Generation of Cerebral Organoids with Enriched Cortical Cellular Diversity and Outer Radial Glial Cell Identity from Human Pluripotent Stem Cells

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Abstract

Cerebral organoids display broad regional heterogeneity and limited capacity to generate cortical cell diversity due to incompetent patterning signals within early developing organoid neural stem cells (NSCs). Here we describe a method to overcome this limitation by applying a short and early dual SMAD/WNT inhibition course, which results in enrichment for cortical NSCs and their progeny while suppressing non-cortical fates. The enhanced cortical cellular diversity achieved with our method is further mirrored by the facilitated emergence of outer radial glial (oRG) NSCs and appearance of molecularly distinct deep and upper layer neurons. In summary, this protocol yields cerebral organoids with considerable cortical regional homogeneity, depicting a short and early pathway Inhibition as a sufficient step for enriching towards cortical NSC identity in organoids. This protocol accompanies Rosebrock D. et al, *Nature Cell Biology*, 2022.

Introduction

Pluripotent stem cells (PSCs) have emerged as an unlimited source for generating the entire repertoire of differentiated cell types and as such they serve as an invaluable tool for modelling human development, evolution and disease. One highly inspiring avenue of research led by the commencement of PSCs is the establishment of model systems for deriving cortical fates. The pioneering work by Sasai and colleagues in mouse embryonic stem cells served as foundation for recapitulating this mechanism in human PSCs in 2D and 3D culture systems (for review see[1-3]). Particularly, the resurgence of organoids as compelling in-vivo-like 3D model systems to mimic complex fundamentals of tissue development and function has led to an upsurge in methods for derivation of cerebral organoids – 3D models for corticogenesis. However, methods for generating organoids with enriched cortical fates are highly variable. While 3D structures generated by these protocols self-assemble and generate cells that express cortical marker genes and display cortical cytoarchitectural hallmarks under current methods, the lack of method standardization is clearly reflected by the considerable regional heterogeneity within and among organoids as well as across methods, which presumably also underlies the limited cortical cellular diversity shared by the current methods. It is conceivable that the presence of specific inductive signals during early phase organoid development is critical for the correct patterning and purity of the founder cortical NSCs that generate consecutive building blocks for corticogenesis within organoids. Here we show the development of a method to overcome limited cortical cellular diversity in organoids. By employing a comparative study among organoids generated through combinatorial pathway inhibition methods run side-by-side, we revealed that a short and early exposure to Dual SMAD and WNT inhibitors results in enhanced cortical NSC identity with efficient suppression of non-cortical NSC fates. This protocol also facilitates enrichment for oRG cells - an important cortical NSC type playing fundamental evolutional and functional role in the expansion of the primate and human brain - as well as the appearance of molecularly distinct deep and upper layer neuronal subtypes.

Reagents

- DMEM/F-12 (Gibco™, cat no: 11320033)
- DMEM/F-12, HEPES (Gibco™, cat no: 31330038)
- DMEM/F-12 (powder, Gibco™, cat no: 32500035)
- Knockout™ DMEM (Gibco™, cat no: 10829018)
- Neurobasal™ Medium (Gibco™, cat no: 21103049)
- mTeSR™1 Basal Medium (Stem Cell™ Technologies, cat no: 85850)
- KnockOut™ Serum Replacement (Gibco™, cat no: 10828028)
- L-Glutamine (Gibco™, cat no: 21051024)
- GlutaMAX™ Supplement (Gibco™, cat no: 35050061)
- Penicillin-Streptomycin (Gibco™, cat no: 15140122)
- MEM Non-Essential Amino Acids (Gibco™, cat no: 11140050)
- 2-Mercaptoethanol (Gibco™, cat no: 31350010)
- Fetal Bovine Serum, qualified, heat inactivated (Gibco™, cat no: 16140071)
- Dimethyl Sulphoxide (DMSO, Sigma-Aldrich®, cat no: D2650)
- Insulin from bovine pancreas (Sigma-Aldrich®, cat no: I6634)
- Apo-transferin human (Sigma-Aldrich®, cat no: T1147)
- Sodium Selenite (Sigma-Aldrich®, cat no: 214485)
- Putrescine dihydrochloride (Sigma-Aldrich®, cat no: P7505)
- Progesterone (Sigma-Aldrich®, cat no: P0130)
- D-(+)-Glucose (Sigma-Aldrich®, cat no: G8270)
- Sodium bicarbonate (Sigma-Aldrich®, cat no: S5761)
- Matrigel® Membrane Matrix (Corning®, cat no: 354234)

