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Human neural organoids for studying brain cancer and neurodegenerative diseases

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TITLE:

Human Neural Organoids for Studying Brain Cancer and Neurodegenerative Diseases

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SUMMARY:

This study introduces and describes protocols to derive two specific human neural organoids as a relevant and accurate model for studying 1) human glioblastoma development within human neural organoids exclusively in humans and 2) neuron dopaminergic differentiation generating a three-dimensional organoid.

ABSTRACT:
The lack of relevant in vitro neural models is an important obstacle on medical progress for neuropathologies. Establishment of relevant cellular models is crucial both to better understand the pathological mechanisms of these diseases and identify new therapeutic targets and strategies. To be pertinent, an in vitro model must reproduce the pathological features of a human disease. However, in the context of neurodegenerative disease, a relevant in vitro model should provide neural cell replacement as a valuable therapeutic opportunity.
Such a model would not only allow screening of therapeutic molecules but also can be used to optimize neural protocol differentiation [for example, in the context of transplantation in Parkinson's disease (PD)]. This study describes two in vitro protocols of 1) human glioblastoma development within a human neural organoids (NO) and 2) neuron dopaminergic (DA) differentiation generating a three-dimensional (3D) organoid. For this purpose, a well-standardized protocol was established that allows the production of size-calibrated neurospheres derived from human embryonic stem cell (hESC) differentiation. The first model can be used to reveal molecular and cellular events occurring during in glioblastoma development within the neural organoid, while the DA organoid not only represents a suitable source of DA neurons for cell therapy in Parkinson's disease but also can be used for drug testing.

INTRODUCTION:
The World Health Organization (WHO) classifies astrocytomas as low grade (grade I to II) or high grade (grade III and IV). Glioblastoma multiforme (GBM) is an astrocytoma grade IV, the most lethal of primary brain tumors, that is resistant to all current forms of treatments[1]. Despite standard-of-care therapy including neurosurgery, chemotherapy, and radiotherapy, GBM remains fatal and the 15-month overall survival rate has not dramatically changed over the past 15 years[2]. To make significant progress in understanding GBM pathogenesis, the use of relevant models is key. So far, the study of GBM has relied on cell lines, rodent organotypic slices, and xenotransplantation of patient-derived cells into mice or transgenic mice developing spontaneous tumors[3,4]. Although these models have been useful to study brain metastasis and tumor aggressiveness, they are restricted by differences among species, and resulting conclusions may be incorrectly translated to human tissues. Moreover, existing models with human cells are also limited by the absence of host tissue/tumor interactions[3,4]. Experimental models are critical for the translation from basic science to therapeutic targets. Therefore, describing a protocol to produce in vitro human neural organoids co-cultured with GBM-initiating cells (GICs) can provide a relevant system that mimics morphological and functional features of GBM development. This system reproduces some in vivo features of GBM development such as diffuse migration of invading cells and necrosis areas, and it highlights gene expression relevant to tumor biology. As previously revealed, some critical microRNAs are induced during GIC development within 3D nervous tissue[5,6].

PD is a major neurodegenerative disorder and associated with the degeneration of multiple neuronal subtypes[7]. Even if a progressive onset of symptoms (e.g., bradykinesia, asymmetric rest tremor, rigidity and posture instability) characterizes the disease, its exact etiology is not clearly established. Indeed, many studies have highlighted evidence that major risk factors can

result from a combination of genetic and environmental factors. Parkinsonian symptoms are associated with the bilateral degeneration of dopaminergic neurons in the substancia nigra (SN), leading to the disappearance of dopaminergic (DA) axons projecting to the striatum[8,9]. Therefore, the reduction of striatal dopamine levels is correlated with progression of motor dysfunction in PD patients. Dopaminergic neurons contain tyrosine hydroxylase (TH), a key enzyme in the synthesis of catecholaminergic neurotransmitters that converts the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA, a dopamine precursor) to dopamine[10]. Early loss of TH activity followed by a decline in TH protein expression is a hallmark of PD.

This study describes two protocols using human neural organoids, with one specifically oriented towards a midbrain-like phenotype enriched with TH-positive cells.

PROTOCOL:

This protocol follows the guidelines of University of Geneva's human research ethics committee.

1. Maintenance and culture of undifferentiated human embryonic stem cells (hESCs)

1.1. Perform maintenance and expansion of hSECs on feeder-free conditions by pre-coating dishes with a specific extracellular matrix.

1.1.1. Thaw 300 µL of extracellular matrix at 4 °C (typical range concentration 18–22 mg/mL, keep on ice) and gently mix with 15 mL of cold DMEM medium to avoid a premature gelation of the extracellular matrix. Add 7.5 mL of the extracellular matric to both T150 flasks.

1.1.2. Incubate the dishes coated with extracellular matrix at 37 °C for at least 1 h (maximum overnight).

1.1.3. Remove the medium and seed hESCs to a density of 6.5 x 10^4 cell/cm^2.

1.2. Maintain H1 (hESC cell line) in hESC medium and 1% penicillin/streptomycin.

1.3. Pass the cells with enzymatic procedure: add 7.5 mL of enzymatic solution to a T75 cm^2 flask over a period of 1–2 min at 37 °C. Once cells are completely detached, add 7.5 mL of DMEM-F12 then centrifuge for 5 min at 300 x *g*. To allow for better survival, re-plate cells at the desired density onto extracellular matrix-coated dishes, in the same medium containing Rho-associated protein kinase (ROCK) inhibitor (10 µM) for 24 h.

