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Immunohistochemistry

In 1 collection

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ABSTRACT

This protocol describes the steps for performing immunohistochemistry.

PROTOCOL MATERIALS

VECTASHIELD Mounting Medium **Vector Laboratories Catalog #H-1000**
Step 23

M.O.M.® (Mouse on Mouse) Immunodetection Kit Basic **Vector Laboratories Catalog #BMK-2202**
Step 27

Sodium Phosphate Dibasic Anhydrous **Fisher Scientific Catalog #S375**
Step 1.1

Potassium Chloride **Fisher Scientific Catalog #P217**
Step 1.1

Potassium Phosphate Monobasic **Fisher Scientific Catalog #P285**
Step 1.1

Sodium Chloride **Fisher Scientific Catalog #S271**
Step 1.1

Peel-A-Way® Embedding Mold **Polysciences, Inc. Catalog #18985-1**
Step 11.1

Epredia™ Neg-50™ Frozen Section Medium **Fisher Scientific Catalog #22-046-511**
Step 11.1

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Cardiac Perfusion

1 Prepare solutions and tools.

1.1 Make solutions:

10X PBS

[M] 1400 millimolar (mM) Sodium Chloride **Fisher Scientific Catalog #S271**

[M] 26.8 millimolar (mM) Potassium Chloride **Fisher Scientific Catalog #P217**

[M] 101.4 millimolar (mM)

Sodium Phosphate Dibasic Anhydrous **Fisher Scientific Catalog #S375**

[M] 17.6 millimolar (mM)

Potassium Phosphate Monobasic **Fisher Scientific Catalog #P285**

Bring to 1 L with MilliQ H2O

pH 7.4

1X PBS

100 mL 10X PBS

900 mL MilliQ H2O

pH 7.4

4% Paraformaldehyde (PFA) in 1X PBS.

1.2 Put on ice:

- 1X PBS: approximately 30 mL per mouse
- 4% PFA: approximately 30 mL per mouse
- 5 mL or 15 mL tubes filled with 4% PFA: usually one per mouse, can put up to 3 brains in a larger tube

1.3 Gather tools:

- Perfusion tray (lid for styrofoam box) and secondary container (9" x 13" baking dish)
- Pins or needles
- Butterfly needle
- Dissection tools (e.g. hemostat, fine scissors, decapitation scissors, forceps, spatula)
- Pump

Equipment	
Variable-Flow Peristaltic Pump	NAME
Fisherbrand	BRAND
13-876-2	SKU
https://www.fishersci.com/shop/products/fisher-scientific-variable-flow-peristaltic-pumps-4/p-158215	LINK

2 Prepare pump

2.1 Attach butterfly needle to outlet end of pump tubing

2.2 Rinse pump tubing by running 30-50 mL water through the lines

2.3 While cleaning the lines, measure and adjust flow rate to **5 mL per minute** (dial should be ~3-7 using fast and forward flow).

2.4 Prime the tubing with 1X PBS, running at least 10 mL through the lines; turn off flow, but keep the inlet end of the tubing in the 1X PBS.

3 Anesthetize mouse by placing it in a bell jar containing isoflurane.

Use a 15 mL tube containing isoflurane-soaked paper towels as a nose cone throughout perfusion.

4 Open chest cavity and start perfusion.

4.1 Place nose cone on mouse and confirm mouse is under the plane of anesthesia using a toe pinch.

4.2 Lay mouse on its back on the dissection tray; pin down each foot.

4.3 Using a hemostat or your fingers, pull up the fur and skin in the lower half of the body, just below the sternum. Use the fine scissors to cut into the skin and make an incision across the body (left to right), being careful to not yet pierce the diaphragm.

4.4 Move the skin aside to uncover the sternum and diaphragm. Grab the sternum with the hemostat and cut around the perimeter of the diaphragm and up the sides of the ribs. **Be very careful to not puncture the lungs or heart**.
Pull rib cage towards the head and to one side to reveal the heart. If using a hemostat, keep attached to the sternum and flip it towards the mouse's head to maintain access to the chest cavity. Pins can also be used.

> **Note**
>
> Once you pierce the diaphragm, oxygen flow to the brain is reduced. It is important to move to the following step quickly as to reduce hypoxic damage.

4.5 Make sure you have good access to the heart and begin the perfusion as follows:
- Insert tip of butterfly needle into caudal tip of left ventricle (keep the needle shallow within

the ventricle - do not pierce the wall of the left atrium)
- Start pump flowing at the previously determined setting
- Clip the right atrium using fine scissors

At this point blood should begin to flow out of the right atrium.

