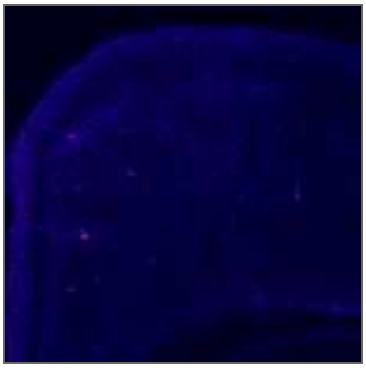

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Protocol status: Working
We use this protocol and it's working

Immunohistochemistry (IHC) Staining Mouse Brain Sections

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ABSTRACT

The Immunohistochemistry (IHC) Staining for Mouse Brain Sections protocol details the blocking, primary, and secondary antibody staining of 50-100 micron mouse brain tissue slices fixed in 4% PFA. The protocol includes suggested staining duration based on slice thickness for each staining step and tables of the most frequently requested blocking serums and primary and secondary antibody stains and dilutions.

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Keywords: Histology, Immunohistochemistry, IHC, Immunolabeling, Antibody

MATERIALS

Sample sections to be stained:

- 50-100 microns thick
- Fixed in paraformaldehyde
- Free floating, stored in PBS or PBS + 0.01% Azide
- Protected from light

Reagents:

10xPBS **Ambion Catalog #**AM9624

Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8787-50ML

Sodium Azide 5% **Ricca Chemical Company Catalog #**71448-16

Milli-Q water

Optional reagents - determined by stain requested:

- Note these reagents and stained tissue must be protected from light

DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) **Thermo Fisher Catalog #**62247

Primary Antibody

Secondary Antibody

Normal Goat Serum **Vector Laboratories Catalog #**S-1000-20

Normal Donkey Serum **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D9663-10ML

Block Aid **Invitrogen - Thermo Fisher Catalog #**B10710

Urea powder **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**U5378-100G

	Materials	Product number
	Serological pipet filler	Thermofisher, 9501
	48 well plate	Costar, 3548
	Cell culture inserts	Netwell, 734-1589
	Serological pipets	Fisher Scientific, 13-678-11E
	Nutating mixer	Fisherbrand, 88-861-043
	Stir plate	Merck MilliporeSigma, Z693510

	Materials	Product number
	Manual single channel pipettes: P20, P200, P1000	Rainin, 30456871
	Manual single channel pipettes: P2	Rainin, 17014393
	Manual dingle channel pipettes: P5000	Rainin, 17011790
	Stir bar	Grainger, 21R590

Recipes - blocking and DAPI solutions should be made depending on experimental need

1L 1xPBS:

Combine the following reagents into a container with a stir bar. A graduated cylinder may be used to measure MilliQ water and a graduated cylinder or serological pipet may be used to measure 10xPBS. Mix well on a stir plate at high speed (300 RPM or higher) for 00:02:00 or until solution is mixed. Store at Room temperature for 1 month.

	Reagent	Volume
	Milli-Q water	900 mL
	10xPBS	100 mL

1L 1xPBS & Sodium Azide 0.01% :

Combine the following reagents into a container with a stir bar, using a graduated cylinder to measure the 1xPBS and a P5000 pipette to measure the 5% sodium azide. Mix well on a stir plate at high speed (300 RPM or higher) for 00:02:00 or until solution is mixed. Store at Room temperature or 4 °C for up to 1 year.

	Reagent	Volume
	1xPBS	998 mL
	0.01% Sodium Azide	2 mL

1L 1xPBS & Triton X-100 0.2%:

Measure 1xPBS into a container with a stir bar using a graduated cylinder and slowly pipet Triton X-100 in using a P5000 pipette. Mix well on a stir plate at high speed (300 RPM or higher) for 00:15:00 to ensure Triton X-100 goes into solution. Store at Room temperature for 1 month.

	Reagent	Volume
	1xPBS	998 mL
	Triton X-100	2 mL

1L 1xPBS & Triton X-100 0.06%:

Measure 1xPBS into a container with a stir bar using a graduated cylinder and slowly pipet Triton X-100 in using a P5000 pipette. Mix well on a stir plate at high speed (300 RPM or higher) for 00:15:00 to ensure Triton X-100 goes into solution. Store at Room temperature for 1 month.

	Reagent	Volume
	1xPBS	994 mL
	Triton X-100	6 mL

2mL 5mg/mL DAPI solution:

Add Milli-Q water to DAPI powder in 10mg vial using P5000 pipette. Vortex until powder completely mixes into solution. Store at 4 °C and vortex before use.

	Reagent	Volume
	Milli-Q water	2 mL
	DAPI powder	10 mg

200 mL 5% Normal Goat Serum & Triton X-100 0.06% & 4M Urea (NGSTU)

Measure urea on scale and add into a container for mixing NGSTU. Add 10x PBS into the container with a serological pipet, then add normal goat serum, Triton and water to container using manual pipette. Add stir bar to container and mix on stir plate at high speed (300 RPM or higher) for 00:15:00 to ensure all reagents fully homogenize. This working solution can be stored at 4 °C and used for up to 1 week. Beyond this time, there would be concern for possible microbial contamination that would affect performance.

