The steps of the intracellular staining protocol are as follows:

1. Perform cell surface staining
2. Fixation and permeabilization, described in detail below
3. Incubate in the dark at room temperature for 15 minutes.
4. Wash the cells by adding 2 mL of PBS (containing 0.1% triton or other permeabilizing detergent), then centrifuge at 300 G (2000 rpm) for 5 minutes. Discard the supernatant and re-suspend the pellet in the remaining volume.
5. Employ an antibody staining procedure (see our direct and indirect protocols). Antibodies should be prepared in permeabilization buffer to ensure the cells remain permeable.

Fixation and permeabilization
For intracellular staining, cells must be fixed first to ensure stability of antigens that are soluble or have a short half-life (see below for important exceptions). This should retain the target protein in the original cellular location. Once the cells are fixed, detection of intracellular antigens requires a cell permeabilization step before staining. Use one of the following three methods: formaldehyde followed by detergent, formaldehyde followed by methanol, or acetone.

**Formaldehyde followed by detergent**

a. Fixate the cells in 100 μL of 0.01% formaldehyde for 10-15 minutes to stabilize proteins.

b. Disrupt the membranes using 100 μL of one of the following detergents:

   - **Triton or NP-40 (0.1-1% in PBS)**: These will also partially dissolve the nuclear membrane and are therefore very suitable for nuclear antigen staining. It should be noted that loss of cell membrane and cytoplasm will result in decreased light scattering and also in reduced non-specific fluorescence.

   - **Tween 20, Saponin, Digitonin, and Leucoperm**: These are mild membrane solubilizers. Use at 0.5% in PBS. These give large enough pores for antibodies to go through without dissolving plasma membranes. They are suitable for antigens in the cytoplasm or the cytoplasmic face of the plasma membrane and for soluble nuclear antigens.

**Formaldehyde (0.01%) followed by methanol**

a. Fixate the cells in 100 μL of 0.01% formaldehyde for 10-15 minutes to stabilize proteins.

b. Add 1 mL ice-cold 100% methanol to each sample.

c. Mix gently. Place at -20°C for 10 minutes.

d. Centrifuge, then wash twice in 1% BSA/PBS.

**Acetone**

*Polystyrene/plastic tubes are not suitable for use with acetone.*

c. Add 1 mL ice-cold 100% acetone to each sample.

d. Mix gently. Place at -20°C for 5–10 minutes.

e. Centrifuge, then wash twice in 1% BSA/PBS
Exceptions

- Antigens close to the plasma membrane and soluble cytoplasmic antigens will require mild cell permeabilization without fixation.
- Cytoskeletal, viral, and some enzyme antigens usually give optimal results when fixed with high-concentration acetone, alcohol, or formaldehyde.
- Antigens in cytoplasmic organelles and granules will require a fixation and permeabilization method depending on the antigen. The epitope needs to remain accessible.

Detection of secreted proteins

Detection of secreted proteins is difficult, as the protein will be released from the cell before detection or may degrade rapidly. Brefeldin A and other compounds are often used as a Golgi block. Incubating cells with Brefeldin A prevents the release of proteins from the Golgi bodies. Any cells expressing the protein can then be detected.

When gating on cell populations, the light-scatter profiles of the cells on the flow cytometer will change considerably after permeabilization.