

Antibody stain – basic protocol
Per S. Sweeney, adapted by F. Wolf /M. Sniffen, 2005

Dissection and fixation

1. Remove brains.
2. Fix brains in 4% Paraformaldehyde/PBT for 30 min. at room temperature (RT).
3. Wash 3x in PBT for 10 min. at RT.
4. Transfer brains to 200 μ l clear-walled microcentrifuge tubes.
5. To continue to primary incubation, Block [PBT + 0.5% Bovine serum albumin (BSA) (5 mg/ml) + 5% Normal goat serum (NGS) + 0.02% NaN₃] for 2 hours rocking at Room Temperature; OR you can store in block at 4°C overnight.

Primary antibody incubation

6. Add 1° Ab(s) in blocking solution.
7. Incubate on rocker ~16-72 hr at 4°C.
8. Remove 1° Ab and store at 4° C for reuse. Record: date/dilution/diluent.
9. Wash 5x 30 min. in PBT (through overnight at 4°, if desired) on rocker. [These can be shorter washes, like 3-5x 10 minutes, if proceeding through secondary antibody in the same day.]

Secondary antibody incubation

10. Block brains overnight at 4°C, or for 1 hour at room temperature on a nutator/rocker.
11. Add 2° Ab(s) in blocking solution at the recommended dilution.
12. Incubate about 2 hrs at room temperature on a rocker.
13. Wash 5x 10 minutes in PBT at room temperature on a rocker.
14. Remove final wash and replace with 2 drops of fluorescent-protective mounting medium (Vectashield or ProLong Gold). Store overnight at 4°, or until brains sink to the bottom of the tube. [The equilibration before mounting is not entirely necessary, but recommended.]

Mounting

14. Transfer brains to a microscope slide using a wide-bore pipette. Add coverslip. Avoid capturing any air bubbles. Add more mounting medium as needed. Soak extra liquid away with a Kimwipe.
15. Allow mounting medium to cure for a couple of hours to overnight in the dark on a flat surface.
16. Seal with nail polish.

Tips for working with antibodies:

- A. Depending on the affinity of the primary antibody, the incubation period at step 7 can be increased up to 3 weeks.
- B. Antibodies should be saved and reused; their background staining is generally reduced.
- C. You must match the host species of the primary antibody (e.g anti-GFP, from mouse) with the antigen for the secondary antibody (e.g. anti-mouse, Cy3, from goat).
- D. If performing a multiple-antibody stain, be sure that (a) the primary antibodies are not from the same host, (b) the secondary antibodies will each recognize only one primary antibody

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each, and (c) the hosts of the secondary antibodies are selected so that they will not cross-react with one another.

- E. The constitution and/or concentration of PBT can be varied to improve antibody penetration. PBS/0.1-0.3% Tween is not such a strong detergent, but PBS/0.1-0.3% Triton X-100 can be used for stronger penetration.
- F. Matching the donor species of the block serum with that of the host of the secondary antibody can help reduce background levels of staining.
- G. Antibody dilutions are generally determined by trial and error. For typical monoclonal primary antibodies, dilutions of 1:100 to 1:500 are common. For typical secondary antibodies, dilutions of 1:500 to 1:1000 or more are generally sufficient.