Differentiation of pluripotent stem cells into neural organoids

Introduction

Recent advances in cell culture techniques have focused on creating 3-dimensional (3D) systems in an attempt to represent *in vivo* cell–cell relationships and microenvironments *in vitro*. Various tissue engineering technologies such as bioprinting, microfluidics, and organs-on-chips have been used successfully to generate 3D cultures [1,2]. Remarkable progress has also been made utilizing adult and pluripotent stem cells (ASCs and PSCs) to generate 3D organ-like (i.e., organoid) cell models [3-5]. PSC-based methods frequently start by aggregating cells in suspension culture to form clusters called embryoid bodies (EBs). Cells in these clusters are capable of differentiating into many types and can undergo self-organization and self-morphogenesis to create a complex cell model that better mimics the *in vivo* cell–cell interactions and microanatomy of a given tissue type. Some PSC-based approaches also require the encapsulation of cells within a natural or synthetic extracellular matrix (ECM)-like substrate [6-8]. In all methods, the application of growth factors, small molecules, and other media supplements is used to guide the formation of organoid systems based on principles inferred from studies of embryogenesis and adult stem cell biology. There are now many published methods for generating a variety of organoid types that resemble different parts of the brain, as well as the liver, intestine, and kidney, to name a few.

The unknown compatibility of multiple reagents from different vendors that span the organoid workflow is an issue that many researchers experience. This issue can have dramatic consequences for the successful generation of the desired organoid system and its reproducibility between laboratories. Established workflows for generating neural organoids from PSCs typically follow a specific sequence of steps that begin with standard PSC culture followed by EB formation, neural induction, neural patterning, and organoid growth [7, 9-12] (Figure 1). The composition of the cell culture medium at each of these steps is critical for the successful differentiation of PSCs. Importantly, the differentiation capacity of a given PSC line must be determined empirically, and some optimization of the differentiation method may be needed for the PSC line of choice. In this application note, we demonstrate the use of feeder-free Gibco™ StemFlex™ Medium, Gibco™ Geltrex™ matrix, and Thermo Scientific™ Nunclon™ Sphera™ Microplates to create neural organoids and spheroids.

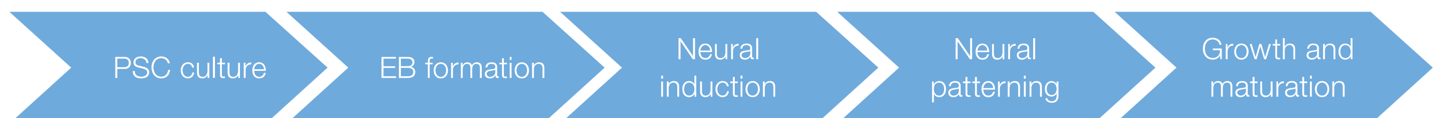

Figure 1. The essential steps of neural organoid formation from PSC cultures.

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Experimental details and results

PSC culture

Prior to differentiation, H9 human embryonic stem cells (ESCs) and Gibco™ Human Episomal Induced Pluripotent Stem Cells (iPSCs, Cat. No. A18945) were maintained using StemFlex Medium and grown on Thermo Scientific™ Nunclon™ Delta tissue cultureware coated with a 1:100 dilution of Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413301). PSC clumps were routinely passaged using Gibco™ Versene™ Solution (Cat. No. 15040066).

EB formation

PSCs were cultured in feeder-free conditions using StemFlex Medium (Cat. No. A3349401). When PSC cultures reached 70–80% confluency, they were dissociated into single-cell suspensions using Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501), Trypsin/EDTA Solution (Cat. No. R001100), or TrypLE™ Select Enzyme (Cat. No. 12563011). Cell counts and viability were determined using Gibco™ Trypan Blue Solution (Cat. No. 15250061) and the Invitrogen™ Countess™ II FL Automated Cell Counter (Cat. No. AMQAF1000). About 6–9 x 10^3 viable cells per well were seeded in StemFlex Medium with Gibco™ RevitaCell™ Supplement (Cat. No. A2644501) in Nunclon Sphera 96-well U-bottom microplates (Cat. No. 174925). Nunclon Sphera microplates exhibit virtually no cell attachment, promoting consistent formation of spheroids. EBs formed overnight equally well with all dissociation methods but most efficiently with the addition of RevitaCell Supplement (Figure 2). In the absence of RevitaCell Supplement, small EBs did form but with poor efficiency, as most cells either did not survive or did not self-aggregate (Figure 2). EBs were then cultivated for 3–4 days, with a 75% medium change every other day with StemFlex Medium with RevitaCell Supplement. The resulting EBs were of consistent size that was directly proportional to the number of cells seeded (Figure 3).

