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DAB immunostaining of thin, fixed mouse brain tissue sections using HNA or NCAM to characterize human iPSC-derived cell xenografts

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We use this protocol and it's working

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Abstract

This protocol describes our use of chromogenic 3,3′-diaminobenzidine (DAB) immunohistochemistry to identify human iPSC-derived cells within thin, fixed mouse brain tissue section series′. We apply this workflow for post-mortem assessment of the survival and growth of human iPSC-derived cells which have been transplanted into the living brain of athymic mice.

Attachments

DOCX

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100KB

Materials

Equipment:

- Horizontal rocker
- Vortex
- Microcentrifuge
- Glass petri dish
- Oven

Consumables:

- 20 mL scintillation vials
- Paint brushes
- Gelatin-Chrom Alum-coating microscope slides
    1. See related protocol - Coating superfrost microscope slides with gelatin-chromium potassium sulfate
- Microscope slide coverslips (no. 1.5, 25 mm x 75 mm)
- Glass pipettes
- Rubber teats
- Transfer pipettes

Key reagents:

- Optimal Cutting Temperature (OCT) compound
- Bovine Serum Albumin (BSA)
- Casein
- Sodium citrate
- Tween-20 and Triton X-100
- Ethanol
- Hydrogen peroxide (H_2O_2)
- DEPEX
- 3, 3'-diaminobenzidine (Sigma #D5905)
- Antibodies
    1. NCAM **Abcam Catalog #**ab75813
    2. HNA (Novus #NOVNBP-313912)
    3. Biotinylated anti-rabbit secondary antibody (Vector Labs #BA-1000)
    4. Avidin/Biotin HRP Complex **Vector Laboratories Catalog #**PK-6100

Solutions:

- 1x PBS, pH 7.4

	A	B
	Antigen retrieval (AR) buffer	
	Sodium citrate	2.94 g (10 mM)
	Tween-20	500 µL (0.05%)
	Up to 1L with dH2O, pH 6.0	

- Quenching solution
  10mL (3.3%) 33% H_2O_2, 50mL (50%) ethanol up to 100mL with 1x PBS
- 1x PBST
  500 µL (0.05%) Tween-20 in 1L 1x PBS

	A	B
	Blocking solution	
	Casein	1 g (1% w/v)
	Triton X-100	250 µL (0.25% v/v)
	Glycine	1.5 g (1.5% w/v)
	BSA	5 g (5% w/v)
	Up to 100mL with 1xPBS	

Material input (animal, cell, tissue, fraction details):

Thin, fixed athymic mouse brain tissue sections prepared from whole mouse brains grafted with human iPSC-derived neural progenitor cells.

Troubleshooting

Day 1 (~4-6 hrs)

1 Pre-heat oven and AR buffer to 70 °C.

2 Label scintillation vials to match labels on section storage plates (mouse and section series IDs, name, date etc.).

3 Transfer sections into scintillation vials using a transfer pipette or fine paintbrush.

4 Remove anti-freeze solution and perform 3× 7 min washes in 1x PBS at Room temperature with gentle agitation.

- Slow shaking on an orbital rocker recommended for washes/incubations to ensure even contact with solutions.
- Use a glass pipette and rubber teat to remove solution during wash changes.
- Anti-freeze solution must be rinsed off prior to immunostaining.

4.1 Remove anti-freeze solution and wash in 1x PBS at Room temperature for 00:07:00 (1/3). 7m

4.2 Remove anti-freeze solution and wash in 1x PBS at Room temperature for 00:07:00 (2/3). 7m

4.3 Remove anti-freeze solution and wash in 1x PBS at Room temperature for 00:07:00 (3/3). 7m

Antigen retrieval (AR)

