

Production of Human Induced Pluripotent Stem Cell-Derived Cortical Neurospheres in the DASbox[®] Mini Bioreactor System

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Abstract

Stem cell-derived cortical neurospheres are of great interest in basic neurobiology research and drug research. Cultivation in controlled, stirred-tank bioreactors can improve the homogeneity of neural spheroid cultures and allows culture scale-up. We used a DASbox Mini Bioreactor System for the generation of human induced pluripotent stem cell (hiPSC) spheroids

and subsequent neural differentiation. Results from three independent bioprocesses demonstrate the reproducibility of the neural differentiation protocol. In a single DASbox Mini Bioreactor, we obtained 2×10^8 cells, demonstrating the potential of stirred-tank bioreactors for the generation of high numbers of iPSC-derived neurospheres.

Introduction

In recent years, a wide range of neural differentiation protocols for human embryonic and induced pluripotent stem cells have been published [1]. There is substantial interest in the scientific community to use these cells to study basic mechanisms of brain development, neuronal function, and drug-induced effects. Conventional monolayer differentiation protocols are well suited for large-scale screenings because they are easily scalable and provide a high degree of cell homogeneity. Nevertheless, there are several limitations of 2D cell culture systems, mainly due to their limited complexity. To overcome these limitations, protocols for *in vitro* brain organoid generation were developed. They allow generation of highly structured organoids consisting of multiple regions with distinct features of different regions of the central nervous system [2]. These systems can be kept in culture for up to 20 months [3] and show a remarkable degree of maturation, which makes them promising models for studying the development of the human brain. On the downside, there is a high degree

of inter-organoid heterogeneity caused by stochastic effects during the initial formation of the organoids. Furthermore, production of these models requires a considerable amount of manual lab work, which limits their scalability. These factors make current organoid technologies unsuitable for the development of standardized models or assays. Using bioreactors that allow tight regulation of process parameters improves homogeneity of neural spheroid cultures [4,5] and allows scale-up to produce sufficient cell numbers for novel pharmacological testing devices such as Multi-Organ Chip systems [6].



TissUse has developed a unique “Human-on-a-Chip” technology platform to accelerate the development of pharmaceutical, chemical, cosmetic, and personalized medicinal products.

Material and Methods

Media and small molecules

We used the following cell culture media: Initial cultivation of iPSCs was performed in StemMACS® iPS-Brew XF (Miltenyi® Biotec, Germany). Neural adaptation medium (NAM) consisted of KnockOut™ DMEM supplemented with 15 % KnockOut Serum Replacement (both Thermo Fisher Scientific®, USA), 1 % MEM nonessential amino acids, 2 mM L-glutamine, 1 % penicillin-streptomycin, and 50 µM 2-mercaptoethanol (all Corning®, USA). Neural induction medium (NIM) contained DMEM/F-12 supplemented with 1 % N-2 Supplement, 2 % B-27 Supplement Minus Vitamin A (all Thermo Fisher Scientific), 1 % MEM nonessential amino acids, 2 mM L-glutamine and 1 % penicillin-streptomycin.

We added the following inhibitors as indicated (Table 1, Figure 2).

Table 1: Small molecule inhibitors used in this study

Compound	Inhibitor	Concentration	Supplier
Y-27632	ROCK-inhibitor	10 µM	Cayman Chemical®
SB431542	Inhibitor TGF-β signaling	10 µM	Miltenyi Biotec
LDN193189	Inhibitor of the bone morphogenetic pathway	1 µM	Selleckchem®
XAV939	Inhibitor of Wnt/β-catenin signaling	2 µM	Cayman Chemical

Generation and characterization of iPSCs and initial cell culture

The iPSC line StemUse101 was derived from human peripheral mononuclear cells (PBMCs) of a 52 year-old male donor. Blood samples were donated with informed consent and ethics approval (Ethic committee, Berlin Chamber of Physicians, Germany) in compliance with the relevant laws. The episomal vector Epi5™ Kit (Thermo Fisher Scientific), containing the factors Oct4, Sox2, Lin28, Klf4, and L-Myc, was used for reprogramming PBMCs into iPSCs. We cultivated the iPSCs under feeder-free conditions on cell culture plates coated with growth factor-reduced 1 % Matrigel® (Corning) in StemMACS iPS-Brew XF. Cells were passaged as single cells every five to seven days, and reseeded with 4000 cells/cm². For the first 48 hours after passaging, we added 10 µM ROCK Inhibitor Y-27632. We

exchanged the medium every day, except for the first day after passaging. We used the iPSCs in passages 18 to 30 for spheroid formation and differentiation into neurospheres.

