Perfusion and fixation of brain tissue for fresh frozen sections followed by immunofluorescence staining

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

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ABSTRACT

This protocol describes the generation of fresh frozen sections from mouse brain, and subsequent immunofluorescence staining.

MATERIALS

Perfusion and fixation

- Anesthetic (ketamine 50 mg/Kg and xylazine 4.5 mg/Kg – varies according to institutional protocols)

- Peristaltic pump (e.g., Gilson) with tubing and connectors

- Dissection tools (scissors, fine scissors, spring scissors, tweezers, spatula, according to preferences)

- Dissection tray

- 50ml falcon tubes

- crushed ice

- PFA stock solution (e.g., 16% PFA solution, Electron Microscopy)

- 10X PBS

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- pH-meter and related reagents/tools

- Perfusion needle (preferred: 27 gauge)

- Extra needles

- Small plastic bags for storage of brains in -80°C freezer

- Solid PFA waste collection bin

- Liquid PFA waste collection bin

- Water wash bottle

- Carcass bag

Tissue processing and sectioning

-Tissue freezing medium (e.g., Tissue-Tek O.C.T. Compound, Sakura)

-Cryostat (e.g., Leica CM3050S with accessories)

- Small amount of dry ice

-Antifreeze solution 1l (300 ml Glycerol, 300 ml Ethylenglycol, 400 ml dest. Water, 5.18 g Na_2HPO_4, 1.57 g NaH_2PO_4;

-30% Sucrose solution 100 ml (100 ml 0.1 M PB, 30 g Sucrose)

-Cryotubes (e.g., CryoPure tubes, Sarstedt)

Immunofluorescence staining

-Pre-cut brain sections (30-50 µm thick)

-0.1 M PB

-Triton-X100 (detergent)

-Normal donkey serum (S30-100ML, Sigma-Aldrich)

-Primary antibodies

-Secondary antibodies

-12-well-plates with netwell inserts (e.g. Corning Costar Netwell)

-Hard-drying mounting medium (recommended ProLong Diamond, ThermoFisher Scientific)

-Aluminum foil

-Microscopy slides

-Glass coverslips

-Designated container for storage of microscopy slides

- Water bath with integrated shaker, temperature adjustable (e.g. GFL-1083)

- Orbital shaker (e.g. Heidolph Duomax 1030)

-Platform shaker (e.g. Heidolph Unimax 1010)

-Optional: DAPI, Sigma-Aldrich, D9542-5MG, 1:10,000 of 5 mg/ml

SAFETY WARNINGS

Perfusion and fixation

Recommended PPE:
- Performing the procedure and handling PFA under a fume hood is strongly recommended, and mandatory in some cases - varies according to institutional protocols.

- Lab gown/disposable gown

- Face mask

- Face shield/goggles

- Examination gloves (cut-resistant gloves are recommended)

Tissue processing and sectioning

Recommended PPE:
- Lab gown/disposable gown

- Safety goggles

- Examination gloves and cut-resistant gloves

Immunofluorescence staining

Recommended PPE:
- Lab coat/disposable gown

- Safety goggles

- Examination gloves

Perfusion and fixation-Before the Procedure

30m

1 Set up equipment and Solutions:
- PBS can be prepared from 10X concentrated solution

- 4% PFA solution is prepared by diluting the concentrated PFA stock and PBS 10X stock.
For better results, it is recommended to prepare a fresh 4% PFA solution in PBS right before the

procedure. Adjust pH of PBS and PFA solutions to 7.3-7.4

2 - Under the hood, pour PBS and PFA solutions in 50ml tubes.

3 - Attach the perfusion needle to the connector at the end of the tubing.
-Start running PBS through the tubing.

3.1 - If possible, it is recommended to have a connector system with a switch that allows to pre-load the PFA solution and the PBS solution in the respective collection tubing and quickly switch from one to the other avoiding the need to move a single collection tube from one solution to the other, interrupting the procedure.