5 Incubate sections in AR buffer for 00:30:00 at 70 °C. 30m

6 Preheat AR buffer to 70 °C prior to addition to sections.

7 After antigen retrieval, allow sections to cool for 00:30:00 before proceeding with staining. 30m

8 Perform 3× 7 min washes in 1x PBST with agitation.

8.1 Wash in 1x PBST with agitation for 00:07:00 (1/3). 7m

8.2 Wash in 1x PBST with agitation for 00:07:00 (2/3). 7m

8.3 Wash in 1x PBST with agitation for 00:07:00 (3/3). 7m

Quenching step

9 Incubate sections in quenching solution for 00:30:00 at Room temperature with gentle agitation. 30m

10 Perform 3× 7 min washes in 1x PBST with gentle agitation.

10.1 Wash in 1x PBST with gentle agitation for 00:07:00 (1/3). 7m

10.2 Wash in 1x PBST with gentle agitation for 00:07:00 (2/3). 7m

10.3 Wash in 1x PBST with gentle agitation for 00:07:00 (3/3). 7m

Blocking step

11 Incubate sections in blocking solution for 01:00:00 at Room temperature with gentle agitation. 1h

Primary antibody step

12 Incubate sections with NCAM (1:20,000) or HNA (1:15,000) primary antibodies diluted in blocking buffer Overnight at 4 °C with gentle agitation.

Day 2 (~8hrs)

13 Perform 3× 7 min washes in 1x PBST with gentle agitation.

13.1 Wash in 1x PBST with gentle agitation for 00:07:00 (1/3). 7m

13.2 Wash in 1x PBST with gentle agitation for 00:07:00 (2/3). 7m

13.3 Wash in 1x PBST with gentle agitation for 00:07:00 (3/3). 7m

Secondary antibody step

14 Incubate sections in anti-rabbit biotinylated secondary antibody 1:500 diluted in blocking buffer that has been diluted 2-fold for 02:00:00 at Room temperature . 2h

15 Perform 3× 7 min washes in 1x PBST with gentle agitation.

15.1 Wash in 1x PBST with gentle agitation for 00:07:00 (1/3). 7m

15.2 Wash in 1x PBST with gentle agitation for 00:07:00 (2/3). 7m

15.3 Wash in 1x PBST with gentle agitation for 00:07:00 (3/3). 7m

Tertiary complex step

16 Incubate sections in Avidin-Biotin Complex (ABC) kit solution (Vector Laboratories) for 02:00:00 at Room temperature . 2h

17 Prepare tertiary complex 00:30:00 prior to use according to the manufacturer's instructions. 30m

- 100 µL A + 100 µL B + 9800 µL 1x PBS (1:100 A + 1:100 B in 1x PBS).

18 Perform 3× 7 min washes in 1x PBST with gentle agitation.

18.1 Wash in 1x PBST with gentle agitation for 00:07:00 (1/3). 7m

18.2 Wash in 1x PBST with gentle agitation for 00:07:00 (2/3). 7m

18.3 Wash in 1x PBST with gentle agitation for 00:07:00 (3/3). 7m

Chromogen step

19 Perform DAB staining as follows;

20 Prepare DAB solution by dissolving 10 mg DAB tablet into 20 mL PBS ([M] 0.5 mg/mL).

21 Filter through Whatman paper #1 or 0.22 um syringe filter before use.

> Note
>
> NB: DAB is a suspected mutagen and should be handled with care.

22 Prepare DAB-H2O2 solution **immediately** prior to use by adding 10 µL 30% H2O2 per 5 mL DAB solution and mix thoroughly.

23 Incubate sections with DAB-H2O2 solution for 00:08:00 . 8m

24 Perform 3× 7 min washes in 1x PBST with gentle agitation.

24.1 Wash in 1x PBST with gentle agitation for 00:07:00 (1/3). 7m

24.2 Wash in 1x PBST with gentle agitation for 00:07:00 (2/3). 7m

24.3 Wash in 1x PBST with gentle agitation for 00:07:00 (3/3). 7m

25 **Mount tissue sections** onto super-frost slides pre-coated with gelatin-chrome alum and allow to dry at Room temperature Overnight .

Day 3 (2 days later) 33m

26 Process slide-mounted tissue sections through the following solutions;

27 dH2O 00:03:00 . 3m

28 50% ethanol 00:03:00 . 3m

29 70% ethanol 00:03:00 . 3m

30 95% ethanol 00:03:00 . 3m

31 100% ethanol 00:03:00 . 3m

32 100% ethanol 00:03:00 . 3m

33 Xylene 00:05:00 . 5m

34 Xylene 00:05:00 . 5m

35 Xylene 00:05:00 . 5m

36 **Coverslip slides with DEPEX mounting media** and allow to dry in the fume hood Overnight before proceeding with microscopy.

37 Image sections using bright field microscopy for subsequent xenograft characterization.