- Paraformaldehyde (Sigma-Aldrich®, cat no: 158127)
- Sucrose (Sigma-Aldrich®, cat no: S0389)
- Optimal Cutting Temperature compound (O.C.T. Compound, Tissue-Tek®, cat no: 4583)
- Bovine Serum Albumin (Sigma-Aldrich®, cat no: A9418)
- Triton™ X-100 (Sigma-Aldrich®, cat no: X100)
- Trypan Blue Solution (Gibco™, cat no: 15250061)
- DPBS, no calcium, no magnesium (Gibco™, cat no: 14190169)
- Recombinant Human FGF basic/FGF2/bFGF (146 aa) Protein (R&D Systems, cat no: 233-FB)
- Recombinant Mouse Noggin Fc Chimera Protein, CF (R&D Systems, cat no: 719-NG)
- SB-431542 (Tocris, cat no: 1614)
- XAV 939 (Tocris, cat no: 3748)
- B-27™ Supplement minus vitamin A (Gibco™, cat no: 12587010)
- B-27™ serum free (Gibco™, cat no: 17504044)
- Y-27632 dihydrochloride (ROCK inhibitor, Tocris, cat no: 1254)
- Neutral protease (Dispase, Worthington, cat no: LS02100)
- Accutase® solution (Sigma-Aldrich®, cat no: A6964)
- UltraPure™ EDTA, pH 8.0 (Invitrogen™, cat no: 15575020)
- Dry ice

Equipment

Instruments

- CO_2 incubators (Heracell VIOS, Thermo Scientific™, cat no: 50145515)
- Class II Biological Safety Cabinet (Herasafe™ KS, Thermo Scientific™, cat no: 51022734)

· Inverted microscope (Nikon SMZ1270, cat no: MNA52110)

· Celltron Orbital Shaker (Infors HT, cat no: 69455)

· Fluid aspiration system BVC control (Vacuubrand, cat no: 20727200)

· Aqualine AL 12 Water bath (LAUDA, cat no: 92635)

· Serological pipettes (25ml, 10ml, 5ml, Sarstedt, cat no: 86.1685.020, 86.1254.025 and 86.1253.025)

· Pipettes (P1000, P200, P10, Eppendorf Research Plus 3-Pack Option 2, cat no: 3120000917)

· Portable Pipet-Aid® XP Pipette Controller (Drummond, cat no: 4-000-101)

· Scissors, pointed/blunt, 130 mm, 37 mm (Roth®, cat no: 3543.1)

· Standard Pattern Forceps (Fine Science Tools®, cat no: 1000-12)

· Laboratory Spatula (Roth®, cat no: 3066.1)

· Counting Chamber (Marienfeld, cat no: 0610010)

· Sealing film PARAFILM® M, 50 mm, 75 m (Roth®, cat no: H951.1)

Plasticware

· Sterile filter tips (1000, 200, 20, 10 ul, Biozym, SafeSeal SurPhob®, cat no: VT0270, VT0250, VT0220 and VT0200)

· 500 mL Vacuum Filter/Storage Bottle System, 0.22 µm Pore 33.2cm² PES Membrane, Sterile (Corning®, cat no: 431097)

