Novel workflow for matrix-free generation and fast track, high-throughput characterization of human brain organoids by lightsheet microscopy

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Introduction

Due to its complexity, the process of human brain development and its functions are incompletely understood. New *in vitro* models and implementation of novel analytical workflows are urgently required to improve our understanding of neurological diseases and to advance therapeutic developments. To overcome current limitations, we established a simple, standardized workflow, including matrix-free generation of human pluripotent stem cell (hPSC)-derived brain organoids followed by detailed characterization of the cellular composition using 3D lightsheet microscopy. Combination of our fast, non-toxic clearing protocol with sample staining using REAfinity™ conjugates optimized for 3D-IF and our newly designed multi-sample holder facilitates for the first time parallel imaging of 48 organoids in a single imaging run using the UltraMicroscope Blaze™.

Methods and Results

1 Generation of stem cell-derived brain organoids

We developed a simple differentiation protocol: PSCs are seeded in 96-well ultra-low attachment plates and cultured for 15 days. At day 15 of differentiation, 48 organoids are transferred to one 10 cm dish and further cultivated on an orbital shaker until day 30 and beyond. Embryoid body formation and further differentiation is induced by four defined, xeno-free cell culture media: the EB Formation Medium allows for seeding and EB formation between d0-1. Neural Induction Medium I induces neuroectodermal differentiation between d1-5 followed by neuroepithelial expansion in Neural Induction Medium II. From d15-30, organoids undergo maturation in the Cultivation Medium. Due to an optimized media formulation organoids do not require Matrigel embedding, substantially simplifying organoid generation (Figure 1).

Figure 1

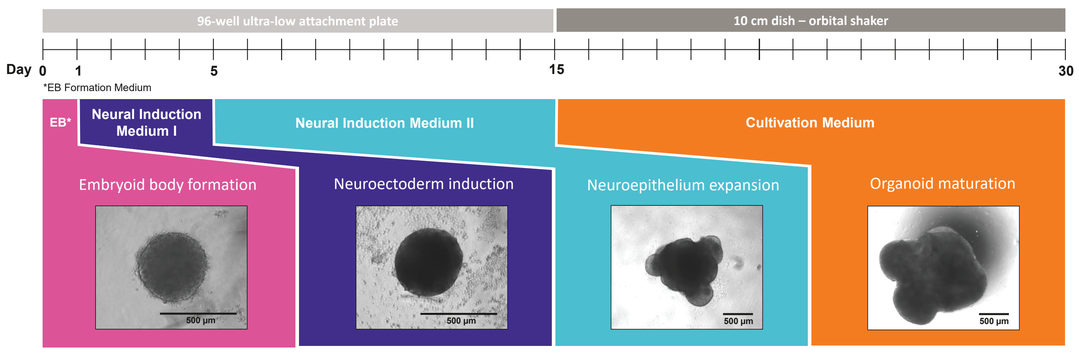
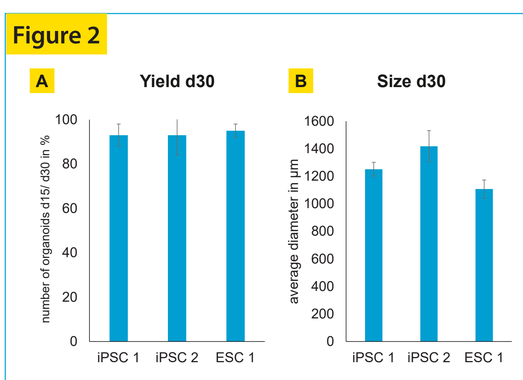


Figure 2



Comparing organoid size and yield at day 30 of differentiation indicates an average diameter of 1100 – 1400 µm per organoid and a yield (number of organoids at d15 / number of organoids at d30) of over 90%. These data indicate reproducible and standardized generation of hPSC-derived organoids with high efficiency (Figure 2A and 2B).

2 Automated dissociation of brain organoids enabling for flow cytometric analysis

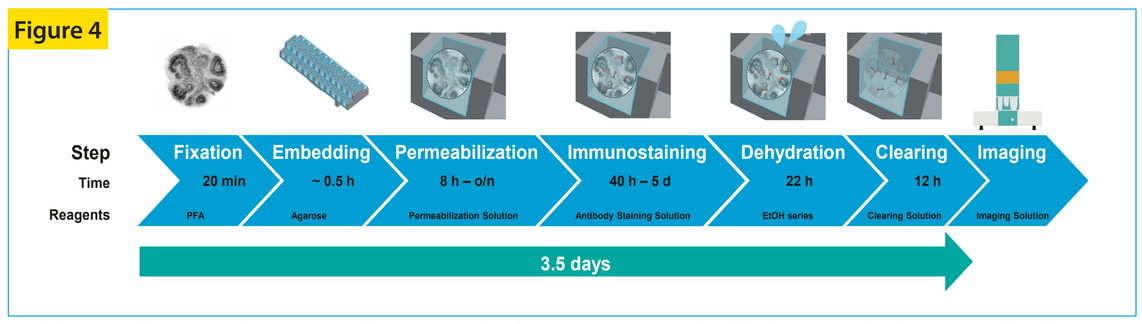
In order to enable for flow cytometry-based single cell analysis as well as monolayer cultivation of neural cells derived from brain organoids at several differentiation stages, a workflow for automated dissociation of brain organoids was developed (Figure 3A). We were able to successfully combine the Neural Tissue Dissociation Kit, originally developed for dissociation of neonatal mouse brains ≤ PD7, and mechanical dissociation using the gentleMACS™ Dissociator. Single cells derived from automated single cell generation showed high viability (Figure 3B). Data from flow cytometry analysis after dissociation and immunofluorescence analysis after 7 days of 2D cultivation in MACS® Neuro Medium incl. MACS® NeuroBrew®-21 confirmed expected neural marker expression patterns of neural cells (Figure 3B-D).

