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Brain processing, slicing and immunohistochemistry protocol

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We use this protocol and it's working

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Abstract

This is a step by step procedure from collecting brain samples to immunohistochemical staining and mounting brain slices.

Materials

Reagents, Instruments, and Materials

For 4% PFA in 0.1M PB pH7.4 preparation: (D1)

- Corning stirrer/Hot Plate
- 500mL Fixative-labelled Erlenmeyer flask with stir bar
- 250mL PFA-designated graduated cylinder
- Glass funnel
- Filter paper
- Thermometer
- 1L volumetric flask
- 500mL storage bottle for fix
- Distilled H2O
- Electron Microscopy Sciences Granular Paraformaldehyde

For perfusion fixation

Phosphate Buffered Saline, pH 7.4

- 4% Paraformaldehyde solution (4% PFA)
- WPI SP100I Syringe Pump
- Steel surgery bin
- Surgical tools
- (scissors, scalpel, forceps, spatula,)
- Ketamine / Xylazine cocktail
- Isoflurane
- 1mL Disposable syringe + 25g PrecisionGlide needle
- 60mL syringe
- 10mL Syringe
- 1ft surgical tubing x 2
- Filed 25g needle tip (files using sandpaper to flat tip)
- Glass vials w labeling tape
- Proper PFA / Hazardous material disposal containers: solid & liquid PFA Waste
- Biohazard / mouse carcass bags

For Vibratome slicing:

- Leica VT1000 S vibratome
- Vibratome blade holder and placement knob
- Vibratome slicing blade
- Vibratome hex-screw tightening tool
- Removable stage holder with tightening screw
- Removable stage
- Personna 0.012 HD Heavy Duty Single Edge Razor blades

- Hard plastic guillotine brain holder w blade slits
- Scalpel + blade
- Super glue
- spatula
- 6 well plate
- Glass vials + labelling tape
- Loew-Cornell size 0-3 round brush
- Zeiss Axioskop 2 Plus

For Antibody staining & mounting:

- Triton X-100 Normal
- Normal Donkey Serum (NDS)
- Primary Antibody (refer to attached table)
- Secondary Antibody (refer to attached table)
- Plastic transfer pipettes
- Foil wrap
- Glass petri dish x2
- Loew-Cornell size 0-3 round brush
- Glass vial (1 / required antibody cocktail)
- ProLong Diamond Antifade Mountant
- FisherScientific Premium Frosted Microscope Slides 3″ x 1″ x 1mm Glass
- FisherScientific Premium Cover Glass 24 × 50, Thickness: 1
- Heathrow Scientific Slide Box

Solutions:

- 0.2 M Phosphate Buffer (PB):
- PB solution A: 0.2 M Na2HPO4.7H2O (MW: 268.07; 53.614 g/L)
- PB solution B: 0.2 M NaH2PO4.H2O (MW: 137.99; 27.598 g/L)
- 8% formaldehyde in dH2O

Fixative preparation

Required PPE: Safety glasses or visor, lab coat, 2 x gloves.

Note: Carry out all work in the fume hood. Use dedicated glassware and thermometer. Maintain a separate bottle of dH2O so that formaldehyde-contaminated glassware does not come into close proximity with the water purification system.

1. Make up 0.2 M Phosphate Buffer (PB):

- PB solution A: 0.2 M Na2HPO4.7H2O (MW: 268.07; 53.614 g/L)
- PB solution B: 0.2 M NaH2PO4.H2O (MW: 137.99; 27.598 g/L)
- Mix A & B at ~5:1. Add B gradually to A until the pH 7.4 reached. 0.2 M PB can be stored at 4 ◦C for up to 2 weeks.

2. Make up 8% formaldehyde in dH2O:

- In 500 mL flask, heat 200 mL of dH2Oto ~50◦C (do not go above 53 ◦C)

- Add 20g granular paraformaldehyde
- Add 1M NaOH with a glass pipette until solution is clear (~20–25 drops)
- Let solution cool and filter with glass funnel and filter paper
- Make solution up to 250 mL with dH2O in graduated cylinder

3. Combine 250 mL of 8% formaldehyde and 250mL of 0.2M PB pH7.4 to make 500mL of 4% formaldehyde in 0.1 M PB pH 7.4. Check pH with pH paper. Store at 4 ◦C in fridge designated for PFA storage, use within 3 days of making (ideally same day).