Perfusion and fixation-The Procedure

30m

4 - Terminally anesthetize the mouse according to institutional protocols.

5 -Bring the anesthetized mouse to the dissection tray and verify that the mouse is fully anesthetized. This can be performed by pinching one of the posterior paws and observing the presence (or lack of) pain reflex. The mouse must be fully anesthetized before starting the trans-cardiac perfusion.

5.1 -Once full anesthesia is achieved, the mouse can be positioned on the dissection area and needles can be inserted in its paws to avoid movement.

5.2 -The mouse should be positioned in a supine position, with the head oriented away from the operator. If the operation area is slightly inclined, the mouse should be oriented so that the head is facing downward.

6 -Holding the skin just below the sternum with a tweezer, cut the skin just below, exposing the peritoneal cavity and the rib cage. The diaphragm should remain intact.

7 -Expand the cut and with the scissors cut the fascia connecting the skin to the rib cage.

-Once the rib cage is clearly visible, carefully cut the diaphragm without damaging the beating heart. Cut the chest cage and lift it toward the head. A needle can be used to hold it in position while operating. The liver should be visible in the abdominal cavity.

8 -Carefully insert the needle connected to the peristaltic pump (where PBS is circulating) in the left ventricle of the heart, and rapidly pinch the right atrium with the spring scissor. Dark-red blood should start flowing out of it immediately. Hold the needle in position, while the solution washes out the blood from the mouse. The heart should still be beating. A wash water bottle can be used to remove excess blood and see more clearly.

9 -As soon as the liver should starts looking whiter, shift the perfusing solution to PFA.

9.1 -Maintain the needle in position and keep perfusing.

-Perfuse mouse with 50 ml of PFA solution.

-As PFA reaches the tissues, some appendages of the mouse might appear to be moving or contracting. This normally indicates that the fixation is working. If the perfusion is done correctly, this should start shortly after changing the perfusion solution to PFA.

10 -Once the desired amount of PFA has run through, stop the perfusion, remove the needle and release the mouse. The carcass should be very stiff.

11 -Decapitate the mouse with the scissor.

12 -With the fine scissors, cut the skin and expose the median line of the skull. Cut off the posterior part of the skull. Then, carefully cut along the median line, towards the rostral part of the head. Past bregma, apply two diagonal cuts toward the eyes, and two other later cuts along the lambdoidal sutures.

13 -Carefully open the skull with the help of the tweezers and expose the brain.

14 -With the spatula, gently remove the brain and slide it into the remaining PFA solution in the falcon tube. Close the falcon tube and gently shake it.

Perfusion and fixation-After the Procedure

1w 1d 0h 10m

15 Disposal & Clean up:
-Properly dispose of the mouse carcass in the carcass bag.

-Dispose of all the PFA waste (liquid and solid) and sharps according to institutional guidelines.

-Clean/wash all the tools/equipment.

16 -Keep the brain in PFA for post-fixation for a variable time depending on experimental needs. We recommend post-fixation in PFA for 4 days at 4°C 4d

17 -Once the post-fixation time has passed, remove PFA solution, substitute with 30% sucrose solution for cryoprotection.

18 -Store brain in 30% sucrose solution for 4 days for proper cryoprotection at 4°C. 4d

19 -Remove brains from sucrose solution and let it dry for a couple of minutes. 2m

20 -Place brain onto crushed dry ice and cover it gently, let it rest for 3 minutes. 3m

21 -Place frozen brain into small plastic bag and label it clearly.

22 -Place bag into -80°C freezer to store brain until further processing.

23 -Let brain rest and accommodate to warmer chamber temperature for 5 min. 5m

Cryosectioning Procedure 3m

24 Mount brain onto specimen stage using Tissue Tek medium.

25 -Let mounted brain freeze for 3 min. 3m

26 -Place specimen stage into object head and adjust specimen orientation as desired.