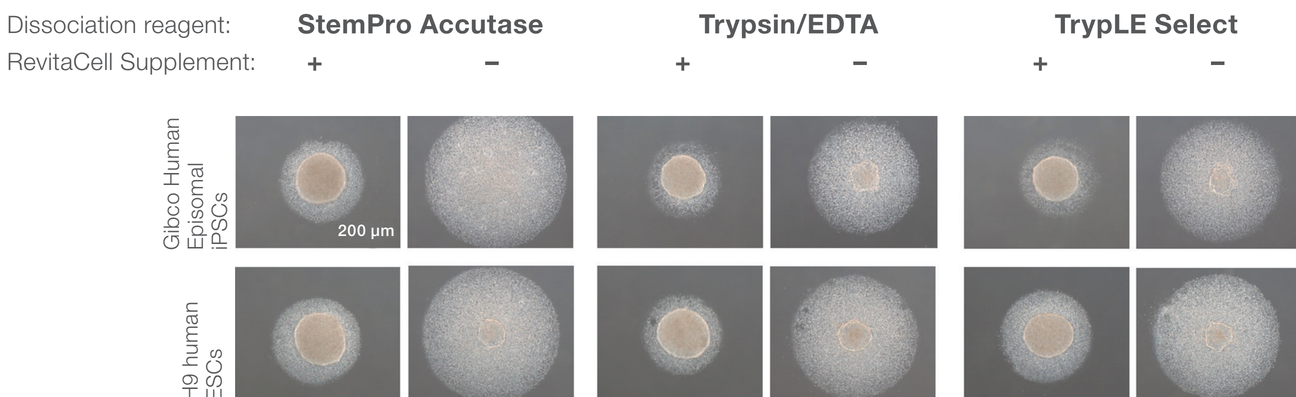


Figure 2. RevitaCell Supplement dramatically improves EB formation. A comparison of EB formation after isolation of PSCs by different methods demonstrated that EBs formed equally well with each dissociation reagent but only if RevitaCell Supplement was included in the culture medium. Cells that do not contribute to the EB are eventually washed away during media changes and do not typically interfere with subsequent steps; here they were not washed away, to illustrate the efficiency of EB formation.

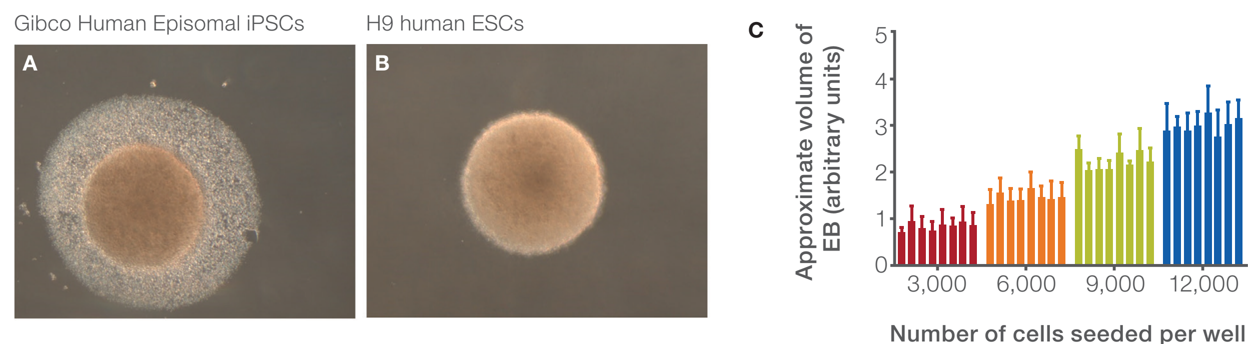


Figure 3. Evaluation of EBs formed in StemFlex Medium with RevitaCell Supplement. (A, B) These images show representative EBs from two different PSC lines after 4 days of culture. **(C)** EB size is directly proportional to the number of cells seeded. The graph shows the consistency in size between 8 replicates for each cell density that was evaluated. Data were calculated by measuring the area of each EB using ImageJ software. The area was then used to calculate the approximate EB volume, which is plotted on the y-axis.

