IHC: Staining Protocol - mCLING

General considerations

mCLING can also be used to study synaptic vesicle recycling in more complex tissue preparations. Of course, tissue dissection and treatment must maintain the sample in a viable state to ensure the ongoing of membrane recycling events.
The main practical difficulty when labeling tissues is ensuring that mCLING homogeneously reaches the plasma membrane of the cell or structure of interest. Therefore, it is recommended to make preliminary tests with different incubation times and concentrations. Once efficient penetration is ensured, preparations can be electrically stimulated or depolarized with a solution containing a high concentration of K^+.

Thin tissues can be imaged as a whole. In cases where a very detailed analysis on protein-mCLING colocalization is required, samples should be embedded in melamine and should be cut into thin slices (200 nm thick). If sample labeling is too dense, thickness can be reduced to 50 to 100 nm.

For efficient fixation of mCLING, a mixture of formaldehyde (FA) and glutaraldehyde is required. Scientists use self-made FA fixation solutions produced by dissolving paraformaldehyd (PFA) in phosphate buffered saline (PBS) or they apply a ready-to-use FA fixation solutions containing different amounts of methanol for stabilization.

Materials and reagents

- **Physiological buffer** (e.g. Hank's balanced salt solution or Jan & Jan's buffer depending on the tissue to be stained)
- **Physiological buffer** without Ca^{2+}
- **mCLING** lyophilized
- **PBS:** Phosphate buffered saline, pH 7.4
- **Fixation solution:** 4% FA, 0.2% glutaraldehyde in PBS
- **Ice**
- **Quenching buffer:** 100 mM NH_4Cl, 100 mM Glycine in PBS
- **Blocking buffer:** 0.5% Triton X-100, 2.5% bovine serum albumin (BSA) in PBS
- **Mounting medium**

Procedure

1. Dissect tissue using ice-cold **physiological buffer** without Ca^{2+}. Make sure to remove and clean muscles or cells of interest from other structures like connective, bone, and fat tissue. In poorly cleaned samples, mCLING binds to surrounding connective and fat tissue creating a strong background fluorescence signal.
2. Prepare two working **mCLING** solutions with a concentration between 2 to 1 nmol/ml, one with the standard **physiological buffer** and one with **physiological buffer without Ca^{2+}**. For mammalian samples, keep solutions at 37°C for Drosophila larvae at room temperature. Working mCLING concentrations must be determined via preliminary test experiments. Mammalian tissues require higher concentrations (sometimes up to 2 µM), as molecules are retained by the connective tissue layers lowering the effective concentration of the solution that reaches the cells of interest.
3. Apply **mCLING** solution without Ca^{2+} for 5 to 10 min (at 37°C for mammalian samples or at room temperature for Drosophila larvae experiments) to allow mCLING to penetrate into the tissue and to reach the membranes of the cell or structure of interest. Incubation times should also be established in preliminary test experiments. To determine optimal mCLING concentration and incubation time, perform parallel preparations and vary these two parameters. Samples can be embedded in melamine and cut into thin sections to be imaged in a conventional epifluorescence microscope. In this way, penetration of mCLING into the particular tissue can be evaluated.
4. Replace **mCLING** solution without Ca^{2+} for the standard solution with mCLING and apply electrical stimulation of your choice. Alternatively, the standard solution can be replaced with a high K^+ solution containing the same concentration of mCLING. Briefly wash excess of mCLING with dye-free and Ca^{2+}-free solution.
5. Immediately place the tissue in **fixation solution** and incubate for 30 min on ice. Incubate for another 30 min at room temperature.
6. Wash briefly with **PBS**.
7. Incubate for 30 min at room temperature in **quenching buffer**.
8. Wash briefly with **PBS**.
9. Incubate 3 times for 10 min each in **blocking buffer**.
10. Incubate with primary antibody in **blocking buffer** for 1 h at room temperature. Overnight incubation is not recommended, as this might affect the stability of mCLING on the labeled membranes.
11. Wash 3 times for 10 min each with **blocking buffer**.
12. Incubate with secondary antibody for 1 h at room temperature in **blocking buffer**.
13. Wash 3 times for 10 min each with **PBS**.
14. Mount coverslips.

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