2. hESC-derived neural organoids for GBM studies

2.1. 24 h before starting the 3D culture, replace the hESC medium with a serum-free medium supplemented with 10 µM ROCK inhibitor (both components are necessary to support cell survival and spontaneous neurosphere formation during the aggregation phase in a microwell

plate). The cells should be at 60% confluency. The next day (day 0), detach hESC colonies as single cells: remove the medium, then rinse with PBS without Ca^{2+}/Mg^{2+}, add 5 mL of enzymatic dissolution solution, and incubate at 37 °C for 1–2 min.

2.2. Collect the cells in serum-free medium with 10 µM of ROCK inhibitor and centrifuge the cells at 300 x *g* for 5 min. Remove the supernatant and count the cells in 10 mL of serum-free medium supplemented with 10 µM of ROCK inhibitor.

2.3. In parallel, rinse the microwell plate with 2 mL of serum-free medium per well and centrifuge the plate at 1200 x *g* for 5 min to remove all bubbles, which can prevent neurosphere formation.

2.4. Prepare 28.2 x 10^6 of cells in 12.5 mL of serum-free medium supplemented with 10 µM ROCK inhibitor. Dispense 1000 cells/microwell. Centrifuge the cells at 300 x *g* for 5 min and place the plate in the incubator at 37 °C overnight (maximum 36 h). For example: to obtain 30 human neural organoids, use one T150 flask at 70%–80% of confluence (about 30 million cells).

2.5. The next day (day 1), collect the spheres (with a P1000) and place them in a 6 well plate. In each well, add 2 mL of B27 medium and DMEM-F12 GlutaMAX and Neurobasal medium (mix at 1:1), supplemented with 1% B27 supplements and 1% non-essential amino acids (NEAA). To promote fast neural induction, supplement the medium with dual-SMAD inhibition cocktail, composed of 10 µM TGFβ/Activin/Nodal inhibitor and 0.5 µM bone morphogenic protein (BMP) inhibitor. From this step forward, the spheres are cultured in rotation (60 rpm, orbital shaker). The rotation is critical to prevent the spheres from sticking together or to the plate.

2.6. Change the medium every 2–3 days: bend the plate and let the spheres fall down for 5 min, remove half of the medium (2 mL), and add 2 mL of fresh B27 medium supplemented with growth factors and inhibitors. Do not centrifuge the spheres.

2.7. Perform neural induction according to the following time course:

2.7.1. From days 1–4, culture the spheres in B27 medium supplemented with dual-SMAD. The dual-SMAD inhibition cocktail (10 µM TGFβ/Activin/Nodal inhibitor and 0.5 µM BMP inhibitor) promote the neural induction.

2.7.2. From days 4–11, promote proliferation of hESC-derived neural rosettes (into the spheres), by adding 10 ng/mL epidermal growth factor (EGF) and 10 ng/mL basic fibroblast factor (bFGF) to the B27 medium supplemented with dual-SMAD cocktail.

NOTE: On day 11, most of the cells should be positive for Nestin.

2.7.3. From days 11–13, culture the spheres in B27 medium supplemented with 0.5 µM BMP inhibitor.

2.7.4. From days 13–21, culture the spheres in B27 medium supplemented with 10 ng/mL glial derived neurotrophic factor (GDNF), 10 ng/mL brain derived neurotrophic factor (BDNF) and 1 µM of γ-secretase inhibitor. GDNF and BDNF promote neuronal and glial differentiation. The γ-secretase inhibitor allows for greater neural maturation.

2.7.5. On day 21, plate the spheres (about 1,000 spheres) on a hydrophilic polytetrafluoroethylene (PTFE) membrane (6 mm diameter, 0.4 µm) deposited on a culture plate insert designed for 6 well plate. Stop any rotation from this step. The presence of rosettes, observed with a bright-field microscope, indicate the initiation of neural differentiation. The neural rosettes can be observed 2–3 days after plating spheres on the PTFE membrane.

2.7.6. Add 1 mL of B27 medium supplemented with growth factors and inhibitors (as followed) to each well underneath the membrane insert, every 2–3 days (usually on Monday, Wednesday and Friday), for a following 3 weeks of differentiation.

2.7.7. From days 21–25, cultivate human neural organoids in the same neural maturation medium (Cf. step 2.7.4).

2.7.8. From days 25–28, only complement B27 medium with 1 µM γ-secretase inhibitor.

2.7.9. From days 28–39, stop adding the γ-secretase inhibitor and continue human neural organoid culture in B27 medium only.

NOTE: After 3 weeks, neural organoids are ready to use for GIC implantation. Along the neural maturation, a decrease of neural immature marker Nestin and increase of mature neural markers β3-tubulin and GFAP were observed.

3. Isolation and cultivation of glioblastoma-initiating cells (GICs)

3.1. Isolate GICs by fragmenting a high grade human GBM biopsy. Transfer the piece of tumor in a beaker containing 0.25% trypsin in 0.1 mM EDTA (4:1) and slowly stir at 37 °C for 30–60 min (depending on tumor size).

3.2. Plate the dissociated cells in 75 cm^2 tissue culture flasks plated at 2,500–5,000 cells per cm^2 in GIC medium: DMEM/F-12 medium (1:1) containing 1% N2, 1% B27, and 1% G5 supplements (to favor GIC survival), supplemented with bFGF and EGF (both at 10 ng/Ml, to promote stemness) and 1% of penicillin/streptomycin.