Neural induction and patterning

Following EB formation, the cell aggregates were induced to differentiate into neural lineages by performing 3–4 successive 75% volume medium changes to serially dilute and remove the prior culture medium. Neural induction medium was composed of Gibco™ DMEM/F-12 with GlutaMAX™ Supplement (Cat. No. 10565018) and N-2 Supplement (Cat. No. 17502001). EBs were cultured for 8–9 days with a 75% volume medium change every other day until the outer layers of the EB formed a bright "ring" in contrast to the darker center (Figure 4). By day 10, each EB that displayed this phenotype was encapsulated in undiluted Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413201) and incubated at 37°C to gel. The use of Geltrex matrix for this application has been independently demonstrated elsewhere [13]. Droplets of Geltrex matrix containing EBs were then transferred to a differentiation medium consisting of DMEM/F-12 with GlutaMAX Supplement (Cat. No. 10565018) and Gibco™ Neurobasal™ Medium (Cat. No. 21103049) with GlutaMAX Supplement (Cat. No. 35050061), MEM with NEAA (Cat. No. 10370021), 2-mercaptoethanol (Cat. No. 21985023), insulin (Cat. No. 12585014), N-2 Supplement (Cat. No. 17502001), and B-27™ Supplement Minus Vitamin A (Cat. No. 12587010). Encapsulated samples were then transferred to Nunclon Sphera 6-well or 24-well plates (Cat. No. 174932, 174930) with a density of 3–5 or 1–2 droplets per well, respectively.

Growth and maturation

The samples were cultured in a growth and maturation medium of the same formulation as the previous incubation medium except this medium contained B-27 Supplement (Cat. No. 17504044). From this point on, neural organoids were cultured on an orbital shaker at 80–85 rpm and the medium was changed every 2–3 days. Neuroepithelia become easily visible within about a week. These samples can be continuously cultured for many weeks (Figure 5A, B) or until analysis is performed (e.g., cellular organization, marker expression). For example, Figure 5C indicates the presence of multiple neural cell types present at day 39 of culture. Gene expression analysis shows that these organoids still contain neural stem and progenitor cells, based on *SOX1*, *SOX2*, and *PAX6* expression, as well as immature neuronal markers such as *DCX* and *MAP2*. However, markers of specific neural regions such as *TBR1* (deep layer neurons), *FOXG1* (forebrain tissue), and *SLC6A1* (encodes GABA1 transporter expressed in cerebral cortical tissue, hippocampus, and other tissues) were also detected, indicating the presence of more differentiated cell types.

