Generation of Bioengineered Neuronal Organoids (BENOs) from human pluripotent stem cells

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Method Article

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Abstract

Here we present a new directed differentiation and maturation protocol to generate Bioengineered Neuronal Organoids (BENOs) comprised of complex networks of inhibitory and excitatory neurons as well as supporting glial cells (astrocytes and myelinating oligodendrocytes).

Introduction

Brain organoids from human induced pluripotent stem cells (hiPSCs) provide useful tools for developmental studies and drug discovery experiments. For proper recapitulation of fetal brain development, organoids need to demonstrate cellular complexity (i.e. inhibitory and excitatory neurons; supporting glial cells), plasticity and electrophysiological maturation. Aiming to recapitulate human brain network function, we developed Bioengineered Neuronal Organoids (BENOs) from human induced pluripotent stem cells (iPSCs). BENOs consist of functionally integrated excitatory (glutamatergic) and inhibitory (GABAergic) neurons as well as supporting glia. The introduced BENO protocol differs from previously established neuronal organoid cultures by (1) a 1-step casting, directed differentiation approach and (2) the use of fully defined components such as a pluripotent stem cell source, collagen, small molecules, and growth factors. Neuroectodermal commitment is established by addition of SB/LDN (dual SMAD inhibition) and retinoic acid, followed by enhanced- neuronal progenitor growth via FGF2, gliogenesis via TGFß and neuronal differentiation via DAPT supplementation.

Reagents

Cells:

Human pluripotent stem cells (PSCs)

e.g.

a) in-house generated hIPS-G1 (Tiburcy et al. 2017)

b) hiPS LiPSC-GR1.1 (also referred to as TC1133 Lot: 50-001-21; Baghbaderani et al. 2015)

Reagents:

- 100x glutamine, Thermo Fisher #25030-024
- 100x penicillin/streptomycin (P/S), Thermo Fisher #15140-122
- 1x DPBS (- MgCl2, -CaCl2), Gibco #14190169

- Acid soluble Collagen, Collagen solutions #CS028
- All Trans Retinoic Acid, Sigma, R2625-50MG
- B27, Thermo Fisher #17504-044
- bFGF, Miltenyi Biotech #130-093-841
- DAPT, Tocris, #2634
- DMEM powder, GIBCO # 52100021
- DMSO, Sigma, # 276855
- EDTA Solution 0.5 M, pH 8, AppliChem #A4892.0500
- Histofix 4%, Roth, #P087.1
- L-ascorbic acid 2 phosphate sesquimagnesium salt hydrate(ASC-2-P), Sigma #A8960-5G
- LDN 193189, Sigma #SML0559
- Matrigel (growth factor reduced), BD Bioscience #354230
- N2, Thermo Fisher #17502-048
- Neurobasal-A , Thermo Fisher # 10888-022
- ROCKi (Y27632), Stemgent, #04-0012
- SB 431542, Tocris, #1614
- StemMACS™ iPS-Brew XF, human, Miltenyi Biotec #130-104-368
- TGFB1, Peprotech, #100-21
- Trypan blue solution (0.4%), Sigma #T8154

Equipment

- 96-well plate (U-bottom, low attachment), Sarstedt #83.3925.500
- T25 flask, Sarstedt #83.3910.002
- 6-well plate, Greiner #657185
- HERA cell 240i CO2 incubator, Thermofisher

- Steril Gard Hood, Labotect

- Falcon 50mL Conical Centrifuge Tubes, Thermofisher # 352070

Procedure

Important notes:

Perform every step under sterile conditions (e.g. laminar flow cabinet).

This protocol is standardised to human PSC culture in T25 flasks and generation of BENOs with 90.000 cells per organoid (3,000 cells/µL).

Day -4 PSC culture

1. Coat T25 flask with 1:120 (in DPBS) growth factor reduced Matrigel™ (100 µL/cm²) and incubate for 30 min in ambient air incubator with 5.0% CO_2 and 37 °C.

2. Plate cells (optimal cell density will have to be established experimentally for every individual PSC line and clone) with 4 mL of iPS-Brew XF media and 5 mmol/L ROCKi.

3. Place PSC in ambient air incubator with 5.0% CO_2 and 37 °C.

4. Change medium daily (4 mL) until cells reach confluence of 70-80%.

Day -1 BENO generation

iPSC dissociation

1. Aspirate cell culture media in T25 flask

2. Wash with 3 mL 1x DPBS two times, aspirate DPBS after each washing step

3. Add 3 mL of 0.5 mmol/L EDTA to PSCs and incubate for 2-3 min (some lines may require different incubation times and/or higher EDTA concentrations).

4. Carefully aspirate EDTA solution and tap the side of the flask until cell detachment.

5. Resuspend iPSC in 10 mL Brew supplemented with 10 µmol/L ROCKi.

6. Determine cell count (e.g. Neubauer chamber with 1:5 0.4 % Trypan blue or by an automated cytometer) and viability.

7. For pluripotency confirmation via flow cytometry, fix $2x10^6$ cells with 4 % Histofix at a 1:1 ratio and incubate for 15 min. Centrifuge at 300 g for 10 min. Wash two times in 1x DPBS. Store in DPBS at 4 °C for further analysis (up to 1 week).