3.3. Once the GIC is well-established and growing, remove the N2 and G5 supplements from the GIC medium.

3.4. One day before adding the cells onto the organoid, dissociate the GICs and count them.

3.5. Rinse the microwell plate with 2 mL of GIC medium and centrifuge the plate at the maximum speed to remove bubbles (1000 x *g*). Place the GICs at 1,000 cells to obtain one gliomasphere per microwell. Incubate overnight at 37 °C (**Figure 1C**). This step is key and allows for well-calibrated GICs (an example of necrotic and over-sized GICs is shown in **Figure 2A,C**).

3.6. To initiate GBM invasion, add one gliomasphere on top of the neural tissue with a large bore pipet tip (**Figure 1F**). Carefully place the 6 well plate back in the incubator.

4. hESC-derived dopaminergic organoids for PD studies

4.1. Day 0: Amplify hESCs in 2D culture up to 60% confluency (day 0), then replace stem cell media used to maintain pluripotency features of hESCs with a serum-free medium. Start neural induction by supplementing culture medium with 0.5 µM BMP inhibitor and 10 µM TGFβ/Activin/Nodal inhibitor (dual-SMAD inhibition cocktail), then add 10 µM ROCK inhibitor for 24 h to increase the survival rate of cells during passage.

4.2. Day 1: Prepare the microwell plate with 2.5 mL per well of serum-free medium supplemented with 0.5 µM BMP inhibitor, 10 µM TGFβ/Activin/Nodal inhibitor, and 10 µM ROCK inhibitor. To specify cells towards the ventral pattern of the neural tube, add 100 ng/mL Sonic Hedgehog (SHH), 100 ng/mL fibroblast growth factor 8 (FGF8), and 2 µM smoothened agonist. Centrifuge the plate (only with medium and without cells) at 1200 x *g* for 5 min to remove air bubbles from the microwells.

4.2.1. After 1 day of neural induction in 2D, remove the medium and quickly wash with PBS without Ca^{2+}/$MgCl^{2+}$. Dissociate the colonies in single cells suspension by adding 7.5 mL of recombinant enzymatic solution in a T75 cm^2 flask. Incubate for 2 min at 37 °C then complete with 7.5 mL of DMEM-F12.

4.2.2. Collect the cell suspension and centrifuge at 300 x *g* for 5 min. Remove the supernatant and count the cells in the same medium used to prepare the microwell plate.

4.2.3. Adjust the medium volume to obtain a cell suspension allowing to form neurospheres containing 1000 cells per microwell (for example, the microwell plate used here contains 4,700 microwells per well). So, prepare 4.7 million cells in 2.5 mL of medium and add it to the previous 2.5 mL of medium already placed in the plate.

4.2.4. In order to correctly distribute the cells in each microwell, gently shake the plate, and centrifuge the microwell plate 300 x *g* for 5 min. Incubate the plate at 37 °C for 24 h to generate spheres.

4.3. Day 2: Gently flush the microwells with the medium and collect then transfer the spheres in tissue-treated six-well plate. Replace medium with Neurobasal medium supplemented with 1% B27, 1x NEAA, 2 mM L-glutamine, and 1% of penicillin/ streptomycin. Additionally, add

regionalization factors SHH, FGF8, smoothened agonist, and dual-SMAD inhibition small molecules.

4.3.1. Place spheres in rotation at 37 °C (60 rpm, orbital shaker) and change half-medium freshly supplemented every 2–3 days.

4.4. Day 3: To enhance neural induction and convert to neural progenitors with a midbrain identity, supplement the medium with 3 µM GSK-3β inhibitor, which activates the Wnt/β-catenin pathway. Maintain GSK-3β inhibitor in the medium up to day 13. Split into two new tissue-treated 6 well plates to reduce both sphere density per well and avoid sphere aggregation.

NOTE: At Day 8, most of the cells should be positive for Nestin.

4.5. Day 8: Start the neural maturation: replace regionalization factors SHH, FGF8, smoothened agonist, and dual-SMAD inhibition cocktail with 0.5 mM dibutyryl cAMP (to favor maturation), 20 nM inhibitor of histone deacetylase (for cell cycle exit), 1 µM γ-secretase inhibitor and growth factors, 10 ng/mL GDNF, 10 ng/mL BDNF, 1 ng/mL transforming growth factor β3 (TGFβ3), and 5 ng/mL FGF20 (both favor DA progenitor survival). Change the medium every 2–3 days.

4.6. Day 21: Generate the neural organoid: seed around 100 neurospheres under air-liquid interface conditions on PTFE membrane (6 mm diameter). Transfer the membrane to a culture plate insert (0.4 mm) and add 1.2 mL of neural maturation medium used for neurosphere differentiation as previously described.

4.6.1. Stop any rotation from this step. Change the medium every 2–3 days until the required differentiation time point is achieved.

NOTE: Regarding neural maturation, a decrease of the neural immature marker Nestin and increase of mature neural markers β3-tubulin and GFAP were observed. High TH and NURR1 expressions were observed (**Figure 3C**) and confirm neural organoid maturity[11].

5. Quantification of TH and Nurr1 gene expression for validation of dopaminergic differentiation

5.1. RNA extraction: On the indicated day of differentiation, lyse 40 neurospheres with 350 µL of RLT buffer (provided in RNA extraction kit) supplemented with 3.5 µL of 2-mercaptoethanol. Extract the RNA from the lysed neurospheres using an RNA extraction kit following the manufacturer's instructions.

