Protocol for mCLING (Cat. No. 710 006DY654)
Immunocytochemistry (ICC)
Fluorescence Staining - Fixed Cells

General considerations

For homogeneous labeling of the plasma membrane in immunocytochemistry experiments, make sure to dissociate cells before plating and use the cells at a confluence rate not above 50% to 70%. Make sure to work with healthy viable cells. mCLING can strongly bind to cell debris.

The coverslips must have the least interaction possible with mCLING molecules. This is particularly important when the cells directly imaged from the coverslip where they are cultured. mCLING is a positively charged molecule. Chemical procedures used to clean glass coverslips typically leave the surface negatively charged, which leads to binding of mCLING molecules and a strong background signal. Similarly, some extracellular matrix molecules used for coverslip coating, like collagen, fibronectin and laminin, have both positive and negative charges, resulting also in a strong background signal that makes it difficult to distinguish the borders of the labeled cells. A good solution to this problem is to use a positively charged coating like poly-L-lysine (PLL).

mCLING has to be stably fixed to the membranes of the stained cells. Especially for subsequent immunostaining, it is important to cross-link mCLING to membrane proteins, via amine-fixative reactions. mCLING offers seven amine groups for its cross-linking with aldehyde fixatives. However, paraformaldehyde does not provide sufficient fixation. Therefore, a mixture of 4% paraformaldehyde (PFA) and 0.2% glutaraldehyde is strongly advised. Unfortunately, antibody binding may be affected by this stronger fixation and a careful evaluation of the antibody used in this approach is mandatory.

Materials and reagents

- **Physiological buffer:** (e.g. Tyrode's buffer for cultured neurons and Ringer's buffer for cultured cell lines like COS-7)
- **mCLING (cat. no. 710 006DY654)**
- **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
- **Fixation buffer:** 4% PFA, 0.2% glutaraldehyde in PBS
- **Ice**
- **Quenching buffer:** 100 mM NH_4Cl, 100 mM Glycine in PBS
- **Primary antibody**
- **Secondary antibody:** Fluorophore conjugated species specific secondary antibody
- **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)
- *Optional: DAPI nuclear stain*
- **Mounting medium**

Procedure for labeling membrane trafficking

1. Pre-warm the **physiological buffer** of choice to 37°C.

2. Prepare a working **mCLING** solution with a concentration between 1-0.2 nmol/ml, diluted in **physiological buffer**. Keep the

solution warm at 37°C. Cells cultured on feeder layers may require higher mCLING concentrations up to 0.8 µM. Never use mCling at a higher concentration as 2 µM since it has a toxic effect.
Note: Avoid bright light when working with fluorescently labeled mCling to minimize photo bleaching of the fluorescent dye.

3. Remove culture medium and carefully wash the cells with pre-warmed **physiological buffer**, three times briefly.

4. Apply the mCLING solution. The labeling protocol will depend on the aim of the experiment, and on the cell type:
   - For endocytosis and membrane trafficking in stable cell lines: Incubate the cells with **mCLING** solution for 5 to 10 min at 37°C. This should allow the visualization of the plasma membrane and endocytosed organelles. Longer incubation periods (e.g. 20 min) might be necessary to see molecular recycling back to the plasma membrane. At this point, mCLING incubation can be combined with fluorescently labeled ligands for endocytosis, like transferrin, low-density lipoprotein (LDL) or epidermal growth factor (EGF).
   - Synaptic vesicle recycling during active release in neurons: Incubate the neurons with the mCLING solution for 1 min to ensure homogeneous plasma membrane labeling. Immediately after, apply the stimulation of your choice (e.g. electrical stimulation or high K^+ solution). Be aware that mCLING is not a washable molecule, as it is the case for other endocytosis markers typically used to study synaptic vesicle recycling in neuronal preparations (e.g. FM 1-43 or FM 4-64). Thus, fluorescent signal coming from the plasma membrane cannot be removed by perfusing the cells with buffer.
   - Synaptic vesicle recycling during spontaneous release in neurons: Incubate the neurons in 500 µl physiological buffer containing 1 µM tetrodotoxin and mCLING for 15 min at 37°C.

5. Wash excess mCLING with **physiological buffer**.

6. Immediately place the coverslip in **fixation buffer**. Incubate for 20 min on **ice** followed by an additional 20 min at room temperature (RT).

7. Wash with **PBS**.

8. Incubate for 30 min in 2 ml **quenching buffer**.

9. Wash with **PBS**.

10. Block for 5 min in **blocking buffer** at RT. Repeat this step 3 times.

11. Incubate with primary antibody for 1 h at RT in **incubation buffer**. **Note:** Overnight incubation at 4°C is not recommended as it might affect the stability of mCLING on the labeled membranes, resulting in a loss of signal.

12. Wash 3 times 10 min each with **PBS** at RT.

13. Incubate with **secondary antibody** diluted in **incubation buffer** according to the manufacturer's recommendation for 1 h at RT.
    Optional: Add DAPI to the secondary reagent solution.
    Note: Avoid bright light when working with the secondary antibody to minimize photo bleaching of the fluorescent dye.

14. Wash 3 times for 10 min each with **PBS** at RT.

15. Mount coverslips and microscope.

Procedure for labeling only the outer plasma membrane

1. Prepare 100 ml of **physiological buffer** pre-chilled to 4°C and 100 ml equilibrated to RT.
2. Prepare a multi-well plate with **fixation buffer** and place it on ice for 30 min.
3. Prepare a working mCLING solution with a concentration between 0.1 to 0.2 µM diluted in cold **physiological buffer**. Keep the solution on ice for 30 min.
   Note: Avoid bright light when working with fluorescently labeled mCling to minimize photo bleaching of the fluorescent dye.
4. Gradually reduce the temperature of the cells by dipping the coverslips once into the **physiological buffer** at RT, and twice into the cold **physiological buffer**. Immediately transfer the coverslips to the multi-well plate with pre-chilled **physiological buffer** and incubate the cells on ice for 5 min.
5. Remove the **physiological buffer** and immediately add the previously cooled mCLING solution and incubate for 10 to 15 min. **Note:** At low temperature, mCLING requires longer incubation times to homogeneously reach the cell plasma membrane.
6. Remove the mCLING solution and briefly wash excess of mCLING two times with cold **physiological buffer**.
7. Immediately transfer the coverslips into ice-cold **fixation buffer** and incubate for 20 min on ice followed by 20 min at RT.
8. Wash with **PBS**.
9. Incubate for 30 min in 2 ml **quenching buffer**.
10. Wash with **PBS**.
11. Block for 5 min in **blocking buffer** at RT. Repeat this step 3 times.
12. Incubate with primary antibody for 1 h at RT in **incubation buffer**. **Note:** Overnight incubation at 4°C is not recommended as it might affect the stability of mCLING on the labeled membranes, resulting in a loss of signal.
13. Wash 3 times 10 min each with **PBS** at RT.
14. Incubate with secondary antibody diluted in **incubation buffer** according to the manufacturer's recommendation for 1 h at RT.
    Optional: Add DAPI to the secondary reagent solution.
    Note: Avoid bright light when working with the secondary antibody to minimize photo bleaching of the fluorescent dye.
15. Wash 3 times for 10 min each with **PBS** at RT.
16. Mount coverslips.

Note: For more background information and more application specific protocols, refer to Revelo NH and Rizzoli SO, 2016.