Uniform cerebral organoid culture on a pillar plate by simple and reproducible spheroid transfer from an ultralow attachment well plate

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Abstract

Human induced pluripotent stem cell (iPSCs)-derived brain organoids could recapitulate the earliest phase of brain development, making them an effective *in vitro* system for simulating both normal brain development and disorders. However, the current techniques for creating brain organoids are low throughput, suffer from high variability in organoid generation, and frequently involve lengthy processes of transferring and encapsulating the cells in hydrogels. These limitations hinder the application of brain organoids for high-throughput assessment of compounds in academic and industry settings. Here we demonstrate a simple method of generating multiple cerebral organoids from iPSCs on a unique 36PillarPlate with sidewalls and slits without tedious manual intervention that leads to the reproducible generation of cerebral organoids. Embryoid bodies (EBs) formed in an ultra-low attachment (ULA) 384-well plate were transferred to the 36PillarPlate with Matrigel on one-to-one basis by a simple sandwiching and inverting method. The success rate of spheroid transfer was 95 – 100%. Cerebral organoids were generated robustly with the coefficient of variation (CV) below 19% by differentiating EBs on the pillar plate, which demonstrate the robustness of our approach to support organoid culture. The spheroid transfer method on the pillar plate could overcome the potential limitation of organoid variability, reduce cell culture volume, and enhance assay throughput as compared to conventional organoid culture in petri dishes, spinner flasks, and 6-/24-well plates.

Keywords: 36PillarPlate, 384DeepWellPlate, induced pluripotent stem cells (iPSCs), embryoid bodies (EBs), spheroid transfer, cerebral organoid

Introduction

The human brain is one of the most complex organs in terms of its structural development and function[1,2]. Studies on understanding the brain development and function have been carried out extensively over the past decades. Animal models have been widely used to understand the principle of human brain development. However, there are significant structural differences between humans and animals such as rodents[3,4]. The lack of reliable *in vitro* models and the accessibility of human brain tissue have severely hindered the research on modeling brain development and disease[5]. Recent advancements of pluripotent stem cells (PSCs) to self-organize and differentiate into three-dimensional (3D) organ-like tissue aggregates (a.k.a., organoids) have provided new opportunity to uncover development, structure, and function of the human brain *in vitro*[6]. Organoids are 3D cell aggregates that resemble the native tissues or organs with similar cell type, function, architecture, and complexity, bridging the gap between traditional 2D cell culture models and animal models[7,8]. Brain organoids differentiated from induced pluripotent stem cells (iPSCs) resemble the human brain not only at the cellular level but also at the level of tissue structure and developmental pathway. Thus, brain organoids could be a useful tool to investigate human brain evolution, development, function, and disease[6,9].

Several protocols have been published so far, including guided and unguided methods to differentiate iPSCs into brain organoids. Unguided differentiation methods are used to generate whole brain organoids whereas guided differentiation methods apply certain patterning factors to generate specific regions of the brain such as the hindbrain, midbrain, and forebrain[10]. Unguided methods follow the development of embryoid bodies (EBs) from iPSC aggregates, embedding them into extracellular matrices (ECMs), and subsequently culturing them in spinning bioreactors to promote neuroepithelial expansion and differentiation[11]. Existing approaches of brain organoid generation go through multiple processes including EB formation, manual picking of the EBs for embedding in ECMs, neuroepithelial expansion, and later transferring the organoids to orbital shakers[12], spinner flasks[13], miniature bioreactors[14] or microfluidic devices[15] for further maturation. The conventional organoid culture platforms such as petri dishes, 6-/24 well plate, and spinner flasks are unaffordable and not well suited for high-throughput culture and analysis[16] due to the need for a large amount of resources such as cell culture media and soluble factors. Although microfluidic devices require less media volume, cells, and reagents, they are inherently low throughput due to tedious manual steps required for cell loading, media change, and imaging. They also need highly skillful experts and expensive proprietary equipment to operate[17]. Thus, current organoid culture systems face significant technical challenges including low throughput, high variability, incompatibility with existing equipment, and user unfriendliness, which should be overcome for widespread usage of organoids for disease modeling and predictive compound screening.

To enhance throughput, reproducibility, and maturity of brain organoids for the assessment of compounds, we have developed a unique pillar plate and a complementary deep well plate by plastic injection molding, including a 36-pillar plate with sidewalls and slits (36PillarPlate) and a clear bottom 384-deep well plate (384DeepWellPlate). In addition, we have developed a unique protocol of spheroid transfer and encapsulation in biomimetic hydrogels for robust and reproducible organoid culture on the 36PillarPlate. The pillar/deep well plate platform offers several advantages over conventional organoid culture systems, which includes scale-up production of multiple organoids in hydrogels on the pillar plate with minimal resources and rapid *in situ* analysis of organoids for high-throughput screening (HTS). Since the pillar plate is built on the footprint of standard 384-well plates, it is compatible with existing lab equipment such as fully automated fluorescence microscopes and microtiter well plate readers. In addition, data points can be generated from organoids on each pillar in a high-throughput manner, potentially providing more predictive information on toxicity and efficacy of compounds. Thus, the pillar/deep well plate could be used for static organoid culture and analysis in academic and industry settings.

Materials and Methods

Surface functionalization of the 36PillarPlate

The 36PillarPlate and the 384DeepWellPlate were manufactured by injection molding of polystyrene (Bioprinting Laboratories Inc., Texas, USA). For surface functionalization, the pillar plate was coated with poly(maleic anhydride *alt*-1-octadecene) (PMA-OD; 419117, MilliporeSigma) by stamping the pillar plate in a lid with 0.01% (w/v) PMA-OD in ethanol. After complete drying for 2-3 hours at room temperature, a mixture of 0.0033% (w/v) poly-L-lysine (PLL; P4707, MilliporeSigma) and 5 mM calcium chloride ($CaCl_2$; C8106, MilliporeSigma) was dispensed manually at 3 µL per pillar and allowed to completely dry at room temperature. Finally, 2 µL of 1.5% (w/v) alginate in distilled water was dispensed on the PMA-OD-coated pillar plate, completely dried, and rehydrated right before using the pillar plate for cell culture. The pillar plate with dried alginate was rehydrated by sandwiching it with the 384DeepWellPlate containing 20 µL of distilled water and incubating for 2 hours at 37°C in a CO_2 incubator. The two-step coating process with PMA-OD and PLL/$CaCl_2$ was performed to make the pillar surface hydrophilic for robust attachment of hydrogels, avoid bubble formation, and ensure complete gelation of the alginate layer. The alginate bottom layer was added to prevent the 2D spreading of cells on the pillar surface and minimize the bubble formation in Matrigel spot.

Human iPSCs culture and maintenance

Human iPSCs (EDi029A) were obtained from Cedar Sinai Biomanufacturing Center and maintained by following the manufacturer's recommended protocol. Briefly, iPSCs were cultured in a 6-well plate coated with 0.5 mg Matrigel™ (354230, Fisher Scientific) in complete mTeSR plus media (100-0276, Stem Cell Technologies). The iPSCs were passaged every 4-5 days when it reached 80-90% confluency using the StemPro® EZPassage™ disposable stem cell passaging tool (23181-010, Life Technologies). The cells were passaged as a small colony with a split ratio of 1:6 and cultured by changing the media every day. iPSCs at the early passage number up to 10-12 stored in a frozen vial were suited for generating cerebral organoids.

Formation of neural stem cell (NSC) spheroids

Human NSC ReNCell VM (SCC008, MilliporeSigma) was cultured in ReNcell NSC maintenance medium (SCM005, MilliporeSigma) supplemented with 20 ng/mL basic fibroblast growth factor (bFGF, EMD Millipore), 20 ng/mL epidermal growth factor (EGF, EMD Millipore), and 1 % (v/v) penicillin/streptomycin (P0781, MilliporeSigma) with medium change every alternate day. Cells were passaged when they reached 90% confluency using Accutase® (A6964, MilliporeSigma), resuspended in the complete maintenance medium, and centrifuged to remove supernatant. The cells were passaged as single cell suspension in the range of 1-1.5 million cells in a freshly coated T-75 flask with 20 µg/mL laminin (CC095, EMD Millipore). ReNcell was detached from the T-75 flask with Accutase, dissociated into single cells, and seeded in an ultralow attachment (ULA)-384 well plate (ULA-384U-020, Nexcelom) at different seeding density of 500, 1000, 3000, and 9000 cells/well to form spheroids in the complete maintenance medium with medium change every alternate day. The spheroids were cultured in the ULA 384-well plate for 4 days.