The wings of the butterfly needle can be used to hold the needle in place by tucking them under the intestinal organs and/or arranging pins/needles around them. Placing the scissors on top of the needle may also help to hold it in place. **Be very careful to keep the tip of the butterfly needle within the left ventricle and do not allow it to fall out or pierce through the atria.**

5 Complete perfusion

5.1 Start timer and allow 1X PBS to flow for 4-5 minutes (~20 mL 1X PBS). You should notice the liver turning pale.

5.2 Move the tubing from the 1X PBS to the 4% PFA, without allowing any bubbles into the line. This is achieved by pinching the tubing tightly while moving or stopping the pump for a few seconds to switch solutions.

5.3 Start timer and allow 4% PFA to flow for at least 5 minutes (i.e. 25 mL 4% PFA). During perfusion it is normal for the muscles and tail to begin to twitch/spasm and at the end of the perfusion, the tail should be stiff.
Halfway through this step, adjust the tip of the needle slightly to ensure that the cardiac tissue has not stiffened around the tip.

6 Pull the inlet of the tubing out of solution and run the remainder of the liquid within the tubing to perfuse through the mouse, being sure to remove the needle from the heart before you reach the end to prevent air entering the system.
Unpin the mouse from the perfusion tray.

7 Clean the perfusion lines (if desired, use 'Prime' setting or increase the speed):
- If another mouse will be perfused, clean the lines by running 30-50 mL water through and then prime the lines for the next mouse with at least 10 mL 1X PBS
- If this is the final mouse, clean the lines by running 50 mL water followed by ~20 mL 70% ethanol

Note

While running solution through the lines you can harvest the brain from the last perfused animal as described in the following section

Brain Harvesting and Storage

8 Remove brain.

8.1 Decapitate mouse; dispose of body.

8.2 Hold the mouse's nose and manually pull back scalp to reveal top of skull.

8.3 Remove skull using dissecting tools. There are multiple ways to achieve this, with one option described below:

- Using scissors, cut along the midline (posterior to anterior)
- Cut across skull (midline going lateral on both sides) at groove between forebrain and cerebellum and at the anterior extent (above olfactory bulbs)
- Using forceps, peel back each side of the skull with a flicking motion

Note

Throughout this process, be careful to not nick or gouge the brain

8.4 Using a spatula, carefully scoop out the brain and drop into tube of cold 4% PFA.

9 Incubate brain in 4% PFA at 4 °C for at least 2 hours and at most overnight.

> **Note**
>
> Depending on how the tissue will be used, a shorter or longer incubation in PFA is recommended. For some proteins, immunostaining is more effective with LESS fixation (e.g. nuclear proteins such as cFos). Conversely, overnight incubation is recommended to ensure complete fixation before sending tissue to another institution.

10 Incubate brain in 30% sucrose in 1X PBS at 4 °C until brain sinks (~24-48 hours), indicating that the brain has fully absorbed the sucrose.

> **Note**
>
> If storing the brain in sucrose for longer than 48 hours, add 0.05% Sodium Azide **Sigma Aldrich Catalog #S2002** to the sucrose solution; this includes for shipping samples between institutions.

11 Freeze brain.

11.1 For long term storage:

- Fill the bottom of an ice bucket with dry ice; you may want to use a hammer to crush the dry ice in order to create a more stable base
- Label specimen molds Peel-A-Way® Embedding Mold **Polysciences, Inc. Catalog #18985-1** and fill the bottom of the molds with frozen section medium Epredia™ Neg-50™ Frozen Section Medium **Fisher Scientific Catalog #22-046-511**; place on dry ice until frozen
- Using a razor blade, trim brain tissue as desired; if possible, cut a flat surface on at least one end (e.g. cut off cerebellum)

- Place flat end of brain tissue in specimen cup onto frozen medium. The orientation will vary based on the intended target brain region(s) and slice orientation; for coronal sections of the striatum, cut off the cerebellum in the previous step and place the resulting caudal end of the brain onto the frozen medium, with the rostral tip of the brain pointing up
- Keeping the specimen cup in the dry ice, fill around the brain with medium until completely covered; be careful to avoid bubbles, especially on the surface of the tissue.
- Once the medium has solidified, move the sample to -80 °C for long-term storage until the tissue will be further processed.

