Protocol for Brn3a Antibody (Cat. No. 411 005) Immunohistochemistry Formaldehyde (IHC) Tissue Preparation and Fluorescence staining - Retina

This protocol has been specially developed for the immunohistochemical analysis of formaldehyde (FA) fixed retinal sections. The protocol is based on publications by our academic cooperation partners (Dick et al. 2001; Gierke et al. 2023). The tissue sections are stained on microscope slides.

Materials and reagents

- **PBS:** Phosphate buffered saline, 0.01 M, pH 7.4
- **Fixation buffer:** 4% FA in PBS, pH 7.4
- **Tissue-Tek®** or alternative freezing compound
- **Blocking buffer:** 10% normal serum, 1% bovine serum albumin, 0.5% 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:** 3% normal serum, 1% bovine serum albumin, 0.5% Triton X-100 in PBS (if secondary antibodies are used, normal serum from the host-species of the secondary antibodies is recommended for blocking)
- **Primary antibody:** Brn3a antibody (cat. no. 411 005)
- **Secondary antibody:** Fluorophore conjugated Guinea pig specific secondary antibody
- *Optional: DAPI nuclear stain*
- **Hydrophobic barrier pen**
- **Slides SuperFrost® plus**
- **Mounting medium**

Procedure: Tissue preparation

1. Sacrifice animals according to local guidlines.
2. Remove cornea along the ora serrata before removing lens and vitreous body.
3. Immersion-fix the posterior eyecups with the retinae for 15-30 min in **fixation buffer** at room temperature (RT).
4. Dissect retinae and cryoprotect in rising sucrose series (1 h 10%, 1 h 20%, overnight 30% sucrose in **PBS** at 4°C).
5. Embed retinae in **Tissue-Tek®** or an alternative freezing compund, freeze it, and store it at -80°C until sectioning.
6. Cut retinae vertically into 12-14 µm thick sections with a cryostat and put on slides.
7. Let the cryosections dry for 15 min at RT.

Procedure: Fluorescence staining

1. Surround tissue with hydrophobic pen.
2. Rehydrate and wash section 2 x 10 min in **PBS** at RT.

3. Add **blocking buffer** and block for 1 h at RT in a wet chamber.

4. Remove the **blocking buffer** and add the **incubation buffer** with the **primary antibody** at a dilution of **1:500**.

5. Incubate with the **primary antibody** overnight in a wet chamber at 4°C.

6. Wash three times for 10 min in **PBS** at RT (orbital shaker: 70 - 80 rpm).

7. Transfer the slides back to the wet chamber and apply the **incubation buffer** with the **secondary antibody** diluted to the manufacturer's recommended concentration. *Optional: Add DAPI to the secondary antibody solution.*

8. Incubate for 1 h at RT.
   Notes:
   - *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.*
   - *Avoid bright light when working with the secondary antibody to minimize photo bleaching of the fluorescent dye.*

9. Wash slides once for 10 min in **PBS** in staining dishes at RT (orbital shaker: 70 - 80 rpm).

10. Wash slides twice for 10 min in **PBS** in staining dishes at RT (orbital shaker: 70 - 80 rpm).

11. Mount slides.

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.