Bioreactor control and process parameters

We performed iPSC spheroid cultivation and neuronal differentiation in a DASbox Mini Bioreactor System (Eppendorf, Germany; Figure 1). The setup of the system and process parameter setpoints and control are summarized in Table 2.



Fig. 1: DASbox Mini Bioreactor System

Table 2: Overview of process parameters and setpoints

Parameter	Device/setpoint
Bioprocess system	DASbox Mini Bioreactor System
Vessel	DASbox Mini Bioreactor; coated with siliconizing agent Sigmacote® (Sigma-Aldrich®, USA) before starting the process to prevent cell adhesion
Agitation	8-blade impeller with 60 ° pitch; 80 rpm
Temperature	37 °C
Dissolved oxygen (DO)	19 %; maintained through gassing with 5 % CO ₂ /variable percentage of O ₂ at 3 sL/h
Sampling	Submerged tube
Medium addition	Non-submerged tube connected to system's pump
Medium withdrawal	Submerged tube equipped with 10 µm porous filter connected to system's pump. The filter allows removal of single cells while retaining spheroids in the vessel

Spheroid formation, growth, and neural differentiation

Initial spheroid formation of iPSCs in the DASbox Mini Bioreactor System was performed as described by Abecasis

et al. [7]. Neural differentiation was induced as described by Rigamonti *et al.* [5]. The workflow is summarized in Figure 2.

