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## Synapse Staining - IHC - VGluT1 and PSD95 - Mouse Brain Sections

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**We use this protocol and it's working**

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## Abstract

Instructions to stain for pre- and post-synaptic markers used to quantify the number of synapses in mouse brain. This protocol specifically labels VGluT1+ inputs which in the mouse cerebral cortex are intracortical. The post-synaptic marker is PSD95 which in the mouse cerebral cortex labels excitatory post-synapses. This protocol has been used to label synaptic compartments at several developmental ages and has been paired with standard confocal microscopy and super-resolution light microscopy (STED). The analysis is facilitated by an in-house ImageJ macro taking advantage of the diffraction limit of standard confocal microscopy to quantify synapses as the observed colocalization of pre- and post-synaptic markers.

## Guidelines

Starting material should be 25-50 micron thick mouse brain sections that have been freshly cut or previously stored in 50% glycerol in 1X TBS at -20 degrees Celsius.

If using a 24-well plate to stain free-floating sections, the volumes listed here are recommended for no more than brain sections per well. This is to ensure even and deep penetration of the antibodies into the tissue sections. To stain thicker sections or more sections consider scaling up the volumes described.

## Materials

	Antibody Name	Host Species	Supplier	Catalog #	Dilution for IHC
	VGLUT1	Guinea Pig	Millipore	AB5905	1:7500
	PSD-95	Rabbit	Invitrogen/Thermo Fisher Scientific	51-6900	1:300
	Anti-Guinea pig Alexafluor 647	Goat	Invitrogen/Thermo Fisher Scientific	A-21450	1:200
	Anti-Rabbit Alexafluor 488	Goat	Invitrogen/Thermo Fisher Scientific	A-11008	1:200

Antibody Reagents used for this protocol

	Chemical Reagent	Supplier	Catalog #	Notes
	Triton™ X-100 Surfact-Amps™ Detergent Solution	Thermo Fisher Scientific	28314	
	Tris	VWR	EM-9230	
	Sodium Chloride	VWR	BDH9286-2.5KG	
	Goat Serum	Thermo Fisher Scientific	16210064	
	10X Tris Buffered Saline (TBS)	Made in house	N/A	
	1X TBS	Made in house	N/A	
	Vectashield with DAPI Mounting Media	Vector Laboratories	H-1200	

Chemical Reagents used for this protocol

	Material	Supplier	Catalog #
	Glass Pasteur pipette	VWR	14672-380
	24 well plate	VWR	353047
	Fine tip paint brush	Any art supply store	N/A
	PAP Pen	VWR	99990-104
	Frosted glass slides	VWR	48311-703

	Material	Supplier	Catalog #
	Cover glass (No. 1)	VWR	48393241
	Clear nail polish	VWR	100491-940

Non-chemical reagents that were used for this protocol. These materials are likely interchangeable with many other products. The only recommendation is to use sterile materials to avoid contamination that could introduce background to antibody-based staining.

## Troubleshooting

## Safety warnings

- ! Paraformaldehyde (PFA) is used to fix tissues and should be handled according to its available MSDS and lab SOP.

## Ethics statement

All animal-related procedures were conducted following an IACUC-approved animal protocol.



## Before start

Tissue must be prepared as follows:

1. Mice were anesthetized with a lethal dose of avertin (1.25% solution in water or 12.5mg/mL in water).
2. After mice are no longer responsive to toe pinch, they were transcardially perfused with 1X Tris Buffer Saline (TBS) until they run clear (~ 5 minutes).
3. Following transcardial perfusion with 1X TBS, mice were then perfused with 4% Paraformaldehyde (PFA) dissolved in 1X TBS for ~ 5 minutes (until stiff).
4. Finally, brains were dissected from the mouse and stored overnight (~12-18 hours) at 4 degrees Celsius in 4% PFA (enough to cover the brain).
5. The following day, brains were removed from PFA, rinsed with 1X TBS, and then stored at 4 degrees Celsius in a 30% sucrose solution in 1X TBS.
6. The brains are stored until they sink to the bottom of their container and can then be frozen and cryosectioned.

Any alterations to tissue preparation should be tested empirically to be certain the resulting staining is still comparable to the staining resulting from the previously described tissue preparation.

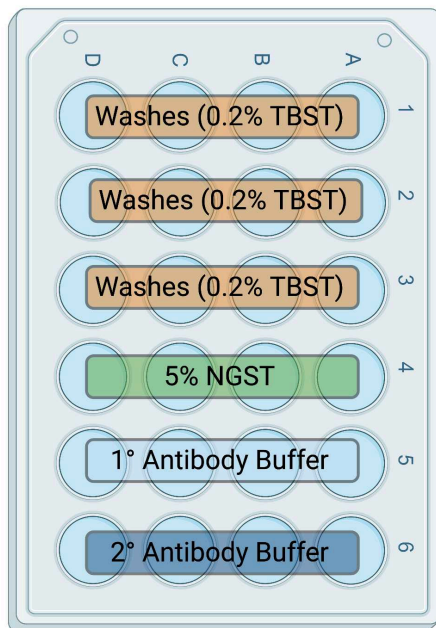
STED Mounting media was made following the recipe here: <https://nic.med.harvard.edu/resources/media/>  
In brief, the final solution is 50mM N-Propyl Gallate, 20 mM Tris, and 90% Glycerol in water.