*Amount of blocking serum made should be determined by number of sections to be stained.

	Reagent	Amount
	Normal Goat Serum	10 mL
	Urea	48 g
	10X PBS	20 mL
	Triton X-100	120 uL
	Milli-Q Water	Fill to 200 mL

200 mL 5% Normal Donkey Serum & Triton X-100 0.2% in PBS (NDST)

Measure 10X PBS into container with a serological pipet, then add Normal Donkey Serum, Triton, and water to container using manual pipette. Add stir bar to container and mix on stir plate at high speed (300 RPM or higher) for 00:15:00 to ensure all reagents fully homogenize. This working solution can be stored at 4 °C and used for up to 1 week. Beyond this time, there would be concern for possible microbial contamination that would affect performance.

*Amount of blocking serum made should be determined by number of sections to be stained.

	Reagent	Amount
	Normal Donkey Serum	10 mL
	10X PBS	20 mL
	Triton X-100	400 uL

	Reagent	Amount
	Milli-Q Water	Fill to 200 mL

200 mL 5% Normal Goat Serum & Triton X-100 0.06% (NGST):

Measure 10X PBS into container with a serological pipet, then add normal goat serum, Triton, and water to container using manual pipette. Add stir bar to container and mix on stir plate at high speed (300 RPM or higher) for 00:15:00 to ensure all reagents fully homogenize. This working solution can be stored at 4 °C and used for up to 1 week. Beyond this time, there would be concern for possible microbial contamination that would affect performance.

*Amount of blocking serum made should be determined by number of sections to be stained.

	Reagent	Amount
	Normal Goat Serum	10 mL
	10X PBS	20 mL
	Triton X-100	400 uL
	Milli-Q Water	Fill to 200 mL

SAFETY WARNINGS

DAPI is a mutagen and should be handled with care. Wear PPE and dispose into hazardous waste stream. Please consult your immediate supervisor or the EH&S manager/representative if you have questions or concerns.

Sodium Azide is toxic and carcinogenic. It should be handled and prepared with care. Do not breathe dust, do not use metal utensils. Wear gloves when handling this chemical.

Paraformaldehyde is carcinogenic. Wear gloves at all times when handling specimen tissue slices fixed in paraformaldehyde, as the tissue may contain trace amounts of the chemical.

Personal Protective Equipment (PPE) should be used at all times while operating this protocol. If you are unsure what PPE you should be using, see your immediate supervisor.

BEFORE START INSTRUCTIONS

This protocol details staining of mouse brain tissue that has already been sectioned. Reference the following protocol for instructions on sectioning a whole mouse brain fixed in 4% PFA: Sectioning Mouse Brain with Sliding Microtome

For all steps, protect tissue from light in order to preserve fluorescence by wrapping well plate in foil between steps and during washes and stains.

Setup

1 Well plate setup:

1.1 Select either a plastic well plate or Netwell cell culture inserts for tissue section staining.

Note

A plastic well plate may be helpful for conducting multiple different stains simultaneously, while a Netwell kit may be efficient for applying the same stain to a large number of tissue sections.

1.2 Fill wells with 1xPBS and place tissue sections to be stained into wells.

If there are a large amount of tissue sections, the sections may be double or triple stacked in each well in the well plate (or more if using Netwell insert), but ideally sections in the same well should be separated by at least a few hundred microns (ex: every 6th section or more) so it is easier to see anatomical differences and determine section order when mounting.

1.3 If tissues will be stained in different conditions (ex: staining sections with different primary or secondary antibodies) it is best to draw a map of these conditions on the plastic well plate lid and a corresponding map in lab notebook.

Staining

3d 0h 10m

2 Wash mouse brain sections three times in 1xPBS for 00:05:00 at Room temperature on shaker 15m using either a plastic well plate or Netwell kit with mesh inserts.

Note

In this step and all subsequent steps, protect tissue from light to prevent bleaching by wrapping well plate in foil while it is on the shaker.

