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Generating 3D and 2D Neuronal Cultures with a Forebrain Specific Identity

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ABSTRACT

While organoid development is not new technology, generating neural organoids with specific developmental identities in an efficient manner remains a challenge. Previously we reported a protocol to generate organoids with hindbrain and midbrain specific identities using our established kit. Here we report a protocol to generate organoids with forebrain specific identity. While most protocols in circulation require over 40 days to generate neural organoids, we established a novel protocol to obtain cortical organoids with forebrain specific identity, within 28 days. Large scale PSC culture either in defined medium (Essential 8, Essential 8 Flex), or in enriched medium (StemFlex, StemScale) can be differentiated into a forebrain phenotype and maintained either in 2D or 3D configuration using this protocol. Our protocol is specifically designed to overcome the hurdle of excessive cell death during differentiation, especially for PSC cultured in defined media. In addition, this protocol utilizes fewer steps to obtain mature organoids, with fully optimized conditions designed to promote healthy and observable growth at each stage. First, PSCs are induced to form spheroids in PSC medium to reach 300-400 microns. Next, spheroids are specified to develop a forebrain identity in a two-step induction media. Finally, forebrain spheroids are differentiated to form mature cortical organoids with forebrain specific identity in Neuronal Maturation Medium. To confirm the phenotype, we designed a gene expression panel to verify forebrain, midbrain and hindbrain regions. After generating region specific organoids with respective phenotypes, their relative gene expression profiles were compared using this custom designed Taqman qPCR panels. In addition, to verify electrophysiological integrity of the neural organoids, we examined the ability of the differentiated forebrain organoids to fire action potentials. Our protocols demonstrate rapid, healthy growth of organoids grown from PSC cultured in multiple media systems, with mature organoids forming in as little as 21 days using a simplified protocol. This protocol completes a series generating organoids with specific developmental identities: forebrain, midbrain and hindbrain, verified by immunohistochemistry, qPCR panel testing and electrophysiology.

INTRODUCTION

Differentiation of human pluripotent stem cells (hPSCs) into fully differentiated, mature neural organoids is a significant endeavour. These organoids serve as neuron banks which may be dissociated and used in research, disease modeling, drug discovery and cell therapies. For specific utility, neural organoids should duplicate regional specificities and developmental markers characterizing the human brain. To do this in an efficient manner, while remaining reproducible in a larger scale, we have developed a protocol to generate organoids with identity specific to the neuronal forebrain. Our protocol is compatible with both defined and enriched media systems and can be performed in both adherent and suspension culture formats. Generation of fully mature forebrain specific organoids can be completed within 28 days, with minimal interference.

MATERIALS

Neuronal Induction Medium	
Product	Cat. No.
Essential 6 Medium	A1516401
RepSox Solution	CAS No. 446859-33-2
LDN-193189 Solution	CAS No. 1062368-24-4
XAV-939 Solution	CAS No. 284028-89-3
N2 Supplement	17502048
Gentamicin	15710064

Neuronal Expansion Medium	
Product	Cat. No.
DMEM/F12 with Glutamax	10565018
Neurobasal Medium	21103049
N2 Supplement	17502048
B27 Supplement	17504044
Glutamax	35050061
Non-Essential Amino Acids (100X)	11140050
Insulin Solution (4 mg/mL)	12585014
Sodium Pyruvate Solution (100 mM)	11360070
RepSox Solution	CAS No. 446859-33-2
DMH1 Solution	41-261-0
Gentamicin	15710064

Neuronal Maturation Medium	
Product	Cat. No.
Neurobasal Plus	A3582901
B27 Plus	A3582801
DA Neuron Maturation Supplement (50X)	A3147401
Antibiotic-Antimycotic (100X)	15240062

RESULTS

Figure 1. Forebrain Differentiation in 2D and 3D

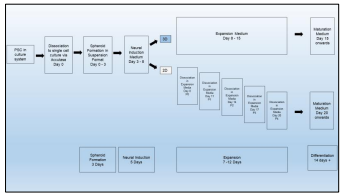
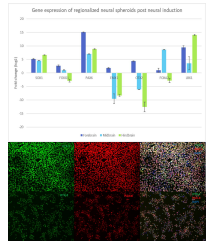