Formation of embryoid bodies (EBs) with iPSCs

iPSCs colonies were harvested with Accutase® and dissociated into single cells using the complete human embryonic stem cell (hESC) medium consisting of DMEM/F12 (11320033, ThermoFisher), 5x knockout serum replacement (10828010, ThermoFisher), 100x GlutaMAX (35050-038, Invitrogen), 100x MEM-nonessential amino acids (MEM-NEAA; M7145, MilliporeSigma), 33x hESC quality FBS (10439001, ThermoFisher), 2-mercaptoethanol (21-985-023, Fisher Scientific) with a final concentration of 4 ng/mL basic fibroblast growth factor (bFGF; 100-18B, Peprotech) and rho kinase inhibitors such as a CEPT cocktail consisting of 5 µM Emricasan (50-136-5235, Fisher Scientific), 50 nM Chroman 1 (7163, Tocris), 0.7 µM Trans-ISRIB

(5284, Tocris), and polyamine supplement diluted at 1:1000 (P8483, MilliporeSigma), or 10 µM Y-27632 (SCM075, Fisher Scientific). Single cells were suspended in the complete hESC medium containing either the CEPT cocktail or Y-27632, seeded in the ULA 384-well plate at a seeding density of 1000 cells/well, maintained in the hESC medium with CEPT or Y-27632 until day 4 of culture, and then cultured in the hESC medium without CEPT or Y-27632 for three more days.

Spheroid transfer from the ULA 384-well plate to the 36PillarPlate

Multiple parameters were optimized to establish the spheroid transfer method for rapid and robust encapsulation of EBs in Matrigel on the pillar plate. Briefly, four parameters were investigated, including (1) the size of spheroids transferred, (2) the time of spheroid transfer, (3) the concentration of Matrigel on the pillar plate, and (4) pre-gelation of Matrigel on the pillar plate prior to pillar plate sandwiching onto the ULA 384-well plate. ReNcell spheroids cultured for 4 days at seeding density of 500, 1000, 3000, and 9000 cells/well in the ULA 384-well plate were transferred to the functionalized pillar plate with 5 µL of 4-6 mg/mL of Matrigel by a simple sandwiching and inverting method. The pillar plate with Matrigel was sandwiched onto the ULA 384-well plate with spheroids, and then the sandwiched plates were inverted and incubated for 20-30 mins at 37°C in a CO_2 incubator for spheroid transfer and encapsulation in Matrigel. The spheroids on the pillar plate were cultured in the complete NSC medium in the 384DeepWellPlate. Similarly, EBs formed for 6 days at seeding density of 1000 and 3000 cells/well in the ULA 384-well plate with the complete hESC medium were transferred to the pillar plate with 5 µL of 6-8 mg/mL of Matrigel by the sandwiching and inverting method. For robust EB transfer and encapsulation on the pillar plate with minimal dilution of Matrigel in the ULA 384-well plate, the pillar plate with Matrigel was pre-incubated at room temperature for 0, 5, and 10 mins. After pre-incubation, the pillar plate with Matrigel was sandwiched onto the ULA 384-well plate with EBs, and then the sandwiched plates were inverted and incubated for 30-40 mins at 37°C in the CO_2 incubator for EB transfer and encapsulation. The EBs on the pillar plate were cultured in the neural induction medium (NIM) consisting of DMEM-F12, N2 supplement (17502001, ThermoFisher), GlutaMax, MEM-NEAA, and heparin (H3149, MilliporeSigma) in the 384DeepWellPlate for 5-6 days with medium change every alternate day.

Differentiation and maturation of cerebral organoids on the 36PillarPlate

After 5-6 days of EB culture in the NIM to generate neuroectoderm (NE), NE on the pillar plate was differentiated in cerebral organoid differentiation and maturation medium without vitamin A (CDM-vit A) in the 384DeepWellPlate for 31 days with medium change every other day. The CDM-vit A consists of an equal volume of DMEM-F12 and neurobasal medium (21103049, Fisher Scientific) with N2 supplement, B27 supplement without vitamin A (12587010, ThermoFisher), insulin (I9278, MilliporeSigma), GlutaMax, MEM-NEAA, penicillin-streptomycin, and 2-mercaptoethanol.

Differentiation and maturation of cerebral organoids in Matrigel dome

EBs were formed at seeding density of 1000 cells/well in the ULA 384-well plate with the complete hESC medium as described above. From day 6, EBs were differentiated in the NIM in the ULA 384-well plate for additional 6 days for NE formation. Each NE was mixed with 20 µL of 6-8 mg/mL Matrigel and then reseeded in a ULA 24-well plate to generate 5 of 20 µL Matrigel dome in each 24-well (single NE in each Matrigel dome). The Matrigel domes were incubated at 37°C for 20 minutes for complete gelation of Matrigel and then cultured with 400 µL of the NIM. The NIM was changed to the CDM-vit A after 2 days of culture, and the CDM-vit A was used for cerebral organoid differentiation until day 31 with medium change every alternate day.

Assessment of EB and organoid viability

The viability of EBs and organoids were measured with luminescence-based Cell Titer-Glo 3D® Cell Viability Assay Kit (G9681, Promega) by following the manufacturers' recommended protocol. For the viability assessment of EBs in the ULA 384-well plate, half the volume of the culture medium in the well was removed and replaced with CellTiter-Glo® reagent, and the EBs were incubated with the 2-fold diluted reagent for 1 hour at room temperature on an orbital shaker. After 1 h incubation, 50 µL of the reagent solution was transferred to an opaque white 384-well plate, and luminescence intensity reading was obtained using Synergy H1 microplate reader (Agilent Technologies). Similarly, for the assessment of organoid viability on the pillar plate, CellTiter-Glo reagent was diluted with the culture medium at 3:1 ratio and dispensed in the opaque white 384-well plate. The pillar plate with organoids was sandwiched onto the opaque white 384-well plate with the reagent, and the sandwiched plates were incubated at room temperature for 1 hour on the shaker. After 1 h incubation, the pillar plate was detached, and luminescence intensity reading was obtained from the opaque well plate using the microplate reader. The viability assessment with CellTiter-Glo assay was performed three times to determine the reproducibility and uniformity of organoid generation on the 36PillarPlate.
In addition to the luminescence-based viability assay, a fluorescence-based cell viability assay was performed with calcein AM and ethidium homodimer-1. Briefly, organoids on the pillar plate on day 31 were rinsed with 1x PBS and stained with 2 µM calcein AM and 4 µM ethidium homodimer-1 in DMEM/F-12 medium for 2 hours at 37°C. After staining, the organoids were rinsed three times with PBS, and the fluorescence images were obtained with a fully automated bright-field and fluorescence microscope (BZ-X800E, Keyence)

Immunofluorescence (IF) staining of whole brain organoids
All the solutions and reagents used for staining were dispensed in the 384DeepWellPlate at 80 µL/well. The pillar plate with brain organoids was sandwiched with the deep well plate to carry out organoid staining. Briefly, brain organoids on the pillar plate were rinsed with 1x PBS and treated with 4% paraformaldehyde (PFA) in the deep well plate overnight at 4°C. The organoids were permeabilized with 1% Triton X-100 in PBS for 1 hour at room temperature and then exposed to a blocking buffer (consisting of 4% normal donkey serum in 1x PBS with 0.5% Triton X-100) overnight at 4°C. Primary antibodies were diluted to recommended concentrations using the blocking buffer. The organoids were incubated with primary antibodies overnight at 4°C with gentle shaking. After primary antibody staining, organoids were rinsed with the blocking buffer three times and incubated with appropriate secondary antibodies diluted in the blocking buffer for 2-3 hours at room temperature or overnight at 4°C with gentle shaking. The stained organoids were washed with 0.5% Triton X-100 in PBS three times for 20 minutes each and incubated with 1 µg/mL DAPI in 0.5% Triton X-100 in PBS for 30 minutes. This was followed by final washing with PBS for 10 minutes three times and incubation with a tissue clearing solution (Visikol Histo-M) for 1 hour. Images were obtained with a confocal microscope (LSM710, Zeiss) with 10x magnification and were analyzed with ImageJ software. The primary and secondary antibodies used are listed in **Supplementary Tables 1 and 2**, respectively.

Gene expression analysis *via* qPCR
Total RNA was isolated from iPSCs and organoids on days 16 and 31 by using the RNeasy Plus Mini Kit (74134, Qiagen) with the manufacturer's recommended protocol. cDNA was synthesized from 1 µg of RNA by following the protocol of cDNA conversion kit (4368814, Applied Biosystems). Real time PCR was performed using SYBR™ Green Master Mix (A25742, ThermoFisher), forward/reverse primers from IDT Technology. Primer pairs were designed by us. The cycle was run 25-35 times at 95°C denaturation for 30 sec, 58-62°C annealing for 45 sec, depending on primer pair, and 72°C extension for 30 sec. The primers used are listed in **Supplementary Table 3**.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.3.1 (GraphPad Software, Inc., CA, USA). The data were expressed as mean ± SD. P-values were calculated using unpaired two-tailed t-test and one-way ANOVA. The statistical significance threshold was set at *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Sample sizes are mentioned in the figure legends.

