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Immunocytochemistry of human iPSC-derived Dopamine Neurons (DaNs)

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We use this protocol and it's working

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Abstract

This protocol outlines the immunocytochemical staining of multiple protein targets for characterisation and synapses in Paraformaldehyde (PFA)-fixed cells plated in a 96-well format plate.

Materials

Reagents:

- Phosphate-buffered saline, pH 7.4 (PBS) (Life Technologies, CAT# 10010056)
- **Triton X-100** (Sigma- Aldrich, SKU# X100)
- **Paraformaldehyde (PFA)** (Sigma- Aldrich, CAT#P6148)
- DAPI (4',6-Diamidino-2-Phenylindole, Dilactate) (Thermo Fisher Scientific, CAT# D1306)

Primary Antibodies:

- Rabbit anti-Foxa2 (Abcam, CAT# ab108422)
- Rabbit anti-Homer1 (Synaptic Systems, CAT# 160 003)
- Guinea Pig anti-synapsin I/II (Synaptic Systems, CAT# 106 004)
- Chicken anti-Map2 (Abcam, CAT# ab92434)
- Sheep anti-TH (Sigma-Aldrich, CAT#ab1542)
- Rabbit anti-vGLUT2 (Synaptic Systems, CAT# 135 403)
- Rabbit anti-vMAT2 (Thermo Fisher Scientific, CAT# PA5-22864)

Secondary Antibodies:

- Donkey anti-mouse IgG Alexa Fluor 488 (Molecular Probes, CAT# A-21202)
- Donkey anti-chicken IgG Alexa Fluor 555 (Thermo Fisher Scientific, CAT# A78949)
- Donkey anti-rabbit IgG Alexa Fluor 647 (Molecular Probes, CAT# A-31573)
- Goat anti-guinea pig IgG Alexa Fluor 488 (Abcam, CAT# ab96954)
- Goat anti-chicken IgG Alexa Fluor 555 (Thermo Fisher Scientific, CAT# A32932)
- Goat anti-rabbit IgG Alexa Fluor 647 (Molecular Probes, CAT# A-21245)

Equipment:

- 96 well tissue culture treated plate (Greiner Bio-One Ltd, CAT# 655090)
- Opera Phenix™ Plus high-content imaging system (Revvity; CAT# HH14001000) - https://www.revvity.com/gb-en/product/opera-phenix-plus-system-hh14001000

SOLUTIONS

Preparing f 4% PFA fixing solution:

Mix 10 mL of 16% PFA with 30 mL PBS.

Preparing 10% Normal Serum:

Add 1 mL of Normal Serum (NS) in PBS.

Donkey Serum for characterisation staining and Goat serum for synaptic staining

Preparing 0.01% Triton X-100 solution:

Add 2 µL of Triton X-100 solution in 20 mL 10% Normal Serum (NS).

Safety warnings

Cells must be fixed with PFA under a fume hood. Any materials (*e.g.*, pipette tips, reservoirs, or waste containers) that come in contact with PFA should remain inside the **fume hood** for a minimum of **24 hours** before discarding. PFA waste should be discarded as per local guidelines.

Ethics statement

The protocols.io team notes that research involving animals and humans must be conducted according to internationally-accepted standards and should always have prior approval from an Institutional Ethics Committee or Board.

Before start

Cells should be plated at a density of 50 000 cells/well in a 96 area full area plate which has been precoated with Geltrex.

Additionally, care should be taken when adding and removing liquid as these adherent cells are prone to peeling. If the plates are stored prior to staining, a **plastic strip** should be used to seal the wells to prevent evaporation or contamination, and the plates should be kept at **4°C.**

PFA Fixation

1 Remove the media from the wells to be fixed.

2 Add 50ul of 4% PFA solution to the wells and keep it for 5 minutes for synaptic markers and incubate for 5 minutes for synaptic markers or 10 minutes for other markers at room temperature (RT).

3 Remove PFA and wash the cells twice with PBS.

Permeabilisation and Blocking

4 Permeabilise the cells with 0.01% Triton X-100 for 1 hour.

5 Remove the solution and wash with PBS.

Primary Antibody Incubation

6 Prepare the primary antibody solution in 1% NS in PBS, supplemented with the appropriate primary antibodies (see **Materials**) at their respective working concentrations as in **Table 1**.

	A	B
	Primary Antibodies	**Dilution**
	Rabbit anti-Foxa2	1:500
	Rabbit anti-Homer I	1:500
	Guinea Pig anti-Synapsin I/II	1:500
	Chicken anti-Map2	1:500

	A	B
	Sheep anti-TH	1:500
	Rabbit anti-vGLUT2	1:500
	Rabbit anti-vMAT2	1:500

Table 1 - Dilution factors for Primary Antibodies used.

7 Add 50 µl of primary antibody solution to each well and incubate overnight at 4°C.

Secondary Antibody Incubation

8 Remove the primary antibody solution and wash twice with PBS.

9 Incubate in species-appropriate Alexa Fluor® secondary antibodies as mentioned in **Table 2** with DAPI (1:10000) in 1% NS in PBS for 1 hour at room temperature.

	A	B
	Secondary Antibodies	**Dilution**
	Donkey anti-mouse IgG Alexa Fluor 488	1:1000
	Donkey anti-chicken IgG Alexa Fluor 555	1:1000
	Donkey anti-rabbit IgG Alexa Fluor 647	1:1000
	Goat anti-guinea pig IgG Alexa Fluor 488	1:1000
	Goat anti-chicken IgG Alexa Fluor 555	1:1000
	Goat anti-rabbit IgG Alexa Fluor 647	1:1000

Table 2 - Dilution factors for Secondary Antibodies used.

Image Acquisition

10 Acquire wells images using a 40x magnification water immersion objective Opera Phenix High content screening system, capturing images at a focus level of +1 µm and a binning factor of 1 for synaptic imaging.

11 For characterisation, acquire images using 40x magnification water immersion objective, capturing images at five focal planes ranging from +1 µm to +9 µm and a binning factor of 2.

12 Take 20 images on average per well, with 3-5 wells analysed per cell line per differentiation.