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We use this protocol and it's working

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Immunofluorescence and confocal imaging protocol

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ABSTRACT

In this protocol we detail the steps needed to obtain fixed brain tissue from mice to perform immunohistochemistry and image acquisition. In particular, we detail the perfusion, dissection, brain slicing, mounting and anti-ChAT immunolabeling. Here we also detail the steps for confocal images acquisition.

MATERIALS

- Ketamine
- Xylazine
- Styrofoam tray
- Large surgical scissors
- Small surgical scissors
- Blunt forceps
- Barbed forceps
- hemostat
- 27G needles for IP injection
- Scalpel
- Borosilicate glass tube
- Super glue
- 20° agarose wedge

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Tissue Fixation Through Perfusion:

1 Anesthetize the mice with a mixture of ketamine (50 mg/kg) and xylazine (4.5 mg/kg).

2 Use toe pinch-response method to determine depth of anesthesia.

3 Place the animals on a styrofoam tray lying on the back with face upward.

4 Make an incision through the abdominal skin.

5 Make two additional skin incisions from the xiphoid process along the base of the ventral ribcage laterally.

6 Gently reflect the two flaps of skin to expose thoracic field completely.

7 Grasp the cartilage of the xiphoid process with blunt forceps and raise it slightly to insert pointed scissors. Cut through the thoracic musculature and ribcage between the breastbone and medial rib insertion points and extend the incision rostrally to the level of the clavicles.

8 Separate the diaphragm from the chest wall on both sides with scissor cuts.

9 Clamp the reflected ribcage laterally with a hemostat to expose the heart.

10 Secure the beating heart with fingers or blunt forceps, and immediately insert a blunt 27G syringe needle.

11 Cut the right atrium with scissors, and at the first sign of blood flow, begin the infusion of 1x slicing solution at 2-4 ml/min.

12 Turn on the pump and perfuse 3 minutes with PBS-heparineat at 8 ml/min rate.

13 Shift the pump tubing to PFA 4% and perfuse during 8 minutes.

Dissection:

14 Decapitate the mouse with large surgical scissors.

15 Cut down the midline to expose the skull.

16 Make two lateral and one dorsal cut using sharp surgical scissors on the base of the skull.

17 Cut the olfactory bulbs/optic nerve at rostral end of skull.

18 Gently peel off the skull using blunt forceps.

19 Once brain is fully exposed, remove it from the skull and place it in a vial containing PFA 4%.

20 Leave the brain in PFA 4% for 4 hrs for post-fixation.

21 Rinse the brain with PBS and transfer it to a vial containing PBS and store at 4 °C until slicing.

Brain Section:

22 Cut a 2 cm piece of the 20° agarose wedge and super glue it to the specimen plate of *Leica VT1200S vibrotome.*

23 Insert razor blade into blade holder.

24 Set parameters (speed, amplitude, and section thickness) on the Vibratome control panel.

25 Cut the brain in two halves along the midline.

26 Super glue the flat section (midline) onto the agarose wedge glued to the specimen plate so that the vibratome cuts along parasagittal plane with a 20° angle. Place the posterior part of the brain in the top of the agarose wedge. If bilateral sections are needed, super glue both hemispheres to the agarose wedge.

27 Play start to the vibrotome and cut the desired slices at 80 um thick.

28 With a pencil transfer the slices to PBS fillet 1.5 tubes.

29 Store the slices at 4 °C, in darkness, until mounting or immunolabeling.

Mounting:

30 Choose the desired section and carefully mount them in a slide with a pencil.

31 Wait until PBS is almost completely dry.

32 Cover with mounting solution ProLong Diamond.

33 Place a cover slip over the mounting solution and carefully distribute it below the cover slip, trying to avoid bubbles over the brain sections.

34 Maintain all the slides in darkness.

Immunolabeling alpha-ChAT: Primary ab

35 Wash at RT 3-5 x 5 minutes in PBS

36 1 hr blocking at RT on shaker in 1x PBS + 5% Normal horse serum + 0.3% Tx-100.

37 Overnight at 4 degrees in the blocking solution + primary (1:1000) (goat anti-chat - millipore AB144).

38 Wash at RT 3-5 x5 minutes in PBS.

Immunolabeling alpha-ChAT: Secondary ab

39 Secondary in PBS (1:500) at RT for 2-4 hours (closer to 2, 4 is pushing it) (Alexa Green or Red anti-goat).

40 Wash at RT 3-5 x 5 minutes in PBS.

41 Mounting.

Confocal Imaging:

42 Place the slide in the slide holder of the Olympus FV10i confocal laser scanning microscope.

43 Turn on the power of laser, miscroscope, and computer.

44 Open the software.

45 Set lightpath to locate brain slice under 10x lens.

46 Once the brain slice is located, switch the lightpath to camera.

47 Check for lasers of interest and make sure they are in use.

48 Switch to 40x or 60x lens for higher magnification images.

49 Save images.

50 Use FIJI (NIH) to adjust images for brightness, contrast, and pseudo-coloring.