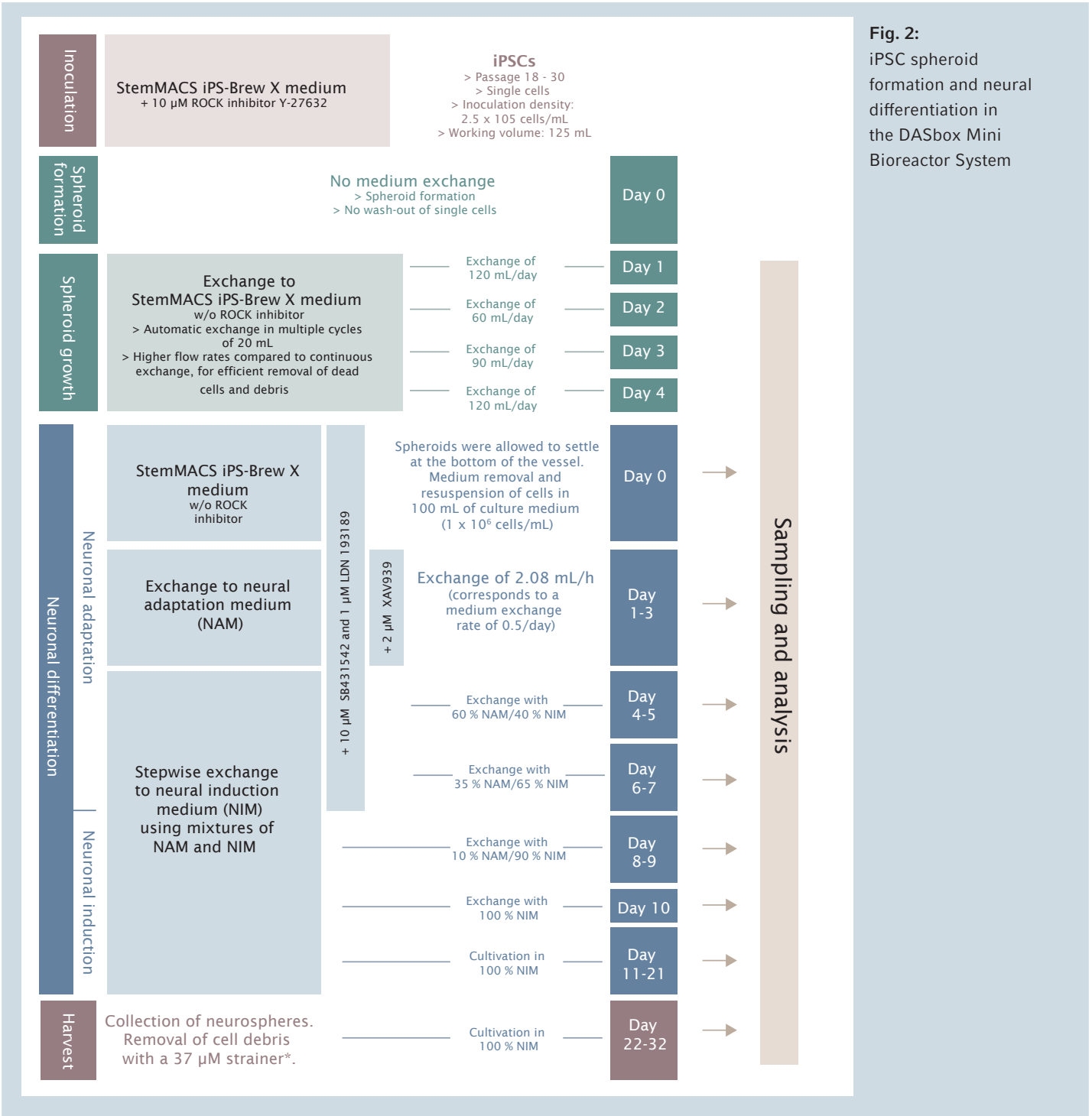


Fig. 2:
iPSC spheroid formation and neural differentiation in the DASbox Mini Bioreactor System

Sampling and analysis

At several points in time, 2 mL of the spheroid suspension were withdrawn from the bioreactor via the submerged tube.

Assesment of pluripotency

To assess the pluripotency of the iPSCs, we dissociated the spheroids with Accutase® (Innovative Cell Technologies, USA), reseeded the cells on Matrigel-coated cell culture plates, and cultured them for two additional passages as described above. We analyzed the iPSC marker expression before and after spheroid formation by staining with the following antibodies: TRA-1-60-PE, SSEA-1-PE-Vio® 770, SSEA-5-VioBlue®, Sox2-FITC, Oct3/4-APC and NANOG-APC (all Miltenyi Biotec) and subsequent flow cytometric analysis on the MACSQuant® Analyzer 10 (Miltenyi Biotec). Gating was performed with Flowlogic® software (Invai Technologies, Australia) and gates were adjusted to the respective isotype controls.

Cell counting

We dissociated a defined volume of spheroid suspension with Accutase for cell counting and viability measurement with an automated cell counter (Nucleocounter® NC-200, ChemoMetec®, Denmark).

Microscopical analysis of spheroid size

Image acquisition was performed on a BZ-X700E fluorescence microscope (Keyence®, Japan) and spheroid size was analyzed using automated particle analysis with Fiji software. Pictures were transformed into binary images to allow automated particle recognition. The individual spheroid area and the roundness were measured. We used the roundness as an secondary parameter to assess the overall vitality of the spheroids, as it was observed that when local cell death occurred spheroids lost their round shape. Roundness was automatically calculated as:

$$\text{Roundness} = \text{Area} / (\pi \times [\text{Major axis}]^2)$$

The average diameter and the average volume of the spheroids were calculated from their area, assuming their sphericity.

Microscopical analysis of cell viability in the spheroids

After image acquisition, we stained a small portion of the spheroids for five minutes with 5 µg/mL propidium iodide (PI) and 10 µg/mL fluorescein diacetate (FDA) to distinguish dead and living cells.

Microscopical analysis of spheroid cell types and status

We embedded multiple spheroids in Tissue-Tek® O.C.T.™ compound (Sakura Finetek, The Netherlands) and immediately froze them at -80 °C. We prepared sections of 8 µm thickness, fixed them in ice-cold acetone for 10 min, and permeabilized them for 10 min with a 0.2 % Triton-X100 solution. We performed immunohistochemical analyses as summarized in Table 3.

Table 3: Microscopic detection

Detection of	Staining with
Proliferating cells	anti-Ki-67
Live cells	Fluorecein diacetate
Dead cells	Propidium iodide
Neural stem cells	anti-PAX6
Neural stem cells	anti-Nestin
Neuronal cells	anti-beta-3 Tubulin
Apoptotic cells	TUNEL assay
Nuclei	DAPI

We used the following primary antibodies: Ki-67 (eBioscience®, USA, mouse anti-human), PAX6 (BioLegend®, USA, rabbit anti-human), Nestin (eBioscience, mouse anti-human), beta-3 Tubulin (eBioscience, mouse anti-human). The following secondary antibodies were used: CF488A goat anti-rabbit IgG, CF594 goat anti-mouse, CF488A donkey anti-mouse and CF594 donkey anti-rabbit (all Biotium®, USA).

