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Immunohistochemistry (IHC) on mouse brain slices

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Transcriptional crosstalk

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We use this protocol and it's working

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Abstract

This protocol provides a starting point for immunohistochemical localization and visualization of proteins in mouse brain tissue. This can be useful for marking cell types of interest or investigating the effect of experimental manipulations on protein expression and/or localization.

This protocol is compatible with free-floating tissue or tissue that has already been mounted onto a slide.

Materials

- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023
- PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #**AM9625
- Triton X-100, 10% solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**93443
- Phosphate buffered saline powder, pH 7.4, for preparing 1 L solutions **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P3813
- BlockAid™ Blocking Solution **Thermo Fisher Scientific Catalog #**B10710
- ProLong Diamond Antifade Mountant **Thermo Fisher Scientific Catalog #**P36970
- Normal Goat Serum **Jackson ImmunoResearch Laboratories, Inc. Catalog #**005-000-121
- Normal Donkey Serum **Jackson ImmunoResearch Laboratories, Inc. Catalog #**017-000-121
- Diethyl pyrocarbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D5758
- Dimethyl dicarbonate (dimethyl pyrocarbonate) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**8187580100
- Citrate Buffer pH 6.0 10× Antigen Retriever **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**C9999-1000ML
- Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water **Thermo Fisher Scientific Catalog #**H3570
- DAPI **Thermo Fisher Scientific Catalog #**62248

Primary antibodies - multiple suppliers, a literature search for IHC against the same target is a good way to identify promising antibodies to test
Secondary antibodies - multiple suppliers

Before start

This protocol assumes that you are starting from free-floating brain slices in 1x PBS, or from sections mounted on a glass slide.

We typically process free-floating sections 30 µm to 100 µm thick, and mounted sections 5 µm to 100 µm thick.

Free-floating sections may allow for better penetration of antibodies into thick sections (as diffusion occurs from both sides). Mounting sections onto a slide may be more convenient for some sectioning methods (cryosectioning) and can enable thinner sections to be collected without risk of damaging sections during processing. Depending on the size of well-plate used, mounted sections may use less antibody, and may thus be cheaper to process. Mounting sections on slides is also recommended if incorporating an antigen retrieval step.

Reagent set up

1 General note on reagents and consumables

> **Note**
>
> If only performing IHC, it is not necessary to use RNase-free reagents. However, if combining IHC with other readouts (e.g. HCR-FISH) it may be necessary to use RNase-free reagents and/or to treat reagents with an RNase-inhibitor (DEPC or DMPC).

2 Prepare 1x PBS, by combining: 2h

- 1 PBS packet
- MilliQ water (or equivalent): 1 L

Shake to combine and store at Room temperature for up to 3 months.

(Optional) Treat 1x PBS with DEPC- or DMPC-treat to inactivate RNases. Add 1 mL of DEPC or DMPC to 1 L of 1x PBS, shake with lid tightly closed, then loosen lid and let sit Overnight (or if needed same day, for 02:00:00 at 37 °C). After incubation, autoclave the 1x PBS to inactivate the DEPC or DMPC. Allow to cool to room temperature before using.

> **Note**
>
> Dimethyl pyrocarbonate (DMPC) is a less toxic alternative to DEPC that can be used in the same manner.

3 Prepare blocking / antibody dilution buffer. First determine how much will be needed. For every well, you will need 1.5 mL . For every slide, you will need 1 mL . This blocking buffer will be used for blocking, primary antibody dilution, and secondary antibody dilution. Make extra (~10%) to ensure there is enough for each well.

Example: for 6 wells with free-floating sections, prepare 10 mL of blocking buffer, by combining:

- BlockAid Blocking Solution: 9.9 mL
- 10% Triton X-100: 100 µL

Prepare on first day of protocol, and store at 4 °C until use.

Note

We have found BlockAid Blocking Solution to be a reliable alternative to normal serums for this protocol. However, normal serums are a valid substitution for BlockAid solution. If using normal serums, ensure that the serum species is the same host as the secondary antibodies (i.e. use normal donkey serum if using donkey secondary antibodies).

For using normal serums, the following blocking buffer can be made. For 10 mL combine:

- Normal donkey serum: 1 mL
- 10x PBS: 1 mL
- 10% Triton X-100: 100 µL
- UltraPure water: 7.9 mL

Use the same as BlockAid Blocking Solution.

4 This protocol diverges depending on whether you are processing free-floating sections or mounted sections. If you are incorporating an antigen retrieval step, we recommend using slide mounted sections or mount free-floating sections on a slide before proceeding.

Once samples have some fluorescence, keep samples in the dark. E.g. if samples have endogenous fluorescence (e.g. GFP or tdTomato-expression), keep samples in dark through the entire protocol. If fluorescence is introduced with secondary antibody, keep samples in dark during and after secondary antibody incubation.

STEP CASE

Free-floating sections 12 steps

5 Note on scaling and volumes

Note

The volumes in this protocol are appropriate for a 24-well plate with 12 or fewer sections per well. If processing more sections per well, consider using a larger well size (e.g. a 12-well plate), and scale up volumes appropriately. If processing fewer sections per well, consider using a smaller well size (e.g. a 48-well plate) and scale down volumes appropriately.

