

METHOD FOR STAINING OF INTRACELLULAR ANTIGENS

MATERIALS:

1. 2% formaldehyde solution (preparation method attached)
2. 1 X PBS (without sodium azide and serum)
3. 1 X PBS + 2% newborn calf serum + sodium azide (buffer)
4. Polyoxyethylensorbitan monolaureate (Tween 20)
5. Human AB serum, heat-inactivated (HAB)
6. 37°C water bath, refrigerated centrifuge.

METHOD:

Fixation

1 x 10⁶ PBS-washed cells from a single cell suspension are pelleted in a 12 X 75 mm culture tube. The pellet is re-suspended in 0.875 mL of cold PBS and the suspension is mixed gently. Then, 0.125 mL of cold 2% formaldehyde solution is added and the mixture is immediately vortexed briefly. The suspension is incubated for at least 30 min or for up to 1 h at 4°C, centrifuged for 5 min at 250g, then the supernatant is removed.

Permeabilization

The pellet is gently re-suspended in 1 mL of room temperature Tween 20 solution (0.2% in PBS) and the mixture is incubated for 15 min in a 37°C water bath. One mL of buffer is added and the suspension is spun for 5 min at 250g. The supernatant is removed and the internal staining then proceeds as described in the direct staining method for antibodies directly conjugated to a fluorochrome or in the indirect staining method for unlabelled antibodies.

Direct Staining Procedure

1. Re-suspend cell pellet first in 50 µL of HAB for approximately 1 min, then add 50 µL of buffer and the appropriate amount of the fluorochrome-conjugated antibody.
2. Vortex briefly and incubate for 30 min at 4°C in the dark.
3. Wash twice with 1 mL of 0.2% Tween 20 solution by centrifugation at 250g for 5 min.
4. Resuspend samples in 0.5 mL of buffer and hold them at 4°C protected from light prior to analysis.

Indirect Staining Procedure

- 1-3. Process samples as above using a working dilution of unlabelled antibody.
4. Resuspend cell pellet first in 50 μ L of HAB for approximately 1 min, then add 50 μ L of a working dilution of the fluorochrome-conjugated second antibody.
5. Vortex briefly and incubate for 20 min at 4°C in the dark.
6. Wash twice with 1 mL of 0.2% Tween 20 solution by centrifugation at 250g for 5 min.
7. Resuspend samples in 0.5 mL of buffer and hold them at 4°C protected from light prior to analysis.

Note: do not use HAB for staining of immunoglobulin chains. Whenever available, use a monoclonal antibody and/or a reagent directly conjugated to a fluorochrome to minimize unspecific binding. Always use isotypic controls of the same heavy chain class at the same protein concentration as your relevant antibody for determination of background staining. For storage of samples longer than overnight and for biohazard considerations the samples can be re-suspended in 1% formaldehyde solution after the internal staining procedure has been completed.

PREPARATION OF 2% FORMALDEHYDE STOCK SOLUTION (2 METHODS)

METHOD 1:

Formaldehyde preservative – 2% formaldehyde solution in protein-free PBS.

Prepare as follows:

10% formaldehyde*	20 ml
10 x PBS	10 ml
Distilled water	70 ml

* 10% formaldehyde solution (e.g., Polysciences, Warrington, PA, ultrapure, Cat.#04018), depolymerized paraformaldehyde, EM grade, methanol-free solution.

METHOD 2:

Formaldehyde preservative – 2% formaldehyde solution in protein-free phosphate-buffered saline (PBS).

Prepare as follows:

Add 2 g paraformaldehyde powder (e.g., Sigma-Aldrich, St. Louis, MO) to 100 ml of 1 X PBS. Heat to 70°C (do not exceed this temperature) in a fume hood until the paraformaldehyde goes into solution (note that this happens quickly as soon as the suspension reaches 70°C). Allow the

solution to cool to room temperature. Adjust to pH 7.4 using 0.1 M NaOH or 0.1 M HCl, if needed. Filter and store at 4 °C protected from light.

Background information for intracellular staining by flow cytometry

For correlation of surface immunofluorescence with intracellular antigen expression the surface antigens are stained according to the "Monoclonal Antibody Staining Procedure" before fixation and permeabilization. However, please note that if a particular antigen is expressed on the cell surface as well as internally the surface antigens will also be detected by the antibody staining procedure after the fixation and permeabilization procedure. If you have stained surface antigens with mouse monoclonal antibodies, you either have to use a directly labelled antibody, an antibody made in a different species, or the avidin-biotin system for intracellular staining, because any anti-mouse second antibody that you are using for internal staining will also react with the mouse monoclonal antibody bound to surface proteins.

For correlation of intracellular antigen expression with DNA content please refer to the procedure "Staining Procedure for Correlation of Surface Antigen Expression Simultaneously with DNA content". Staining for internal antigens is done on fixed and permeabilized cells before staining for DNA content.

The fixation and permeabilization method described here employs a very mild treatment of cells with 0.25% buffered formaldehyde and Tween 20. Thus it offers a valuable alternative to alcohol fixation in cases where the immunoreactivity of the antigen is compromised by conformational changes due to excess crosslinking and harsh fixation conditions. In addition, it allows the preservation of cell surface immunofluorescence and the light scatter profiles of cell clusters for gating purposes in flow cytometry and gives very low coefficients of variation on DNA histogram distributions. It is therefore particularly suited for correlation of internal staining with surface antigen staining and DNA content.

The method described here was successfully used for internal staining of differentiation antigens, intracytoplasmic m , and vimentin in leukemic cell lines. In addition, the current method was able to permeabilize a variety of other cell types including human lymphocytes and thymocytes, and murine thymocytes and spleen cells for DNA staining. Thus it appears that the fixation and permeabilization procedure may be broadly applied to many different cell types as well as tissue culture lines. However, the current method may not be applicable to all cells and internal antigens.

Whenever the results you are obtaining with this method are not satisfactory, please consider the following: the precise optimal conditions for staining of intracellular antigens may be influenced by the nature of the antigen and its localization. It might be necessary to modify the length of the incubation with the antibody and/or the temperature of the reaction. Keeping the antibody in the detergent solution during the incubation step has also been described as a measure to improve penetration of the reagent to the reaction site. In addition, it is also known that some cytoplasmic or nuclear antigens require a higher degree of fixation and/or a different fixative. Therefore, modification of the concentration of the fixative and/or the temperature of the fixation step can improve staining of some internal proteins.

For any quantitation of expression of intracellular proteins the results should be evaluated critically in each case to verify the specificity of the antibody-antigen reaction. The antibodies and their controls should be used at the same protein concentration. Antibodies from different manufacturers directed at the same antigen can display different properties when they are used for intracellular staining. All reagents should be titrated for optimal internal staining and may require different amounts than those used for detection of surface antigens. The flow cytometric results should be verified by a comparison to results obtained with non-flow cytometric methods. Known negative cell controls should be fixed, permeabilized and stained to detect non-specific reactivity of the antibodies with fixed cells. The localization of the fluorescent reaction should always be verified by fluorescence microscopy.

For further information on this method please refer to:

Schmid I, Uittenbogaart CH, Giorgi JV: A gentle fixation and permeabilization method for combined cell surface and intracellular staining with improved precision in DNA quantitation. *Cytometry* 12, 279-285, 1991.

Schmid I and Giorgi JV: Preparations of cells and reagents for flow cytometry, Intracellular Staining. *In: Current Protocols in Immunology, Vol1, Unit 5.3, Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, eds., John Wiley & Sons, 1995, pp 5.3.1-5.3.23.*