Results

Reproducibility of iPSC culture

Maintaining healthy and undifferentiated culture of iPSCs is highly critical for the reproducible generation of brain organoids. EDi029A iPSCs were assessed for their uniformity in maintaining pluripotency and compact colonies in our hand in four different batches. We observed compact colony formation with smooth edges, with higher nucleus to cytoplasmic ratio, throughout the culture period in multiple batches, demonstrating their applicability for uniform cerebral organoid generation **(Supplementary Fig. 1A)**. In addition, the iPSCs maintained their pluripotency with less than 10% differentiated cells when characterized for pluripotency with representative pluripotency makers such as OCT4 and SSEA4[18,19] (**Supplementary Fig. 1B**).

High viability of embryoid bodies (EBs) with the CEPT cocktail

The differentiation of cerebral organoids can be initiated by forming EBs with Rho-kinase inhibitors such as CEPT and Y-27632 in the cell culture medium, which improve viability of the cells, and thus potentially improve reproducibility of organoid generation[19–22]. Cellular stress during trypsinization and cryopreservation could reduce EB formation and compromise cell differentiation. Here, we compared the effect of recently published CEPT and traditional Y-27632 on cell viability and uniform EB formation. The CEPT cocktail consisting of Chroman, Emricasan, Polyamine supplement, and Trans-ISRIB was developed to improve viability of cells after cryoprotection[23]. Interestingly, the addition of CEPT in the culture medium resulted in compact and round EB formation with a bigger size, suggesting improved cell survival and proliferation as compared to Y-27632 (**Fig. 1A & 1B**). More dead cell debris was observed around the EBs formed with Y-27632. Higher viability of EBs formed with CEPT was confirmed with the ATP-based luminescence assay, as compared to that with Y-27632. (**Fig. 1C).** With this result, we decided to use CEPT instead of Y-27632 for EB formation in the brain organoid differentiation protocol.

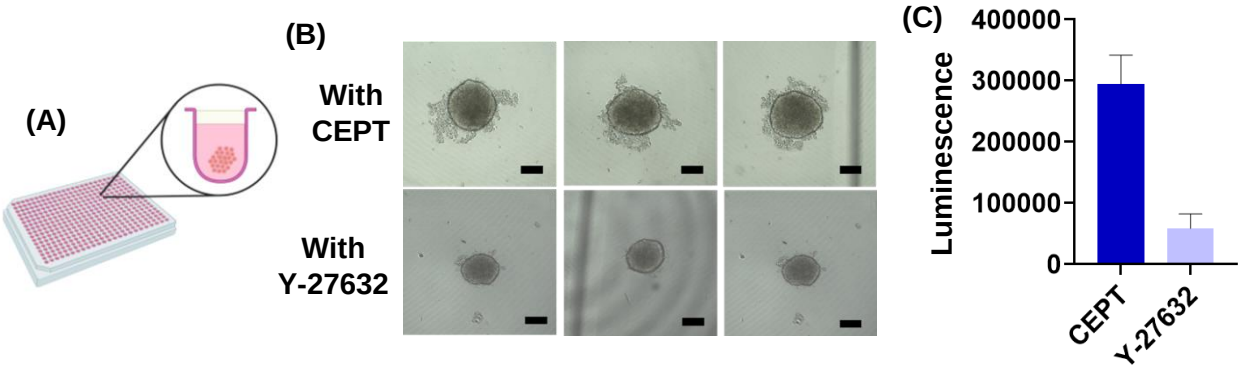
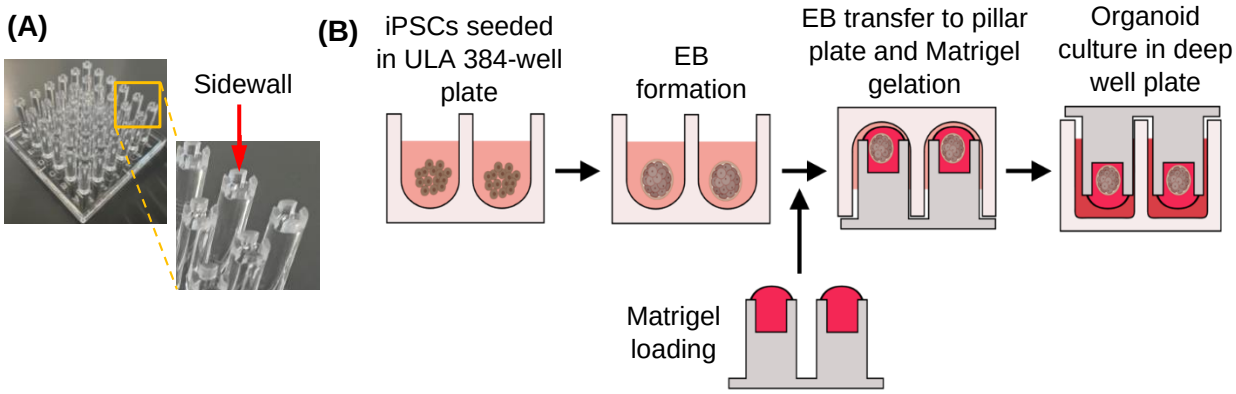


Figure 1. Embryoid body (EB) formation in a ULA 384-well plate: (A) Formation of EBs with single iPSC suspension in a ULA 384-well plate. **(B)** Morphology of day 7 EB formed in hESC medium supplemented with CEPT or Y-27632 for 4 days. Scale bar: 200 µm. **(C)** Viability of EBs formed with CEPT or Y-27632 measured by using the ATP-based luminescence assay kit (n=18).

Robust transfer of EBs to the pillar plate *via* a sandwiching and inverting method

For brain organoid generation, individual EBs need to be encapsulated in biomimetic hydrogels such as Matrigel and differentiated to neuroectoderm (NE) in a 6-/24-well plate, which is labor-

intensive and error-prone. To address this issue and improve reproducibility of organoid formation, we developed an efficient method of spheroid transfer from the ULA 384-well plate to the pillar plate on an one well-to-one pillar basis (**Fig. 2A & 2B**). The working principle of the spheroid transfer method is simple and straight-forward. The pillar plate with 5 µL of cold Matrigel can be sandwiched onto the ULA 384-well plate with EBs. The sandwiched plates can be immediately inverted to induce an EB in each well settled on each pillar. By incubating the sandwiched plates at 37°C in a CO_2 incubator, EB transfer followed by Matrigel gelation can be achieved. The unique structure of the pillar plate with sidewalls and slits allowed robust spheroid transfer and encapsulation in Matrigel with minimal dilution. For optimization of the spheroid transfer protocol, several parameters including incubation time at 37°C for Matrigel gelation, spheroid size, and pre-gelation of Matrigel at room temperature were tested to achieve high success rates of spheroid transfer. Among the incubation time at 37°C tested, 30-40 min incubation of the sandwiched plates was necessary to have complete Matrigel gelation on the pillar plate in the ULA 384-well plate. The effect of the spheroid size on transfer efficiency was tested with ReNcell spheroids with cell seeding density ranging from 500 - 9000 cells/well in the ULA 384-well plate. The size of spheroids formed was between 120 - 350 µm after 4 days of culture with the seeding density (**Fig. 2C**). The success rate of spheroid transfer was > 95% at all the size of spheroids tested on the pillar plate with 4-6 mg/mL of Matrigel and 20-30 mins of Matrigel gelation at 37°C in the CO_2 incubator (**Fig. 2D**). This result indicates that the speed of spheroid precipitation on the pillar plate is much faster than that of Matrigel gelation, leading to successful encapsulation of spheroids. Since cerebral organoids differentiated from EBs at 1000 cells/well seeding density showed more complex morphology as compared to those generated with 3000 cells/well (**Supplementary Fig. 2**), the established spheroid transfer protocol can be used for cerebral organoid culture. The Matrigel concentration for EB encapsulation was increased to 6-8 mg/mL to account for potential dilution of Matrigel after sandwiching the pillar plate into the ULA 384-well plate during spheroid transfer. Finally, partial gelation of Matrigel on the pillar plate at room temperature (i.e., pre-gelation) before spheroid transfer was investigated due to the concern for potential Matrigel dilution in the medium in the ULA 384-well plate during the sandwiching (**Fig. 2E & 2F**). A high success rate of EB transfer was obtained when the pillar plate with Matrigel was sandwiched into the ULA 384-well plate without pre-gelation, indicating that premature polymerization of Matrigel prohibits successful spheroid transfer and encapsulation on the pillar plate (**Fig. 2G**).