27 - Collect the sections in the cryotubes. It is important that the sections are collected consecutively in the correct sorting.

Cryosectioning - After the Procedure

28 - After the whole brain has been sectioned, close all cryotubes and store them at 4°C, preferably upright

in a designated box.

29 - Remove the specimen stage from the object head and remove the remaining Tissue Tek medium.

30 - Clean the Cryostat from sample residues according to the manufacturer's manual.

Immunofluorescence staining - Before the Procedure

31 -Prepare 0.1 M PB

-Prepare PBT (0.1 M PB with 0.3% Triton X-100)

-Prepare NDS solution (10% normal donkey serum diluted in PBT)

Immunofluorescence staining - Procedure

1d 12h 13m

32 -Place brain sections (30-50 µm thick) in 12-well-plates with netwell inserts (up to 6-10 sections per netwell insert, depending on the size of the sections).

33 -Place 12-well-plates on platform shaker and wash sections for 4x 5 min in 0.1 M PB. Exchange PB solution in between washing steps. 20m

34 -Block sections for 60 min at room temp. in NDS solution in 12-well-plates. We recommend 4 ml soluti 1h per well for good results.

35 -For incubation with primary antibodies, transfer sections in a new 12-well-plate 16h
but without netwell inserts. This allows better shaking **overnight** (time can vary). Solution for incubation with primary antibodies should contain NDS solution and the respective primary antibodies diluted according to manufacturer recommendation. Incubate sections on orbital shaker with gentle shaking at

4°C overnight. We recommend at least 1 ml solution for each well.

36 -On the next day, transfer sections into 12-well-plate with netwell inserts and wash sections 4x 5 min 20m
PBT on platform shaker at room temperature. Change washing solution after each washing step.

37 -Next, transfer sections back in 12-well-plate without netwell inserts for incubation with secondary antibodies.

38 -Incubate sections with fluorophore-conjugated, species-specific secondary antibodies in NDS solution 2h
for 2 hours on orbital shaker at room temperature. For dilution of the secondary antibodies we generally use 1:500. We recommend at least 1 ml solution for each well. Cover the plate with aluminum foil during antibody incubation to avoid light exposure.

39 -Thereafter, transfer sections back to 12-well-plate with netwell inserts and wash sections for 5x 5mi 30m
PBT. In case you want to additionally stain with DAPI, perform only 2 washing steps with PBT and add DAPI (1:10.000 of 5 mg/ml) to the third PBT washing step and let sections wash for 10 min. Perform a final washing step without DAPI in PBT solution. For all washing steps, 12-well-plate should be covered with aluminum foil to avoid light exposure.

40 -After washing, mount sections on microscopy slides using a fine brush.

41 -Let sections dry a few minutes. 3m

42 -Apply a small amount of hard-drying mounting medium sufficient to cover the sections. Carefully avoid the formation of air bubbles. Gently apply a coverslip over the sections and the mounting medium.

43 -Let cure **overnight** (time can vary) in the dark at room temperature. 16h

44 -General note: This staining protocol can be expanded with a signal amplification step before the incubation with the secondary antibodies on the second day. Before incubating the sections with fluorophore-conjugated, species-specific secondary antibodies, incubate the sections with a biotinylatedspecies-specific secondary antibody in NDS solution for 1 hour at RT on the orbital shaker. Thereafter, wash sections 3 x 5 min in PBT and incubate with fluorophore-conjugated species-specific secondary antibodies as described above, except that one of the species-specific secondary antibodies needs to be
coupled to streptavidin. From this point on follow the protocol described above
for all the remaining steps.

Immunofluorescence staining – After the Procedure:

45 -Dispose of waste and excess reagents/solution according to institutional guidelines.

46 -Clean tools/working station.

47 -Once the mounting medium is cured, slides are ready for observation.

48 -Fully mounted microscopy slides should be stored in a designated container at 4C until time of observation.