Tissue Sectioning (Cryostat)

12 Prepare cryostat:
- Temperature in the chamber and at the chuck holder set to -20 °C
- Replace blade if needed

Equipment	
Cryostat	NAME
Leica	BRAND
Cryostat	SKU
https://www.leicabiosystems.com/histology-equipment/cryostats/	LINK

13 Mount sample:
- Cover surface of a chuck with a thin layer of frozen section medium
- Remove sample from embedding mold and place onto chuck, flat side down
- Keep in cooled chamber of cryostat for at least 5 minutes to freeze sample onto chuck

14 Mount chuck and adjust slicing conditions:
- Mount chuck in holder
- Advance sample to the blade; cut off layers of ~100 µm frozen section medium until brain sample is visible
- Adjust chuck holder to straighten the tissue with respect to the blade
- Adjust thickness to desired value (30-50 µm)
- Adjust roll plate to ensure sections come off the blade flat

15 Start collecting tissue at the desired plane:
- For free floating sections: transfer slices from the cryostat to 1X PBS

- For slide mounted sections: touch front surface of slide to the surface of brain sections; use heat from a finger on the back of the slide to encourage attachment of the section. Floating sections in 1X PBS can be mounted on a slide for immunostaining.

Immunostaining and Mounting Sections

16 Wash sections three times in 1X PBS, 00:05:00 each in the orbital shaker 70-80 rpm 5m

17 Block sections in blocking buffer 01:00:00 Room temperature in the orbital shaker 70-80 rpm 1h

- **Blocking buffer**: 5-10% normal serum (from the host-species of the secondary antibodies), 0.3% Triton X-100 in PBS.

Note

Mouse on Mouse Immunohistochemistry (MOM IHC):

M.O.M.® (Mouse on Mouse) Immunodetection Kit Basic **Vector Laboratories Catalog #BMK-2202**

18 Incubate the sections with an appropriate dilution of the primary antibody in 1X PBS or blocking buffer, 4 °C Overnight (orbital shaker 70-80 rpm if possible).

19 Wash sections three times in 1X PBS, 00:05:00 each in the orbital shaker 70-80 rpm 5m

20 Incubate the sections with an appropriate dilution of the secondary antibody in 1X PBS or blocking buffer 01:00:00 Room temperature in the orbital shaker 70-80 rpm and covered from light. 1h

21 Wash sections three times in 1X PBS, 00:05:00 each in the orbital shaker 70-80 rpm covered from light. 5m

22 If sections were free floating in 1X PBS, transfer them to a slide.

23 Add mounting media VECTASHIELD Mounting Medium **Vector Laboratories Catalog #H-1000** and put the coverslip on top.

24 Store short-term at 4 °C or long-term at -80 °C

Imaging

25 Acquire fluorescent images using a slide scanner microscope (VS120, Olympus) or confocal microscope.

26 Analyze images in Fiji (ImageJ).

Mouse on Mouse Immunohistochemistry (MOM IHC)

27 When the secondary antibody host specie is mouse, the IHC is Mouse on Mouse (MOM). Steps for IHC from 16-24 are the same, with some modifications stated below, according to manufacturer's directions:
M.O.M.® (Mouse on Mouse) Immunodetection Kit Basic **Vector Laboratories Catalog #BMK-2202**

- Blocking:

Avidin/biotin blocking step (if required): Avidin/Biotin Blocking Kit, Cat. No. SP-2001). Incubate sections with Avidin Solution for 15 minutes. Rinse briefly with buffer, then incubate in the Biotin Solution for 15 minutes. Wash sections 2 x 2 minutes in buffer. This blocking step may be eliminated if suitable controls have determined this step to be unnecessary.
Mouse Ig blocking step: Incubate sections for 1 hour in working solution of M.O.M. Mouse Ig Blocking Reagent.
Protein blocking step: incubate tissue sections for 5 minutes in working solution of M.O.M. diluent.

- Incubation with primary antibody:

Dilute primary antibody in M.O.M. diluent to the appropriate concentration. Incubate section in diluted primary antibody for 30 minutes. Wash sections in PBS as previously described.

- Incubation with secondary antibody:

Apply working solution of M.O.M. Biotinylated Anti-Mouse IgG Reagent. Incubate sections for 10 minutes. Wash sections in PBS as previously described.

- Incubation with avidin- or streptavidin- conjugate:

Incubate sections in fluorochrome-conjugated Avidin/Streptavidin diluted in 1X PBS for 30 minutes at room temperature in dark. Wash sections in PBS as previously described. Mount and coverslip the slides.