α-GFP 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-minutes washes while nutating. (Option: 4 X 15-minute washes.) 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 (36-48 hours).
    - Mouse nc82 (1:30 or 33.3 µL/mL)
    - Rabbit polyclonal α-GFP (1:1000 or 1 µL/mL)
6. **Post-primary washes.** Remove the 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. (Option: 4 X 15-minute washes.)
7. **Secondary antibodies**. Remove PBT and add 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.
    - AF568 Goat α-Mouse (1:400 or 2.5 µL/mL)
    - AF488 Goat α-Rabbit (1:800 or 1.25 µL/mL)
8. **Post-secondary washes.** Remove the secondary antibodies and rinse briefly 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. (Option: 4 X 15-minute washes.) If needed, store tissue in 0.5% PBT at 4°C while nutating or lay tube flat and rotate.

    Option: For mounting in a glycerol medium, such as SlowFade Gold, proceed to FlyLight Protocol - Glycerol Mounting. For xylene clearing and DPX embedding follows steps 9-14, below.

9. **Pre-embedding fixation**. Remove PBT and add 1.75 mL 4% PFA in PBS at RT. Fix for 4 hours at RT while nutating.
10. **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.
11. **Mount**. Mount the tissue on a poly-L-lysine (PLL) coated cover glass.
    - For making PLL see FlyLight Recipe – Poly-L-Lysine.
12. **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.
13. **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.
14. **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

- AF488 Goat α-Rabbit. Life Technologies. # A11034
- AF568 Goat α-Mouse. Life Technologies. # A11031
- 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
- 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
- Rabbit polyclonal α-GFP Fraction. Life Technologies. # A11122
- S2 – Schneider's Insect Medium. Sigma Aldrich. # S01416
- Triton X-100. Sigma Aldrich. # X100
- Xylenes. Fisher Scientific. # X5-500

Imaging Protocol

Track 1 Ch 1	AF488	498-552 nm	Neuron
Track 2 Ch2	AF568	588-733 nm	Neuropil (reference)
Dichromatic Mirror	MBS 488/561		
		20X	**63X**
	Resolution	1024 x 1024	1024 x 1024
	Pixel size	.52 x .52	.19 x .19
	Speed (pixel dwell)	7 (1.58 µs)	9 (0.79 µs)
	Bit	12	12
	Direction	Bidirectional ↔	Bidirectional ↔
	Average	1	1
	Zoom	0.8	0.7
	Pinhole (488)	38	68
	Interval	1 µm	0.38 µm