MCFO Hybrid Chemical Tag & IHC for Adult CNS

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Abstract

The use of genetically encoded 'self-labeling tags' with chemical fluorophore ligands enables rapid labeling of specific cells in neural tissue. To improve the chemical tagging of neurons, we synthesized and evaluated new fluorophore ligands based on Cy, Janelia Fluor, Alexa Fluor, and ATTO dyes and tested these with recently improved *Drosophila melanogaster* transgenes. We found that tissue clearing and mounting in DPX substantially improves signal quality when combined with specific non-cyanine fluorophores. We compared and combined this labeling technique with standard immunohistochemistry in the Drosophila brain.

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Guidelines

- 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.
- Protocols at https://www.janelia.org/project-team/flylight/protocols
- **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, please refer to Kohl, et al., 2014. http://www.pnas.org/content/111/36/E3805.long doi: 10.1073/pnas.1411087111

Before start

Reagents and Supplies

- AF594 Donkey α- 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 ligand, Luke Lavis, JRC
- NMS – Normal Mouse Serum. Jackson Immuno Research. # 015-000-120

- PBS – Phosphate Buffered Saline, 1X**.** # 21-040
- PFA – Paraformaldehyde. 20% PFA. Electron Microscopy Sciences. # 15713-S
- Poly-L-Lysine. Sigma Aldrich. # P1524-25MG
- Protein LoBind Microcentrifuge Tubes - 2 mL. # 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
- Fisher Scientific. # X5-500

Protocol

Step 1.
Dissect. Dissect adult brains or CNS in cold Schneider's Insect Medium (S2).

Step 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.

Step 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 10-15 minute washes 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.

Step 4.
Chemical tag labeling. Remove PBT and add 200 µL 2 µM Cy2 Snap ligand in PBT per tube. Incubate for 15 minutes at RT on a rotator with tubes upright.

Cy2 Snap ligand (2 µL/mL for final concentration of 2 µM)

Step 5.
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.

Step 6.
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.

Step 7.
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.

Antibodies:
Rat α-FLAG Tag (1:200 or 5 µL/mL)
Rabbit α-HA Tag (1:300 or 3.3 µL/mL)

Step 8.
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 5 X 15-minute washes while nutating.

Step 9.
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.

Antibodies:
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)

Step 10.
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 5 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.

Step 11.
Pre-embedding fixation. Remove PBT and add 1.75 mL 4% PFA in PBS at RT. Fix for 4 hours at RT while nutating.

Step 12.
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.

Step 13.
Mount. Mount the tissue on a poly-L-lysine (PLL) coated cover glass.• For making PLL see FlyLight Recipe – Poly-L-Lysine.

Step 14.
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.

Step 15.
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.

Step 16.
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.