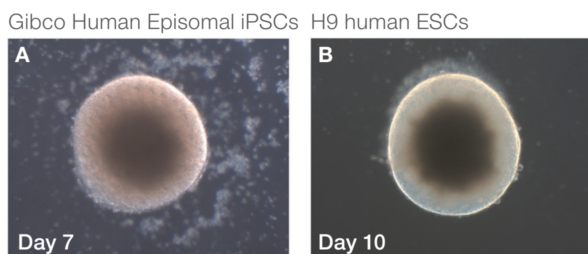
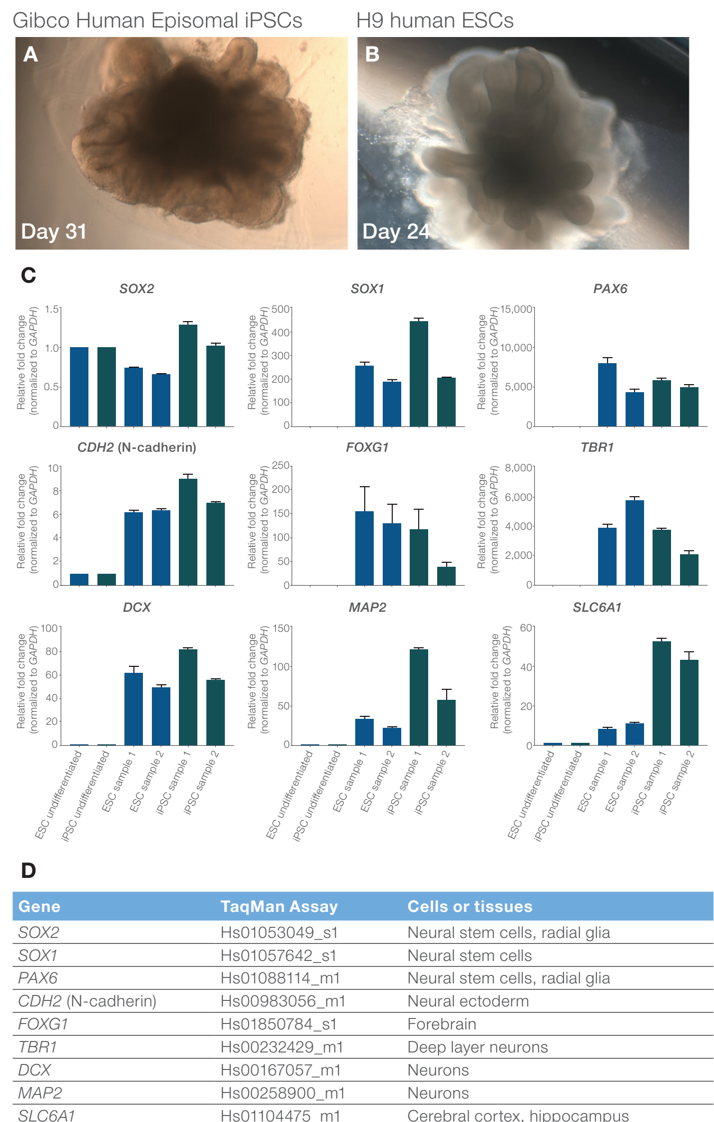


Figure 4. Neural induction and patterning. (A) Brightfield image showing a day 7 EB. **(B)** Day 10 neuralized EB immediately before encapsulation in Geltrex matrix.



Gene	TaqMan Assay	Cells or tissues
SOX2	Hs01053049_s1	Neural stem cells, radial glia
SOX1	Hs01057642_s1	Neural stem cells
PAX6	Hs01088114_m1	Neural stem cells, radial glia
CDH2 (N-cadherin)	Hs00983056_m1	Neural ectoderm
FOXG1	Hs01850784_s1	Forebrain
TBR1	Hs00232429_m1	Deep layer neurons
DCX	Hs00167057_m1	Neurons
MAP2	Hs00258900_m1	Neurons
SLC6A1	Hs01104475_m1	Cerebral cortex, hippocampus

Figure 5. Phenotypic characterization and gene expression analysis of neural organoids. (A, B) Brightfield images of neural organoids on day 31 or day 24 of culture show convoluted neuroepithelial structures. **(C)** Gene expression analyses of day 39 neural organoid cultures indicate the presence of multiple neural cell types, including neural stem cells and neurons. Expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method, relative to undifferentiated H9 human ESCs or Gibco Human Episomal iPSCs. Samples from two experiments are shown. **(D)** Summary of Applied Biosystems™ TaqMan® Assays used for gene expression analysis.

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Conclusions

Together, these data demonstrate the compatibility of feeder-free StemFlex Medium and Nunclon Sphera 96-well U-bottom microplates with EB formation and neural organoid differentiation. Furthermore, we demonstrate the effectiveness of Geltrex matrix for the encapsulation and morphogenesis of neural organoids. In all, the results indicate that these three products can be successfully integrated with existing Gibco basal media and supplements that are commonly used for studies involving neural organoids.

References

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Ordering information

Product	Cat. No.
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	A1413201
B-27 Supplement (50X), Serum Free	17504044
B-27 Supplement (50X), Minus Vitamin A	12587010
DMEM/F-12, GlutaMAX Supplement	10565018
N-2 Supplement (100X)	17502001
Neurobasal Medium	21103049
StemFlex Medium	A3349401
RevitaCell Supplement	A2644501
Nunclon Sphera Microplates, 96-Well U-Bottom	174925
Nunclon Sphera 24-Well Plate	174930
Nunclon Sphera 6-Well Plate	174932

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