Free floating immunofluorescent staining protocol on mouse brain sections V.2

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

ABSTRACT

This protocol describes our free-floating immunofluorescence staining protocol used to investigate the fate and pathology
of human iPSC-derived cells grafted in the mouse brain. This protocol allows for the post-mortem visualisation and analysis of the morphology and pathological inclusions of the transplanted human iPSC-derived cells carrying different PD-related mutations in different regions within mouse brain tissue sections.

GUIDELINES

IMPORTANT: perform all antibody incubation steps and steps following in minimal light so as not to bleach signals prior to imaging

MATERIALS

Antibodies

- TH(IgG2b): TH Monoclonal Antibody (OTI3G3),TrueMAB™ #TA506549, Ms, IgG2b, clone OTI3G3, 1:200
https://www.thermofisher.com/antibody/product/TH-Antibody-clone-OTI3G3-Monoclonal/TA506549
- Syn204(IgG2a): Anti-α-Synuclein Antibody, Biolegend #838201, Ms, IgG2a, clone Syn204 (aa 87-110) 1:1, 1:200
https://www.biolegend.com/en-us/products/anti-alpha-synuclein-antibody-10995?GroupID=BLG15651
- S129(Rb): Recombinant Anti-Alpha-synuclein (phospho S129) antibody [EP1536Y] (ab51253), Rb, Mono, 1:500https://www.abcam.com/products/primary-antibodies/alpha-synuclein-phospho-s129-antibody-ep1536y-ab51253.html

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	A	B	C	D	E
	Comb#1 ***Primary***	**TH IgG2b**	**Syn204**	**S129**	
	Cat #	**TA506549, MS IgG2b**	**838201, Ms IgG2a**, 1:1	**ab51253, Rb**	
	dilution	200	200	500	
	Comb#1 ***Secondary***	Goat @ mouse IgG2b 647	Goat @ mouse IgG2a 568	Donkey @ rabbit 488	Hoechst33342
	Cat #	A-21242	A-21134	A-21206	
	dilution	1:250	1:250	1:200	1:1000

Equipment

- Orbital shaker
- black porcelain spot plate

Consumables

- microscope slides
- 6-well plates and net inserts
- Microscope slide coverslips (no. 1.5 thickness, 22x50mm)

Key reagents

- Blocking buffer for IF in tissue section: 2% Donkey serum, 1%BSA, 0.2% TritonX-100, 0.1% gelatine, 0.1% Tween-20 in 1XPBS
- Citrate buffer (0.01M pH 6.0): 2.94g/L tri-sodium citrate (dihydrate) in deionised water
- sodium borohydride
- Tween-20 and Triton X-100
- DAKO Fluorescence Mounting Medium

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).
NOTE: Sodium borohydride is highly toxic and flammable

Experimental outline

1 Briefly, the mouse brain tissue sections are prepared by washing off the cryoprotectant medium and then antigen retrieval is performed followed by quenching, blocking and primary antibody incubation. Sections are then washed and incubated in the appropriate secondary antibody solution and are then mounted, cover-slipped and sealed.

Day 1 - Tissue preparation

2 30 um mouse brain sections were stored in anti-freeze solution at -20 °C until required. 35m

1. Remove samples from freezer and equilibrate at Room temperature for 00:10:00 - 00:20:00
2. Pour sections into a well insert in a 6-well plate to separate storage solution from section
3. Move the well insert to another well containing approximately 6 mL of 1x PBS. Wash at least 5x with 1x PBS for 00:05:00 each on an orbital shaker using low speed at Room temperature

Antigen retrieval

3

1. Incubate the sections in 10mM sodium citrate buffer (pH 6.0) for 00:30:00. Let it cool to Room temperature 35m
2. Rinse the sections 3x 00:05:00 each in 1X PBS

Quenching aldehyde group

4

1. Weigh $NaBH_4$ to make 0.1~0.5% in 1X PBS, made fresh 35m
2. Move the insert with sections into the fresh-made solution for 00:30:00 at Room temperature
3. Wash 2x 00:05:00 in 1X PBS

Blocking

5

1. Incubate sections in normal donkey serum IF blocking buffer 02:00:00 Room temperature on shaker 60 rpm 2h

Primary antibody incubation

6 **Make primary antibody cocktails in blocking buffer** 3d

1. Prepare ~ 300 µL per sample of primary antibody solution consisting of selected primary antibody (diluted appropriately) in home-made normal donkey serum IF blocking buffer
2. Transfer sections from well insert into wells of black porcelain spot plate containing

primary antibody solution to bind to the antigen(s) of interest

3. Place the plate on a rotating mixer using low speed (speed 7 rpm) and incubate 72:00:00 at 4 °C (or 3X night/ over weekend)

Day 2 - Secondary antibodies

7

2h 15m 30s

1. The following day, pour sections into a well insert in a 6-well plate to separate sections from primary antibody solution.
2. Wash sections 3 times with 1x PBST at Room temperature. Note: 00:00:30 for the first two rinses, 3x 00:10:00 for additional washing
3. Prepare 300 µL per sample of secondary antibody solution consisting of appropriate secondary antibody + Hoechst 33342 (diluted accordingly) in blocking buffer (shield solution from light)
4. Transfer sections into the black porcelain spot plate containing 300 µL secondary antibody cocktail
5. Incubate for 02:00:00 at Room temperature on orbital shaker using low speed (shield solution from light).
6. Pour sections into a well-insert in a 6-well plate containing 1X PBST to separate sections from the secondary antibody solution
7. Continuing to shield samples from light, wash 3 times with 1x PBS for 00:05:00 at Room temperature

Mounting

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1. Pour sections into a glass petri dish
2. Submerge a glass slide into the 1x PBS and use a fine paintbrush to coax the sections towards the slide
3. Gently tap the sections onto the slide, making sure there are no wrinkles or folds
4. Repeat until all sections are mounted onto the slide(s)

Cover-slipping

15m

9

15m

1. After sections are dried onto the slide(s), about 00:15:00 at Room temperature or until sections look opaque (remember to shield slides from light), apply an appropriate aqueous mounting medium (hardening or non-hardening). Antifading (DAKO Fluorescence Mounting Medium is preferred if using a fluorescent conjugated secondary antibody
2. Using tweezers, place a coverslip on top of the medium. Cover with filter paper and press down firmly to remove excess mounting medium

3. Image sections using an appropriate microscope. Store in a dark slide box at 4 °C