Gene expression analysis

Spheroids were collected, washed, and frozen at -80 °C for subsequent RNA isolation. We performed RNA isolation and cDNA synthesis with the MultiMACS™ cDNA Synthesis Kit (Miltenyi Biotec) or with a combination of the NucleoSpin® RNA kit (Macherey-Nagel, Germany) and reverse transcription with TaqMan® Reverse Transcription Reagents (Roche Diagnostics, Germany). Real-time qPCR experiments were conducted using the QuantStudio® 5 Real-Time PCR System with 384-well thermal block (Life Technologies, USA) and the SensiFAST® SYBR® Lo-ROX Kit (Bioline, UK) according to the manufacturer's instructions. We normalized the measured cT values by the cT values of the housekeeping gene TBP. Fold-change was calculated compared to iPSC spheroids. We sorted the fold-changes into five categories: 0 – 0.75: downregulation; 0.75 – 1.25: constant expression; 1.25 – 4: light upregulation; 4 – 10: moderate upregulation, and >10: high upregulation.

Outgrowth morphology

At day 14 and day 22 of cultivation, we plated spheroids on Matrigel-coated cell culture plates or chambered cell culture slides (Falcon®, USA) to observe outgrowth morphology. Attached spheroids were then cultured in neural maturation medium (Neurobasal™ Medium, Thermo Fisher Scientific) with 1 % N-2 Supplement, 2 % B-27 Supplement Minus

Vitamin A, 1 % MEM nonessential amino acids, 2 mM L-glutamine, 1 % penicillin-streptomycin, 10 ng/mL BDNF (PeproTech®, USA) and 10 ng/mL GDNF (PeproTech), with medium exchanges every two to three days until neural rosette formation or neurite outgrowth was visible.

Results

iPSC spheroids

Spheroid formation and growth

The iPSCs rapidly formed spheroids in the bioreactor during the first 24 hours of culture and increased in size until day five of cultivation to an average diameter of 136.5 μm and average volume of 1.75 nL (Figure 3B-C). We stained sampled spheroids daily with propidium iodide to label dead cells and fluorescein diacetate to label living cells. For the first 48 hours of culture, many dead single cells were visible, but were subsequently washed out, while spheroids appeared highly vital (Figure 3A). After five days, iPSC expanded five fold up to a total of 1.58×10^8 cells with a viability of 86 %. The iPSC spheroid expansion was adapted from Abecasis *et al.* [7], who reported an expansion factor of 12.4 and an average diameter of 136 μm after 4 days of cultivation in a DASGIP® Parallel Bioreactor System. The higher expansion rate in this protocol might be caused by a different expansion medium or a higher medium exchange rate. Therefore, further optimization of the expansion

protocol might be possible, but is not necessary for this protocol, because a sufficient number of cells was produced for subsequent neural induction.

Expression of pluripotency markers

Expression of pluripotency marker genes was not affected by 3D cultivation in the bioreactor, as shown by flow cytometric measurement before and after bioreactor cultivation (Figure 3D). The iPSC spheroids were dissociated and recultured on Matrigel-coated cell culture plates for two additional passages. The expression of the iPSC markers SSEA-5, TRA-1-60, SOX-2 and OCT-3/4 was stable over the whole time frame, while the early differentiation marker SSEA-1 was not expressed at any measured time point (Figure 3D). However, the replated iPSCs showed a higher degree of spontaneous differentiation (as seen by morphological changes, data not shown), which is unfavorable if iPSCs were used for subsequent monolayer expansion. Nevertheless, this behavior is not problematic for this protocol, because iPSC spheroids were used directly for differentiation.

