

IMMUNOHISTOCHEMISTRY

Immunohistochemical localization of cellular molecules exploits the ability of antibodies to bind specific antigens (usually proteins) with high affinity. The technique may be used to localize antigens to subcellular compartments or individual cells within tissues. The chapter begins with a discussion of fixation and sectioning of mouse tissues. Fixation and sectioning which critically affect the success of immunohistochemistry experiments must frequently be optimized for each experimental application.

Perfusion of adult mice:

Perfusion of animals is essential for achieving good morphology and presentation of brains, kidneys, hearts and many other organs.

Materials:

1x phosphate-buffered saline (PBS)
4% paraformaldehyde (PFA) fixative, freshly prepared at 4°C.
2 syringes (20-30 ml) equipped with 23-G needles.
Chamber for mouse and Methofane
Dissection instruments (scissors, forceps).

1. Fill a syringe with PBS and another with 4% PFA fixative 4°C
2. Mice are sacrificed by Methofane (Methoxyfluorene) in a chamber. Immediately after respiratory arrest, lay mouse on its back and open the thorax carefully to avoid bleeding. Cut quickly through rib cage and remove diaphragm to access the heart.
3. Insert syringe filled with PBS into the left ventricle and at the same time cut open the right ventricle for drainage, allowing the PBS to be slowly but constantly perfused into heart.
4. After most of the blood has been flushed out, remove the syringe with 1x PBS and insert a syringe filled with 4% PFA into the same puncture in the left ventricle. If the perfusion is working well, blood rich organs such as the liver, spleen and kidney will turn grayish-white.
5. Following perfusion, dissect out organs and tissues, transfer into labeled glass vials filled with PFA at 4°C and store on ice.

Frozen sections:

1. Fix in 4 % PFA overnight in refrigerator or 3 hours at room temperature.

2. The next day do several washes (4-5) with cold PBS 20-30 min. each.
3. Decalcify, if the mouse is > 5 days old.
RDO: 4-5 hours for frozen sections. Test until the bone is soft enough.
(Skip this step if you have tissues without bone).
4. Put the tissues in 25 % Sucrose/dH₂O until tissue settles in the bottom (equilibrates). Place the tissue in 25 % Sucrose at 4°C overnight.
5. The next day block the tissues in O.C.T. compound in acetone / dry ice bath and store the blocked tissues at -70°C until cryosectioning or continue with the sections using a cryostat.

Paraffin sections:

1. After perfusion in situ put the tissues in Bouin's solution and leave them overnight in the refrigerator.
2. The next day rinse the tissues with 50% EtOH, 20-30 min each time, 2-3 times. Between each change rinse the tissues with running water 10-15 min.
3. Rinse with 70 % EtOH 4 times, 15 min each time. At this point tissues can be stored for several days in the refrigerator
4. Rinse with 95 % EtOH 2 times, 30 min each time.
5. Rinse with 100% EtOH 4-6 times, 15 min each time.
6. Rinse the tissues with EtOH /HISTOSOL 3 times, 20 min each time.
7. Melt paraplast in 3 disposable bakera in the vacuum oven at 60°C. Since this takes time you may start this the night before or early in the morning.
8. Flame forceps for transferring the tissues.
9. Incubate for 1 hour in the oven.
10. After 1 hour, transfer to the second baker with melting paraplast and incubate for 1 hour.
11. Incubate again for 1 hour .

12. Do this for a total of 3 hours.
13. After the 3 hours of baking, put the samples in embed molds.
14. Allow to cool and label with the date. Store in the cold.

Aminopropyltrioxy-silane treated slides:

For frozen and paraffin sections we suggest silane treated slides.

1. Make 2% silane (3-aminopropyltrioxysilane) in acetone.
2. Set up 3 staining dishes in the hood.
 - a. acetone
 - b. 2 % silane in acetone
 - c. dH₂O
3. Place the slides in slides holders and dip the slides in the above dishes, 2 min for each one.
4. Put the slides upright in a test tube rack and put them at 37°C to dry.
5. Put dry slides back in the box and label.

The silane must be used the same day it is made and cannot be kept overnight.

Staining procedure:

For frozen sections:

Label the slides, label one slide“PBS” and use it as a control.

1. Wash the slides in a dish with PBS for 20 min.
Lay the slides on a paper towel and put cement around the sections.
For staining use the VECTASTAIN Elite ABC kit (VECTOR LAB).
2. Prepare the blocking buffer by adding 3 drops (150 ul) of stock Normal serum to 10 ml of PBS. The preferred serum for blocking is prepared from the same species in which the biotinylated secondary antibody is made.
3. Add 2-3 drops over each slide, and incubate the sections for 30 min in a 37°C incubator.

4. Incubate sections for 2 hours with primary antiserum diluted in the blocking serum.
5. Wash the slides twice in PBS for 10 min.
6. Incubate sections for 30 min with diluted biotinylated secondary antibody solution (1 drop or 50 ul of biotinylated antibody stock in 10 ml blocking buffer).
7. Wash the slides twice in PBS for 10 min..
8. Incubate sections for 30-45 min with VECTASTAIN Elite ABC Reagent (Add exactly 2 drops of Reagent A to 5 ml of PBS and then add 2 drops of Reagent B to the same mixture, mix immediately, and allow Vectastain ABC Reagent to stand about 30 min before use.
9. Wash the slides with PBS and dH₂O, 10 min each.
10. Incubate in peroxidase substrate solution DAB for 2-7 min or until desired stain intensity develops. Prepare 5 ml 0.1 M Tris pH 7.5 + 0.1 ml of the following solution: (0.5 ml 0.1 M Tris + 8.5 ul 30 % H₂O₂). Combine 1 ml of DAB and this solution and add dropwise over the sections. When the color is brown wash the slides with dH₂O for 5 minutes. Repeat twice.
11. Wash with 5 % P.T.A. for 10 min.
12. Wash the sections 3 times with dH₂O for 5 min each.
13. Treat the slides with 1% Fast green in EtOH.
14. Destain the slides twice with 95% EtOH, twice with 100% EtOH, once with 100 % EtOH / Xylene, and twice with Xylene. 2 min each time.
15. Add 1 drop Permount, cover with coverslips, and put the slides in the hood overnight.

For paraffin sections:

Wash the slides with:

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| Xylene I | 5min |
| Xylene II | 5 min |
| 100% EtOH/ Xylene | 3 min |
| 100% EtOH | 3 min |

95% EtOH 3 min

70 % EtOH 3 min

Tap water 3 min.

Now follow steps 1-15 of the procedure recommended for frozen sections.