Mounting / Storage details

- Proper slide labelling format is
  mouse ID – hemisphere
  series
  primary antibody
  secondary antibody
  genotype
  initials – date

- Insert new slide data and location into slide census spreadsheet

Antibodies

Table 2: Primary antibodies

Name	Host	Supplier	Cat#	Dilution (/1 mL)
Anti-NeuN (A60)	Mouse	Millipore-Sigma	MAB377	1:200 (5 µL)
Anti-NeuN (EPR12763)	Rabbit	Abcam	ab177487	1:1000 (1 µL)
Anti-Tyrosine Hydroxylase (LNC1)	Mouse	Millipore-Sigma	MAB318	1:5000 (0.2 µL)
Anti-Huntington Protein (mEM48)	Mouse	Millipore-Sigma	MAB5374	1:100 (10 µL)[2]
Anti-GFAP (polyclonal)	Chicken	Abcam	ab4674	1:1000 (1 µL)
Anti-Iba1 (polyclonal)	Rabbit	Wako	019-19741	1:1000 (1 µL)
Anti-c-Fos (9F6)	Rabbit	CST[3]	2250S	1:500 (2 µL)
Anti-Parvalbumin (polyclonal)	Guinea	SynapticSystems	195 004	1:1000 (1 µL)
Anti-vGluT1 (polyclonal)	Guinea	SynapticSystems	135304	1:1000 (1 µL)
Anti-vGluT2 (polyclonal)	Rabbit	SynapticSystems	135403	1:1000 (1 µL)
Anti-vGAT (117G4)	Mouse	SynapticSystems	131011	1:250 (4 µL)
anti-Gephyrin (mAb7a)	Mouse	SynapticSystems	147021	1:300 (3.33 µL)
Anti-Bassom (polyclonal)	Guinea	SynapticSystems	141004	1:1000 (1 µL)
Anti-$GABA_A$-R γ2 (polyclonal)	Rabbit	SynapticSystems	224003	1:1000 (1 µL)

Table 3: Secondary anitbodies

Fluorophore	Host	Target	Supplier	Cat#	Dilution (/1 mL)
Alexa 488	Donkey	Anti-Mouse	Jackson ImmunoResearch	715-545-150	1:250 (4 µL)
Alexa 568	Donkey	Anti-Mouse	Fisher Scientific	A10037	1:333 (3 µL)
Alexa 594	Donkey	Anti-Mouse	Jackson ImmunoResearch	715-585-150	1:250 (4 µL)
Alexa 647	Donkey	Anti-Mouse	Jackson ImmunoResearch	715-605-150	1:250 (4 µL)
Alexa 488	Donkey	Anti-Rabbit	Jackson ImmunoResearch	711-545-152	1:250 (4 µL)
Alexa 568	Donkey	Anti-Rabbit	Fisher Scientific	A10042	1:333 (3 µL)
Alexa 594	Donkey	Anti-Rabbit	Jackson ImmunoResearch	711-585-152	1:250 (4 µL)
Alexa 647	Donkey	Anti-Rabbit	Jackson ImmunoResearch	711-605-152	1:250 (4 µL)
Alexa 488	Donkey	Anti-Chicken	Jackson ImmunoResearch	703-545-155	1:250 (4 µL)
Alexa 594	Donkey	Anti-Chicken	Jackson ImmunoResearch	703-585-155	1:250 (4 µL)
Alexa 647	Donkey	Anti-Chicken	Jackson ImmunoResearch	703-605-155	1:250 (4 µL)

Troubleshooting

Perfusion fixation

1 While wearing proper PPE, set up surgical area in fume hood

2 Once a suitable period post-surgery has occurred (3+ weeks) the animal can be perfused

3 Set up surgical area

4 Load ketamine-xylazine cocktail into injection syringe (0.2mL/mouse)

5 In physiological fume hood (separate from PFA-exposed hood) load isoflurane into holding chamber

6 Place mouse in isoflurane holding chamber until mouse breathing has slowed and running has stopped

7 Inject 0.2mL ketamine/xylazine IP and place animal back into original cage

8 Monitor breathing and foot-pinch twitch reflex

9 Once mouse reflex has stopped (should still be breathing) quickly move mouse to the surgical area

10 Restrain mouse using PrecisionGlide needles to hold down limbs

11 Cut mouse skin from to reveal fascia layer, do not yet make incision to reveal pleural space

12 Using fine surgical scissors, cut fascia to reveal organ space

13 Cut diaphragm and up sides of rib cage to reveal heart

14 Using resistance clippers, cut right atrium of the heart

15 Grip heart and insert cold PBS flat needle into left ventricle from the apex of the heart, begin to perfuse 10mL of PBS by hand at ~2mL/min