Appropriate scaling factors can be found in https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/cell-culture-useful-numbers.html, based on ratio between surface areas.

For free-floating sections, incubations and washes can be done in the wells.

6 Note on serial sections

Note

In some cases, it may be desirable to arrange sections on a slide serially. If processing free-floating sections, it can be helpful to seperate sections across multiple wells during sectioning (e.g. Starting at well A1, collect section 1. Then collect section 2 in well A2. Section 3 in A3, Section 4 in A4, etc. When collecting section 7, start back at the beginning and place in A1. Section 8 in A2, etc.)

This ensures that serial sections in the same well are separated by some distance and can be easily placed on the slide in the correct order.

If left and right side are important, mark one side. E.g. trim tissue off in a region outside of the region of interest.

7 If necessary, transfer the desired slices to a new well plate filled with 1x PBS. Use a paintbrush to transfer sections.

8 Aspirate and dispose of 1x PBS in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal. 1h

Add 500 µL of blocking buffer to each well. Incubate for 01:00:00 at Room temperature with gentle shaking.

9 In last 5 minutes of the blocking step, prepare the primary antibody solution. Dilute the primary antibody in blocking buffer and mix by inverting gently.

Note

Optimal dilution may need to be determined empirically. Previous literature using the same antibody in the same application can provide good starting points for optimal dilution.

10 Once blocking is complete, aspirate and dispose of blocking buffer in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

Add 500 µL of primary antibody dilution to each well. Incubate. Overnight at 4 °C with gently shaking.

Note

For some primary antibodies, incubation for 01:00:00 to 02:00:00 at Room temperature may be sufficient. This should be determined empirically.

11 Following primary antibody incubation, aspirate and dispose of primary antibody solution in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal. 15m

Wash samples 3 times, with 1 mL of 1x PBS per wash. Each wash should be 00:15:00 at Room temperature, with gentle shaking.

Note

In some cases, a primary antibody conjugated to a fluorophore may be used. This can mitigate some confounds due to the amplification when using both a primary and secondary antibody, and thus may be desirable when a quantitative readout of protein level is desired. However, this will also produce a lower signal. In this case, no secondary antibody is needed.

If a fluorophore-conjugated primary antibody was used, skip to Step 15 following washes.

12 During last 5 minutes of washes, prepare secondary antibody solution. Dilute the secondary antibody in blocking buffer and mix by inverting gently.

Note

Optimal dilution may need to be determined empirically. Manufacturer suggested dilutions for your particular application can provide good starting points.

Multiple secondary antibody formats are available (https://www.thermofisher.com/us/en/home/life-science/antibodies/secondary-antibodies/fab-fab2-fragment-secondary-antibodies.html). Smaller formats [F(ab) and $F(ab')_2$] may be useful in cases where penetration into tissue is necessary.

13 Once washes are done, aspirate and dispose of 1x PBS in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal. 1h

Add 500 µL of secondary antibody dilution to each well. Incubate for 01:00:00 at Room temperature with gently shaking.

14 Following secondary antibody incubation, aspirate and dispose of secondary antibody solution in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal. 15m

Wash samples 3 times, with 1 mL of 1x PBS per wash. Each wash should be 00:15:00 at Room temperature , with gentle shaking.

15 (Optional) Stain sections with Hoechst 33342 or DAPI to label nuclei. For each well, prepare 500 µL of 1x PBS with 1/10000 Hoechst 33342 or DAPI. 20m

Aspirate and dispose of 1x PBS in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

Add 500 µL of Hoechst or DAPI solution. Incubate 00:15:00 at Room temperature with gentle shaking.

Following incubation with Hoechst or DAPI solution, aspirate and dispose of Hoechst or DAPI solution in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

Wash samples 3 times, with 1 mL of 1x PBS per wash. Each wash should be 00:05:00 at Room temperature , with gentle shaking.

16 Mount sections on slide. Use paintbrush to transfer and flatten sections onto surface of slide. Once all sections are placed, excess 1x PBS can be removed with a Kimwipe and/or by placing

slide in a fume hood. Allow sections to dry on slide (sections are dried once the tissue appears transparent).

Once tissue is dry, pipette 100 µL of mounting media in a line on the surface of the slide, near the tissue sections. Spread the mounting media over the tissue by gently lowering a clean coverslip over the tissue, being careful not to introduce bubbles.

Once all coverslips are mounted, gently press down on each coverslip to push out excess mounting media. Store in the dark at Room temperature for at least Overnight until mounting media cures. Once cured, excess Prolong Diamond can be easily removed by running slides under a gentle stream of MilliQ water, being careful to not displace coverslips.

Note

Be careful not to dry tissue too long. If the edges of the tissue are turning white, the tissue is becoming too dry.

For mounting coverslips, we use Prolong Diamond, but any mounting medium compatible with fluorescence signal should work. If using another mounting medium, follow manufacturer directions for that mounting medium.