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We use this protocol and it's working

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Immunocytochemistry of cultured human Medium Spiny Neurons (MSNs) V.1

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ABSTRACT

This protocol describes the immunolabelling of one or several protein targets on PFA-fixed cell culture on glass coverslips.

GUIDELINES

Adherent cells are prone to peeling, and hence, addition and removal of any liquid should be performed slowly with care.

At no point during the entire procedure prior to mounting should the sample be left to dry.

Fluorescent-dye conjugated Secondary Antibodies are light-sensitive and hence should always be protected from light.

All PBS washes should be at least 10 mins, and plates are left to incubate on the bench at room temperature.

MATERIALS

Reagents:

- Activin A (Sigma-Aldrich, SKU# SRP3003)
- B-27™ Supplement (50X), serum free (ThermoFisher Scientific, CAT# 17504044)
- β-Mercaptoethanol (ThermoFisher Scientific, CAT# 21985023)
- CaCl2 (sigma, SKU# C5670)
- CHIR 99021 (Bio-Techne/Tocris/R&D, CAT# 4423)
- Citrate buffer solution, pH 6.0 (Sigma- Aldrich, SKU# C9999-100ML)
- DAPT, gamma-Secretase inhibitor (Abcam, CAT# ab120633, CAS# 208255-80-5)

Keywords:
Immunocytochemistry, Cell Culture, Human Neurons

- dCAMP (Sigma-Aldrich, CAT# D0627. CAS# 16980-89-5)
- DMEM/F12 basal medium (ThermoFisher Scientific, CAT# 11320033)
- DMEM/F-12, GlutaMAX™ supplement (ThermoFisher Scientific, CAT# 10565018)
- Donkey Serum (Sigma- Aldrich, SKU# D9663-10ML)
- GABA (Sigma-Aldrich, SKU# A2129)
- LM22A4 (Tocris, CAT# 4607)
- Matrigel (CAT# 354277)
- MEM Non-Essential Amino Acids Solution (100X) (NEEA) (ThermoFisher Scientific, CAT# 11140050)
- Neurobasal (CAT# 21103049)
- Paraformaldehyde (PFA) (Sigma- Aldrich, CAT#P6148)
- PBS with Azide (Insight Biotechnology, CA# sc-296028)
- Penicillin-Streptomycin (10,000 U/mL) (ThermoFisher Scientific, CAT# 15140122)
- PD0332991 (Selleck Chemicals, CAT# S1579)
- Phosphate-buffered saline, pH 7.4 (PBS) (Life Technologies, CAT# 10010056)
- Poly-D-Lysine (ThermoFisher Scientific, CAT# A3890401)
- Recombinant Human/Murine/Rat BDNF (BDNF) (PeproTech, CAT# 450-02)
- ROCK inhibitor Y-27632 (ROCKi) (Bio-Techne, CAT# 1254)
- SlowFade Diamond Antifade mountant (ThermoFisher Scientific, CAT# 36963)
- Triton X-100 (Sigma- Aldrich, SKU# X100)

Equipment:

- Adhesion slides, SuperFrost Plus, White (VWR International LTD, CAT# 631-0108)
- Fisherbrand™ Glass Circle Coverslips (ThermoFisher Scientific, CAT# 12323138).
- ImmEDGE™ Hydrophobic Barrier Pen (Pap Pen) (ThermoFisher Scientific, CAT# NC9545623)

Preparing Striatal neuronal induction base medium (sNIM):

- DMEM/F12 basal medium
- 1% MEM Non-Essential Amino Acids (NEAA)
- 1% Glutamax
- 1x B27 without vitamin A
- 1% penicillin/Streptomycin (P/S)
- 0.05% β-mercaptoethanol

Preparing Day 16 (D16) media:

1. Add to sMM1 base media:

- 100 ng/ml BDNF (1:10000)
- 200 µM Ascorbic acid (1:1000)
- 10 µM DAPT (1:10000)
- 2 µM PD0332991 (1:500)
- 0.6 mM $CaCl_2$ (1:1000)
- 1 µM LM22A4 (1:1000)
- 200 nM cAMP (1:500)

- 3 µM CHIR (1:3333)
- 300 µM GABA (1:1000)
- 25 ng/ml Activin A (1:1000)
- 10 µM ROCKi (1:1000)

Replating human Medium Spiny Neurons (MSNs) onto glass...

1 Prepare glass coverslips and plates for replating

1.1 Add 10 or 100 µg/mL of Poly-D-lysine onto plastic wells and glass coverslips, respectively and incubate at 37^{o}C overnight.

Note

These glass coverslips should have been sterilised in 70% ethanol for at least 1 hour and air dried completely in a tissue culture hood.

