

## Midbrain-like Organoids generation from hiPSCs

In this protocol we describe the differentiation of human induced pluripotent stem cells (hiPSCs) into human midbrain-like organoids (hMLOs). This protocol has been developed based from several published protocols

### Day 0:

- Dissociate iPSC colonies to single cells with Accutase for 7 minutes at 37°C.
- Re-suspend cells in day0 medium and plate 8.000 cells/well in 96Wells Plate U-round-Bottom
- Low Attachment.

Day 0-Medium composition:

- **DMEM F12 (w/o HEPES) /Neurobasal (1:1)**
  - **N2** 0.5% 250 µL/50 mL
  - **B27** 1% 500 µL/50 mL
  - **NEAA** 1% 500 µL/50 mL
  - **B-MercaptoEtOH** 0,1%
  - **Heparin** 1 µg/mL (100 mg/mL) 1:100.000
  - **SB431542** 10 µM (10mM) 1:1000
  - **Noggin** 200 ng/mL (200µg/mL) 1:1000 or (**LDN** 1:100.000)
  - **CHIR99021** 0,7 µM (3mM) 1:4285 11,7 µL/50 mL
  - **Rock Inhibitor (APOI)** 50 µM (10mM) 1:200

### Day 4:

Carefully exchange the medium, without touching the EBs.

Day 4-Medium composition:

- **DMEM F12 (w/o HEPES)/Neurobasal (1:1)**
  - **N2** 0.5% 250 µL/50 mL
  - **B27** 1% 500 µL/50 mL
  - **NEAA** 1% 500 µL/50 mL
  - **B-MercaptoEtOH** 0,1%
  - **Heparin** 1 µg/mL (100 mg/mL) 1:100.000
  - **SB431542** 10 µM (10mM) 1:1000
  - **Noggin** 200 ng/mL (200µg/mL) 1:1000 or (**LDN** 1:100.000)
  - **CHIR99021** 7.5 µM (3mM) 1:400
  - **SHH-C25II** 100 ng/mL (100µg/mL) 1:1000
  - **FGF8** 100 ng/mL (100µg/mL) 1:1000
  - **PMA** 1µM (Stock: 1mM)

### Day 7:

Matrigel embedding:

- Dilute Matrigel in a 3:2 ratio with day 4 medium (used as an embedding mixture).
- Wash EBs in day 4 medium. Transfer and mix 5-8 EBs into the embedding mixture and plate onto a 6-well ultra-low-attachment plate.
- Incubate for 30 min at 37°C and add day 7 medium.
- Incubate at least 24h between d7 and d8.

Day 7-Medium composition:

- **Neurobasal**
  - **N2** 0.5%
  - **B27** 1%
  - **Glutamax** 1%
  - **NEAA** 1%
  - **B-MercaptoEtOH** 0,1%
  - **Insulin** 2,5 µg/mL (5mg/mL) 1:2000
  - **Laminin** 200 ng/mL (100µg/mL) 1:500
  - **SHH-C25II** 100 ng/mL (100µg/mL) 1:1000
  - **CHIR99021** 7.5 µM (3mM) 1:400
  - **FGF8** 100 ng/mL (100µg/mL) 1:10.000
  - **PMA** 1µM (Stock: 1mM)

### Day 8:

Add fresh differentiation medium without disrupting the embedded EBs.

Final Differentiation Medium composition:

- **Neurobasal**
  - **N2** (250 µL/50 mL)
  - **B27** (500 µL/50 mL)
  - **Glutamax** 1% (500 µL/50 mL)
  - **NEAA** 1% (500 µL/50 mL)
  - **B-MercaptoEtOH** 0,1% (50 µL/50 mL)
  - **BDNF** 10 ng/mL (20µg/mL) 1:2000
  - **GDNF** 10 ng/mL (20µg/mL) 1:2000
  - **Ascorbic Acid** 100 µM (200mM) 1:2000
  - **db-cAMP** 125 µM (500mM) 1:4000
  - **CHIR99021** 7.5 µM (3mM) 1:400

After Day 8 change medium twice a week.

### Day 10-13

At day 10 reduce CHIR to 3,0 µM (1:1000)

### Day 13-15

Reduce CHIR to 0,7 µM.

### Day 20-25

Manually dissociate organoids from Matrigel, using two surgical needles.

Place on orbital shaker after dissociation (80 RPM).

Cytokines and factors:

| Company          | Cat.Nr.      | Name                          | Size    |
|------------------|--------------|-------------------------------|---------|
| ThermoScientific | 12-587-010   | <b>B27</b>                    | 10ml    |
| ThermoScientific | 17502-048    | <b>N2</b>                     | 5ml     |
| R&D System       | 3400-010-03  | <b>Laminin I</b>              | 1mg     |
| Corning          | 354230       | <b>Matrigel</b>               | 10ml    |
| Selleckchem      | S1049        | <b>ApoI (Y-27632 2HCl)</b>    |         |
| AppliChem        | A0455,1000   | <b>dbCAMP (C)</b>             | 1mg     |
| Sigma-Aldrich    | 540220-5MG   | <b>Purmorphamine (PMA)</b>    |         |
| R&D System       | 1614         | <b>SB 431542</b>              | 10mg    |
| PeproTech        | 450-02-500   | <b>BDNF</b>                   | 500µg   |
| PeproTech        | 450-10-500   | <b>GDNF</b>                   | 500µg   |
| PeproTech        | 100-25-500   | <b>FGF8</b>                   | 500µg   |
| PeproTech        | 100-45-100   | <b>Sonic hedgehog</b>         | 100µg   |
| Sigma-Aldrich    | A6964-100 ml | <b>Accutase</b>               | 100ml   |
| Gibco            | 11320074     | <b>DMEM/F12 w/o HEPES</b>     | 500ml   |
| Gibco            | 21103049     | <b>Neurobasal Medium</b>      | 500ml   |
| Gibco            | 11140035     | <b>MEM NEAA</b>               | 100ml   |
| Gibco            | 35050038     | <b>Glutamaxx</b>              | 100ml   |
| Sigma            | SML1046-5MG  | <b>CHIR 99021</b>             | 3mM     |
| Sigma            | 25556-4      | <b>L-Ascorbic Acid</b>        | 200mM   |
| ThermoScientific | 12585014     | <b>Insulin, human recomb.</b> | 4 mg/ml |
| Axon             | 1509         | <b>LDN 193189HCl</b>          | 100µM   |

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