8. Centrifuge remaining cell resuspension at 100 g for 3 min.

9. Resuspend cell pellet in Brew supplemented with 20 ng/mL FGF2 and 10 µmol/L ROCKi to achieve cell concentration of 4500 cells/µL.

BENO generation

All steps are performed on ice:

10. To generate BENOs, add a 1:1 ratio of Collagen and 2x DMEM to a pre-cooled vessel.

11. Neutralised by 0.1 mol/L NaOH.

12. Add an appropriate amount of cell resuspension to achieve a concentration of 3,000 cells/µL in the final BENO reconstitution mixture.

13. Pipette 30 µL of the BENO reconstitution mixture into a single well of a 96-well plate (U-bottom, low attachement); fill as many wells as experimentally desired.

14. Incubate for 30 min in ambient air incubator with 5.0% CO_2 and 37 °C.

15. Add 300 µL of Brew supplemented with 10 ng/mL FGF2 and 10 µmol/L ROCKi.

16. Incubate overnight in ambient air incubator with 5.0% CO_2 and 37 °C.

Differentiation Day 0 to 3: Neuronal commitment phase in 96 well plate

1. Prepare sufficient amounts of neuronal commitment medium (NCM):

Add 10 µmol/L SB 431542, 50 ng/ml LDN and 1 µmol/L RA to Basal Medium (Neurobasal-A containing 2 mmol/L glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 2% B27, 1% N2 supplement, 200 µmol/L ascorbic acid).

2. Carefully remove 200 µL of medium per well and add 200 µL of pre-warmed NCM. Change medium daily.

3. Tap plate to make sure that BENOS sediment to the bottom of the wells. Incubate BENOs in ambient air incubator with 5.0% CO_2 and 37 °C.

4. On day 3, transfer BENOs into 6-well plates (10 BENOs / well) with 5 mL NCM per well.

Differentiation Day 6 and 8: Neuronal commitment phase in 6 well plate

1. Carefully aspirate 4 mL of NCM per well.

2. Add 4 mL of fresh, pre-warmed NCM to each well.

Differentiation Day 10 and 13: Neuronal progenitor growth phase

1. Prepare sufficient amounts of neural progenitor expansion medium (NPEM):

Basal Medium complemented with 10 ng/mL FGF2 and 5 ng/mL TGFB1.

2. Carefully aspirate 4 mL of NPEM per well.

3. Add 4 mL of fresh, pre-warmed NPEM to each well.

Differentiation Day 15, 17, 20, 22, 24, and 27: Neuronal differentiation phase

1. Prepare sufficient amounts of neural progenitor differentiation medium (NDM):

Basal medium complemented with 5 ng/ml TGFB1 and 2.5 µM DAPT.

2. Carefully aspirate 4 mL of NDM per well.

3. Add 4 mL of fresh, pre-warmed NDM to each well.

Differentiation Day 29 onwards: Neuronal maturation phase

1. Assess BENOs on day 28 (e.g. by immunofluorescence or gene expression analysis).

2. Carefully aspirate 4 mL of Basal Medium per well.

3. Add 4 mL of fresh, pre-warmed Basal Medium to each well.

4. Change medium every second day.

BENOs can be cultured according to your experimental needs. Neuronal network maturation occurs at day 40 onwards. Palpable gliogenesis can be observed from day 60, with first notable astrocyte formation followed by the appearance of myelinating oligodendrocytes from day 90 onwards. Substantial myelination can be observed from culture day 150.

Troubleshooting

Time Taken

Day -4 PSC culture: 30 min

Day -1 BENO generation: 2 hours

Differentiation Day 0 to 3: Neuronal commitment phase in 96 well format: ~30 min (depending on the amount of BENO cultures)

Differentiation Day 6 and 8: Neuronal commitment phase in 6 well format: ~30 min (depending on the amount of BENO cultures)

Differentiation Day 10 and 13: Neuronal progenitor growth phase: ~30 min (depending on the amount of BENO cultures)

Differentiation Day 15, 17, 20, 22, 24 and 27: Neuronal differentiation phase: ~30 min (depending on the amount of BENO cultures)

Differentiation Day 29 onwards: Neuronal maturation phase: ~30 min (depending on the amount of BENO cultures)

Total time to observe first neurogenesis: ~20 days

Total time to observe first neuronal network formation: ~20 days

Total time to observe neuronal network maturation: ~40 days

Total time to achieve cortical layering: ~40 days

Total time until astrocyte formation in BENOs: ~60 days

Total time until myelinating oligodendrocytes form in BENOs: ~90 days

Anticipated Results

BENOs should demonstrate increased condensation from day 3 onwards. From day 15 onwards, a rapid loss of pluripotency (e.g. *POUF51*) and an increase in neurogenesis markers (e.g. *PAX6, MAP2, GRIN1, GABBR2*) as well as neuronal markers (e.g. *NF-H, SYP, MAP2*) should be observed. BENOs at day 40 onwards will show signs of neuronal network function and plasticity. Endodermal and mesodermal markers should be of relative low abundance. BENOs will demonstrate an increase in astrocytes (day 60 onwards) and in myelinating oligodendrocytes (day 90 onwards).

References

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