· 250 mL Vacuum Filter/Storage Bottle System, 0.22 µm Pore 19.6cm² CN Membrane, Sterile (Corning®, cat no: 430756)

· 60 mm TC-treated Culture Dish (Corning®, cat no: 430166)

· Screw cap tube, 50 ml, (LxØ): 114 x 28 mm, PP, with print (Sarstedt, cat no: 62.547.254)

· Screw cap tube, 15 ml, (LxØ): 120 x 17 mm, PP, with print (Sarstedt, cat no: 62.554.502)

· 96-well Clear Round Bottom Ultra-Low Attachment Microplate, Individually Wrapped, with Lid, Sterile (Corning®, cat no: 7007)

· 24-well Clear Flat Bottom Ultra-Low Attachment Multiple Well Plates, Individually Wrapped, Sterile (Corning®, cat no: 3473)

· 6-well Clear Flat Bottom Ultra-Low Attachment Multiple Well Plates, Individually Wrapped, Sterile (Corning®, cat no: 3471)

· Peel-A-Way® Embedding Mold (Truncated - T8) (Polysciences, cat no: 18985-1)

Procedure

Reagents Setup

hESC medium (500 ml)

· Mix 387 ml of DMEM/F-12/HEPES, 100 ml KnockOut Serum Replacement (KOSR), 2.5 ml of GlutaMAX, 5 ml of MEM-NEAA, 5 ml of Penicillin-Streptomycin and 0.5 ml of 2-mercaptoethanol. Sterile filter using a vacuum-driven 0.2-µm filter unit, store at 4°C and use at room temperature (RT). Add FGF2 and ROCK inhibitor (RI) just before usage.

mTESR1 medium (500 ml)

· Mix 400 ml of mTESR1 basal medium, 100 ml of mTESR1 5X supplement and 5 ml of Penicillin-Streptomycin. Sterile filter using a vacuum-driven 0.2-µm filter unit, store at 4°C and use at room temperature (RT).

Neurobasal (NB) medium (500 ml)

· Mix 484.5 ml of Neurobasal medium, 5 ml of GlutaMAX, 5 ml of MEM-NEAA, 5 ml of Penicillin-Streptomycin and 0.5 ml of 2-mercaptoethanol. Sterile filter it using a vacuum-driven 0.2-µm filter unit, store at 4°C and use at room temperature (RT).

N2 medium (1000 ml)

· In a 2000ml conical flask, add 980 ml of DDW. Along with that, add a magnet and keep the beaker on a magnetic stirrer for mixing the reagents. Add 12 g of DMEM/F-12 powder, 1.55 g of D-Glucose, 2 g of Sodium bicarbonate, 100 mg of Apo-transferrin, 60 µl of 500µM Sodium selenite, 120 µl of 830nM Putrescine, 200 µl of 100µM Progesterone and 10 ml of Penicillin-Streptomycin. Weight 25 mg of insulin

in a 6cm dish and add 10ml of DDW. Keep the solution for 2 minutes and then add 700ul of 0.1N NaOH solution (freshly made) in order to dissolve the insulin particles. Mix the insulin solution thoroughly and then add it into the conical flask. Let the medium mix for 10 minutes. Sterile filter it using a vacuum-driven 0.2-µm filter unit, store at 4°C and use at room temperature (RT).

MEF medium (feeder cells) (500ml)

· Mix 389.5 ml of DMEM-KO, 100 ml of FBS, 5 ml of GlutaMAX, 5 ml of Penicillin-Streptomycin and 0.5 ml of 2-mercaptoethanol. Sterile filter it using a vacuum-driven 0.2-µm filter unit, store at 4°C and use at room temperature (RT).

Dispase solution

· Reconstitute 50 mg of Dispase powder with indicated activity of C units/mg dry weight (=50C units) in a desired volume (=50C/4) of hESC medium to get a working activity of 4 units/ml. Filter the solution using a vacuum-driven 0.2-µm filter unit, store at -20°C and use at RT.

