MCFO Hybrid Chemical Tag & 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. **Prepare chemical tag stock.** Dilute Cy2 SNAP-tag ligand in anhydrous DMSO to a concentration of 200 µM to create a 100x stock solution. Store in aliquots at -20°C. The stock is stable for several months.
2. **Dissect**. Dissect adult brains or CNS in cold Schneider's Insect Medium (S2).
3. **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.
4. **Post-fix wash**. Remove the fix and add 1.75 mL phosphate buffered saline with 0.5% Triton X-100 (PBT) and wash for 10-15 minutes while nutating. Perform up to 3 additional washes if waiting to add chemical tag. Samples may be held in PBT for up to 7 hours prior to chemical tagging.
5. **Chemical tag labeling**. Remove PBT and add 200 µL 2 µM Cy2 SNAP-tag ligand in PBT per tube. Incubate for 15 minutes at RT on a rotator with tubes upright.
    - Cy2 SNAP-tag ligand (10 µL/mL for final concentration of 2 µM)
6. **Post-chemical tag washes.** Remove the chemical tag 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 2 X 10-minute washes while nutating. Samples can be held for at least 3 days prior to beginning IHC steps below.
7. **Block Goat Serum (GS) & Normal Mouse Serum (NMS)**. Remove PBT and add 200 µL 5% GS, 5% NMS in PBT per tube. Incubate for 1.5 hours at RT on a rotator with tubes upright.
8. **Primary antibodies**. Remove block and add primary antibodies diluted in 5% GS, 5% NMS 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.
    - Rat α-FLAG Tag (1:200 or 5 µL/mL)
    - Rabbit α-HA Tag (1:300 or 3.3 µL/mL)
9. **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.
10. **Secondary & direct label antibodies**. Remove PBT and add the secondary antibodies diluted in 5% GS, 5% NMS 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 3-4 overnights.
    - ATTO647N Goat α-Rat (1:300 or 3.3 µL/mL)

- AF594 Donkey α-Rabbit (1:500 or 2 µL/mL)
- DL550 Mouse α-V5 (1:500 or 2 µL/mL)

11. **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. If needed, store tissue in 0.5% PBT at 4°C while nutating or lay tube flat and rotate.
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

- pBPhsFlp2::PEST in attP3; brp-SNAP / CyO; 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. http://www.pnas.org/content/112/22/E2967.long doi: 10.1073/pnas.1506763112
- For details on brp-SNAP chemical tagging, see Kohl, et al., 2014. http://www.pnas.org/content/111/36/E3805.long doi: 10.1073/pnas.1411087111
- For details of hybrid MCFO protocol, see Meissner, et al., 2018. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0200759 doi: 10.1371/journal.pone.0200759

Reagents and Supplies

- AF594 Donkey α-Rabbit. Jackson Immuno Research. # 711-585-152
- ATTO 647N Goat α-Rat IgG (H&L) Antibody. Rockland. # 612-156-120
- 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
- Cy2 SNAP-tag ligand, Luke Lavis, JRC
- DMSO, Hybri-Max, (dimethyl sulfoxide). Sigma Aldrich # D2650-5X5ML
- 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
- Rat α-FLAG Tag (DYKDDDDK Epitope Tag). Novus Biologicals. # NBP1-06712
- Triton X-100. Sigma Aldrich. # X100
- Xylenes. Fisher Scientific. # X5-500

Imaging Protocol - MCFO

Configuration 1	Track 1 Ch 1	AF488	498-543 nm	Neuropil (reference)
	Track 2 Ch 1	AF594	600-638	Neuron
	Dichromatic Mirror	MBS 488/594		
Configuration 2	Track 1 Ch 1	AF488	498-543 nm	Neuropil (reference)
	Track 1 Ch2	AT647	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 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	