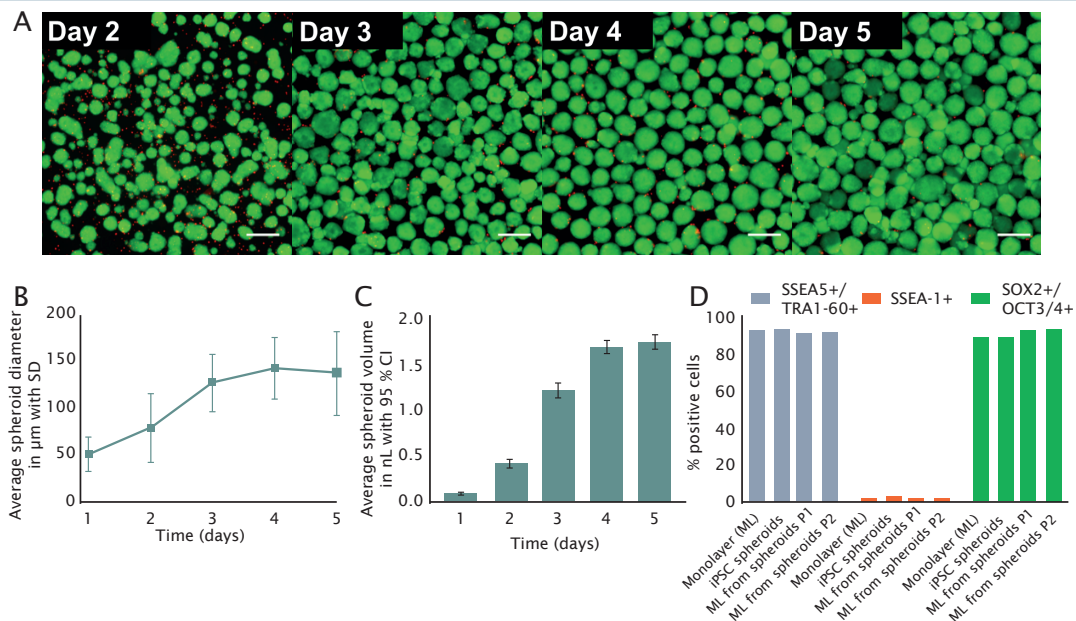


Fig. 3: : iPSC spheroid formation in the DASbox Mini Bioreactor. **A:** Propidium iodide and fluorescein diacetate staining of iPSC spheroids over time. Scale: 250 μm . **B:** Development of the average diameter and **C:** volume during the spheroid formation phase. **D:** Pluripotency marker expression of monolayer (ML) iPSCs, iPSC spheroids, and monolayer iPSCs after replating from spheroids **B-D:** n = 3.

Cortical neurospheres

After 5 days of spheroid formation from single iPSCs, we induced differentiation of iPSC spheroids to cortical neurospheres by inhibition of SMAD signaling by the transforming growth factor- β inhibitor SB431542, the bone morphogenetic signaling inhibitor LDN193189 and inhibition of Wnt signaling by the tankyrase inhibitor XAV939 [8,9] (Figure 4A). The medium composition of the feed medium was changed at multiple time points of the differentiation process which leads to a gradual shift of the medium composition from NAM to NIM and wash out of the small molecule inhibitors (Figure 4A). Thereby, no manual medium exchange was necessary during the differentiation process, after the neural adaptation/induction process was started.

Spheroid size

During the neuronal adaptation phase, spheroid size did not increase further (Figure 4C, D). On day seven, the number of dead cells had increased, as shown by a distinct increase in the PI-stained dead single cells (Figure 4B) and a decrease in roundness of the average spheroids (Figure 4E). This corresponded with the timing of the medium transition, and is therefore likely to be an effect of dying cells that had not

yet adapted to the new culture conditions. After this phase, spheroid size increased considerably up to an average diameter of 230 μm and an average volume of 11.4 nL (Figure 4D). After 21 days, the cell number increased twofold up to a total of 2×10^8 cells with a viability of 82 %. The total number of neuronal cells in the adult human brain is roughly 100×10^9 . In our Multi-Organ-Chip systems, we usually use a scaling factor of 1:100,000, which results in a cell number of 1×10^6 cells per neuronal model. With one bioreactor run, 200 neuronal models can be generated, which is sufficient even for large-scale experiments. It was not possible to estimate the total number of cells or the number of spheroids during the differentiation, because the spheroids were not distributed homogeneously in the bioreactor. As a result, the cell concentrations in the acquired medium samples were not representative of the whole culture. To improve homogeneity of the spheroid distribution further optimization of stirrer speed is necessary.

Spheroid viability

Spheroids stayed highly vital over the whole time, as shown by PI/FDA staining (Figure 4B). At the end of the process, cryosections were stained with a TUNEL assay and for Ki-67 to test for apoptotic and proliferating cells, because PI/FDA

