

### **Immunofluorescence Labeling of Free-Floating Perfusion-Fixed Brain Sections**

Note: protocol can be completed in one day by shortening primary antibody incubation (Step 3).

Day 1.

- 1) Section preparation: wash sections four times with ice-cold (4°C) 0.1 M PB (recipe below) with gentle rocking/agitation at room temperature (RT) for 5 min each.
- 2) Block: incubate sections in vehicle (recipe below) with gentle rocking/agitation at 4°C for 1 h.
- 3) Primary antibody incubation: transfer sections into ice-cold (4°C) vehicle containing diluted NeuroMab(s) and incubate with gentle rocking/agitation at RT for 3 h (or at 4°C overnight).

Note: antibody concentrations and incubation conditions should be determined empirically for each combination of target sample, NeuroMab, secondary antibody and detection system but, as a general guide, NeuroMabs should be used between 0.1 and 10 µg/mL.

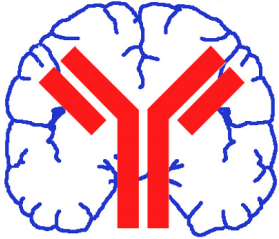
Day 2.

- 4) Wash: transfer sections into fresh ice-cold (4°C) vehicle and wash a total of four times with gentle rocking/agitation at RT for 5 min each.
- 5) Secondary antibody incubation: transfer sections into ice-cold (4°C) vehicle containing fluorescently-conjugated anti-mouse secondary antibodies diluted as per manufacturer's recommendations (e.g., 0.5 µg/mL of Alexa Fluor 488 conjugated anti-mouse IgG1 antibody, Thermo Fisher Scientific catalog # A-21121) and incubate with gentle rocking/agitation protected from light at 4°C for 1 h.

Note: use subclass-specific secondary antibodies matched to the NeuroMab IgG subclass (*i.e.*, IgG1, IgG2a or IgG2b) for optimal signal detection and lowest background (Manning et al., PLoS One 7:e38313, 2012, <http://www.ncbi.nlm.nih.gov/pubmed/22675541>).

- 6) Wash: transfer sections into 0.1 M PB, wash twice with gentle rocking/agitation at RT for 5 min each and wash once with 0.05 M PB with gentle rocking/agitation at RT for 5 min.
- 7) Mounting: transfer sections into 15 mL Petri dish containing 0.05 M PB, carefully mount onto gelatin-coated microscope slides and allow to air dry.
- 8) Sudan Black B treatment (optional, see below).
- 9) Coverslipping: pipet anti-fade medium (e.g., ProLong Gold Antifade Mountant, Thermo Fisher Scientific catalog # P36930) on each section according to manufacturer's instructions, place

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glass coverslip on top, cure for at least 24 hours and seal edges with nail polish.

Note: tissue autofluorescence can be reduced with an optional Sudan Black B treatment (SBB, Oliveira et al., *Histol Histopathol* 25:1017, 2010, <https://www.ncbi.nlm.nih.gov/pubmed/20552552>).

- 1) Completely rehydrate slide-mounted sections with ddH<sub>2</sub>O
- 2) Completely submerge slide in a tray containing 0.05% SBB (recipe below).
- 3) Incubate with gentle rocking/agitation at RT for 5 min. Sections will become blue.
- 4) Remove slide from tray and rinse well with ddH<sub>2</sub>O, paying attention to remove any SBB particulates.
- 5) Air dry and proceed to coverslipping step.

### Recipes:

0.1 M PB: Dilute 500 mL of stock 0.4 M PB (see perfusion protocol for recipe) into ≈1400 mL ddH<sub>2</sub>O, adjust pH to 7.4 and bring to final volume of 2 L.

Vehicle: 10% normal goat serum and 0.3% Triton X-100 in 0.1 M PB

SBB solution:

- 1) Prepare stock solution of 1% SBB (Electron Microscopy Sciences catalog # 21610) in 100% ethanol.
- 2) Vortex or place into a sonicating water bath for 1 h.
- 3) Vacuum filter SBB through 0.45 μM cellulose acetate, low protein binding membrane (Corning catalog # 430314).
- 4) Prepare working 0.05% SBB solution. Dilute 2.5 mL SBB stock in 47.5 mL 70% ethanol **immediately** prior to each use in order to minimize particulates. If autofluorescence levels are high, then higher SBB concentrations (up to 0.15%) may be justified but increasing SBB concentrations may also reduce immunofluorescence signal.

Note: stock SBB solution can be reused but must be sonicated for at least 1 h prior to preparing working solution.

Refs: Oliveira et al., *Histol Histopathol* 25:1017, 2010 (<https://www.ncbi.nlm.nih.gov/pubmed/20552552>);  
Manning et al., *PLoS One* 7:e38313, 2012 (<https://www.ncbi.nlm.nih.gov/pubmed/22675541>);  
Bishop et al., *J Neurosci* 35:14922, 2015 (<https://www.ncbi.nlm.nih.gov/pubmed/26538660>)