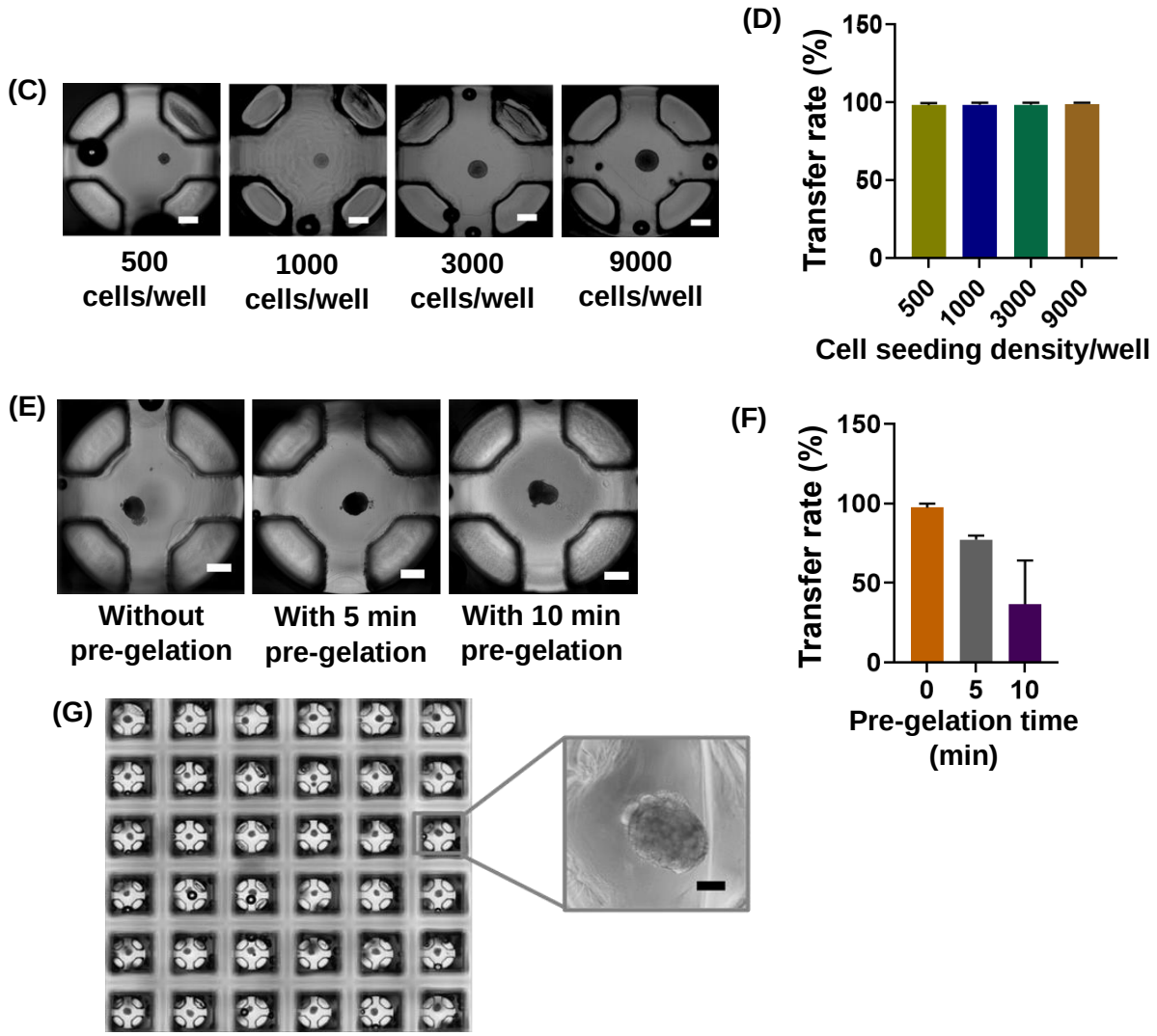


Figure 2. Optimization of spheroid transfer to the 36PillarPlate from the ULA 384-well plate: **(A)** SolidWorks design of the 36PillarPlate with sidewalls and slits for robust spheroid transfer. **(B)** Method of spheroid transfer to the pillar plate from the ULA plate. **(C)** Brightfield images of ReNcell spheroids with different sizes transferred to the pillar plate. **(D)** The success rate of ReNcell spheroid transfer at different seeding density. **(E)** Representative images of iPSC spheroids transferred at different pre-gelation time of Matrigel. Scale bar: 300 µm. **(F)** The success rate of iPSC spheroid transfer at different pre-gelation time. **(G)** Stitched images of EBs encapsulated in Matrigel on the pillar plate after transfer. Scale bar: 200 µm.

Generation of cerebral organoids on the pillar plate

Cerebral organoids were generated on the pillar/deep well plate by following the Lancaster protocol[24] with slight modification for early embedding of EBs in Matrigel on the pillar plate (**Fig. 3A)**. The differentiation process of cerebral organoids started with EB formation and transfer on the pillar plate, followed by neural induction, neural expansion, and maturation without the use of any exogenous factors. EBs formed in the ULA 384-well plate showed clear borders that were visible on day 2 and were brighter on day 4 (**Fig. 3B)**. EBs showed brighter morphology with smooth edges on the day of transfer on day 7. EBs can be stimulated to develop germ layers including ectoderm from which the neural tissues are developed and can be differentiated with low bFGF in hESC medium[25–27]. After transferring on the pillar, they were cultured in the neural

induction medium for 5-6 days which led to uniform neuroectoderm (NE) formation by the suppression of mesodermal tissue development[11]. The organoids were then cultured in cerebral organoid differentiation medium (CDM) without vitamin A until day 31. Cerebral organoids at early stages (day 15-20) on the pillar plate showed several small neural rosette, which are reminiscent of neuroepithelia (**Fig. 3B**), similar to previously published data[11]. CDM without vitamin A supports the developmental progression of neural progenitors and their progeny[11]. Neuronal development and differentiation are supported by neurobasal medium and B27 supplement[28]. Additional supplements such as 2-mercaptoethanol and insulin maintained the NSCs[29,30]. They were cultured until day 31 only, which was enough to observe some of the representative markers related to cerebral organoids for analyzing their formation on the pillar plate. These cerebral organoids generated on the pillar plate have expression levels of crucial genes related to brain development, similar to that from conventional Matrigel dome culture with slight increment in most of the genes, demonstrating the compatibility of the pillar/deep well plate platform for robust brain organoid culture (**Supplementary Fig. 3**).

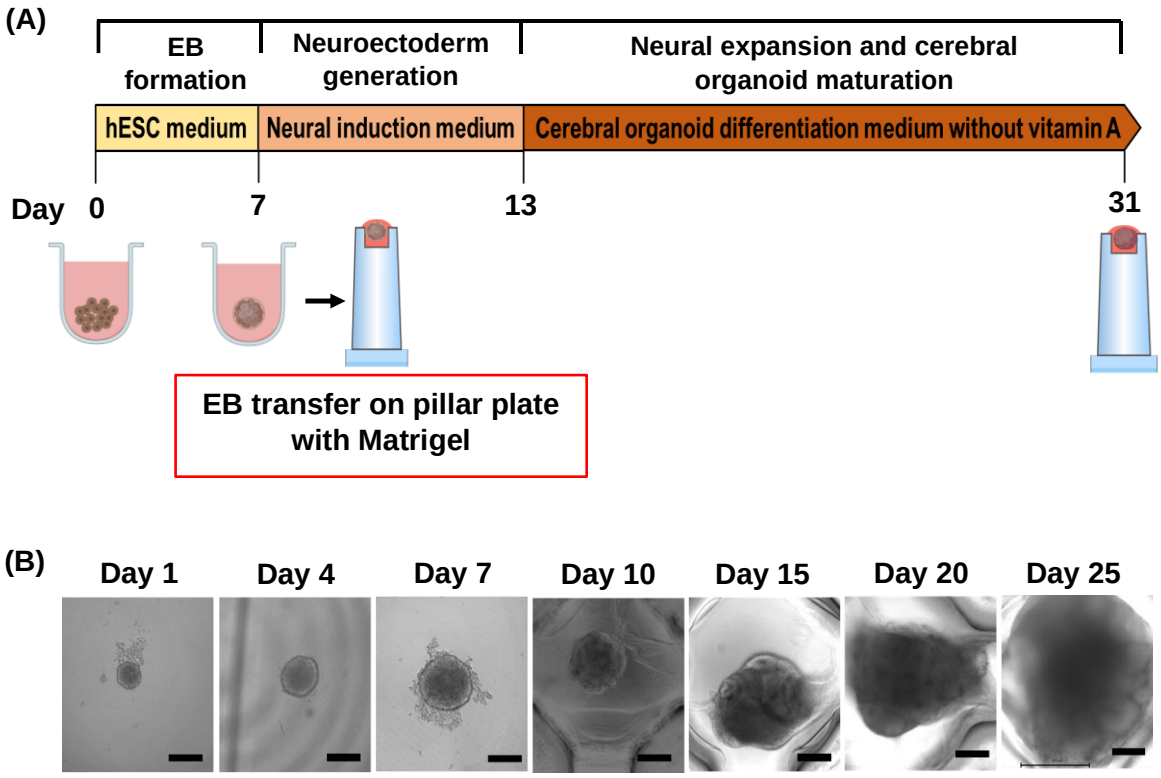


Figure 3. Generation of cerebral organoids from EDi029A iPSCs: (A) Differentiation process of cerebral organoids on the pillar plate. **(B)** Cerebral organoids generated on the pillar plate over time. Scale bar: 200 µm.

