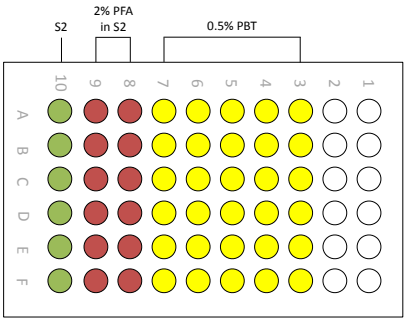
Split GAL4 Screen IHC for Adult CNS

- Protect samples from light at all steps.
- Handle tissue with forceps or MicroSieve to avoid transferring solution from well-to-well.

- 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.

Prepare MicroWell plates. Make only the plates needed for one day. Fill wells in sequential columns with 10 µL each of Schneider's Insect Medium (S2), 2% paraformaldehyde (PFA) in S2 (2 wells), and phosphate buffered saline with 0.5% Triton X-100 (PBT) (5 wells). Store extra plates at 4°C during the day. To reduce evaporation if the tissue will be held for more than two days prior to mounting, add a moistened 6 x 40 mm piece of filter paper to the far-right side of the plate.



1. **Dissect**. Move a plate to room temperature. Dissect adult brains or CNS in cold S2 and transfer to individual S2-filled wells until plate is full.
2. **Fix**. Transfer tissue from S2-filled well to 2% PFA-filled well. Rinse briefly then transfer to second fix well and incubate at RT for 65 minutes. Wash off the fix by passing the tissue through each of the 5 PBT-filled wells. Rinse briefly in the first well, then allow the tissue to soak in remaining wells for 10-15 minutes. Tissue can be stored in the last well of PBT for up to two days prior to mounting. If the tissue will be held longer, every two days fill an additional column with fresh PBT and transfer all tissue to that column.
3. **Mount**.

   For mounting instructions refer to FlyLight Protocol – DPX Mounting of *Drosophila* CNS.

   - *Day before*: Prepare cover glass by dipping in poly-L-lysine (PLL) and place in slide holder to protect from dust while drying. One to two dips each dried overnight works well.
     - For making PLL see FlyLight Recipe – Poly-L-Lysine.
   - *Day of*: Transfer each tissue to a PBS-filled well on the mounting dish to remove the PBT. Tissue won't stick to the PLL if Triton in PBT is present. Mount the tissue (posterior down) on a PLL-coated cover glass while keeping the tissue submerged in PBS.
4. **Block in Goat Serum (GS)**. Move the cover glass to a cover glass staining jar filled with 10 mL of 5% GS in PBT. Incubate while rotating for 1-1.5 h at RT.
5. **Primary antibodies**. Move the cover glass to a jar with 10 mL of primary antibodies diluted in 5% GS in PBT. Incubate 4 hours at RT, then at 4°C for 2-3 overnights on a rotator.
   - Mouse nc82 (1:30 or 33 µL/mL)
   - Rabbit α-GFP (1:1000 or 1 µL/mL)

6. **Wash**. Move the cover glass to a jar with 10 mL of 0.5% PBT and soak at RT for 10 minutes while on the rotator. Pour off PBT and replace with new PBT. Repeat for a total of 5 X 10-minute washes while rotating.
7. **Secondary antibodies**. Move the cover glass to a jar with 10 mL of secondary antibodies diluted in 5% GS in PBT. Incubate for 4 hours at RT on a rotator. Continue incubation at 4°C on a rotator for 2-3 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**. Move the cover glass to a jar with 10 mL of 0.5% PBT and soak while on the rotator for 10 minutes. Pour off PBT and replace with new PBT. Repeat for a total of 5 X 10-minute washes while rotating.
9. **Pre-embedding fixation**. Move the cover glass to a jar of 10 mL 4% PFA in PBS. Fix for 4 hours at RT on a rotator.
10. **Post-4% PFA washes**. After 4% PFA fixation is complete, move the cover glass to a jar with 10 mL of 0.5% PBT and soak while on the rotator for 15 minutes. Pour off PBT and replace with new PBT for a total of 3 X 15 minute washes. Rotate jar during washes.
11. **Dehydration**. Briefly dip the cover glass in MilliQ water and then move the cover glass through a series of 7 cover glass jars filled with increasing concentrations of ethanol (30%, 50%, 75%, 95%, 100%, 100%, 100%). Soak the cover glass for 10 minutes in each jar. Take care to maintain the orientation of the cover glass (tissue toward the front of the jar).
12. **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.
13. **DPX embedding**. Add 7 drops of 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.

Reagents and Supplies

- AF488 Goat α-Rabbit. Invitrogen. # A11034
- AF568 Goat α-Mouse. Life Technologies. # A11031
- Cover glass staining jars with plastic screw cap. Electron Microscopy Sciences. # 72242-24 (4 pack)
- Extra Thick Western Blotting Filter Paper 8cm x 13.5cm. Thermo Scientific. #88615
- GS - Goat Serum. Life Technologies. # 16210-064
- MicroSieve. MiTeGen. Contact company for ordering information.
- nc82 – Mouse α-bruchpilot. Developmental Studies Hybridoma Bank. # nc82-s
- Nunc MicroWell MiniTrays. Fisher Scientific. (60 well, case of 100) # 439225
- PBS - Phosphate Buffered Saline, 1X**.** Cellgro. # 21-040
- PFA – Paraformaldehyde, 20% PFA. Electron Microscopy Sciences. # 15713
- Poly-L-lysine hydrobromide (PLL). Sigma Aldrich. # P1524-25MG
- Protein LoBind MicrocentirifugeTubes - 2 mL. Eppendorf. # 022431102
- Rabbit Polyclonal α-GFP. Life Technologies. # A11122
- S2 – Schneider's Insect Medium. Sigma Aldrich. # S01416
- Triton X-100. Sigma Aldrich. # X100

Protocol modified from Wu and Nern (2013), Immunostaining of Adult Brains on poly-L-Lysine (Rubin lab, HHMI-Janelia).

Imaging Protocol

Track 1 Ch 1	AF488	498-543 nm	Neuron
Track 2 Ch2	AF568	588-735 nm	Neuropil (reference)
Dichromatic Mirror	MBS 488/561		
		20X	
	Resolution	1024 x 1024	
	Pixel size	.52 x .52	
	Speed (pixel dwell)	7 (1.58 µs)	
	Bit	12	
	Direction	Bidirectional ↔	
	Average	1	
	Zoom	0.8	
	Pinhole (488)	38	
	Interval	1 µm	