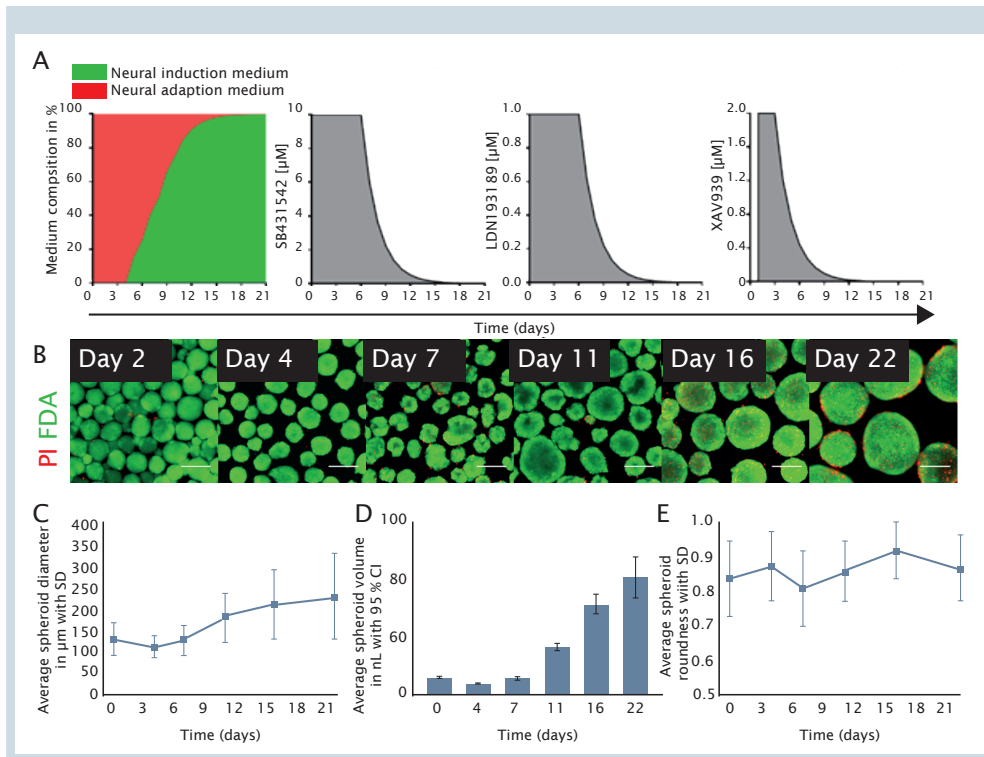


Fig. 4: Neural induction of iPSC spheroids.

A: Gradual dilution of neural adaptation medium and small molecule inhibitors **B:** Propidium iodide/fluorescein diacetate staining of neuronal spheroids. Scale: 250 μm . **C:** Development of the average diameter, **D:** volume and **E:** roundness of neuronal spheroids during the neuronal adaptation and induction phase. **C–E:** n = 3.

staining only allows observation of cells close to the surface of the spheroid. As expected, most dead cells were located close to the core of the spheroid, but constituted only a small part of the whole spheroid. Ki-67-positive, proliferating cells were evenly distributed in the whole spheroid (Figure 6A). Based on these observations, nutrient and oxygen supply seemed to be sufficient, even in the inner regions of the spheroid.

Marker gene expression

To assess the efficiency of the neural induction protocol the $\Delta\Delta cT$ mRNA fold-change of selected marker genes, normalized to the housekeeper TBP and compared to iPSC-spheroids was measured at the end of the neural induction. To test reproducibility of the protocol, we performed the measurement for three independent cultivations in the bioreactor (Figure 5A). The pluripotency marker genes NANOG and OCT3/4 were downregulated, which was expected due to the cell differentiation and subsequent loss of pluripotency. The gene expression of the neuronal stem cell marker gene Nestin (NES) increased in all three cultivations, while SOX2 expression stayed constant in two out of three cultivations. The constant or light increase of SOX2 expression is likely due to the fact that this transcription factor is already highly expressed in iPSCs and thus was not further upregulated.

Marker genes that are known to play an important role in corticogenesis and development of cortical layering such as OTX1, TBR1 and SATB2 were upregulated, reflecting the cortical identity of the spheroids as shown by Rigamonti et al. [5]. Multiple pan-neuronal markers such as MAP2, TUBB3, Synaptophysin (SYP), Enolase-2 (ENO2) and the more specific neuronal markers SLC6A4 (serotonergic neurons) and TH (dopaminergic neurons) were also upregulated, indicating the differentiation of neural stem cells into neuronal progenitor cells. We did not observe a distinct expression increase of the neuroglia markers ALDH1A1 (Astrocytes) and MAG (Oligodendrocytes), indicating that the spheroids had a predominantly neuronal phenotype at that point in time. This is supported by the absence of the astrocyte marker GFAP in immunofluorescence staining (data not shown). This was expected, because differentiation into neuroglia takes notably longer timespans [3] than the relatively short induction protocol presented here.