EDTA solution

· Add 200 µl of 0.5M EDTA to 200 ml of DPBS and filter it using a vacuum-driven 0.2-µm filter unit, store and use at RT. Note: Careful not to use EDTA if it has white precipitates.

Matrigel working solution (for dish coating)

· For maintaining human iPSCs (hiPSCs) (feeder free culture), 0.5ml of Matrigel is diluted in 50 ml of DMEM-KO medium and before splitting plate was coated for 1 hour at 37°C in the incubator.

Sucrose 30% working solution

· Add 15 g of sucrose to 30 ml of PBS in a 50 ml falcon. Vortex aggressively for 5 minutes, until sucrose dissolves. Complete the volume up to 50 ml with PBS.

Small molecules stock solutions

SB-431542: 10mM stock solution

· 10mg powder is reconstituted by adding 2.378 ml of 100% ethanol in order to obtain 10mM working stock concentration. Wait for 5 mins or more until the particles have been dissolved completely. Aliquots are made and stored at -80°C.

XAV 939: 20mM stock solution

· 10mg powder is reconstituted by adding 1.6 ml DMSO in order to get 20mM working stock concentration. Wait for 5 mins or more until the particles have been dissolved completely. Aliquots are made and stored at -80°C.

Noggin: 250ug/ml stock solution

· 1mg stock was supplied as 3.77 ml in PBS/ 1mM EDTA with a concentration of 268ug/ml. Add 271ul of 0.2um filtered PBS in order to get 250ug/ml working stock concentration. Aliquots are made and stored at -20°C.

FGF2 stock solution

· 10 µg/ml solution prepared by reconstituting 25 µg FGF2 in 2.5 ml filtered PBS containing 0.1% BSA. Aliquots are stored in -80 °C.

Rock Inhibitor (RI) stock solution

· 10 M solution prepared by reconstituting 10 mg FGF2 in 3.03 ml filtered PBS. Aliquots are stored in -20 °C.

Procedure

Feeder-dependent hESC culture

· Feeder-dependent hESCs were cultured on mitotically-inactivated mouse embryonic fibroblasts (MEFs) (Globalstem). Undifferentiated human PSCs were maintained in hESC medium. Cells were passaged weekly using Dispase to maintain their undifferentiated state.

Feeder-independent human PSC culture

· hiPSC lines used in this study were cultured and maintained on Matrigel-coated dishes in mTesR1 medium and passaged every 3-4 days using 0.5mM EDTA to maintain their undifferentiated state.

Derivation of cerebral organoids (Figure 1)

Day 0 (Generating EBs)

hESCs and hiPSCs cells were grown in a 60mm dish until 65-70% confluent. Whole or part of the plate can be used for EB generation. Follow option A for feeder dependent and B for feeder independent culture.

A. For feeder-dependent hESCs

· hESCs colonies were washed with DPBS without calcium and magnesium and 1 ml of Dispase enzyme was added. Cells were kept in the incubator for 7-10 minutes at 37°C until the colonies started detaching from feeder cells (MEFs). Note: If required gently tap the plate from the sides.

· Dispase enzyme was neutralized by adding 7 ml of hESC medium. Colonies were collected in a 15 ml falcon and were allowed to sink at the bottom of the tube.

· Medium was carefully aspirated until 500ul were left. Colonies were again washed with 7 ml of fresh hESC medium and were allowed to settle down in the bottom of the tube (~1-2 minutes).

· Medium was carefully aspirated without disturbing the settled colonies, and 1 ml of Accutase was added along with ROCK inhibitor (1:1000). Cells were kept in 37°C water bath for 4 minutes. Colonies were then triturated using a p1000 tip for 15 times until single cells were obtained. Single cells were confirmed by observing under the microscope and residual cell clumps were further triturated 5-6 times and observed again under the microscope.