Characterization of cerebral organoids generated on the pillar plate

The cerebral organoids generated on the pillar plate were characterized by qPCR and immunofluorescence staining. Gene expression analysis of cerebral organoids done by qPCR at day 16 and 31 showed decrease in pluripotency marker OCT4 by more than 1000-fold with time of organoid differentiation (**Fig.4**). By day 16 of differentiation, the organoids also showed more than 30-fold increase in expression of neural progenitor marker, paired box protein 6 (PAX6), indicating successful neural induction. Decrease in the pluripotency marker and increase in the neural progenitor marker during differentiation was consistent with previously published data[9]. PAX6 expression was maintained throughout the differentiation until day 31. The forebrain marker, forkhead box protein G1 (FOXG1) expression was increased by 10-fold at day 16 which

was maintained until day 31, showing earlier brain regionalization. Similarly, neuronal marker, microtubule-associated protein 2 (MAP2) was increased from nearly 20-fold to 50-fold from day 16 to day 31 of differentiation. MAP2 expression was increased by more than 2-fold from day 16 to day 31, indicating the maturation of brain organoids with time of culture. The expression level of cortical plate precursor, intermediate progenitor marker, T-box protein (TBR2) was decreased by 30-fold to 20-fold from day 16 to day 31, which might be because of the transition of intermediate progenitor cells to post mitotic neurons[31]. The expression of SOX2, neural stem cell marker was expressed by 2.5-fold which was maintained at day 16 and day 31. Deep cortical neuronal marker, COUP-TF-interacting protein 2 (CTIP2) and choroid plexus marker, TTR was expressed only on day 31 as seen in previous study[11] by 3.5-fold wheareas the neuronal marker TUBB3 was expressed by 10-fold on day 16 and increased their expression to 16-fold on day 31. TBR1, a preplate marker, was expressed by 115-fold on day 16, and their expression was increased by 3 times on day 31, indicating the formation of the subventricular zone. Overall, the cerebral organoids generated on the pillar plate recapitulated neurogenesis, cortical organization, and brain regionalization, revealing early features of brain development.

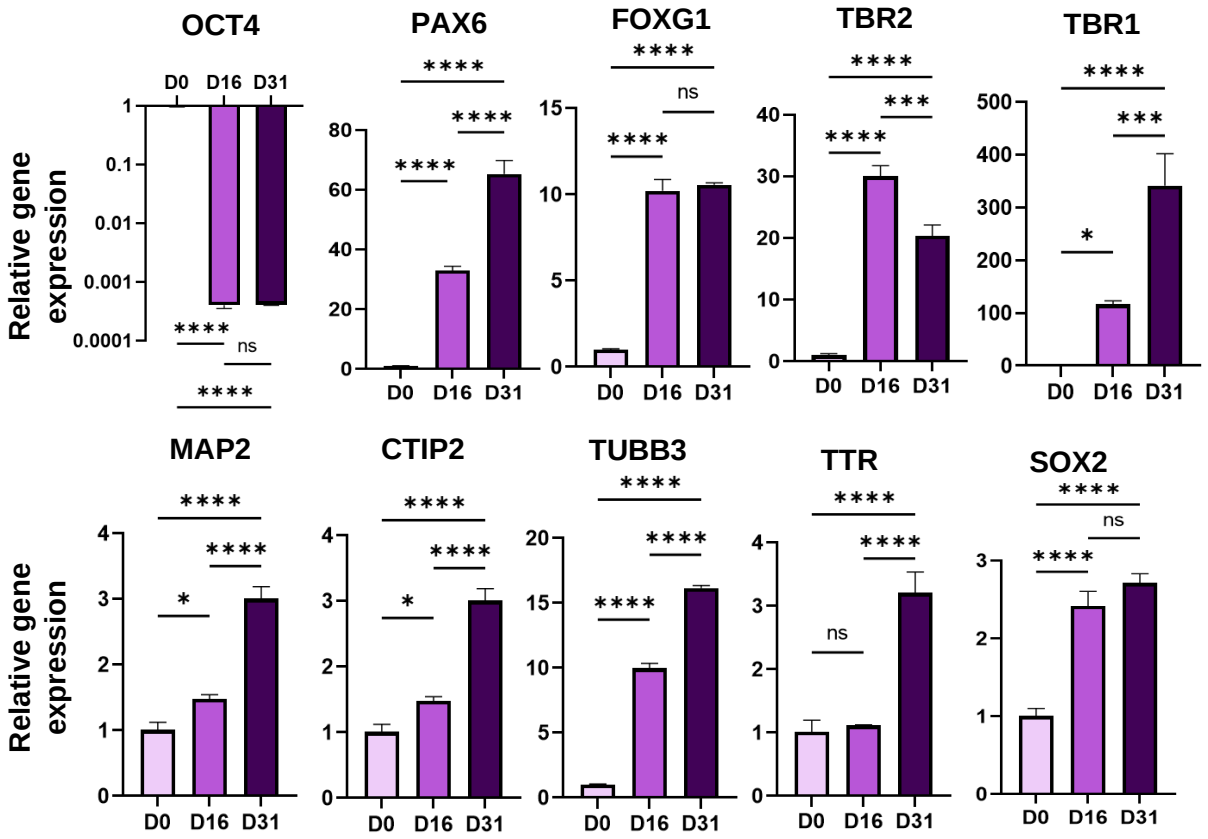


Figure 4. Characterization of days 16 and 31 cerebral organoids. Gene expression of OCT4 pluripotency marker, PAX6 neuroprogenitor marker, FOXG1 forebrain marker, TBR2 intermediate progenitor marker, TBR1 preplate marker, MAP2 neuronal marker, CTIP2 deep cortical neuronal marker, TUBB3 neuronal cytoplasm marker, TTR choroid plexus marker, and SOX2 neural stem cell marker analyzed by qPCR. Statistical significance was performed using one-way ANOVA. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, n. s. = not significance ($p > 0.05$).

In addition to qPCR analysis, immunofluorescence staining was performed on day 31 to determine whether the cells were randomly scattered or developed with discrete regions within whole cerebral organoids (**Fig. 5A**). The stained organoids on day 31 revealed the higher

expression of several forebrain region identities, marked by PAX6 neural identity marker[9]. On the other hand, localized expression of FOXG1 forebrain region marker was seen in the organoids[11]. In addition, we analyzed the genes related to cortical organization such as CTIP2 which showed in deeper region of the organoids with discrete arrangement. This suggested the initiation of cortical plate layer formation within the brain organoids. There was a mixed population of CTIP2+ and SOX2+ cells, which referred to the presence of the subventricular zone as defined in the previous study[32]. The organoids were also stained with TBR2 intermediate progenitor marker which is also a precursor of cortical plate[11]. The expression of TBR2 was concentrated at the center of the organoid showing a separate layer in the organoids. MAP2 neuronal marker revealed the appearance of the basal neural layer that represents a superficial preplate zone-like layer in day 31 organoids (**Fig. 5A**).

In addition, live/dead cell staining of day 31 cerebral organoids on the pillar plate with calcein AM and ethidium homodimer-1 (EthD-1) showed very few dead cells (**Fig. 5B**). This result indicates that the pillar/deep well plate platform could support the culture of cerebral organoids with sufficient diffusion of oxygen and nutrients to the core.