We performed staining of cryosections on spheroids from three independent cultivations to test reproducibility of the differentiation. In all differentiations, neural stem marker

Nestin and PAX6 positive cells were present (Figure 5B), which shows the robust generation of neuronal stem cells. Nestin and PAX6 positive cells were evenly distributed throughout the whole spheroid, indicating a homogeneous differentiation of the spheroids.

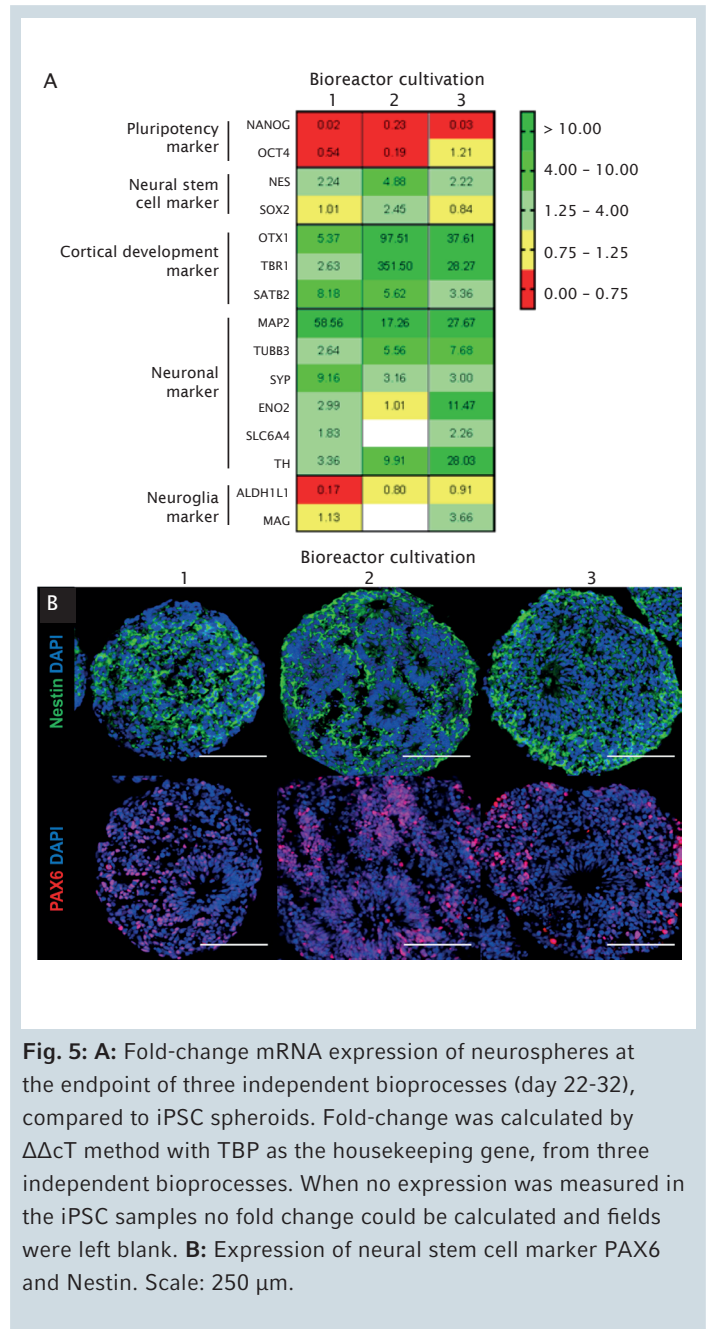


Fig. 5: A: Fold-change mRNA expression of neurospheres at the endpoint of three independent bioprocesses (day 22-32), compared to iPSC spheroids. Fold-change was calculated by $\Delta\Delta cT$ method with TBP as the housekeeping gene, from three independent bioprocesses. When no expression was measured in the iPSC samples no fold change could be calculated and fields were left blank. **B:** Expression of neural stem cell marker PAX6 and Nestin. Scale: 250 μ m.

Neurite outgrowth

When we replated neurospheres on Matrigel-coated cell culture plates at day 14 of bioreactor differentiation, most cells were PAX6 and Nestin-positive (Figure 6B), indicating that the spheroids contained mostly neural stem cells. We observed the formation of neural rosettes with sporadic neurite outgrowth (Figure 6C). Neurite outgrowth was more

pronounced when plated at day 21 of differentiation, as shown by phase contrast images and staining for TUBB3 (Figure 6D, E). Neural stem cells were still present and appeared as clusters of PAX6 positive cells (Figure 6F). Outgrowth of TUBB3 positive neurites from there shows the differentiation potential into neuronal lineages.