16 Begin flow of PFA at 120mL/hr, switch PBS needle for PFA needle using the same hole formed from the initial insertion

17 Set timer for 10min, when expires reduce flow to 100mL/hr for 15min

18 Set timer for 15 min, when expires reduce flow to 90mL/hr until 50mL is reached, 25min

19 Remove needle, turn mouse over (should be rigid due to PFA perfusion) and use thick scissors to sever head

20 Using fine surgical scissors, remove scalp to reveal skull

21 Use resistance clippers to gently cut skull without damaging brain, make incisions on skull at most rostral section of brain to enable skull to be peeled from brain

22 Use spatula to delicately remove brain and place into glass vial labelled with mouse ID, 4% PFA, initials and date

23 Refrigerate brain at 4°C for 24 hours

Brain Slicing - Vibratome Operation (24 hours post-perfusion)

24 Set up work-station by gathering 6-well plate with PBS, a petri dish, and 6/12 glass vials (depending on if collecting both hemispheres)

25 Fill all of these with PBS, label the glass vials with the mouse ID, hemisphere genotype, initials, and date

26 Remove vibratome blade from manufacturer packaging and wash with ethanol followed by distilled water to remove protective oils

27 Use hex-tool to tighten blade to the blade-holder so that it is straight and extends several millimeters beyond the black blade-guards

28 Do not attach blade to vibratome at this time

29 Plug in vibratome and turn on, check settings: Slice thickness is 70um, slice frequency is set at 9, slicing speed should be between “4” and “5”

30 Retrieve brain from 4°C Fridge, wash in PBS x 3 and dispose of waste in Liquid PFA waste disposal unit

31 Place brain into holding chamber and using a brush, gently orient the brain to be equally distributed along the sagittal axis

32 For sagittal slicing, take one Personna 0.012 HD Heavy Duty Single Edge Razor blade and insert into the sagittal guide slits, push down through brain while maintaining even force between sides of the blade, this will cut the brain into two halves

33 Take one hemisphere and place into a petri dish filled with PBS, put the other half back into glass vial of PBS and store at 4°C until ready to slice

34 Equip the scalpel with its blade and prepare to use

35 Using spatula, pick up the hemisphere by the bisected plane on the flat edge of the spatula

36 Using filter paper, dry the bisected plane, absorbing residual PBS

37 Paint a thin line of super glue onto the vibratome stage, then quickly use the flat side of the scalpel blade to push the onto the stage so that the bisected plane comes into full contact with the glue

38 Place stage in stage holder and fill with PBS

39 Attach vibratome blade to vibratome

40 Raise the stage so that the blade is several millimeters above the slice

41 Using V-Max, set front and back of continuous cutting using the limit-set button, should be set a few millimeters in front of and behind the most forward and back parts of the brain

42 Begin slicing, placing the first brain slice in well 1, and the following in well 2, going in 6 slice groups (slice 7 will be in well 1) to generate a 1/6 series of the brain per vial

43 Once complete, take slices from ONE WELL that should contain your ROI and observe for viral expression using the epifluorescent microscope (if applicable)

44 Return slices to their original well

45 Transfer slices

Primary incubation

46 Place each series in a glass vial and rinse sections with PBS 3 times

47 Add 1 mL of PBS-T with 2% Normal Donkey Serum (20 µL/mL) to each series and swirl briefly

48 Optional blocking step: leave slices in PBS-T and 2% Normal Donkey Serum at room temperature for 45–60 min

49 Add primary antibody to each series (see table 2)

50 Shake gently for 48–72 h at 4 ◦C (sections should barely revolve around the vial)

Secondary incubation

51 Rinse sections with PBS 3 times before starting secondary reactions

52 Create necessary “Secondary Antibody Cocktail” consisting of 1 mL of PBS-T with 2% Normal Donkey Serum (20 µL/mL) and corresponding secondary antibody (refer to suggested antibody concentration). Volume of cocktail should be +1 to all reagents to avoid lack of volume due to pipette error (meaning 12 vials = 13mL PBS-T, 20uL NDS x 13, 4uL 2° x 13)

53 Protect from light for all remaining steps.

54 Shake gently for 90 min at room temperature (sections should barely revolve around the vial)

55 Rinse sections with PBS 3 times before mounting

56 Using a glass petri dish, gently remove brain slices from 1 vial at a time and mount onto “name of glass slide”

Mounting slices and Labelling

57 Mount sections serially on slides with Prolong Diamond Anti-fade mounting media; protect slides from light and keep at 4 ◦C after 24 h drying at room temperature

58 Proper slide labelling format is
mouse ID – hemisphere
series
primary antibody
secondary antibody
genotype
initials – date

59 Insert new slide data and location into slide census spreadsheet