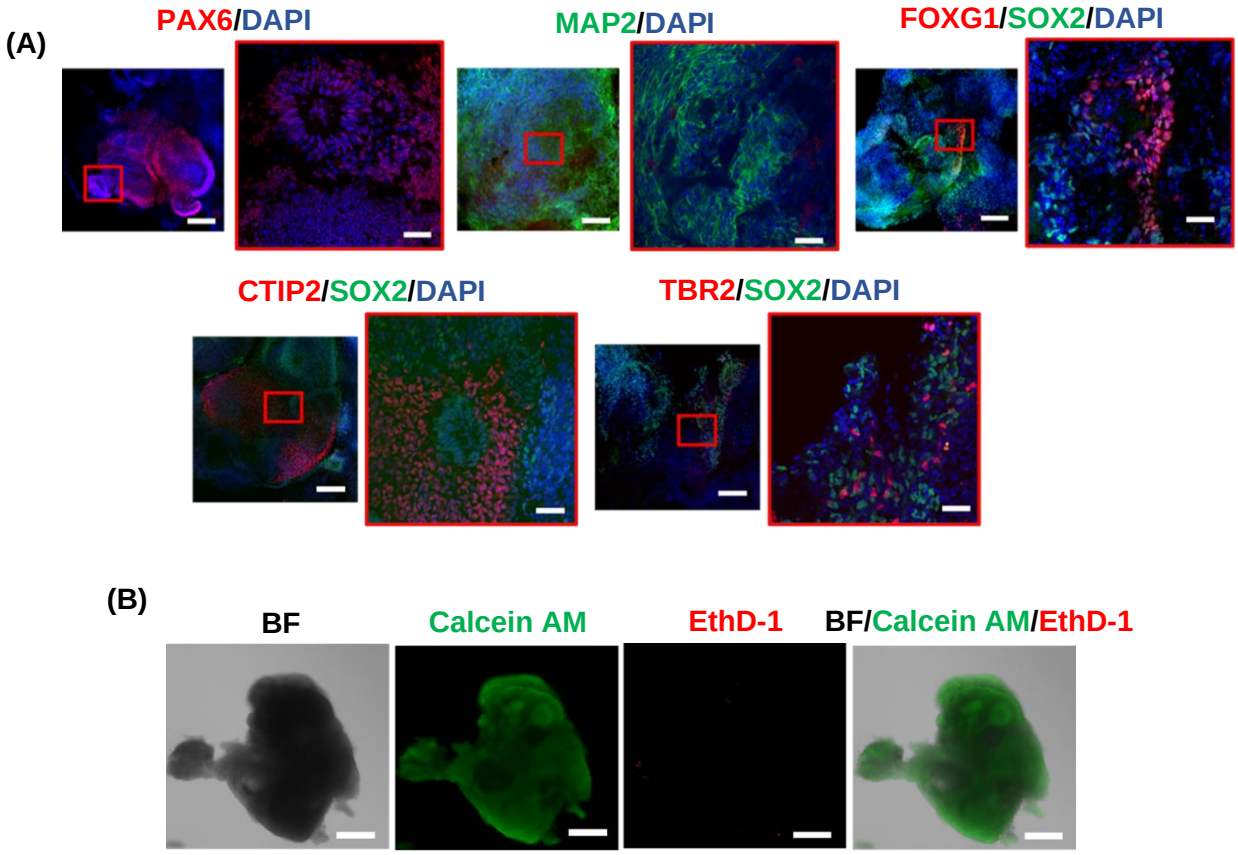


Figure 5. Characterization of day 31 cerebral organoids: (A) Immunofluorescence staining of cerebral organoids for PAX6 neuroprogenitor marker, MAP2 neuronal marker, FOXG1 forebrain markerCTIP2 cortical neuronal marker, SOX2 proliferating neuroprogenitor marker, and TBR2 intermediate progenitor marker. Scale bar: 500 µm. **(B)** Viability of cerebral organoids assessed with calcein AM and ethidium homodimer-1 (EthD-1) staining.

Reproducibility of cerebral organoid generation on the pillar plate

One of technical limitations of current cerebral organoid protocols is poor reproducibility and uniformity, which represent a significant bottleneck for high-throughput assessment of compounds with organoids. In the present work, we generated uniform cerebral organoids on the

pillar plate reproducibly by transferring single EB to each pillar. The EBs were formed uniformly in the ULA 384-well plate (**Fig. 6A**), which were simultaneously transferred to the pillar plate by simple sandwiching and inverting. Due to single, uniform size of EB transferred to the pillar plate, we were able to achieve uniform and reproducible brain organoid generation with the coefficient of variation (CV) values between 7 – 19% in three replicates (**Fig. 6B & 6D**), which was measured by the ATP-based cell viability assay. In every batch of culture, the organoids showed expanded neuroepithelial bud outgrowth (thin arrow) that is optically clear and surrounded by a visible lumen which is the typical morphology of brain organoids (**Fig. 6C**), indicating the successful differentiation as seen in previous study[11,33].

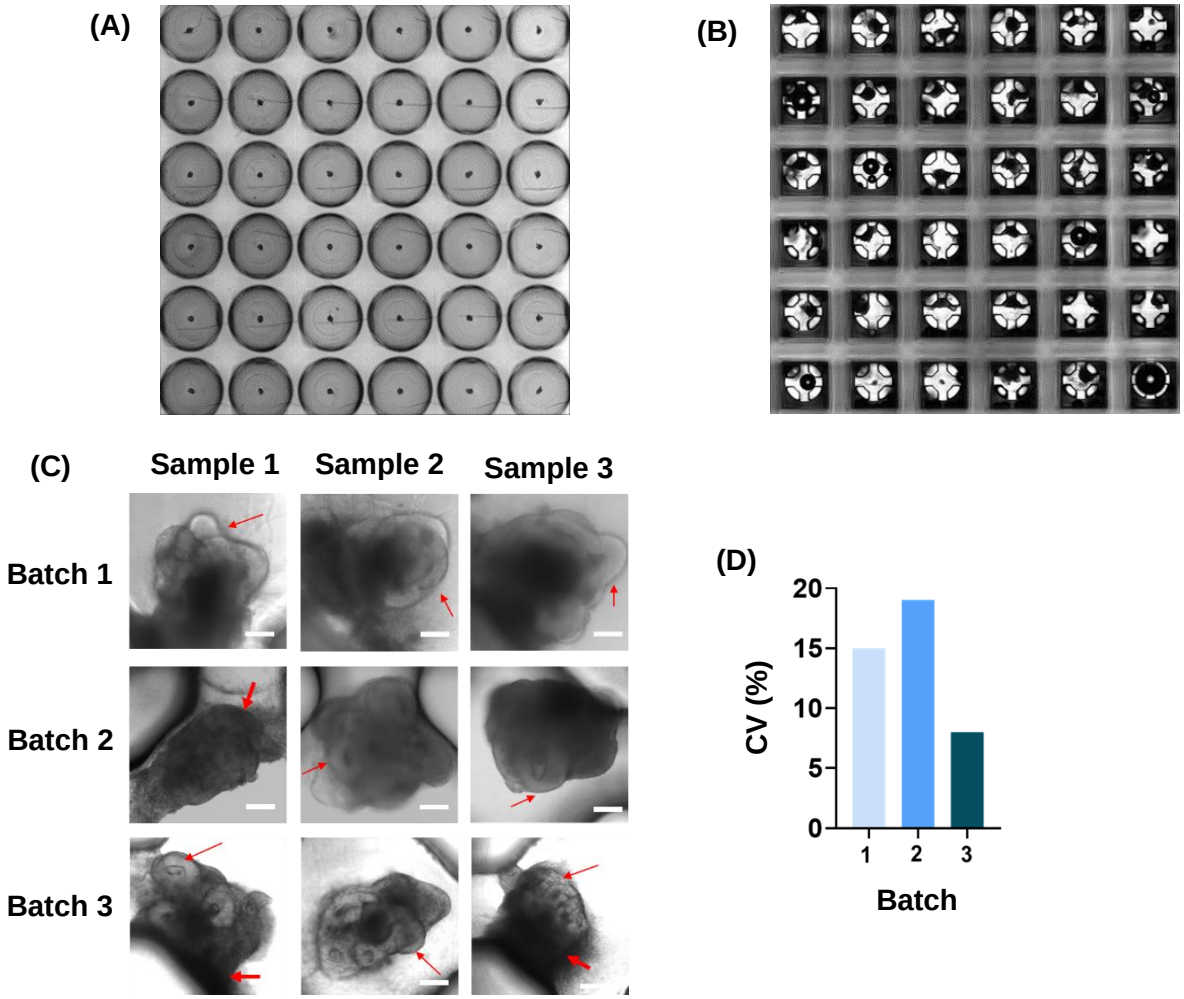


Figure 6. Reproducible generation of cerebral organoids on the pillar plate: (A) Stitched images of EBs in the ULA 384-well plate. **(B)** Stitched images of day 31 cerebral organoids on the pillar plate. **(C)** Reproducibility of early cerebral organoids with expanded neuroepithelium on the pillar plate from three replicates of culture. Early cerebral organoids generated on the 36PillarPlate showed expanded neuroepithelial bud outgrowth (thin arrow) that is optically clear and surrounded by a visible lumen. Other outgrowths and migrating cells are also visible (thick arrow) that are not neuroepithelial. Magnification: 10X, Scale bar: 500 µm. **(D)** CV values of cell viability measured with the ATP-based luminescence assay. The CV values were in the range of 7 - 19% from triplicate trials, indicating the robustness of cerebral organoid generation on the pillar plate.

