

Double Label 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 FlyLight Protocol - Adult Dissection and 2% Fixation.
- For mounting and embedding instructions refer to FlyLight Protocol – DPX Mounting.
- For videos of dissection of adult brains see Adult Brain Dissection or for adult CNS see Adult CNS dissection.
- For videos of mounting for DPX embedding of adult CNS see Adult Mounting or for larval CNS see Larval Mounting.
- For video demonstrations of DPX embedding see the movie DPX Embedding.

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 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-minute 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. **Primary antibodies.** Remove block and add primary antibodies 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)
 - Rabbit α-HA Tag (1:300 or 3.3 µL/mL)
6. **Post-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. **Secondary antibodies.** Remove PBT and add the secondary antibodies 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 3 overnights.
 - Cy2 Goat α-Mouse (1:400 or 2.5 µL/mL)
 - Cy5 Goat α-Rabbit (1:400 or 2.5 µL/mL)
8. **Post-secondary washes.** Remove the secondary 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). If needed, store tissue in 0.5% PBT at 4°C while nutating or lay tube flat and rotate.
9. **Block Normal Mouse Serum (NMS).** Remove PBT and add 200 µL of 5% NMS in PBT per tube. Incubate for 1.5 hours at RT on a rotator with tubes upright

10. **Direct Label α -V5 antibody.** Remove NMS block and add DL550 Mouse α -V5 in 5% NMS in PBT. 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.
 - DL550 Mouse α -V5 (1:500 or 2 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. **Pre-embedding fixation.** Remove PBT and add 1.75 mL 4% PFA in PBS at RT. Fix for 4 hours at RT while nutating.
13. **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.
14. **Mount.** Mount the tissue on a poly-L-lysine (PLL) coated cover glass.
 - For making PLL see FlyLight Recipe – Poly-L-Lysine.
15. **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.
16. **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.
17. **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

- 10XUAS-IVS-myr::smGFP-HA in attP18, 13XLexAop2-IVS-myr::smGFP-V5 in su(Hw)attP8

Reagents and Supplies

- Cy2 Goat α -Mouse. Jackson Immuno Research. # 115-225-166
- Cy5 Goat α -Rabbit. Jackson Immuno Research. # 711-585-152
- DL550 Mouse α -V5 Tag. AbD Serotec. # MCA1360D550GA
- 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
- Kodak Photo-Flo 200 Solution. Electron Microscopy Sciences. # 74257
- nc82 – Mouse α -bruchpilot. Developmental Studies Hybridoma Bank. # nc82-s
- NMS – Normal Mouse Serum. Jackson Immuno Research. # 015-000-120
- 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 - 2 mL. Eppendorf. # 022431102
- S2 – Schneider's Insect Medium. Sigma Aldrich. # S01416
- Rabbit α -HA Tag. Cell Signal Technologies. # 3724S
- Triton X-100. Sigma Aldrich. # X100
- Xylenes. Fisher Scientific. # X5-500

Imaging Protocol – Double Label

Track 1 Ch1	Cy2	498-543 nm	Neuropil (reference)
Track 1 Ch2	Cy5	654-735 nm	Neuron
Track 2 ChS1	DL550	585-623 nm	Neuron
Dichromatic Mirror	MBS 488/561/633		
		20X	63X
	Resolution	1024 x 1024	1024 x 1024
	Pixel size	.52 x .52	.19 x .19
	Speed (pixel dwell)	7 (1.58 μ s)	9 (0.79 μ s)
	Bit	12	12
	Direction	Bidirectional \leftrightarrow	Unidirectional \rightarrow
	Average	1	1 (or 2)
	Zoom	0.8	0.7 (or higher)
	Pinhole (488)	38	68
	Interval	1 μ m	0.38 μ m (or .19)