· Accutase enzyme was neutralized by washing twice with 10 ml hESC medium containing FGF2 (1:5000) and centrifuged at 270g for 5 minutes.

· Single cells were re-suspended in 1ml of hESC medium containing FGF2 (1:5000) and ROCK inhibitor (1:200).

· Cells were counted using hemocytometer by taking 5µl cells with 15µl Trypan Blue and 10 µl of this mixed cell suspension was added to the Hemocytometer chambers.

For calculation: cell count/ml = 4 x 10000 x (cell count from two sides of chamber).

· The volume of the hESC medium was adjusted along with FGF2 and ROCK inhibitor to obtain a concentration of 9000 cells per 150µl or the required number of cells was taken from the cell suspension. For example, for a full 96 well plate, considered taking cells for 100 wells for pipetting error, 900,000 cells were combined in 15ml of hESC medium containing FGF2 and RI, then pipet 150ul of cell suspension in a 96 well U-bottom low attachment plate (Corning).

B. For Feeder-independent hiPSCs

· hiPSCs were first washed with 3 ml of DPBS without calcium and magnesium and then incubated with 1 ml of EDTA for 2 minutes at 37°C in the incubator. EDTA was carefully aspirated and was replaced it with 1 ml of Accutase (per a 60mm culture dish). Cells were incubated for 3 minutes at 37°C.

· After incubation, cells were detached and then triturated using p1000 tips for 10-15 times in order to obtain single cells.

· Single cell suspension was first washed with mTesR1, and afterwards with hESC medium containing FGF2 (1:5000). Cells were centrifuged at 270g for 5 minutes.

· Single cells were re-suspended, counted and plated similarly as done for hESCs as indicated in section A. Suspended single cells (9000/150 µl) were plated on a 96 well U-bottom low attachment plate.

Day 1

· The plate was observed under an inverted microscope for the formation of EBs.

Day 2

· EBs' sizes were measured under the microscope, and FGF2 and RI were either withdrawn from or added (1x times) to the medium for EBs greater or smaller than 350µm, respectively.

· Half of the medium (75µl) was removed using a multichannel pipette under a stereomicroscope without disturbing EBs.

· To the remaining half (75µl), 150µl of hESC medium were added along with 1.5x times the concentration required for each of the three inhibitors as follows:

For SB-431542 (10mM), a dilution of 1:1000 is required to reach a final concentration of 10uM and hence on Day 2, a volume of 1.5x(75ul/1000) was added per each well.

For Noggin (250ug/ml), a dilution of 1:1000 is required to reach a final concentration of 250ng/ml and hence on Day 2, a volume of 1.5x(75ul/1000) was added per each well.

For XAV 939, a dilution of 1:6000 is required to reach a final concentration of 10uM and hence on Day 2, a volume of 1.5x(75ul/6000) was added per each well.

Day 4

· Under the stereomicroscope, 150 µl of the hESC medium were removed from the wells carefully without disturbing the EBs and were replaced with fresh 150µl hESC medium containing 1x concentration factors.

· For EB sizes smaller than 350 µm, FGF2 and RI were added 1x times to the fresh hESC medium added.

Day 6 (Neural Induction)

· EBs were around 500-600µm and hence transferred to 24 well low attachment plates. The minimum size of the EBs should be approximately 400-500 µm. For sizes at the lower end, EBs were kept for one more day in the 96 well plate without any medium change.

· Neural induction medium was prepared by mixing N2 medium along with 1x times corresponding inhibitors., and 500µl medium were distributed to each of the 24 low attachment wells. Medium containing plates were kept at 37°C until the EBs were transferred in the wells.

· Along with medium preparation, edges of 200µl tips were cropped using sterile autoclaved scissors in order to obtain a wide opening of 1-1.5mm in diameter. Caution must be taken to create enough opening to allow free passage and prevent organoid disruption.