3 Blocking:

3.1 Select blocking solution. See examples of frequently requested blocking solutions below:

	Blocking solution	Notes	Example
	BlockAid	Can be paired with any secondary antibody	Example: BlockAid + chicken anti-GFP (primary antibody) + (goat OR donkey) anti-chicken (secondary antibody)
	Normal goat serum with triton (NGST)	Must match secondary antibody	Example: NGST (blocking solution) + chicken anti-GFP (primary antibody) + goat anti-chicken (secondary antibody)
	Normal donkey serum with triton (NDST)	Must match secondary antibody	Example: NDST (blocking solution) + chicken anti-GFP (primary antibody) + donkey anti-chicken (secondary antibody)
	Normal goat serum with triton and urea (NGSTU)	Must match secondary antibody, urea helps with background	Example: NDSTU (blocking solution) + chicken anti-GFP (primary antibody) + donkey anti-chicken (secondary antibody)

3.2 Choose a blocking duration based on section thickness. See table below. Note that suggested blocking durations are minimums and sections may be blocked up to Overnight

	Section thickness	Blocking duration
	50 microns	1 hour
	100 microns	2 hours

3.3 Wash sections in chosen blocking solution for chosen blocking duration on shaker at Room temperature

4 Primary staining:

4.1 Choose a primary antibody and dilution based on tissue labeling. See table below for commonly requested primary antibodies and dilutions. If using NDST, NGST, or NGSTU as blocking serum, dilute the primary antibody in tube with blocking serum. If using Block Aid as blocking serum, dilute the primary antibody in tube with 1xPBS & Triton X-100 0.2%. Mix primary antibody dilution mixture in tube by gently swirling.

	Frequently requested primary antibodies	Suggested dilution
	Immunostar mouse anti-Tyrosine hydroxylase (22941)	1:1000

	Frequently requested primary antibodies	Suggested dilution
	NovusBio rabbit anti-Tph2 antibody (NB100-74555)	1:1000
	Rockland rabbit anti-RFP antibody pre-adsorbed (600-401-379)	1:1000 or 1:800
	Invitrogen mouse anti-HA (26183)	1:500
	AVES chicken anti-GFP (GFP-1020)	1:800
	Immunostar goat anti-5HT (26183)	1:800
	FujiFilm Wako rabbit anti-IBA1 (019-19741)	1:1000
	Sigma mouse anti-GFAP (G6171)	1:1000

4.2 Chose a staining duration based on section thickness. Note that the durations below are minimums and sections may be stained up to 72:00:00 3d

	Section thickness	Primary antibody staining duration minimums
	50 microns	24+ hours
	100 microns	48+ hours

4.3 Remove blocking solution and incubate tissue in chosen primary antibody solution on shaker at 4 °C or Room temperature for chosen staining duration.

> Note
>
> **If no secondary antibody stain is required, skip to step 7.**

5 Wash sections in new wells containing 1xPBS & Triton X-100 0.2% five or more times for 00:05:00 on shaker at Room temperature 25m

6 Secondary staining:

6.1 Choose a secondary antibody and dilution based on the primary antibody used. See table below for commonly requested secondary antibodies and dilutions. If using NDST, NGST, or NGSTU as blocking serum, dilute the secondary antibody in tube with blocking serum. If using Block Aid as blocking serum, dilute the secondary antibody in tube with 1xPBS & Triton X-100 0.2%. Mix secondary antibody dilution mixture in tube by gently swirling.

	Frequently requested secondary antibodies	Suggested dilution
	DAPI (5 mg/mL stock concentration, then diluted to working concentration) combine with secondary antibody	1:1000 or 1:5000
	Invitrogen goat anti-mouse 488 cross-adsorbed (A11001)	1:500
	Invitrogen goat-anti-rabbit 488 (A11012)	1:500
	Invitrogen goat anti-mouse 647 (A-21236)	1:500
	Invitrogen goat anti-rabbit 405 (A-31556)	1:500
	Invitrogen goat anti-rabbit 647 (A21244)	1:500
	Jackson Immuno goat anti-chicken 488 (703-545-155)	1:500
	Invitrogen goat anti-rabbit 594 (A-11012)	1:500
	Invitrogen donkey anti-goat 647 (A-21447)	1:500

6.2

Note

If additional DAPI stain is required in combination with secondary antibody, continue with step 6.2. If not, skip to step 6.3.

Vortex [M] 5 mg/mL DAPI solution and dilute it to preferred concentration in secondary antibody solution. 1:5000 is a common dilution for DAPI.

Safety information

DAPI is a mutagen, so wear PPE (gloves, lab coat, safety goggles) and dispose in hazardous waste stream.

6.3 Choose a staining duration based on section thickness. Suggestion below are minimums and secondary staining duration may run up to 48:00:00 : 2d

	Section thickness	Secondary staining duration minimuns
	50 microns	1+ hour
	100 microns	2+ hours

6.4 Incubate sections in chosen secondary antibody solution for chosen staining duration on shaker at Room temperature

7 Wash sections in 1xPBS & Triton X-100 0.2% three times for 00:05:00 on shaker at Room temperature 25m

8 Wash sections in 1xPBS two times for 00:30:00 on shaker at Room temperature 30m

9 Store samples in 1xPBS for short term use or 1xPBS & Azide 0.01% for long term use (Overnight or up to several weeks) at 4 °C until ready for mounting.

Safety information

Sodium Azide is toxic and carcinogenic. It should be handled and prepared with care. Do not breathe dust, do not use metal utensils. Wear gloves when handling this chemical.

Note

Proceed to Mounting and Coverslipping Mouse Brain Sections protocol.