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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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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

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MATERIALS TEXT

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)

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- Casein
- Sodium citrate
- Tween-20 and Triton X-100
- Ethanol
- Hydrogen peroxide (H_2O_2)
- DEPEX
- 3, 3'-diaminobenzidine (Sigma #D5905)
- Antibodies

  NCAM

  1. **Abcam Catalog #ab75813**
  2. HNA (Novus #NOVNBP-313912)
  3. Biotinylated anti-rabbit secondary antibody (Vector Labs #BA-1000)

     Avidin/Biotin HRP Complex **Vector**
  4. **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.

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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 3x 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  30m

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

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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 3x 7 min washes in 1x PBST with agitation.

8.1 7m

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

8.2 7m

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

8.3 7m

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

Quenching step

9 30m

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

10

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

10.1 7m

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

7m

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10.2

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

10.3 7m

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

Blocking step

11 1h

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

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

13.1 7m

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

13.2 7m

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

13.3 7m

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

Secondary antibody step

2h

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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** .

15

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

15.1 7m

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

15.2 7m

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

15.3 7m

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

Tertiary complex step

16 2h

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

17 30m

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

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

18

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

18.1 7m

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

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18.2 7m

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

18.3 7m

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

Chromogen step

19 Perform DAB staining as follows;

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

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

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 8m

Incubate sections with DAB-H2O2 solution for **00:08:00** .

24

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

24.1 7m

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

24.2 7m

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

24.3 7m

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

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

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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.

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