

MCFO IHC for Larval CNS

- All tissues and solutions are at room temperature (RT), unless noted. Always protect tissue from light exposure.
- For details on dissection and fixation see FlyLight Protocol - Larval Dissection and 4% Fixation.
- For mounting and embedding instructions refer to FlyLight Protocol – DPX Mounting.
- For videos of dissection of larval CNS see Larval CNS dissection.
- For videos of mounting for DPX embedding of larval CNS see Larval Mounting.
- For video demonstrations of DPX embedding see the movie DPX Embedding.

1. **Dissect.** Dissect larval CNS in cold phosphate buffer saline 1X (PBS).
2. **Fix.** Transfer tissue to 2 mL Protein LoBind tubes filled with cold 4% paraformaldehyde (PFA) in PBS 1X kept on ice. Move to RT and incubate for 1 hour at RT while nutating.
3. **Post-fix wash.** Remove the fix and add 1.75 mL phosphate buffered saline with 1% Triton X-100 (PBT) and wash for a total of 4 X 15-minutes washes while nutating. If needed, store tissue at 4°C in 1% PBT while nutating or rotating.
4. **Block Normal Donkey Serum (NDS).** Remove PBT and add 200 µL NDS (1:20 or 50 µL/mL) in PBT per tube. Incubate for 2 hours at RT on a rotator with tubes upright.
5. **Primary antibodies.** Remove block and add primary antibodies diluted in NDS + PBT for a volume of 200 µL per tube. Incubate for 4 hours at RT on a rotator with tubes upright. Then continue incubation at 4°C on a rotator with tubes upright for 2 overnights.
 - Mouse α -Neuroglial (1:50 or 20 µL/mL)
 - Rabbit α -HA Tag (1:500 or 2 µL/mL)
 - Rat α -FLAG Tag (1:500 or 2 µL/mL)
6. **Post- primary washes.** Remove the primary antibodies and add 1.75 mL of 1% PBT. Wash 15 minutes. Repeat for a total of 4 X 15-minute washes while nutating.
7. **Secondary antibodies.** Remove the PBT and add the secondary antibodies diluted in NDS + PBT for a volume of 200 µL per tube. Incubate for 4 hours at RT on a rotator with tubes upright. Continue incubation at 4°C on a rotator with tubes upright for 2 overnights.
 - AF488 Donkey α -Mouse (1:500 or 2 µL/mL)
 - DL549 Goat α -Rabbit (1:800 or 1.25 µL/mL)
 - AF594 Donkey α -Rat (1:700 or 1.42 µL/mL)
8. **Post-secondary washes.** Remove the secondary antibodies and add 1.75 mL of 1% PBT. Wash 15 minutes. Repeat for a total of 4 X 15-minute washes while nutating.
9. **Block Normal Mouse Serum (NMS).** Remove PBT and add 200 µL NMS (1:20 or 50 µL/mL) in PBT per tube. Incubate for 2 hours at RT on a rotator with tubes upright.

10. **Direct Label α -V5 antibody.** Remove NMS block and add AF647 Mouse α -V5 in NMS + PBT for a volume of 200 μ L per tube. Incubate for 4 hours at RT on a rotator with tubes upright. Then continue incubation at 4°C on a rotator with tubes upright for 1 overnight.
 - AF647 Mouse α -V5 Tag (1:200 or 5 μ L/mL)
11. **Post- α -V5 washes.** Remove the α -V5 antibody and do a brief rinse with 1.75 mL 0.5% PBT. Allow the tissue to settle to the bottom and then remove the rinse solution and add 1.75 mL 0.5% PBT. Wash for a total of 3 X 30-minute washes while nutating (or 4 X 15 minutes).
12. **Mount.** Mount the tissue on a poly-L-lysine (PLL) coated cover glass.
 - For making PLL see FlyLight Recipe – Poly-L-Lysine.
13. **Dehydrate.** Move the cover glass through a series of 7 cover glass staining jars filled with increasing concentrations of ethanol (30%, 50%, 75%, 95%, 100%, 100%, 100%). Soak the cover glass for 10 minutes in each jar.
14. **Xylene clearing.** (IN THE HOOD). Move the cover glass through a series of 3 jars filled with xylene. Soak the cover glass for 5 minutes in each jar.
15. **DPX embedding.** Add 7 drops of dibutyl phthalate in xylene (DPX) on top of the tissue mounted on the cover glass. Place the cover glass (DPX down) on a prepared slide with spacers. Use the edge of a glass slide to gently press down on the center of the cover glass to seat the cover glass onto the slide. Let the slide dry in the hood for 2 days before viewing.

Reporter Genotype

- pBPhsFlp2::*PEST* in attP3; ;pJFRC201-10XUAS-FRT>STOP>FRT-myr::*smGFP-HA* in VK0005, pJFRC240-10XUAS-FRT>STOP>FRT-myr::*smGFP-V5-THS*-10XUAS-FRT>STOP>FRT-myr::*smGFP-FLAG* in su(Hw)attP1
- For details on reporter constructs see Nern *et al.* 2015.

Reagents and Supplies

- AF488 Donkey α -Mouse. Jackson Immuno Research. # 715-545-151
- AF594 Donkey α -Rat. Jackson Immuno Research. # 712-585-150
- AF647 Mouse α -V5 Tag. AbD Serotec. #MCA1360A647
- DL549 Goat α -Rabbit. Rockland Antibodies and Assays. # 611-142-002
- DPX Mountant for Microscopy. Electron Microscopy Sciences. # 13512, 500 mL
- Ethanol. ACS reagent, >99.5% (200 proof). Sigma Aldrich. # 459844-1L
- Mouse α -Neuroglian. Developmental Studies Hybridoma Bank. # BP104 anti-Neuroglian-s
- NDS. Jackson Immuno Research. # 017-000-121
- Kodak Photo-Flo 200 Solution. Electron Microscopy Sciences. # 74257
- PBS - Phosphate Buffered Saline, 1X. Cellgro. # 21-040
- PFA – Paraformaldehyde. 20% PFA. Electron Microscopy Sciences. # 15713-S
- Poly-L-Lysine. Sigma Aldrich. # P1524-25MG
- Protein LoBind Microcentrifuge Tubes. Eppendorf. # 022431102
- Rat α -FLAG Tag (DYKDDDDK Epitope Tag). Novus Biologicals. # NBP1-06712
- Rabbit α -HA Tag. Cell Signal Technologies. # 3724S
- Triton X-100. Sigma Aldrich. # X100
- Xylenes. Fisher Scientific. # X5-500