1.2 Wash plenty with Phosphate-buffered Saline (PBS), at least 3 times.

1.3 Add Matrigel and incubate at 37^{o}C for at least 1 hour.

2 Prepare media for thawing and replating

2.1 Pre-warm spinning falcons containing 9 mL of Neurobasal.

2.2 Prepare Day 16 (D16) media + ROCKi (1:1000).

2.3 Allow an aliquot of desired volume to reach room temperature.

3 Thawing and replating of Day 16 Medium Spiny Neurons (MSNs) precursors

3.1 Thaw cryovial containing Day 16 MSN precursors in water bath until only a small component remains frozen.

3.2 Carefully transfer contents of cryovial to pre-warmed spinning tubes.

3.3 Centrifuge at 350g for 5 min.

3.4 While spinning, aspirate Matrigel and replace with D16 media + ROCKi (1:1000).

3.5 Aspirate media from cell pellet in spinning falcon and replace with D16 media + ROCKi (1:1000), slowly and gently resuspending the pellet.

3.6 Transfer an appropriate amount of cell suspension into previously prepared glass coverslips and swirl plate gently in a figure 8 motion.

3.7 Allow cells to be cultured on glass coverslips as described in **step 5** of **Protocol: Differentiation of human medium spiny neurons (MSNs) from induced pluripotent stem cells (iPSCs)** until relevant experimental timepoints for immunocytochemistry.

PFA Fixation

4 Transfer glass coverslips cultured with MSNs matured to relevant experimental timepoints into a 6-well plate.

5 Draw a fitting circle enclosing the entire coverslip with a hydrophobic Pap pen.

Note

This circle serves as a boundary to contain the fluid volume applied onto the cell coverslip.

Note

If performing immunocytochemistry on plastic well-cultured MSNs, omit **steps 4 and 5** and proceed to **step 6** onwards.

6 Fix cells by adding 2% PFA in PBS to each well at room temperature for 20 mins.

7 Remove PFA completely using a pipette.

8 Wash thoroughly 3 times with PBS, at least 10 mins each.

9 Incubate the coverslips in PBS for at least 1 hour at room temperature or at 4°C overnight.

Heat-induced antigen retrieval

10 Perform antigen retrieval by adding 50 mL of 1x citrate buffer (pH 6.0) to each well, and place the 6-well plate in a water bath at 80ºC for 5 mins.

Note

Fluid can quickly dried out during the incubation - a generous amount of citrate buffer should be added.

This step is time- and temperature-sensitive.

Longer incubation and/or higher temperature can damage the cell sample.

11 Leave to incubate at room temperature for 10-20 mins.

12 Prepare blocking solution containing PBS with 10% Donkey Bovine Serum (NBS) and 0.1% Triton-X during the post-antigen retrieval incubation.

13 Remove the citrate buffer and add 50 mL of blocking solution.

14 Leave to incubate at room temperature for 10 mins.

15 Wash twice with PBS, at least 10 mins each.

Primary Antibody Incubation

16 During the previous washes, prepare the Primary Antibody Solution containing PBS with 10% Donkey Bovine Serum (NBS), supplemented with appropriate primary antibodies in their respective working concentrations.

The following primary antibodies (working concentration 1:250) were used in Do, Q. et al. (2023) for immunostaining: anti-CTIP2, anti-DARPP32, anti-DARPP32, GAD67, anti-MAP2, anti-Calbindin, PDYN, PKEN, Substance P, Tyrosine hydroxylase.

17 Remove all the PBS from each well and add 80 mL of the Primary Antibody Solution.

18 Leave to incubate overnight at 4°C.

Secondary Antibody Incubation

19 Remove all the Primary Antibody Solution and add fresh PBS.

20 Wash thoroughly 3 times with PBS, at least 10 mins each.

21 During the last wash, prepare Secondary Antibody Solution containing PBS with 10% Donkey Bovine Serum (NBS), supplemented with appropriate secondary antibodies and DAPI (4',6-Diamidino-2-Phenylindole, Dilactate) in their respective working concentrations.

The following secondary antibodies (working concentration 1:1000) were used in Do, Q. et al.

(2023) for immunostaining: Alexa-Fluor 555 Mouse and Rabbit, Alexa-Fluor 648 Rabbit, Alexa Fluor 488 Rat and Chicken, and DAPI.

Note

Keep pre-made solution wrapped in aluminium foil and/or keep away from direct light during incubation.

22 Remove all the PBS from each well and add 80 mL of the Secondary Antibody Solution.

23 Leave to incubate at room temperature for 1-2 hours.

Note

Keep cell plates wrapped in aluminium foil and/or keep away from direct light during incubation.

24 Once done, wash well with PBS.

Mounting

25 Wrap culture plates with aluminium foil and/or keep away from direct light.

26 Keep immunostained well plates in PBS plus 0.02% Azide for long-term storage.

Note

Samples can be mounted immediately after washes (**step 22**) without PBS storage, if imaging is to be done within the next 48-72 hours.

27 Keep immunostained coverslips in PBS till 24 hours prior to imaging date.

Note

Regularly add extra PBS to protect samples from drying out.

28 At least 24 hours prior to the recording, apply a small drop of SlowFade Diamond Antifade mountant onto each coverslip, and carefully place the coverslip on the glass slides.

29 Leave to incubate the mounted slides at 4°C overnight.

Note

Proceed with imaging within 2 weeks after completing the protocol.

SlowFade Diamond Antifade mountant is non-hard setting mountant, and therefore, **prolonged storage of the mounted sample is not desirable.**