5.2. Quantify total RNA concentrations.

5.3. Perform reverse transcription of 300 ng of the total RNA extraction using reverse transcription kit for quantitative real-time polymerase chain reaction (qPCR) and follow the manufacturer's instructions**.**

5.4. Perform qPCR analysis on real-time PCR detection systems, based on asymmetrical cyanine dye detection. Normalize the data with housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor 1-alpha (EF1). Sequences of primers are described in **Table 1**.

6. High pressure liquid chromatography (HPLC) detection

6.1. Use high pressure liquid chromatography (HPLC) with electrochemical detection to detect the presence of dopamine. Dopamine was extracted by lysing neural organoids in 100 mL of 0.1 N perchloric acid ($HClO_4$) for 15 min at 4 °C with a vigorous vortexing every 5 min. After centrifugation, collect and store the supernatant at -20 °C for dopamine dosage.

6.2. Use a C-18 column (5 µm, 4.6 mm x 150 mm) to separate the analytes by reversed-phase HPLC in isocratic mode at a flow rate of 1 mL/minute. Detection of dopamine should be carried out using a coulometric detector with the conditioning cell set at a potential of +200 mV.

7. Raw data recording with microelectode array (MEA) platform

7.1. Use a dissection microscope to transfer neurospheres to the center of a porous MEA device.

7.2. Use an amplifier and data acquisition system for electrophysiological recordings. Measure the signal-to-noise ratio (SNR) as the standard deviation of the voltage during a 5 min recording, using the signal as the average peak-to-peak voltage of the spikes recorded in the same 5 min periods.

REPRESENTATIVE RESULTS:

The critical steps of this protocol must be well-identified and handled properly. Therefore, a diagram of culture conditions indicating the time-lapse for each step as well as the compounds used for the differentiation protocol are illustrated in **Figure 1A** and **Figure 3A** for NO plus GBM and DA neural organoids, respectively. **Figure 1B,C,D,E,F** illustrates the cells, spheres, and NO and show the typical morphology for each step. **Figure 1G,H,I** illustrates immunofluorescence staining with some neural markers.

FIGURE AND TABLE LEGENDS:

Figure 1: Human neural organoid (NO) differentiation protocol. (**A**) Standardized protocol for the generation of NO derived from human embryonic stem cells (hESC). (**B**) hESCs are maintained on extracellular matrix in hESC medium. (**C**) Microwell plates were used to generate calibrated neurospheres. At 2 weeks, neurospheres were plated onto the insert containing a

PTFE membrane (scale bar = 50 µm). (**D**) Macroscopic view of NO into the insert in one well of a 6 well plate. During the first days, rosettes were observed (black arrow) (**E**). (**F**) Macroscopic view of a NO plus GIC sphere on the top. (**G–I**) Immunofluorescence analysis of NO plus GIC sphere (EGFR-positive; scale bar = 50 µm) (**G**) and NO alone, which showed immune reactivity for the neuronal marker βIII-tubulin and slightly positive for nestin; however, synapsin 1 showed a weak signal (**H,I**) (scale bars = 100 µm and 50 µm, respectively).

Figure 2: Illustration of necrotic spheres and immature NO. The neurospheres **(A)** and NO (**B**) can undergo necrosis when they are too numerous in the well or oversized (**C**) (scale bar = 10 µm). (**D**) One GIC infected with a tomato reporter help to track tumor cell invasion in NO, scale bar, 10 µm. Example of immature NO with neural tubes (**E**) and no neural tubes (**F**) (scale bar = 50 µm).

Figure 3: Standardized protocol for generation of DA neural organoid and electrophysiological and morphological analysis. (**A**) Standardized protocol for the generation of DA neural organoids. (**B**) Immunofluorescence analysis of DA neural organoid; TH-immunoreactive cells co-expressing Nurr1, a midbrain specific marker (scale bar = 50 µm). Data are represented as mean ± SEM (n = 3). (**C**) Graphs represent kinetics of TH and Nurr1 gene expression evaluated by qRT-PCR. (**D**) Representative HPLC: dopamine peak (arrow) was detected by HPLC from DA neural organoid lysate. (**E**) Example of raw data recorded with MEA platform. Each spike is displayed by a vertical line (time stamps), whereas the remaining trace is noise. (**F**) Picture representing a neurosphere deposited on the MEA. (**G**) Superposition of typical spikes (blue and red curves) detected from the raw data. The black bold curve indicates the average of the corresponding red curves. (**H**) Raster plot showing the time stamps associated with each spike detected. The different colors highlight the different electrodes.

Table 1: Primers used in this protocol.

DISCUSSION:

One of the most critical aspects of this protocol includes the maintenance of hESC pluripotency during cell culturing and close monitoring of the spheres and neural organoid morphology. hESCs are very sensitive, and every manipulation can lead to early uncontrolled differentiation as well as cell death. In order to increase experimental reproducibility and avoid the occurrence of abnormal karyotype events, it is advised to cryopreserve several batches of hESCs at the lowest passage after validation of their chromosome stability. Moreover, it is recommended to thaw a new vial for each experiment and check the behavior of the cells every day. If the spheres are less refractive with abnormal higher size, they will likely start to aggregate and die.

