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Multiplex fluorescent immunostaining of thin, fixed mouse brain tissue sections to characterize human iPSC-derived cell xenografts

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ABSTRACT

This protocol describes our multiplex fluorescent immunohistochemistry protocol used 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, growth and maturation of human iPSC-derived cells which have been transplanted into the living brain of athymic mice.

ATTACHMENTS

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KEYWORDS

Human iPSC, Immunohistochemistry, Fluorescent, Human-to-mouse xenograft

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MATERIALS TEXT

Equipment:

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

Consumables:

- 20mL 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 thickness, 22x50mm)
- 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
- ProLong Diamond Antifade mountant

Primary antibodies:

A	B	C	D
Target	**Species**	**Dilution**	**Company, Catalog #**
alpha-Synuclein	Mouse	1:1000	Santa Cruz, sc-12767
CTIP	Rat	1:1000	Abcam, ab18465
FOXG1	Rabbit	1:500	Abcam, ab18259
HNA	Mouse	1:1000	Novus, #NOVNBP-313912
NCAM/CD56 (ERIC-1)	Mouse	1:100	Santa Cruz, sc-106
NeuN	Chicken	1:1000	Merck-Millipore, ABN91
Pax6	Rabbit	1:1000	ThermoFisher Scientific, #42-660
SOX2	Mouse	1:500	R&D Systems, AF2018
Tbr1	Rabbit	1:1000	Abcam, ab31940
Tyrosine Hydroxylase	Rabbit	1:1500	Pel-freeze, P40101-0

Anti-α-synuclein Antibody (211) **Santa Cruz Biotechnology Catalog #sc-12767**

Anti-Ctip2 antibody [25B6] (ab18465) **Abcam Catalog #ab18465**

Anti-FOXG1 antibody (ab18259) **Abcam Catalog #ab18259**

Anti-NCAM/CD56 Antibody (ERIC 1) **Santa Cruz Biotechnology Catalog #sc-106**

Anti-NeuN Antibody **Sigma Aldrich Catalog #ABN91**

PAX6 Polyclonal Antibody **Thermo Fisher Scientific Catalog #42-6600**

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Human/Mouse/Rat SOX2 Antibody **R&D Systems Catalog #AF2018**

Anti-TBR1 antibody **Abcam Catalog #ab31940**

Rabbit Tyrosine Hydroxylase **Pel-Freez Catalog #P40101-0**

Secondary antibodies:

A	B	C	D
Target	**Species**	**Dilution**	**Company, Catalog #**
anti-mouse AF647	Donkey	1:500	Life Technologies, A31571
anti-rabbit AF647	Goat	1:500	Life Technologies, A11008
anti-chicken CF594	Goat	1:500	Merck, SAB4600094
anti-goat CF488A	Donkey	1:500	Merck, SAB4600032

Donkey anti-Mouse IgG (H L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor™ 647 **Thermo Fisher Scientific Catalog #A-31571**

Goat-anti-rabbit-Alexafluor 488 **Thermo Fisher Scientific Catalog #A11008**

Anti-Chicken IgG (H L) highly cross-adsorbed CF594 antibody produced in donkey **Sigma Aldrich Catalog #SAB4600094**

Anti-Goat IgG (H L) highly cross-adsorbed CF™ 488A antibody produced in donkey **Sigma Aldrich Catalog #SAB4600032**

Solutions:

- 1x PBS, pH **7.4**
- Antigen retrieval (AR) buffer
  2.94 g ([M] **10 millimolar (mM)**) sodium citrate, **500 µL** (0.05%) Tween-20, up to **1 L** with dH_2O, pH **6**
- 1x PBST
  1. **500 µL** (0.05%) Tween-20 in **1 L** 1x PBS
- Blocking solution
  1. **1 g** (1% w/v) casein, **250 µL** (0.25% v/v) Triton X-100, **1.5 g** (1.5% w/v) glycine, **5 g** (5% w/v) BSA up to **100 mL** with 1x PBS

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.

Day 1 (~3-4 hrs)

1 Pre-heat oven and Antigen Retrieval (AR) buffer to **70 °C** .

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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 perform 3x **00:07:00** washes in 1x PBS at **Room temperature** with gentle agitation (1/3). 7m

4.2 Remove anti-freeze solution and perform 3x **00:07:00** washes in 1x PBS at **Room temperature** with gentle agitation (2/3). 7m

4.3 Remove anti-freeze solution and perform 3x **00:07:00** washes in 1x PBS at **Room temperature** with gentle agitation (3/3). 7m

Antigen retrieval (AR) - Day 1

5  30m

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

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

7 

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

7.1 Perform 3x **00:07:00** washes in 1x PBST with gentle agitation (1/3). 7m

7.2 Perform 3x **00:07:00** washes in 1x PBST with gentle agitation (2/3). 7m

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7.3 Perform 3x **00:07:00** washes in 1x PBST with gentle agitation (3/3). 7m

Blocking step - Day 1

8 1h

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

Primary antibody step - Day 1

9 1h

Incubate sections with desired primary antibody combinations (to characterise either cortical or ventral midbrain derived xenografts) diluted in blocking buffer **Overnight** at **4 °C** with gentle agitation.

Day 2 (~4hrs)

10

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

10.1 Perform 3 x **00:07:00** washes in 1x PBST with gentle agitation (1/3). 7m

10.2 Perform 3 x **00:07:00** washes in 1x PBST with gentle agitation (2/3). 7m

10.3 Perform 3 x **00:07:00** washes in 1x PBST with gentle agitation (3/3). 7m

Secondary antibody step 1d 4h 7m

11 2h

Incubate sections in secondary antibodies, that match the species of chosen antibodies, diluted in blocking buffer for **02:00:00** at **Room temperature** . Incubate in a dark room or cover the vials with foil to protect the fluorophores from light.

12

Perform 3x 7 min washes in 1x PBST with gentle agitation keeping vials protected from the light.

12.1 Perform 3x **00:07:00** washes in 1x PBST with gentle agitation keeping vials protected from the light (1/3). 7m

12.2 Perform 3x **00:07:00** washes in 1x PBST with gentle agitation keeping vials protected from the light (2/3). 7m

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12.3 Perform 3x **00:07:00** washes in 1x PBST with gentle agitation keeping vials protected from the light (3/3). 7m

13 **Mount tissue sections** in a dimly lit room (to protect the fluorophores) onto super-frost slides pre-coated with gelatin-chrome alum and allow to dry in the dark at **Room temperature** for **01:00:00** - **02:00:00** . 3h

14 Remove ProLong Diamond Antifade mountant from storage at **4 °C** and allow the reagent to equilibrate to **Room temperature** before use.

15 **Coverslip slides with ProLong Diamond Antifade mountant** and allow to set at **Room temperature** for at least **24:00:00** in the dark before proceeding with microscopy. 1d

16 

Image sections using fluorescent microscopy for subsequent xenograft characterization.

17 Slides can be stored at **4 °C** or **-20 °C** .

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