

### Polarity Sequential IHC for Adult 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 Protocol - Adult Dissection and 2% Fixation.
- For mounting and embedding instructions refer to Protocol – DPX Mounting.

Protocol based on collaboration with R.M. Johnston and based on work described in:

Aso Y, Hattori D, Yu Y, Johnston RM, Iyer NA, Ngo T-TB, et al. The neuronal architecture of the mushroom body provides a logic for associative learning. *Elife*. 2014; 3: e04577. doi: 10.7554/eLife.04577

1. **Dissect.** Dissect adult brains or CNS in cold Schneider's Insect Medium (S2).
2. **Fix.** Transfer tissue to 2 mL Protein LoBind tubes filled with 2% paraformaldehyde (PFA) in S2 at RT. Fix for 55-60 minutes at RT while nutating.
3. **Post-fix wash.** Remove the fix and add 1.75 mL phosphate buffered saline with 0.5% Triton X-100 (PBT) and wash for a total of 4 X 10-minutes washes while nutating. If needed, store tissue in 0.5% PBT at 4°C while nutating or lay tube flat and rotate.
4. **Block Goat Serum (GS).** Remove PBT and add 200 µL 5% GS in PBT per tube. Incubate for 1.5 hours at RT on a rotator with tubes upright.
5. **Reference primary antibodies.** Remove block and add primary antibody diluted in 5% GS in 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 nc82 (1:30 or 33.3 µL/mL)
6. **Post-reference primary washes.** Remove the primary 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).
7. **Reference secondary antibody.** Remove PBT and add the reference primary antibody diluted in 5% GS in 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 2-3 overnights.
  - Cy2 Goat α-Mouse (1:600 or 1.67 µL/mL)
8. **Second Fixation.** Transfer tissue to 2 mL Protein LoBind tubes filled with 2% paraformaldehyde (PFA) in PBS at RT. Fix for 55 minutes at RT while nutating
9. **Post-fix wash.** Remove the fix and add 1.75 mL phosphate buffered saline with 0.5% Triton X-100 (PBT) and wash for a total of 4 X 10-minutes washes while nutating. If needed, store tissue in 0.5% PBT at 4°C while nutating or lay tube flat and rotate.
10. **Block #2 GS.** Remove PBT and add 200 µL of 5% GS in PBT per tube. Incubate for 1.5 hours at RT on a rotator with tubes upright

11. **Neuron primary antibodies.** Remove block and add primary antibodies diluted in 5% GS in PBT for a volume of 200  $\mu$ 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 2 overnights.
  - Rat  $\alpha$ -FLAG Tag (1:100 or 10  $\mu$ L/mL)
  - Rabbit  $\alpha$ -HA Tag (1:600 or 1.67  $\mu$ L/mL)
12. **Post-neuron primary washes.** Remove the neuron primary antibodies 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).
13. **Neuron secondary antibodies.** Remove the PBT and add the secondary antibodies diluted in 5% GS in PBT for a volume of 200  $\mu$ 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 2-3 overnights.
  - ATTO647N Goat  $\alpha$ -Rat (1:150 or 6.6  $\mu$ L/mL)
  - Cy3 Goat  $\alpha$ -Rabbit (1:1000 or 1  $\mu$ L/mL)
14. **Post-secondary washes.** Remove the secondary antibodies 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). If needed, store tissue in 0.5% PBT at 4°C while nutating or lay tube flat and rotate.
15. **Pre-embedding fixation.** Remove PBT and add 1.75 mL 4% PFA in PBS at RT. Fix for 4 hours at RT while nutating.
16. **Post-4% PFA washes.** Remove the 4% PFA 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 4 X 15-minute washes while nutating. If needed, store tissue in 0.5% PBT at 4°C while nutating or lay tube flat and rotate.
17. **Mount.** Mount the tissue on a poly-L-lysine (PLL) coated cover glass.
18. **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.
19. **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.
20. **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

- pJFRC225-5xUAS-IVS-myr::smGFP-FLAG in VK00005, pJFRC51-3xUAS-Syt::smGFP-HA in su(Hw)attP1

## Reagents and Supplies

- Cy2 Goat  $\alpha$ -Mouse. Jackson Immuno Research. # 115-225-166
- Cy3 Goat  $\alpha$ -Rabbit. Jackson Immuno Research. # 111-165-144
- ATTO 647N Goat  $\alpha$ -Rat IgG (H&L) Antibody. Rockland. # 612-156-120
- DPX Mountant for Microscopy. Electron Microscopy Sciences. # 13512, 500 mL
- Ethanol, ACS reagent, >99.5% (200 proof). Sigma Aldrich. # 459844-1L
- GS – Goat Serum. Life Technologies. # 16210-064, 100 mL
- nc82 – Mouse  $\alpha$ -bruchpilot. Developmental Studies Hybridoma Bank. # nc82-s
- 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  $\alpha$ -FLAG Tag (DYKDDDDK Epitope Tag). Novus Biologicals. # NBP1-06712
- Rabbit  $\alpha$ -HA Tag. Cell Signal Technologies. # 3724S
- S2 – Schneider's Insect Medium. Sigma Aldrich. # S01416
- Triton X-100. Sigma Aldrich. # X100
- Xylenes. Fisher Scientific. # X5-500