One improvement upon this system is either perfusion or implementing a vascularized system (by adding endothelial cells or within a 3D fluidic microchip)[12,13]. However, controlling the thickness of the neural organoid (≤300 µm) allows efficient passive perfusion of oxygen and nutriments and prevents necrosis. Another improvement is the introduction of immune cells (microglia). With these limitations in mind, neural organoids plus a GIC system may be a

relevant tool for several reasons. First, this system allows drug screening to monitor how a therapeutic compound may affect an organoid or tumor cell. Second, cell-to-cell interactions can be studied, and micro-environmental determinants underlying individual and collective invasions can be visualized and explored[5,6,13].

In the context of Parkinson's disease, a neural organoid enriched in DA neurons can represent a relevant and accurate 3D model to study disease development. In previous studies, Parkinson's patient-derived induced pluripotent stem cells differentiated towards DA neurons have been used to study the affected neuronal subtypes. Of note, some disease-related phenotypes such as the accumulation of α-synuclein and sensitivity to oxidative stress have been observed[14,15]. Moreover, the neural organoid may be used as a tool to screen therapeutic molecules. However, specific and relevant readouts should be set up to evaluate DA neuron survival and functionality, such as dopamine production and electrophysiological activity. Altogether, this protocol provides two standardized and accurate stem cell-based approaches to generate neural organoids.

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DISCLOSURES:

The authors declare no competing financial interests.

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Figure 1

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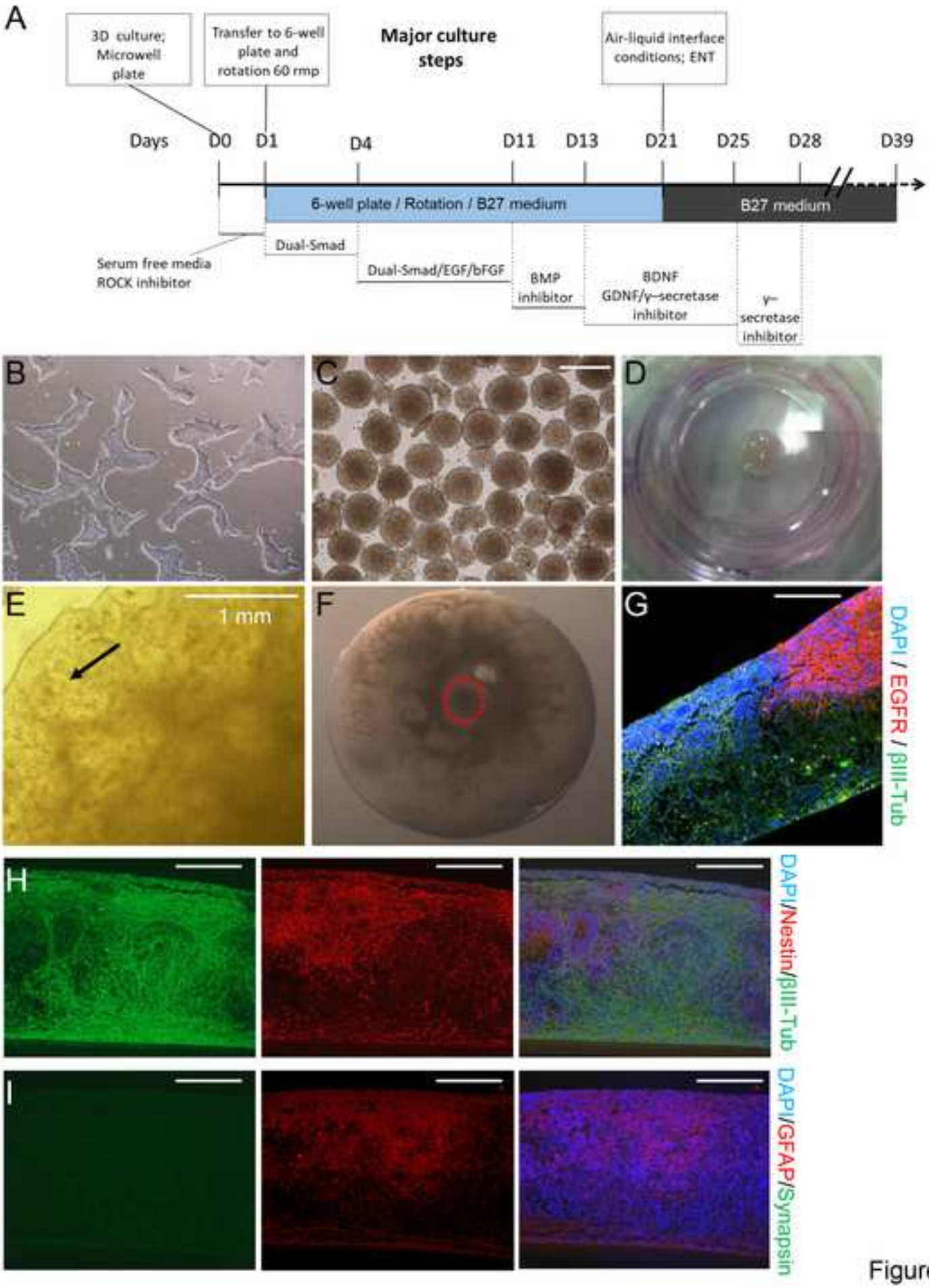