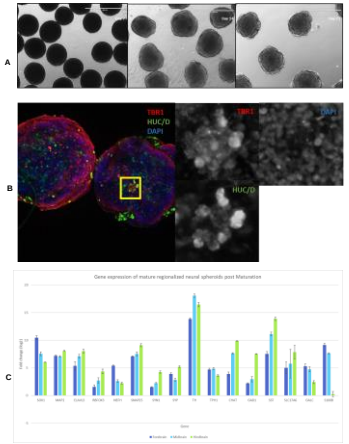
Figure 1: Schematic of the forebrain differentiation protocol. PSC are grown as suspended aggregates in media system of choice. Day 0 is the first passage into single cells before spheroids are allowed to form. Spheroids are induced neurally on day 3, followed by exposure to expansion medium on day 8. Depending on 2D (adherent) or 3D (suspension) format preferences, spheroids will be either dissociated into single cells or allowed to continue in suspension. Culture is introduced to maturation medium on day 15 or day 20, format depending. Length of time required for spheroid formation can vary based on media system, please see detailed protocol for guidance.

Figure 2. Forebrain Specific Neural Induction



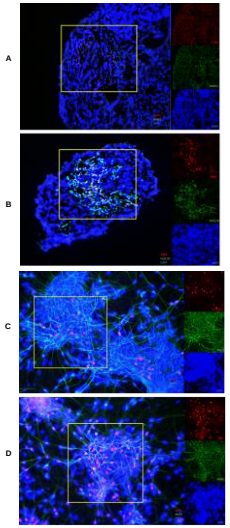
A) qPCR quantification of genes expressed during early forebrain (SOX1, FOXG1, PAX6, EMX1, OTX2), midbrain (FOXA2) and hindbrain (LBX1) differentiation (error bars +/- s.d.).
B) Spheroids grown out of StemFlex (above) and StemScale (below) culture were dissociated, passaged and stained to show early forebrain markers OTX-2 and PAX-6.

Figure 3. Forebrain Specific Maturation in Whole Spheroids



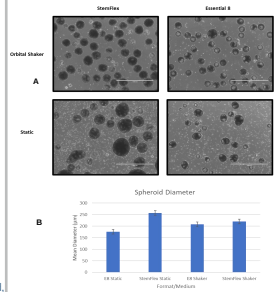
A) Morphological progression of spheroids grown in StemScale, from left to right, after 7, 14 and 21 days in maturation media.
B) Spheroids originating from StemScale culture were differentiated to forebrain (2 weeks) and ICC performed using a pan neuronal (MAP2) and a mature forebrain (TBR1) marker.
C) qPCR quantification of genes expressed after 2 weeks of maturation in forebrain (dark blue), midbrain (turquoise) and hindbrain (green) populations. Markers of neuronal progenitor (SOX1), pan-neuronal maturation (MAP2, ELAVL3, RBFOX3, NEFH, SNAP25, SYN1, SYP), subtype-specific maturation (TH, TPH1, CHAT, GAD, SST, SLC17A6) and non neuronal markers (GALC, S100B) are shown (error bars +/- s.d.).

Figure 4. Markers of Forebrain Maturation in Spheroid Sections and Adherent Culture



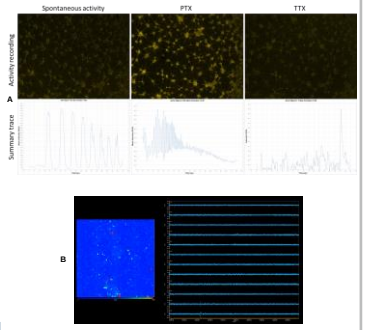
A and B) Spheroids originating from StemScale culture were differentiated to forebrain (2 weeks), sectioned (12 um) and ICC performed using a pan neuronal (HUC/D) and mature forebrain (TBR1, CTIP2) markers.
C and D) Adherent culture originating from StemScale were differentiated to forebrain (2 weeks) and stained using a pan neuronal (MAP2) and mature forebrain (TBR1, CTIP2) markers.

