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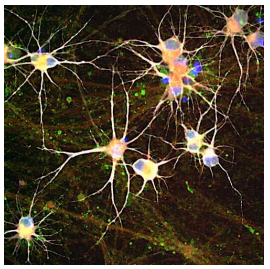
Immunolabeling and Quantitative Analysis of Synapses in Human iPSC-Derived Dopaminergic Neurons



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We use this protocol and it's working

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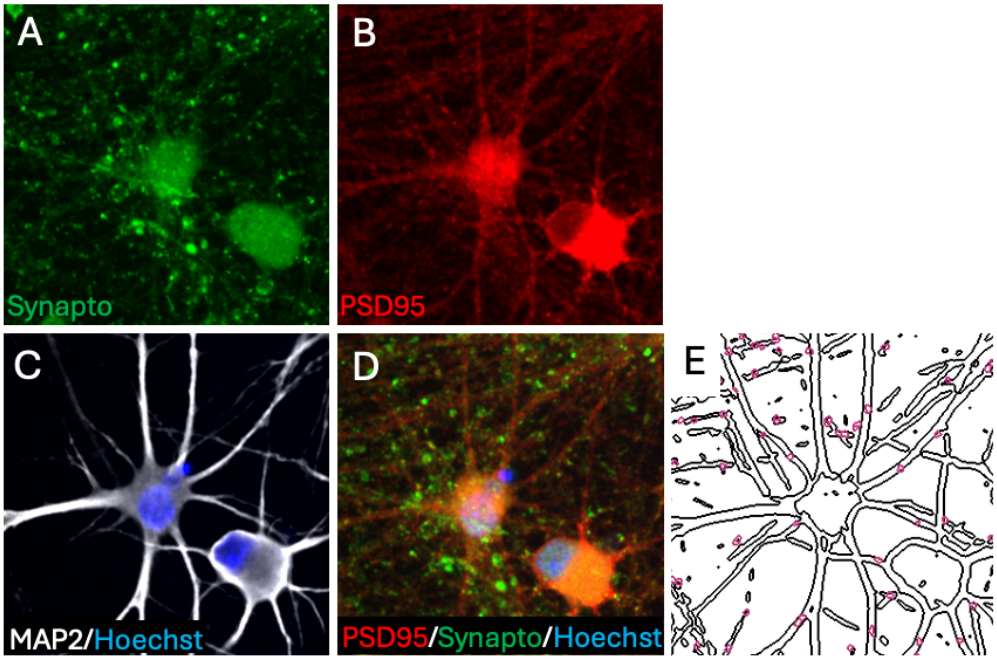
Abstract

This protocol outlines the procedure for labeling synapses with pre-synaptic and post-synaptic markers, synaptophysin and PSD-95 respectively, and microtubule-associated protein 2 (MAP2) in induced pluripotent stem cell (iPSC)-derived dopaminergic neurons using immunofluorescence.



Immunofluorescence labeling of neurons differentiated for 6 weeks was performed using antibodies against Synaptophysin (**A**), PSD-95 (**B**), MAP2, and Hoechst (**C**). (**D**) shows the merged image of Synaptophysin, PSD-95, and Hoechst staining. (**E**) displays representative colocalization of Synaptophysin and PSD-95 (highlighted with magenta circles) overlaid on the MAP2 mask following analysis with CellProfiler.

Materials

Reagents	Company	Cat. Number
Rabbit Synaptophysin antibody	Synaptic Systems	101002
Mouse PSD-95 antibody	Millipore	MABN68
Chicken MAP2 antibody	Encor Biotech	CPCA-MAP2
Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 555	ThermoFisher Scientific	A21437
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	ThermoFisher Scientific	A21235
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher Scientific	A11008
Hoechst 33342	SIGMA	H3570
16% Formaldehyde solution (w/v) Methanol-free	ThermoScientific	28908
Normal goat serum	SIGMA	G9023
Triton X-100	SIGMA	X-100

Before start

iPSC-derived dopaminergic neurons are cultured on 96-well plates for up to 4 to 6 weeks of differentiation. We use black Costar 96-well assay plates (Corning cat. no. 3904), which are compatible with imaging using the Opera Phenix high-content screening system.