Figure 1

Figure 2

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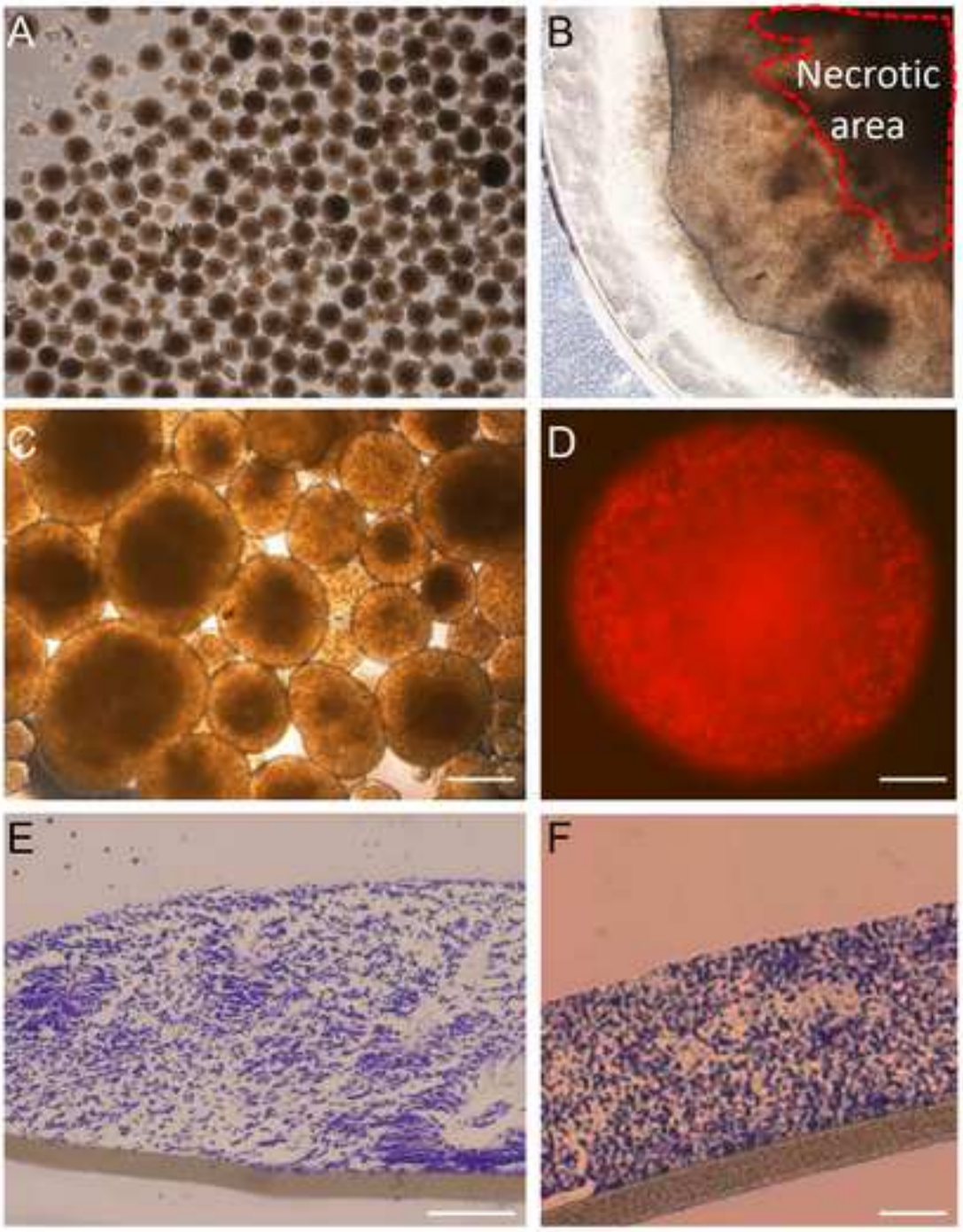

