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Version 2

Unguided Cortical Organoid Protocol V.2

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We use this protocol and it's working

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Abstract

This protocol is based on the original protocol published by Lancaster et al. in 2013 (Lancaster, M., Renner, M., Martin, CA. *et al.* Cerebral organoids model human brain development and microcephaly. *Nature* **501**, 373–379 (2013). <https://doi.org/10.1038/nature12517>) with the following modifications

Guidelines

Make sure to use high quality iPSCs.



Materials

Media used during forebrain organoid culture:

Preparation of mTeSR1 Plus Medium with ROCK Inhibitor:

To prepare mTeSR1 Plus medium with ROCK inhibitor, combine the following components to achieve the specified final concentrations:

- mTeSR1: 100% (Thermo Fisher)
- ROCK Inhibitor (Y-27632, 10 mM stock): 50 μ M final concentration (Stemcell Technologies)

Add the ROCK inhibitor to the mTeSR1 medium to reach a final concentration of 50 μ M. Prepare and mix all components under sterile conditions. Use immediately or store as per manufacturer's recommendations.

Preparation of NIM (Neural Induction Medium):

To prepare NIM, combine the following components to achieve the indicated final concentrations:

DMEM-F12: 100%

N2 Supplement (100X): 1%

GlutaMAX (100X): 1X final concentration

MEM-NEAA (100X): 1X final concentration

Heparin (10 mg/ml): 1 μ g/ml final concentration Add each component under sterile conditions. Mix thoroughly and, if necessary, filter sterilize before use.

Preparation of COD-Vit A / +Vit A Medium:

To prepare the COD-Vit A or +Vit A medium, combine the following components to achieve the specified final concentrations:

- DMEM-F12: 50%
- Neurobasal Medium: 50%
- N2 Supplement (100X): 0.5X final concentration
- B27 Supplement -Vit A: 1X final concentration
- Insulin: 1X final concentration
- GlutaMAX (100X): 1X final concentration
- MEM-NEAA (100X): 0.5X final concentration
- Penicillin/Streptomycin (100X): 1X final concentration
- 2-mercaptoethanol (2-BME) in DMEM-F12: add as a 1:100 dilution

First, prepare the 2-BME working solution by diluting it 1:100 in DMEM-F12, then add to the mixture. Combine all components under sterile conditions, mix thoroughly, and filter sterilize if necessary before use.

Generating embryoid bodies (EBs) from human iPSCs

- 1 Day 0: Add Y-27632 ROCK inhibitor (Selleckchem, #S1049) to iPSCs at 70-80% confluency, at a final concentration of 10µM. Allow cells to incubate at 37°C for at least 20 min
- 2 Add Accutase (Stem Cell Technologies) to facilitate cell detachment and to obtain a single cell solution
- 2.1 Incubate for 10-15 minutes and periodically check on cell detachment and gently tap plate to shake cells. Collect cells with appropriate amount of mTeSR1 Plus media supplemented with 10µM ROCK inhibitor
- 3 Transfer cells to a 15-ml or 50-ml Eppendorf tube.
- 4 Centrifuge cells at 1000g for 3 min.
- 5 Aspirate supernatant, and resuspend cell pellet in 1 ml mTesr1 media + 50 µM ROCK inhibitor.
- 6 Count cells using cell counter. Ideal cell count is at least 2×10^6 cells per ml.
- 7 Calculate and dilute cell suspension with mTesr1+50 uM ROCK to reach 9000 cells in a final volume of 150 ul per well.
- 8 Mix final cell solution by pipetting up and down. Pour cell solution into sterile reservoir.
- 9 Using multi-channel pipet, pipet 150 ul of cell solution into each well of a ULA 96-well plate.
- 10 Centrifuge 96-well plate(s) containing cells at 300 g for 3 min.
- 11 Check plate to ensure every well contains cells in a spherical shape.
- 12 Place in incubator and change media next day.



Day 1 and 2

13 Half media change using mTesr1 + 50 μ M ROCK Inhibitor. Specifically, remove 75 μ l media per well, and replace with 100 μ l fresh media.

13.1 Ideally, use automated liquid handler (i.e. Integra Viaflow or Tecan Fluent).

14 Place back in 37°C incubator.

Day 3

15 Half media change using mTesr1 without ROCK. Specifically, remove 100 μ l media per well, and replace with 100 μ l fresh media.

16 EBs should be 350-600 μ m in size. Also check for brightened smooth edges on EBs.

17 Place back in 37°C incubator.

Day 5: Neural induction of embryoid bodies

18 Carefully remove as much media as possible (full media change) without disturbing the EBs. Add 150 μ l of Neural Induction Media (NIM). Avoid disturbing the EBs.

Day 7: Media Change

19 Half media change using NIM media (i.e. remove 75 μ l old media, add 75 μ l new media).

20 Observe translucent border for each EB.

Day 9: Embedding



- 21 EBs should be at least 800 μm in size.
- 22 Prepare dimpled parafilm sheets ahead of time by using parafilm, 15 ml Falcon tube (bottom), and 200 μl tip box as a tray. Ensure all materials are sterile and further spraying liberally with 70% ethanol.
- 23 Thaw matrigel onto ice
- 24 Place the sterile parafilm sheet onto the 200 μl tip box tray.
- 25 Using wide-bore 200 μl tips or a cut pipet tip, gently aspirate each EB and transfer to a dimpled well onto the parafilm sheet.
- 26 Add 30 μl of cold matrigel onto each EB, on the dimpled parafilm. Immediately use a pipet tip to position the EB to the center of the Matrigel before polymerization starts.
- 27 Gently cover the embedded EBs (on parafilm sheet) with plate lid without crushing the domes. Place into 37°C incubator for 20 minutes. Continue with the rest of the EBs on the multichannel plane.
- 28 Pop each dimpled well of parafilm sheet upside down, and align it with a well plate (sterile ULA 6-well). Carefully flush the embedded EBs into the well using IDM-Vit A media.
- 28.1 Maximum 12 EBs per 6-well, and 5 ml media per well.
- 29 Place embedded EBs back in 37°C incubator. Observe for polarized neuroectoderm.

Day 11

- 30 Half media change by aspirating 2.5 ml old media from each well. Add 2.5 ml fresh IDM-Vit. A media.
- 31 Place embedded EBs back in 37°C incubator.
- 32 Repeat half media change every 2 days.



Day 16

- 33 Remove as much media as possible without aspirating embedded EBs. To do this, gently tilt the 6-well plate, allow EBs to fall to the bottom of the well, and aspirate from the sides or top.
- 34 Replace with IDM+Vit A media (final volume of 5 ml).
- 35 Change half media every 2 days. If media is yellow, perform full media change.

Day 18: Place EBs onto shaker (Shaking phase)

- 36 Full media change using IDM+Vit A.
- 37 Place embedded EBs onto shaker in 37°C incubator at 90 rpm.
- 38 Change half media every 2 days, and full media change every 3 days.

Protocol references

Lancaster, M., Renner, M., Martin, CA. *et al.* Cerebral organoids model human brain development and microcephaly. *Nature* **501**, 373–379 (2013). <https://doi.org/10.1038/nature12517>