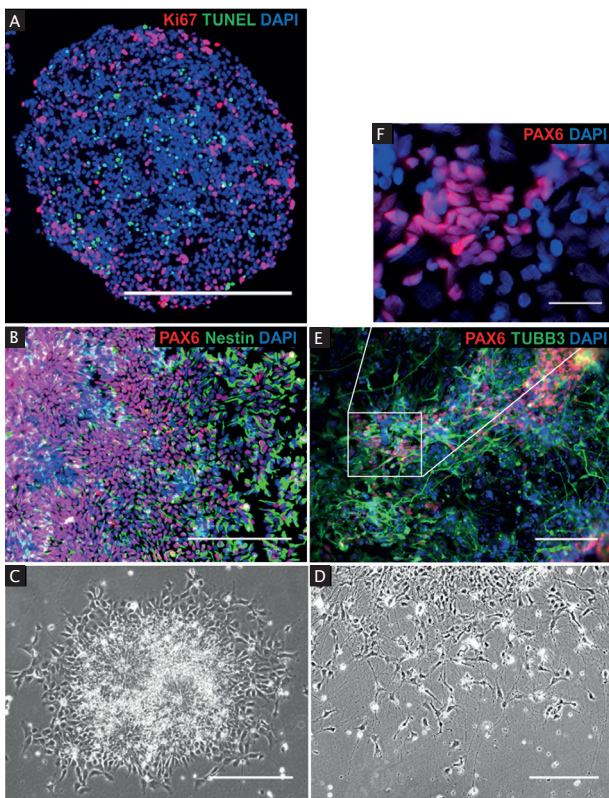


Fig. 6: Staining of necrotic/apoptotic and proliferating cells in the neurospheres at the end of the differentiation.

Neurospheres were replated on Matrigel-coated cell culture plates with subsequent 2D culture. Scale: 250 μ m.

A: Cryosections were stained with a TUNEL kit and an antibody directed against Ki-67 to stain for apoptotic and proliferating cells at the end of bioprocess.

B-C: Neurospheres were replated at day 14 of the bioprocess.

B: Cells were mostly PAX6 and Nestin-positive.

C: They showed formation of neuronal rosettes.

D-F: Neurospheres were replated at day 21 of the bioprocess.

D, E: The cells showed neurite outgrowth of TUBB3-positive cells.

F: Clusters of PAX-positive cells were visible. Scale: 50 μ m.

Conclusion

The DASbox Mini Bioreactor System enabled efficient iPSC-spheroid formation and subsequent neural induction. The ubiquitous presence of PAX6-positive neural stem cells throughout the spheroids indicates that they are likely to be an ideal basis for further maturation or directed differentiation of specific neural or glial cell types. Further development will be necessary to use them for assessment of neurotoxicity or disease-related mechanisms.

The following aspects are especially important, when thinking about the routine use of cell spheroids in drug research.

> **Reproducibility**

Neurospheres generated in three independent bioprocesses showed comparable expression patterns on mRNA and protein level, indicating that the presented protocol robustly induces neural differentiation.

> **Yield**

The yield of 2×10^8 neuronal stem and progenitor cells

in a single bioreactor showed that these technologies are capable of generating a sufficient amount of cells for future applications, like personalized drug testing in microphysiological systems.

> **Scalability**

If higher cell numbers are needed for future applications, upscaling is easily possible by parallel operation of multiple bioreactors. As an alternative approach, upscaling can be achieved by using larger vessels, which would be facilitated by the similarities in functionality and geometry of the DASbox Mini Bioreactors and larger Eppendorf bioreactors.

In summary, the ability to generate high numbers of homogenous human iPSC-derived neurospheres will contribute towards personalized drug testing in microphysiological systems.

Literature

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Ordering information

Description	Order no.
DASbox® Mini Bioreactor System for Cell Culture , max. 5 sL/h gassing, 4-fold system	76DX04CC
DASbox® Mini Bioreactor , for Cell Culture	76DS0250DSS
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Holding Sleeve , for 8-blade impeller, stainless steel with set screw, I.D. 8 mm, for shaft with O.D. 5 mm (DASbox®)	78100595
Sinter Glass Sparger , O.D. 13 mm, tube barb O.D. 4 mm, Porosity 20-40 µm, L35 mm	78903230
Triple-Port , including liquid feed connector assembly, for 1 vessel Pg13.5	76DGTRIP
Stainless steel pipe , with barb, O.D. 4mm, I.D. 2mm, L 225mm	78107023
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