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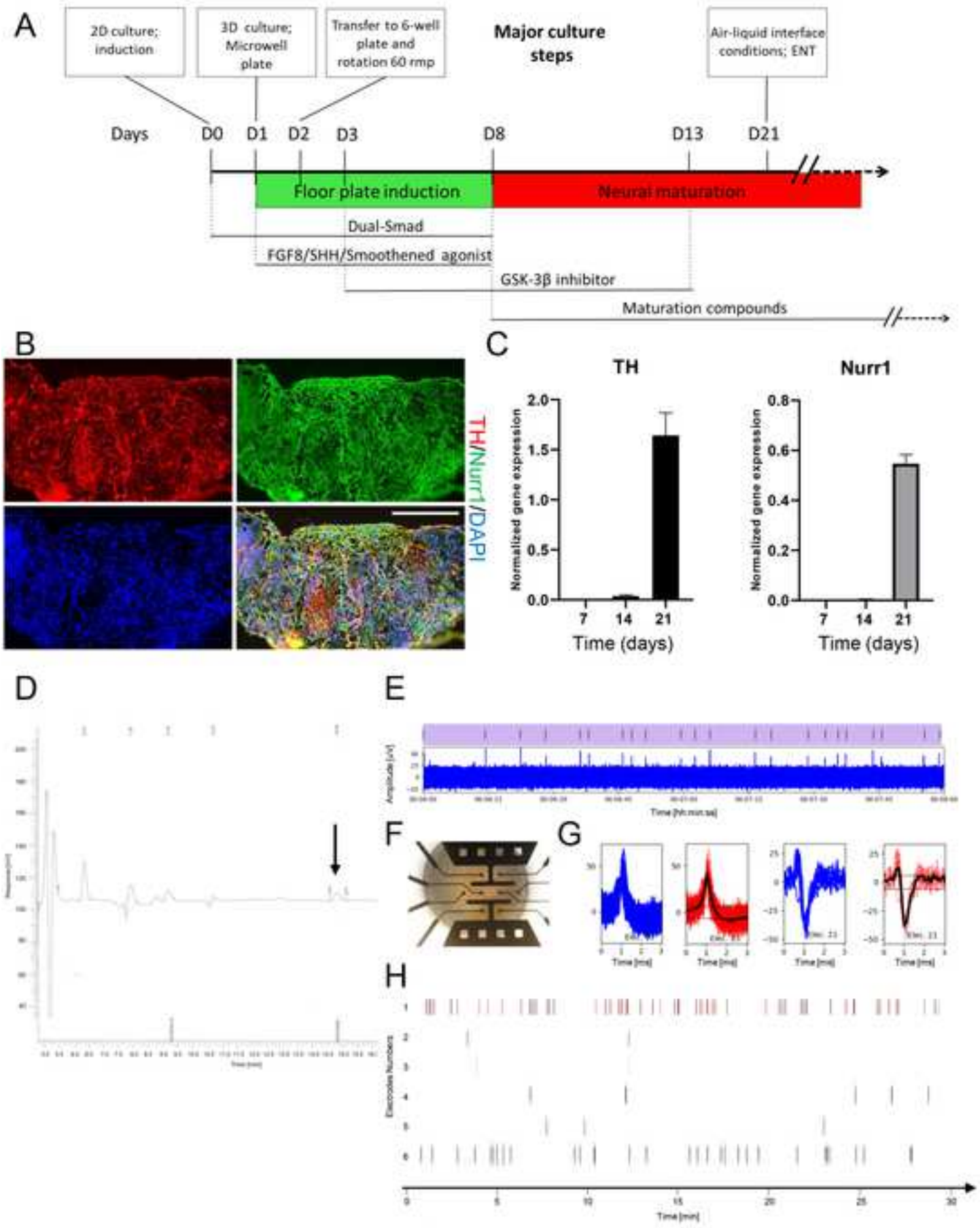

Figure 3

Table 1

Gene	Foward	Reverse
Nurr1	GGCTGAAGCCATGCCTTGT	GTGAGGTCCATGCTAAACTTGACA
TH	GCACCTTCGCGCAGTTCT	CCCGAACTCCACCGTGAA
EEF1	AGCAAAAATGACCCACCAATG	GCCTGGATGGTTCAGGATA
GAPDH	GCACAAGAGGAAAGAGAGAGAACC	AGGGGAGATTCAGTGTGGGT

Name

6-well plate (6-well plate)
ABI Prism 7900 HT detection system (Real-Time PCR detection systems)
Aggrewell 400 (Microwell culture plates)
Amplifier (W2100-HS32) (Amplifier)
Anti-EGFR (phospho Y1101) antibody
Anti-GFAP Antibody
Anti-Nestin, Human Antibody
Anti-Synapsin I Antibody
B27 supplements (B27)
Brain-derived neurotrophic factor (BDNF)
CHIR-99021 (GSK-3β inhibitor)
Compound E a γ–secretase inhibitor (γ–secretase inhibitor)
Coulochem III (Coulometric detector parameters)
Dibutyryl cyclic-AMP (Dibutyryl cAMP)
Dimethyl Sulfoxide Pure (DMSO)
Dulbecco's Modified Eagle Medium (DMEM)
Dulbecco's Modified Eagle Medium Mixture F-12 (DMEM-F12)
EDTA 0.1 mM (EDTA)
Epidermal Growth Factor (EGF)
Fibroblast Growth Factor 20 (FGF20)
fibroblast growth factor 8 (FGF8)
Fibroblast growth factor-basic (bFGF)
G5 supplements (G5)
Glial cell-derived neurotrophic factor (GDNF)
Hydrophilic polytetrafluoroethylene membrane (PTFE membrane)
LDN-193189 (BMP inhibitor)
L-glutamine (L-glutamine)
Matrigel (extracellular matrix)
Millicell-CM Culture plate insert (0.4 µm) (Culture plate insert)
Monoclonal Anti-β-Tubulin III antibody
MS Orbital Shaker, MS-NOR-30 (Orbital shaker)
N2 supplements (N2)
Nanodrop (Nanodrop)
Neurobasal (Neurobasal)
Non-Essential Amino Acids (NEAA)
Nurr1 Antibody (M-196)
Nutristem (hESC medium)
Penicilin / Streptomycin (Penicilin / Streptomycin)
Perchloric acid 0.1N (HCLO4)
Phosphate Buffered Saline without Ca2+/Mg2+ (PBS without Ca2+/Mg2+)
PrimeScript RT-PCR Kit (Reverse transcription kit)
Purmorphamin (smoothened agonist)
Rho-associated Kinase Y-27632 (ROCK)
RNeasy mini kit (RNA extraction kit)
SB-431542 (TGFβ/Activin/Nodal inhibitor)

Sonic Hedgehog (SHH)
StemPro Accutase (hESC enzymatic solution)
Symmetry C-18,5 mm (4.6 150mm2) (Reversed-phase column)
T150 flask (T150 flask)
TH Antibody (F-11)
Transforming Growth Factors beta 3 (TGFβ3)
Trichostatine A (inhibitor of histone deacetylase)
TrypLE (recombinant enzymatic solution)
Trypsin 0.25% (enzymatic solution)
W2100, Multi Channel Systems (Data acquisition system)
X-vivo (serum free medium)