Be extremely gentle when dispensing into the wells, as dopaminergic neurons are prone to detaching with forceful pipetting.

Fixation of Dopaminergic Neurons in 96-well plates

35m

1

35m

- Carefully aspirate the culture media, leaving 50 µL in each well.
- Gently add 50 µL of 8% paraformaldehyde (PFA) to each well to reach a final concentration of [M] 4 % (v/v) PFA.
- Incubate at room temperature for 00:20:00 .
- Remove the fixative and wash each well three times with 75 µL of 1X PBS. Allow each wash to incubate for 00:05:00 .

Permeabilization

25m

2

25m

- Prepare the permeabilization solution by diluting Triton X-100 to a final concentration of [M] 0.5 % (v/v) in 1X PBS.
- Add 60 µL of the permeabilization solution to each well and incubate for 00:10:00 at room temperature.
- Remove the solution and wash wells three times with 75 µL of 1X PBS, allowing each wash to incubate for 00:05:00 .

Blocking

1h

3

1h

- Prepare the blocking buffer by mixing [M] 5 % (v/v) normal goat serum and [M] 0.02 % (v/v) Triton X-100 in 1X PBS.
- Add 60 µL of the blocking buffer to each well and incubate for 01:00:00 at Room temperature .

Primary Antibody Incubation

16h

4

16h

1. Prepare the primary antibody solution in blocking buffer with the following dilutions:

- Rabbit anti-Synaptophysin: 1:500
- Mouse anti-PSD-95: 1:250
- Chicken anti-MAP2: 1:1500

2. Add 60 µL of the antibody mix to each well.
3. Incubate Overnight at 4 °C with gentle shaking.
4. Remove antibody mix and wash four times with 75 µL 1X PBS, allowing each wash to incubate for 00:05:00 .

Secondary Antibody Incubation

2h 15m

5

1. Prepare the secondary antibody solution in [M] 1 % (v/v) normal goat serum diluted in 1X PBS with the following components: 2h 15m

- Alexa Fluor 488 anti-Rabbit: 1:1000
- Alexa Fluor 647 anti-Mouse: 1:1000
- Alexa Fluor 555 anti-Chicken: 1:1000
- Hoechst 33342: 1:5000

2. Add 60 µL of the antibody mix to each well.
3. Incubate for 02:00:00 at Room temperature with gentle shaking, protected from light.
4. Remove secondary antibody mix and wash four times with 75 µL 1X PBS, allowing each wash to incubate for 00:05:00 .

Imaging using the Opera Phenix high-content imaging system

6

1. Insert the prepared plate into the Opera Phenix chamber.
2. Utilize a 40× water immersion objective. Capture images using the Hoechst, 488 nm, 555 nm and 647 nm laser channels. Acquire 15-18 fields of view per well

Synapse Detection and Data analysis using Cell Profiler software

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Images are analyzed using a Cell Profiler automated synaptic quantification pipeline, previously published by Berryer et al., eLife, 2023. https://doi.org/10.7554/eLife.80168

https://github.com/mberryer/ALPAQAS

Protocol references

1. Martin H Berryer, Gizem Rizki, Anna Nathanson, Jenny A Klein, Darina Trendafilova, Sara G Susco, Daisy Lam, Angelica Messana, Kristina M Holton, Kyle W Karhohs, Beth A Cimini, Kathleen Pfaff, Anne E Carpenter, Lee L Rubin, Lindy E Barrett (2023) High-content synaptic phenotyping in human cellular models reveals a role for BET proteins in synapse assembly eLife 12:e80168 https://doi.org/10.7554/eLife.80168