Thick-Section Based Antibody Screening

This protocol is used to validate antibodies for usage in whole mount immunolabeling. This protocol can also be used to examine targets of interest in the brain in thick-section format to obtain three-dimensional information in specific areas of interest. Using delipidated tissue for section staining provides advantages over traditional immunohistochemistry, such as increased antibody access to lipid-enriched regions to achieve complete penetration/coverage (e.g. myelinated axons and white-matter tracts).

I. Tissue Preparation

1. Collect tissue samples. (See "Sample Collection Protocol" for recommended methods.)
2. Delipidate. (See "Delipidation Protocol.")

II. Sectioning

3. Wash brain in 1xPBS for 1hr, 3 times.
4. Cut whole brain along midline to two hemispheres for sagittal sectioning (if not already cut), or cut to other desired orientation.
5. Mount hemisphere in 2% Agarose/1xPBS and cut free floating sections with vibratome to desired thickness (suggested range: 50µm to 250µm).
    a. Our preferred method: Section to 100µm in sagittal plane from medial to lateral sides, resulting in ~36 total sagittal sections.
    b. Note: incubation and wash times should be adjusted based on section thickness. Suggested times provided below are for 100µm thick sections and 200µm thick sections.
6. Store the sectioned brain slices in 1xPBS/**0.2%**NaN3 (high concentration) at 4°C.

III. Standard Immunohistochemistry Protocol for Thick-Section Based Antibody Screening

Note: May choose to perform either sequential immunostaining (Method A) or pre-conjugated immunostaining (Method B). The pre-conjugated method is faster and may improve signal-to-noise ratio of staining, but requires use of Fab fragment secondary antibodies against Fc fragment only.

I. Section selection & blocking – use in both Method A and Method B

Step	Instruction	# buffer changes	wash duration	
			100µm	200µm
1.	Pick a section and remove all agarose for easier antibody staining/permeabilization.			
2.	Place into a 2ml Eppendorf tube containing 1xPBS.			
3.	Block with PTxwH buffer (~1.6ml), nutate at RT.	2x	10-15min 20min	30min 1hr

II. Immunostaining – choose Method A or Method B

A. Sequential Immunostaining					B. Pre-conjugated Immunostaining *(Recommended approach. Only compatible w/ Fab-anti-Fc 2° Ab)*
Step	**Instruction**	**# buffer changes**	**wash duration**		
			100µm	**200µm**	
4.	Add primary antibody to section in PTxwH. Incubate while nutating at RT, o/n. *Tip*: For testing new antibodies, we recommend an initial test dilution of 1µg primary antibody in 800µL PTxwH.	1x	o/n	o/n	*Prepare preconjugation mixture*: In separate tube, dilute primary antibody in PTxwH (1:10 in volume), then add and mix secondary antibody in desired ratio to the same stock solution. Leave at RT, 30 min. *Note*: recommended 1°Ab to 2°Ab ratio is 1:2 by weight.
5.	Wash in PTxwH, RT.	4-5x	5min 10min 15min 30min (1hr opt.)	15min 30min 1hr 2hr	Add the preconjugation mix to sections in 800µl PTxwH. Incubate with nutation at RT, o/n.
6.	Add secondary antibody to section in PTxwH. Incubate with nutation at RT, o/n (or for 6hrs minimum). *Tip*: we typically start with 2x the 1° Ab concentration (in mg/mL).	1x	o/n	o/n	

III. Prepare sections for mounting – use in both Method A and Method B

Step	**Instruction**	**# washes**	**wash duration**	
			100µm	**200µm**
7.	Wash in 1.6mL PTxwH, nutating at RT.	5x	5min 10min 15min 30min	10min 20min 30min 1h
8.	Wash in 1.6mL 20mM PB, nutating at RT	3x	5-10min 30min	15-20min 1hr

IV. Mounting

9. Place brain sections in dish with 20mM PB and mount on slide using mounting media with high RI (e.g. ACB mounting media).
    a. Note: Mounting in high RI solution will help to image thicker sections. For thin sections, other mounting media may yield acceptable results.

10. Leave o/n to curate and clear. Seal the coverslip with nail polisher around edges (or 4 corners first) for later imaging.

V. Imaging

Recommended:

11. Widefield imaging can be done with a conventional epifluorescence microscope to image slides. Set up equipment and camera conditions as suggested by manual. Most common low magnification lenses (5x or below) have enough focal depth to image over 100µm sections. For higher resolution imaging, Z-scan is recommended with regular epifluorescence microscope or other types microscopes (e.g., confocal). This approach is generally adaptable for various imaging setups.
12. Set exposure times and other settings as guided by microscope manual recommendations, experimental design, apparent signal intensity, and secondary antibody fluorophore conjugates.
13. Capture and save images using .tiff format to preserve information.

Tips for choosing secondary antibodies:

- ***Subclass-specific antibodies***: When working with mouse monoclonal primary antibodies, cross-reactivity can occur between anti-mouse secondary antibodies and endogenous mouse immunoglobulins. For this reason, it is highly recommended to use IgG subclass-specific secondary antibodies (e.g. anti-Ms-IgG1, anti-Ms-IgG2b, etc.) to minimize specific background.
    a. *Extra Tip*: Using subclass-specific secondary antibodies will also enable co-labeling using multiple mouse monoclonal primary antibodies in the same tissue section (as long as the mAbs are of different IgG subclasses).
- ***Preabsorption***: It is recommended to pre-absorb secondary antibodies on tissue from your experimental species to reduce nonspecific tissue binding in final immunostaining.
    a. *Extra tip*: Mounting and imaging the tissue sections on which the antibody is preabsorbed can provide a sense of any specific background pattern that may be expected (at a reduced level) in the final staining due to the secondary antibody.
- ***Antibody Format***: If your aim is to screen antibodies for use in whole-mount, choosing Fab or $F(ab')_2$ fragment secondary antibodies is recommended. Because these antibodies are smaller than whole IgG secondaries, they will diffuse more easily into the sample during later whole-mount staining (particularly if the protein target of interest is densely distributed within the target tissue), producing more even staining. For thick-section staining alone, use of whole IgG secondary antibodies is fine and will provide stronger signal strength (with Method A).

Buffer Recipes: See “Buffer Recipes” document.