Protocol: iNeuron differentiation from human iPSCs

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Key words

iPSC, differentiation, iNeuron, i[3]Neuron, NGN2

Abstract

We adapted a previously-described method (Boecker et al., 2020, 2021; Fernandopulle et al., 2018) for differentiating iPSCs stably expressing mNGN2 at a safe-harbor locus into human excitatory glutamatergic neurons. Pre-i[3]Neuron iPSCs (human iPSCs with an integrated doxycycline-inducible mNGN2 transgene in the AAVS1 safe-harbor locus) were a gift from M. Ward (National Institutes of Health, Maryland).

Materials

- 10 cm cell culture dish
- 15 cm cell culture dish

- Cryovials

Reagents

- Growth Factor Reduced Matrigel (Corning, Cat# 354230)
- Essential 8 media (ThermoFisher, Cat# A1517001)
- Accutase (Sigma, Cat# 07920)
- DMEM/F12 media (ThermoFisher, Cat# 11330032)
- N2-supplement (Thermo Fisher, Cat# 17502048)
- NEAA (Thermo Fisher, Cat# 11140050)
- GlutaMAX (ThermoFisher, Cat# 35050-061)
- Doxycycline (Sigma, Cat# D9891)
- Y-27632 ROCK inhibitor (Selleck Chemicals, Cat# S1049)
- Tetracycline-free FBS (Takara, Cat# 631107)
- DMSO (CATALOG)
- BrainPhys media (STEMCELL Technologies, Cat# 5790)
- NT-3 (Pepro Tech, Cat# 450-02)
- BDNF (Pepro Tech, Cat# 450-03)
- B-27 supplement (Thermo Fisher, Cat #17504-044)

Safety warnings

Wear proper PPE when transferring cryovials to liquid N2.

Steps

1. Culture pre-iNeuron iPSCs in a 10 cm dish coated with Growth Factor Reduced Matrigel in Essential 8 media, fed daily. Pre-iNeuron iPSCs should either have doxycycline-inducible NGN2 present in the safe-harbor AAVS1 locus ("i^{3}Neurons") or should stably express doxycycline-inducible NGN2 following piggybac transfection (see protocol: "Piggybac-mediated stable expression of NGN2 in iPSCs for differentiation into excitatory glutamatergic neurons"). Before performing differentiation, iPSCs should be tested for mycoplasma, and cytogenetic analysis of G-banded metaphase cells should be performed to confirm a normal karyotype.
2. Passage iPSCs using warm Accutase and plate 5.5x10^6 cells onto a Matrigel-coated 15 cm dish, in Induction Media (DMEM/F12 supplemented with 1% N2-supplement [GIBCO], 1% NEAA [GIBCO], and 1% GlutaMAX [GIBCO], and containing 2 µg/mL doxycycline and 10 µm ROCK inhibitor).

   Note: DMEM/F12 supplemented with N2-supplement, NEAA and GlutaMAX can be kept at 4°C for 2-3 months. Doxycycline and ROCK inhibitor should always be added fresh.
3. After 24 hours, replace all media with fresh Induction Media, containing 2 µg/mL doxycycline but no ROCK inhibitor. Replace again with the same media after 24 hours (48 hours after plating).
4. 72 hours after plating, dissociate cells with warm Accutase. Count cells in freezing media (70% BrainPhys, 20% FBS, 10% DMSO, supplemented with 10 ng/mL BDNF, 10 ng/mL NT-3, and 1x B-27 supplement). Freeze down cells in a Mr. Frosty

container placed in a -80°C freezer overnight. On the following day, transfer cryo-preserved neurons to liquid nitrogen storage.

Citations

Boecker, C.A., and Holzbaur, E.L.F. (2021). Hyperactive LRRK2 kinase impairs the trafficking of axonal autophagosomes. Autophagy *00*, 1–3.

Boecker, C.A., Olenick, M.A., Gallagher, E.R., Ward, M.E., and Holzbaur, E.L.F. (2020). ToolBox: Live Imaging of intracellular organelle transport in induced pluripotent stem cell-derived neurons. Traffic *21*, 138–155.

Fernandopulle, M.S., Prestil, R., Grunseich, C., Wang, C., Gan, L., and Ward, M.E. (2018). Transcription Factor-Mediated Differentiation of Human iPSCs into Neurons. Curr. Protoc. Cell Biol. *79*, e51.