· Organoids were transferred under the stereomicroscope from one well of a 96 well plate and to one well of 24 well plate, using cut tips together with 30 µl of their own medium.

Day 8

· Organoids were inspected under the microscope to verify appearance of a neuroepithelium at the edge of each organoid.

· 300 µl of medium were removed using p1000 tips and a same volume of fresh N2 medium with inhibitors' concentration similar to that of Day 6 was added to each well.

Day 10

· Organoids were inspected under the microscope for the presence of pseudostratified radially organized neuroepithelium, which forms in the outer most part of the organoid and appears less dense compared to the inner part of the organoid.

· Medium change was given by removing 300µl of medium and adding fresh N2 medium with corresponding inhibitors.

Day 11 (Embedding in Matrigel)

· Organoids with a size of approximately 500-600µm and that contained a pseudostratified radially organized epithelium further proceeded to embedding.

· Before embedding, 6-well low attachment plates were prepared with each well containing 2.5 ml of N2/NB (1:1) medium along with B-27 minus vitamin A (1:100). These plates were kept in the incubator.

· To prepare Matrigel droplets for embedding, a rectangle-shaped parafilm was cut using sterile scissors followed by disinfection using 70% ethanol. Then, the film was laid on head of an empty sterile 10ul tip box and dimples were generated. Film was kept in a sterile 60mm dish.

· Organoids were transferred using 200µl cut tips onto the parafilm dimples under the stereomicroscope. Excess medium that was carried over was removed, and 30µl of Matrigel were added on each organoid. Following that, a 200ul pipette tip was used to place the organoid at the center of the Matrigel drop.

· Matrigel embedded organoids were kept in the incubator at 37°C for 30 minutes (maximum 45 minutes). The parafilm was then taken out of the 60mm dish using sterile forceps and transferred

carefully into the 6 well plates with the help of a sterile spatula.

Day 13

· Organoids were inspected for the presence of vesicle like structure on their periphery.

· Medium change was given using the same medium from day 11. Approximately 1.5ml were removed and freshly made medium (1.5ml) was added.

Day 15

· Medium was removed as completely as possible without disrupting organoids and 2 ml of the fresh medium containing N2/NB (1:1) along with B-27 with vitamin A (1:100) were added.

· Organoid plates were transferred onto an orbital shaker (84 rpm).

· Medium was changed every other day by removing 1.5 ml and replacing it with fresh 1.5 ml medium of the same composition.

Day 45 onwards (Matrigel addition)

For long-term organoid cultures on day 45 and onwards:

· Matrigel (1:100) was added to the N2/NB (1:1) medium along with B-27 with vitamin A.

· 2.5 ml of medium was added to each well in every two days.

· Frequent medium change was given if change in the medium color (from pink to yellow) was observed.

Organoid fixation

· Organoids were transferred into a 15 ml falcon (usually up to 5 organoids per tube) using p1000 cut sterile tips. (Note: p1000 tips diameter should be cut according to the size of the organoids)

· Excess medium was removed using the p1000 pipette tips followed by fixation with 4 ml 4% paraformaldehyde for 20-40 minutes (RT) depending on organoid size. (up to 20 minutes for day 50 organoids and up to 40 minutes for Day 80 organoids).

· Organoids were washed twice with 5ml of DPBS without calcium and magnesium, with 10 minutes incubation between washes. This was followed by addition of 3 ml of 30% sucrose to the tube and storage at 4°C overnight or more days, until organoids sank at the bottom of the tube, after which they were ready for embedding in OCT.

Troubleshooting

Troubleshooting

· Problem: No or small EB formation on Day 0.

· Solution: Verify that PSC colonies are in an undifferentiated state. In addition, PSC colonies must be around 70% confluent when starting the protocol.

· Problem: EB size is greater than 500-600 µm On Day 2.

· Solution: Such experiments are discarded as EBs develop into compromised organoids at later stages.

