 5: Do not store specimens, use immediately
Step 7: Permeabilise with 0.5% Triton X-100 for 30 minutes at 37°C.
Step 10: Incubate with antibody overnight at room temperature instead of 4°C.

If this protocol does not work for intracellular targets, please use the PFA / Methanol intracellular staining method in the next page, which permeabilises better than triton-X-100.

Intracellular FC staining with PFA / Methanol protocol:

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

Western Blot:

1- Load 0.5-1ug cell lysate in a gel (loading above 5ug will cause target band disappearance due to bleaching or quenching effects).
2- Transfer to membrane.
3- Block membrane with 3% BSA in a clean/new Falcon tube or tray for 1 hour (Do not block with milk or serum; 2- Do not use reusable trays/containers (milk stuck on the plastic from previous WBs will deactivate the antibodies).
4- Wash membrane 3 x 5 min in TBS (no tween) - this step is to remove unbound BSA which will mask antibodies.
5- Incubate with fresh antibody in TBS (1:5000 dilution) overnight in a cold room. (Do not include BSA or tween in the antibody dilution mixture; 2- Do not reuse diluted antibodies).
6- Wash membrane 3 x 5 min in TBST
7- Apply ECL reagent and proceed to detection