## Day 1: Buffer preparation

- 1 Prepare 50 mL of 0.2% Triton in 1X TBS (**TBST**) by combining 1 mL of Triton X-100 with 49 mL of 1X TBS. Vortex to mix well.
- 2 Prepare 7.5 mL of 5% Normal Goat Serum in TBST (**NGST**) by combining 375  $\mu$ L of Goat serum with 7.125 mL of 0.2% TBST. Vortex to mix well. Any leftover NGST can be stored at 4 °C until it is needed for the duration of the staining protocol. Do not use NGST older than 1 week.
- 3 Prepare Primary Antibody Buffer (**1° AB**) by aliquoting 2.5 mL of 5% NGST into a new tube. Add appropriate dilution of each antibody to your tube making sure to use new tips for each antibody. For 2.5 mL of 1° AB, add 0.34  $\mu$ L of guinea pig anti-VGLuT1 and 8.33  $\mu$ L of rabbit anti-PSD95. Vortex to mix well. Centrifuge the 1° AB at max rpm at 4°C for 5 minutes. After completing the spin, store the 1° AB on ice or at 4 °C until ready to use.

## Day 1: Primary antibody staining

- 4 Get a fresh 24 well plate, orient the plate as depicted in **Figure 1**, and label the first three rows for TBST. Fill each well with 1 mL of TBST.



**Figure 1:** Example 24 well plate for immunohistochemical (IHC) staining of free-floating mouse brain sections. Typically, one column (A-D) is used for brain sections from a single sample and the wash buffer is vacuumed off and replaced with fresh buffer when necessary.

- 5 Using a flame-shaped glass pipette (as depicted in **Figure 2**), transfer up to 3 brain sections into one well to wash off OCT or glycerol storage buffer. Incubate at room temperature (RT) in this well for 5-10 minutes.



**Figure 2:** Example of a borosilicate glass Pasteur pipette that has been sealed and shaped into a hook using a Bunsen burner flame. This pipette is used to transfer free-floating mouse brain sections between wells.

- 6 Transfer brain sections to the next wash well (keeping each sample within it's own column A,B,C, or D as depicted in **Figure 1**). Incubate at RT for 5-10 minutes.
- 7 Repeat step 6 and allow sections to incubate at RT for 5-10 minutes.
- 8 Aliquot 500  $\mu$ L of 5% NGST into each well labeled for NGST.
- 9 Transfer brain sections to the NGST well and allow them to incubate at RT for 1 hour.
- 10 Aliquot 500  $\mu$ L of 1° AB in each well labeled for 1° Antibody Buffer.
- 11 Transfer brains sections to the 1° AB and allow them to incubate at 4 °C overnight (12-18 hours)

## Day 2: Buffer Preparation

- 12 Prepare Secondary Antibody Buffer (**2° AB**) by aliquoting 2.5 mL of 5% NGST into a new tube. Add appropriate dilution of each secondary antibody to your tube making sure to use new tips for each secondary antibody. For 2.5 mL of 2° AB, add 12.5  $\mu$ L of goat anti-guinea pig Alexafluor 647 and 12.5  $\mu$ L goat anti-rabbit Alexafluor 488. Vortex to mix well. Centrifuge the 2° AB at max rpm at 4 °C for 5 minutes. After completing the spin, store the 2° AB on ice and covered or at 4 °C in the dark until ready to use.

## Day 2: Secondary antibody staining

- 13 Vacuum off the old wash buffer from the first three rows and replace it with 1 ml of fresh 0.2% TBST per well.
- 14 Transfer the sections from the 1° AB solution to the first wash well. Allow them to incubate at RT for 5-10 minutes. Repeat this step twice by transferring the sections to the next wash well for a total of 3 washes.
- 15 Aliquot 500 µL of the 2° AB solution to the wells labeled for 2° AB. Transfer the sections to this well and incubate at RT for 2 hours (light protected).
- 16 After the 2 hour incubation, vacuum off the old wash buffer from the first three rows and replace it with 1 ml of fresh 0.2% TBST per well.
- 17 Transfer the sections from the 2° AB solution to the first wash well. Allow them to incubate at RT for 5-10 minutes. Repeat this step twice by transferring the sections to the next wash well for a total of 3 washes.

## Mount Sections and Coverslip

- 18 Prepare each frosted glass slide by drawing a rectangle on it with a PAP pen and labeling it with the appropriate information.
- 19 Add 1 mL of 1X TBS to the slide you are currently mounting. Using the paint brush, carefully transfer the brain sections onto the slide and flatten into place.
- 20 Once the sections are in place, with a pipette carefully remove excess 1X TBS. Once the sections appear firmly set on the glass, remove residual 1X TBS using either a Kimwipe or a vacuum line. (Be careful not to touch the sections or damage them.)
- 21 When the sections are dried into place, but not fully dried (there should be not salt build-up and the sections should still be slightly opaque not transparent), add 70 µL of mounting media to your slide.
- 22 Carefully, place the coverslip onto the slide. Do not allow bubbles to buildup on the sections.
- 23 Once the coverslip is on the sections, seal the slide using the clear nail polish. You need to ensure the slides are totally sealed to prevent leakage of the mounting media, but do not cover your sections with nailpolish.
- 24 After the slides dry, either image immediately or store at 4 °C in the dark. Best practice is to image the slides within 48 hours after completing secondary to avoid fading of the secondary dye.