Discussion

Brain organoids have capability to recapitulate the architecture and function of the brain in *in vitro* culture system. However, their development and application are significantly hampered by lack of reproducibility, scalability, technical complexity, cost, etc[12]. Currently, brain organoids are cultured in spinner flask, 6-well plates, petri dishes, which require a large volume of cell culture media, growth factors, additives, and expensive equipment[12,13,34]. In addition, they involve a labor-intensive and cumbersome process of encapsulation and dissociation of spheroids and organoids in hydrogels, making the cell culture system low throughput along with higher probability of contamination. For example, in the studies by Lancaster et al.[11], Krefft et al.[35], and Gabriel et al.[36], EBs formed in the ULA well plates were transferred manually one-by-one on each dimple of a parafilm by using a 200 µL pipette. This was followed by adding a droplet of Matrigel to each cell aggregate and culturing them in a petri dish by placing in an orbital shaker or by transferring to a spinning bioreactor with 75-100 mL of cell culture media. In the study by Bodnar et al.[12], organoids were collected in a conical tube, resuspended in Matrigel, and cultured in a ULA 6-well plate. On the other hand, Quian et al.[32] generated brain region specific organoids using a miniature spinning bioreactor, which is developed to generate organoids in a small scale that enhances diffusion of oxygen and nutrients into the core of the organoid. This method needs tedious arrangement of the miniature bioreactor although it could reduce the usage of cell culture media. In addition, microfluidic devices have been implemented in brain organoid culture with the aim to provide a dynamic environment and to enhance the maturity of organoids. However, they also require cumbersome steps of transferring individual spheroids and organoids encapsulated in biomimetic hydrogels into each organoid culture chamber[15,37]. In addition, microfluidic devices are inherently low throughput due to pumps and tubes required. To generate cerebral organoids reproducibly, Sivitilli et al.[38] implemented “V” bottom, non-binding, 96-well plate instead of a surface-treated, “U” bottom, polystyrene plate for robust EB formation. However, the workflow in this study also involved manual embedding of EBs in Matrigel, which was followed by culturing them in an orbital shaker.

In this study, we addressed the technical challenges in brain organoid generation, which include costs of cell culture media, reproducibility and scalability of organoid culture and analysis, and complexity of the culture process. Using our spheroid transfer and encapsulation method on the pillar plate, we simplified the EB encapsulation in hydrogel and organoid culture process with high reproducibility. EBs formed in the ULA 384-well plate were transferred to the pillar plate on one EB-to-one pillar basis by simple sandwiching and inverting with minimal manual intervention. Changing cell culture media during differentiation is simple and straightforward without any concerns for disrupting EBs in hydrogels. Fresh media (80 µL/deep well) can be dispensed into the 384DeepWellPlate using a multichannel pipette or a liquid dispenser, and then the pillar plate with organoids in Matrigel can be sandwiched onto the 384DeepWellPlate all at once for media change. Since the pillar plate requires EBs in 5 µL of Matrigel spot and 80 µL of cell culture media in the 384DeepWellPlate, the cell culture volume necessary for organoid culture is 20-100-fold reduced, as compared to conventional 6-/24-well plates, petri dishes, and spinner flasks. The reduced cell/Matrigel volume could enhance the diffusion of oxygen and nutrients to the core of organoids, potentially leading to high maturity of organoids as compared to conventional Matrigel dome culture (**Supplementary Fig. 3**). The maturity of organoids could be further enhanced by incorporating dynamic culture in the pillar/perfusion plate[39,40]. In addition, organoid staining and analysis can be streamlined on the pillar plate using existing lab equipment. For example, immunofluorescence staining of cerebral organoids with antibodies as well as cell viability assays with fluorescent dyes can be done simultaneously with organoids on the pillar plate and reagents in the deep well plate (**Fig. 5**). Due to the small scale, organoid analysis can be done in high throughput with existing fluorescence microscopes and microtiter well plate readers. The throughput of organoid-based assays can be further enhanced by using a 144PillarPlate with 144 pillars or a 384PillarPlate with 384 pillars per plate, instead of using the 36PillarPlate with 36 pillars. Furthermore, the reproducibility of brain organoid generation was demonstrated with the

CV values below 19% by using the spheroid transfer and encapsulation method on the pillar plate (**Fig. 6D**). Therefore, the pillar/deep well plate platform could reduce costs of cell culture media, enhance reproducibility and scalability of organoid culture and analysis, and simplify the organoid culture process with minimal manual intervention.

There is room for improvement of the spheroid transfer and encapsulation method on the pillar plate. Currently, we load 6-8 mg/mL of diluted Matrigel on the pillar plate with a multichannel pipette, which takes approximately 2 mins for the 36PillarPlate. Aligning pipette tips to the pillars is quite cumbersome and often leads to premature gelation of Matrigel and failure of spheroid transfer, particularly when undiluted Matrigel is used. To avoid this issue, a cell loading plate is under development, which allows hydrogels and cells in hydrogels loaded on the pillar plate by simple one-step stamping. The unique structure of the pillar with sidewalls and slits allows hydrogels and cells in hydrogels loaded reproducibly by stamping. The second issue is detachment of organoid spots from the pillar plate during long-term cell culture which might be because of surface incompatibility between alginate in the bottom layer and Matrigel on the pillar. Alginate was used to prevent 2D cell growth on the surface of the pillars during long-term cell culture due to its low affinity to the integrin-binding domain on the cell surface. With alginate bottom coating, 10-20% of EBs in Matrigel were detached from the pillar plate within a few days of culture, and the remaining 80-90% of cell spots were attached even after 31 days of culture and Matrigel remodeling by organoids. This problem could be resolved by adding alginate in Matrigel or using other non-adherent polymer coatings that have more affinity to Matrigel. Dilution of Matrigel on the pillar plate in the ULA 384-well plate during spheroid transfer and encapsulation would not be a concern because of the pillar located very close to the bottom of the ULA well with small volume of media available and relative fast gelation of small volume of Matrigel (5 µL).

Conclusions

In summary, we developed a simple and robust method of spheroid transfer and encapsulation in hydrogel on the pillar plate, which could reduce costs of brain organoid culture by 20-100-fold miniaturization, enhance reproducibility and scalability of organoid culture and analysis, and simplify the organoid culture process with minimal manual intervention. The spheroid transfer and encapsulation method on the pillar plate can be used for general static 3D cell culture including organoids and spheroids. Conventional methods of spheroid and organoid transfer and encapsulation are tedious, labor-intensive, and thus low throughput. This step is often the critical bottleneck in scale-up organoid culture and analysis for high-throughput screening of compounds with organoids. We envision that our spheroid transfer and encapsulation approach on the pillar plate could be widely adopted in academic and industry settings due to its simplicity and applicability to any 3D cell culture.

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Supplementary Information

Supplementary Table 1. List of primary antibodies

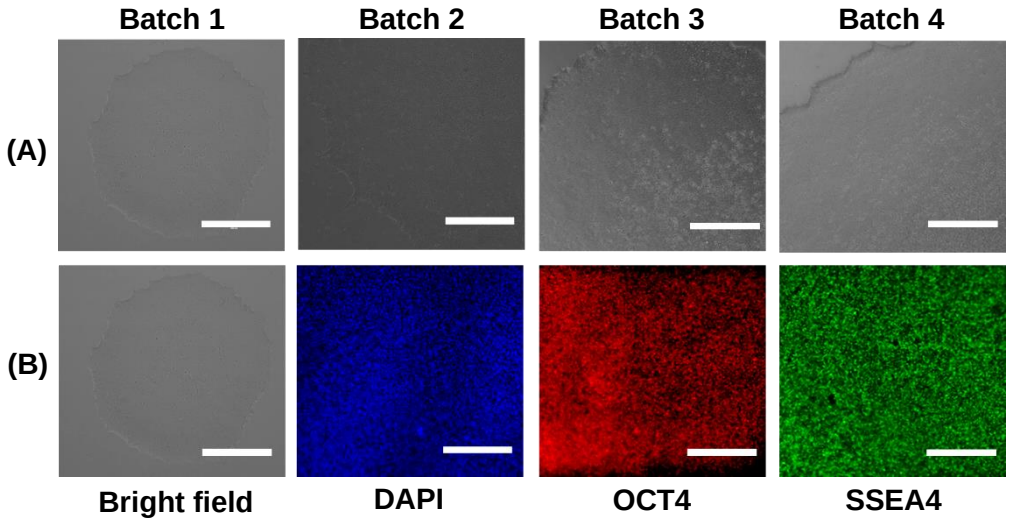
Name	Species	Dilution	Vendor	Catalog #
PAX6	Rabbit	1:350	Abcam	ab195045
FOXG1	Rabbit	1:100	Abcam	196868
SOX2	Mouse	1:40	Santa Cruz Biotech	sc-365823
TBR2	Rabbit	1:200	Abcam	183991
CTIP2	Rabbit	1:200	Abcam	ab 240636
MAP2	Rabbit	1:200	Abcam	183830