Provider	Catalog Number
Falcon / Corning	07-201-588
Applied Biosystems	Discontinued
StemCell Technologies	34421
Multi Channel Systems	
Abcam	ab76195
Dako	Z334
Millipore	ABD69
Chemicon	AB1543P
Life Technologies / Invitrogen	1238
Cell Guidance	GFH1-2
Axon Medchem	ct99021
Calbiochem	CAS 209986-17-4
Thermo scientific	
Sigma	D0627
Sigma-Aldrich	C6164
Life Technologies	12491-015
Gibco	11320033
Life Technologies	AM9912
Gibco	PHG0313
Peprotech	100-41
Peprotech	GFH176-5
Gibco	PHG0024
Invitrogen	17503012
Cell Guidance	GFH2-2
BioCell-Interface	Discontinued
Axon Medchem /Stemgen	04-0072-02 /1509
Gibco	25030081
BD Biosciences	354277
Millipore	PICM03050
Sigma	T8660
Major Science	MS-NRC-30
Invitrogen	17502-048
Thermo Fisher Scientific	Discontinued
Life Technologies / Gibco	21103049
Gibco	11140
Santa Cruz	Sc-5568
Biological Industries	05-100-1A
Life Technologies / Gibco	15140122
Merck	100519
Life Technologies	14190250
Takara	RR014A
Calbiochem	SML0868
Abcam Biochemicals	ab120129-1
Qiagen	74104
Ascent	Asc- 163

Cell Guidance	GFH168-5	
Gibco	A11105-01	
Waters Corporation		
Falcon	08-772-1F	
Santa Cruz	Sc-25269	
Cell Guidance	GFH109-2	
Sigma	T8552	
Invitrogen		12604021
Life Technologies		15050065
	WAT045905	
Lonza	BE04-743Q	

Notes

1/100 dilution
1/1000 dilution
1/400 dilution
1/500 dilution
For both protocol, stock solution 100x, final solution 1x
For both protocol, stock solution 100 µg/mL in pure H2O, final solution 20 ng/mL
For Dopaminergic protocol, stock solution 7.5 mM in DMSO, final solution 3 µM
For both protocol (gamma-secretase inhibitor XXI), stock solution 5 mM in DMSO, final solution 1 µM

For Dopaminergic protocol, stock solution 0.5 M in DMSO, final solution 0.5 mM
Compounds solvent, ready to use
For cell culture, ready to use
For cell culture, ready to use
For cell culture, ready to use
For GIC culture, stock solution 100 µg/mL in pure H2O, final solution 10 ng/mL
For Dopaminergic protocol, stock solution 100 µg/mL in pure H2O, final solution 5 ng/mL
For Dopaminergic protocol, stock solution 100 µg/mL in pure H2O, final solution 100 ng/mL
For GIC culture, stock solution 100 µg/mL in pure H2O, final solution 10 ng/mL
For GIC culture, stock solution 100x, final solution 1x
For both protocol, stock solution 100 µg/mL in pure H2O, final solution 20 ng/mL

Dual/Smad, stock solution 5 mM in DMSO, final solution 0.5 µM
L-Glutamine (200 mM), stock solution 200 mM, final solution 2 mM
hESC-qualified Matrix, stock solution 18-22 mg/mL, final solution 180-220 µg/mL

1/1000 dilution

For GIC culture, stock solution 100x, final solution 1x

Maintenance and maturation embryonic neuronal cell populations , ready to use
Non-essential Amino Acids 100X, stock solution 100x, final solution 1x
1/100 dilution
Stem cell media, ready to use
For cell culture, stock solution 5 mg/mL, final solution 50 µg/mL
For HPLC, ready to use
For cell culture, ready to use

For Dopaminergic protocol, stock solution 10 mM in DMSO, final solution 2 µM
Rock Inhibitor, stock solution 50 mM in DMSO, final solution 10 µM

Dual-Smad, stock solution 50 mM in DMSO, final solution 10 µM

For Dopaminergic protocol, stock solution 100 µg/mL in pure H_2O, final solution 100 ng/mL
hESC enzymatic solution, ready to use

1/200 dilution
For Dopaminergic protocol, stock solution 100 µg/mL in pure ethanol , final solution 1 ng/mL
For Dopaminergic protocol, stock solution 100 µM in DMSO, final solution 20 nM
recombinant enzymatic solution, ready to use
enzymatic solution, ready to use

serum free medium, ready to use

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Author(s):	Cosset Erika, Locatelli Manon, Pierre Lescuyer, Florence Dall Antonia, Olivier Preynat-Seauve, Luc Stoppini and Tieng Vannary

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Editorial comments:

1. I'm assuming the bolded material is the part highlighted for filming (note that it is now highlighted in yellow); if so, it exceeds our limits for filming (2.75 pages). Please reduce the highlighted length.

Accordingly, we reduced the highlighted length to reach 2.75 pages.

2. Figure 1: The scale bar in panel G is not defined. Also, please leave a space between '1' and 'mm'.

The scale bar in now defined and a space has been added.

3. Figure 2: The red arrows in panel A are not explained. Also, there are no black arrows in panel E.

The red arrows have been removed as well as the comments about black arrows.

4. Figure 3: Please use 'days' instead of 'day' in panel C.

We changed this.