· Problem: All or some EBs exhibit a dark (black colored) center

· Solution: Poor neural induction. Such experiments are discarded as EBs develop into compromised organoids at later stages.

· Problem: Newly formed Matrigel drops break while transferring to medium on Day 11.

· Solution: Keep organoids-containing Matrigel drops for a longer time (15 minutes more) in the incubator until drops acquire a gel-like consistency. Re-embed broken Matrigel drops to avoid organoid loss.

· Problem: Organoids escape from Matrigel drops during incubation on orbital shaker after Day 15.

· Solution: Reduce orbital shaker speed slightly below 84 rpm.

Tips

· On Day 1, it is normal to observe many single cells attached to the edges of EBs.

· On Day 8, cell shedding off from organoid edges is normal. However, if progressive shedding leading to significant reduction in organoid size (to half its size) occurs, experiment should be discarded or otherwise these compromised organoids will develop at later stages.

· On day 10, we recommend not to proceed for embedding in Matrigel any organoids exhibiting a very dark / pigmented center, as these will result in compromised cortical identity.

· On Day 11, care must be taken not to break Matrigel droplets during transfer, as this will result in compromised vesicle development within organoids at late stages.

· Organoids developing transparent (one-cell thick) sphere structures will not generate cortical cell types.

· Matrigel addition to the medium can be started earlier than Day 45, if cell shedding is observed from organoids.

Time Taken

Duration of procedures (Time approximation for one 96 well plate)

Day 0: 1-2 hours

Day 2: 1-2 hours

Day 4: 1-2 hours

Day 6: 3-4 hours

Day 8: 1-2 hours

Day 10: 1-2 hours

Day 11: 4-5 hours

Day 13: 1-2 hours

Day 15: 1-2 hours

Anticipated Results

On Day 6, organoid size should be around 500-600µm.

On Day 11, organoids should reach a size of 500-800µm and exhibit a pseudostratified radially organized epithelium-like outer boundary.

On Day 12 and onwards, organoids show expanding protrusions, indicating proper development of cortical organoids towards vesicle structure formation.

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Figures

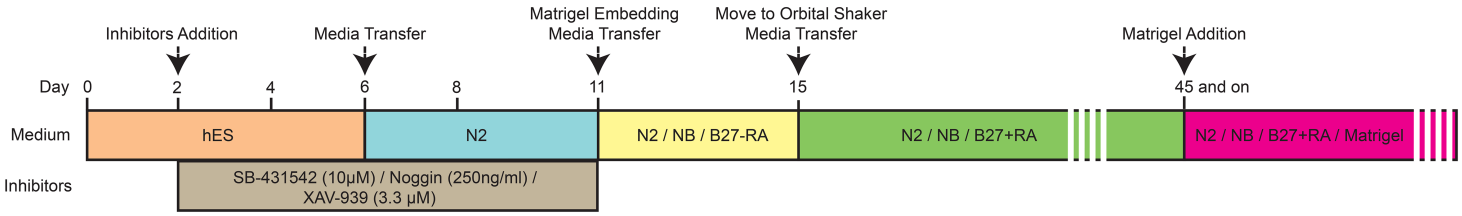

Figure 1

Schematic representation of generation of cortical organoids from hPSCs through short and early SMAD/WNT inhibition

The protocol begins by generating EBs from hPSCs on day 0. Important days for medium change are highlighted. On day 6, organoids are transferred to N2 medium from hES medium. On day 11, organoids are embedded into Matrigel and medium is changed to N2/NB (1:1) along with the addition B-27 without vitamin A. On day 15, medium is changed to N2/NB (1:1) with the addition of B-27 with vitamin A and then organoids are transferred to the orbital shaker. Subsequently medium is changed every alternate day. Matrigel is added directly to the medium from day 45 of organoid development. EB: Embryoid body, hES medium: human embryonic stem cell medium; NB: Neurobasal medium, RA: Retinoic Acid.