Protocol for ENT4 Antibody (Cat. No. 463 005)
Immunocytochemistry (ICC)
Fluorescence Staining - Fixed Cells

Para-formaldehyde (PFA) or formaldehyde (FA) is the most common fixative in immunocytochemistry experiments. It cross-links amino-groups of proteins and guarantees a good preservation of cell morphology. Scientists use self-made FA fixation solutions produced by dissolving paraformaldehyd (PFA) in phosphate buffered saline (PBS), or they apply ready-to-use FA fixation solutions containing different amounts of methanol for stabilization. In our standard immunocytochemistry protocol, we apply a ready-to-use FA solution with a low amount of methanol.

Glyoxal can be an alternative to PFA/FA fixation. It is less toxic and sometimes yields superior results in immunocytochemistry. It penetrates cells faster than PFA, cross-links amino-groups of proteins more effectively, and can improves the maintenance of cell morphology.

Methanol, acetone and methanol-acetone fixation work by denaturing and precipitating proteins, and as such it is a quick method. For most antibodies/proteins, it takes only 5 minutes. This procedure sometimes leads to an unmasking of epitopes. In general the preservation of cell morphology is less good compared to PFA/FA or glyoxal fixation.

Materials and reagents

- **PBS:** Phosphate buffered saline (200 mM NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4), pH 7.4
- **Blocking buffer:** 10% normal serum, 0.1% Triton X-100 in PBS (if secondary antibodies are used, normal serum from the host-species of the secondary antibodies is recommended for blocking)
- **Incubation buffer:** 5% normal serum, 0.1% Triton X-100 in PBS (if secondary antibodies are used normal serum from the host-species of the secondary antibodies is recommended)
- **Primary antibody:** ENT4 antibody (cat. no. 463 005)
- **Secondary antibody:** Fluorophore conjugated Guinea pig specific secondary antibody
- **Mounting medium**
- *Optional: DAPI nuclear stain*
- **Fixation solutions:** The following fixatives are compatible with this antibody:
  - **FA fixation solution**: 4% FA (4% PFA) in PBS, 4% sucrose, pH 7.4

Procedure

1. Wash cells briefly with **PBS** at room temperature (RT).

2. The following fixation steps can be alternatively performed:
   - Fix cells with **FA fixation solution** for 15-20 min at RT.

3. Wash three times with **PBS** for 10 min each.

4. Incubate for 30 min with **blocking buffer**.

5. Apply **primary antibody** in **incubation buffer** at a dilution of **1:500 to 1:2000** for 2 h at RT.

6. Wash three times with **PBS** for 10 min each.

7. Apply fluorophore conjugated **secondary antibody** in **incubation buffer** for 1 h at RT diluted according to the manufacturer recommendations.
    - *Optional: Add DAPI to the secondary antibody solution.*
    - *Notes:*
        - *Avoid bright light when working with the secondary antibody to minimize photo bleaching of the fluorescent dye.*
        - *In multiplex staining, make sure to use secondary antibodies cross-adsorbed against the host species of the other primary antibody used in your experiment. Ideally, all secondary antibodies should come from the same host species. If not, make sure that they have been cross-adsorbed against IgGs of the host-species of the other secondary antibody as well. This avoids cross-reaction between the secondary antibodies.*

8. Wash three times with **PBS** for 10 min each.

9. Mount coverslips.

Note: This protocol has been validated in the SYSY Antibodies laboratories to ensure consistent and reliable staining results. However, for achieving the best specific signal with minimal background, the optimal antibody concentration, incubation temperature, and incubation duration should be optimized for each experiment.