Supplementary Table 2. List of secondary antibodies

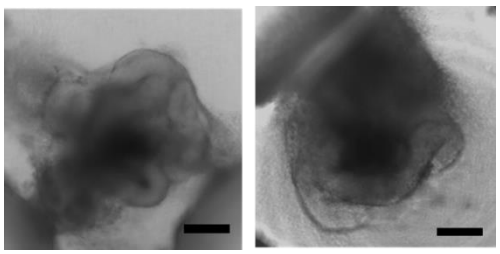
Name	Dilution	Vendor	Catalog #
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	1:200	ThermoFisher	A-31573
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:200	ThermoFisher	A-21202
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:200	ThermoFisher	A21206

Supplementary Table 3. List of primers

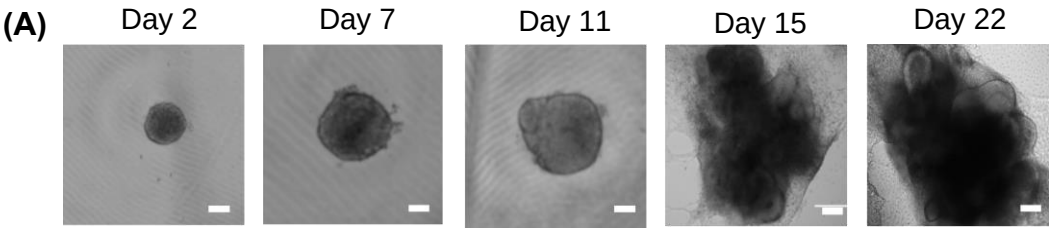
S.N.	Genes		Primer Sequence (5'- 3')
1.	OCT4 (POU5F1)	F	CCTGAAGCAGAAGAGGATCACC
		R	TCTTGAAGCTAAGCTGCAGA
2.	SOX2	F	GCTACAGCATGATGCAGGACCA
		R	TCTGCGAGCTGGTCATGGAGTT
3.	PAX6	F	CTGAGGAATCAGAGAAGACAGGC
		R	ATGGAGCCAGATGTGAAGGAGG
4.	FOXG1	F	CCGCACCCGTCAATGACTT
		R	CCGTCGTAAAACTTGGCAAAG
5.	MAP2	F	AGGCTGTAGCAGTCCTGAAAGG
		R	CTTCCTCCACTGTGACAGTCTG
6.	TBR2	F	TGGGTGACCTGTGGCAAAG
		R	CTCCTGTCTCATCCAGTGG
7.	GAPDH	F	GAAGGTGAAGGTCGGAGTC
		R	GAAGATGGTGATGGGATTTC
8.	CTIP2	F	TCCAGCTACATTTGCACAACA
		R	GCTCCAGGTAGATGCGGAAG
9.	TUBB3	F	GGCCAAGGGTCACTACACG
		R	GCAGTCGCAGTTTTCACACTC
10.	TTR	F	GTGCATGTGTTCAGAAAGGCTG
		R	CTCCTCAGTTGTGAGCCCATGC
11.	TBR1	F	CACTGGAGGTTTCAAGGAGG
		R	TCTTGGCGCATCCAGTGAGC

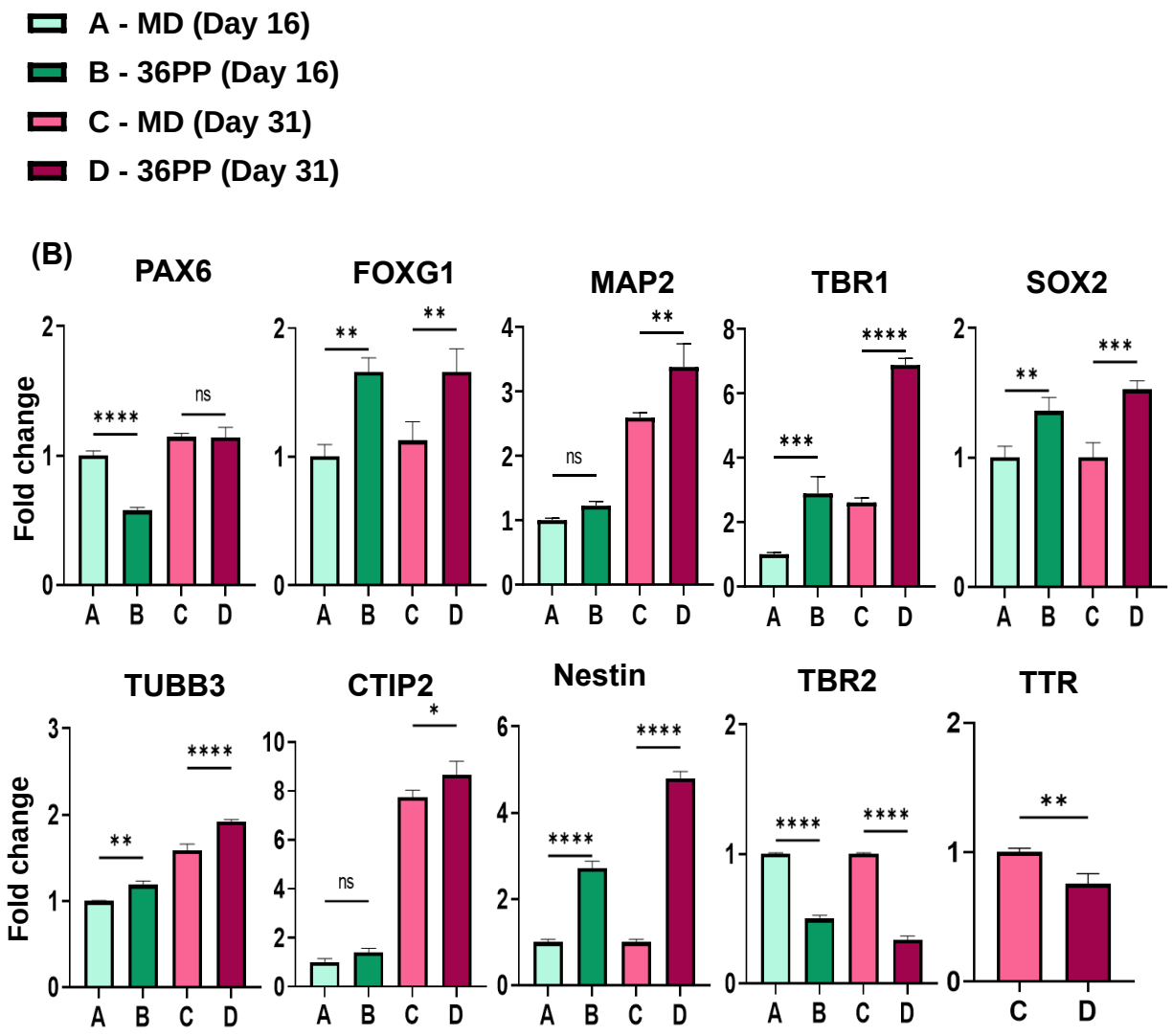


Supplementary Figure 1. Culture and characterization of EDi029A iPSCs: (A) Brightfield images of iPSC colony from different batches. **(B**) Immunofluorescence images of iPSC colony showing the expression of pluripotency markers, including OCT4 and SSEA4. Magnification: 10X, Scale bar: 400 µm.



Supplementary Figure 2: Brightfield image of day 16 cerebral organoids on the pillar plate with seeding density of 1000 (left) and 3000 (right) cells per EB. Scale bar: 200 µm.





Supplementary Figure 3. (A) Generation of cerebral organoids in Matrigel dome in a 24-well plate, Scale bar: 200 µM. **(B)** Comparison of gene expression for days 16 and day 31 cerebral organoids cultured in Matrigel dome (MD) and on the 36PillarPlate (36PP). Statistical significance was performed using unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.