Figure 5. StemFlex directly improves efficiency of spheroids grown out of Essential 8 PSCs



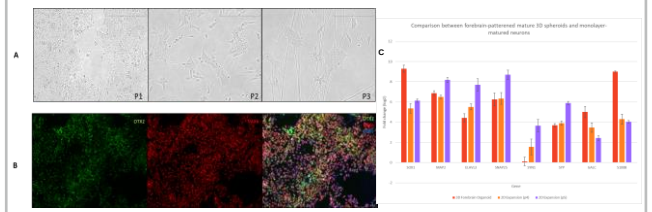
A) PSC cultured in Essential 8 were dissociated and replated in either Essential 8 or StemFlex medium and induced to form spheroids. Both static and orbital shaker (70 RPM) formats were tested.
B) Mean diameter comparison of spheroids grown in Essential 8 and StemFlex media systems in both static and shaking conditions (error bars +/- s.e.m.).

Figure 7. 3D and 2D cultures demonstrate replicable electrophysiological activity on multiple platforms



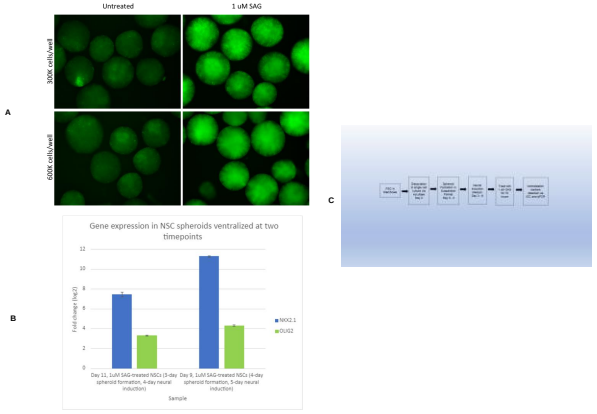
A) Left Panel: Spontaneous calcium oscillations recorded from a partially dissociated spheroid culture after transfection with a lentivirus based labeling reagent, which consists of a synapsin promoter that drives expression of a genetically encoded calcium indicator. Trace diagrams summarizing activity shown below. Middle panel: Changes in spontaneous electrical activity after exposure to Picrotoxin. Right panel: Changes in activity after exposure to Tetrodotoxin. All populations were incubated for 3 weeks in maturation media. The cultures were imaged on a live imaging system from Sartorius.
B) Recordings of multiple whole organoids seeded onto an electrical chip using a Multi Electrode Array (MEA) system provided by 3Brain. Cultures were incubated for 3 weeks in maturation media.

Figure 6. Additional passages in expansion media increases yield of neurons in 2D culture



In order to increase yield of neurons in 2D post neural induction, spheroids can be dissociated and replated for three consecutive passages in expansion media.
A) Phase images showing yield after first (P1), second (P2) and third (P3) passages of spheroids cultured out of StemScale.
B) Expression of early forebrain markers (OTX2, PAX6) after completion of neural induction and P1 in expansion media.
C) qPCR quantification of genes expressed after 2 weeks of maturation in 3D spheroids (red), monolayer neuronal culture after 4 passages in expansion media (orange) and 5 passages in expansion media (purple). Markers of neuronal progenitor population (SOX1), pan-neuronal maturation (MAP2, ELAVL3, SNAP25, SYN1, SYP) and non neuronal markers (GALC, S100B) are shown (error bars +/- s.d.).

Figure 8. Ventralization of forebrain spheroids is compatible with established protocol



A) Expression of reporter line OLIGO2-GFP after treatment with 1 uM Sonic hedgehog (Shh) pathway agonist (SAG) post completion of neural induction.
B) qPCR quantification of ventral progenitor markers NKX2.1 and OLIG2.
C) Schematic of the ventralization process. After neural induction is complete, spheroids are exposed to 1 uM SAG for 72 hours.

CONCLUSIONS

- Human PSC cultured into aggregates in suspension can be successfully differentiated into functional forebrain specific organoids.
- Our established protocol is compliant with multiple media systems, both defined and enriched.
- Markers indicative of a mature forebrain population are expressed in as little as 28 days from start of protocol.
- After completion of neural induction, aggregates can be dissociated and expanded in 2D to increase neuronal yield prior to maturation.
- Both dorsally and ventrally specified forebrain organoids can be obtained using our protocol.
- Cultures in both 3D and 2D formats demonstrate robust electrical activity, detected using both MEA and live imaging systems.

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