**Immunofluorescence Staining Protocol (GFP + Orexin)**

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**Cryosection procedure:**

1. Perfuse animals with ice cold ***or*** RT 4%PFA (temp does not seem to matter)

2. Fix the brain with 4% PFA @ 4°C O/N.

3. Transfer brain sample to 30% sucrose/1xPBS (0.01M PBS), allow sample sink @ 4°C for about 48hr. If brain doesn’t fully sink, it’s OK.

4. Section on freezing microtome and store tissue in 0.01M PBS+ 0.01% NaAz @ 4°C until ready for IHC

**Staining procedure:**

5. Block tissue in 1% BSA in 1X PBS or 1% BSA + 0.2% Triton X-100 for 30 minutes to 1 hour, rocking/shaking @ RT

6. Incubate the sections with primary antibody (1:500 for mouse anti-orexin-A; 1:1000 for rabbit anti-GFP) in 1% BSA + 0.2% Triton X-100 rocking @ RT O/N.

7. Wash the section with 1X PBS three times @ RT, 5 min/each. Save primary antibody: add sodium azide (NaAz) to a final concentration of 0.01% in antibody solution.

8. Incubate the sections with secondary antibody (1:200) in 1X PBS rocking @ RT for 30’ to 1 hour ***or*** 1:500 for 2 hours.

9. Wash the sections with 1X PBS three times at RT, 5 min/each.

10. Mount the sections on subbed slides, don’t let them totally dry

11. Rehydrate tissue briefly if necessary with drops of 1X PBS and wipe off excess liquid around sections with a kimwipe

12. Add 3-4 drops of aqueous mounting medium and coverslip (I prefer Prolong Gold from Invitrogen—don’t use mount with DAPI!).

**Antibody:**

**Primary antibodies:**

1. Thermo Fisher Scientific, anti-GFP (A-11122) Rabbit mAb; STORED IN FRIDGE (4-8 degrees)
2. R&D Systems, anti-Orexin-A MAB763, mouse mAb; STORED IN FREEZER (-20)

**Secondary antibody:** Stored in fridge (4 degrees)

1. Invitrogen, Alexa Fluor® 488 goat anti-rabbit IgG (H+L), Cat# A-11008
2. Invitrogen, Alexa Fluor® 594 goat anti-mouse IgG (H+L) Cat# A-11032