Figure 3

3 Imaging-based characterization of organoids

For the analysis of 3D cellular topography by lightsheet microscopy, we have developed an optimized workflow for tissue clearing using the MACS® Clearing Kit, which is based on a non-toxic, organic solvent-based clearing procedure. After fixation organoids are transferred to the 48 slots of the multi-sample holder, MACS® UltraMount 48, and embedded in agarose. The holder is compatible with all following sample preparation steps, including permeabilization, immunostaining, dehydration and clearing. Thereafter, the entire holder is loaded into the UltraMicroscope Blaze™ for rapid imaging. MACS® iQ View 3D large volume software serves for reliable data processing.

Figure 4



Highly specific, recombinant REAfinity™ antibodies conjugated to Vio® dyes were used for whole mount labeling of brain organoids (Figure 5A and 5B). Subsequent multiplex analysis with up to three different conjugates was performed using the UltraMicroscope Blaze™. Overview and detailed images of cleared and stained brain organoids are depicted in Figure 5C, highlighting multiple ventricular-like zones with dorsal forebrain neural progenitor cells (Sox2$^+$), apical membrane (N-Cadherin$^+$) and newly generated immature neurons (β-Tubulin 3$^+$). Besides single plane images also 3D rendered data were generated in high resolution, which enable deep insight into the 3D organoid cellular architecture (Figure 6).

Figure 5

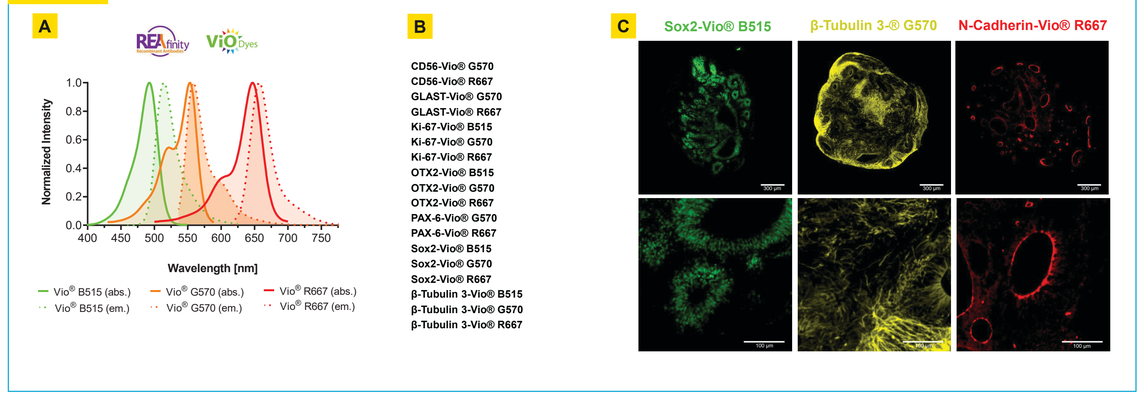


Figure 6

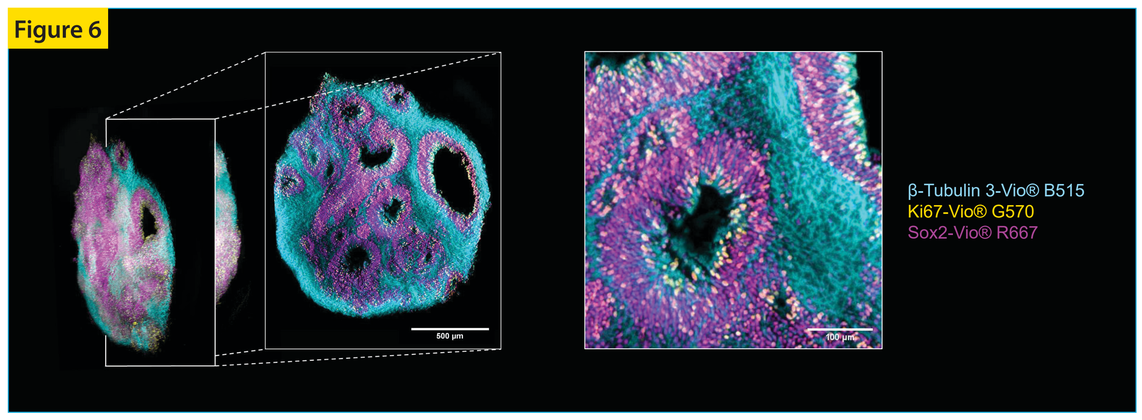
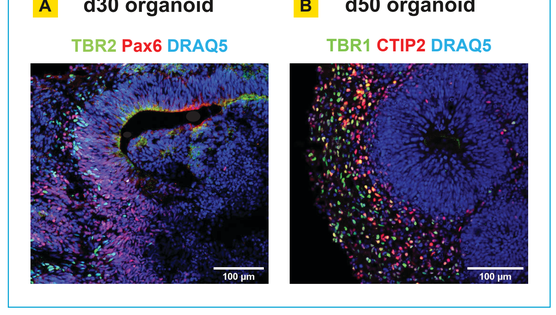


Figure 7



Additionally, staining on cryosections revealed the generation of various neuronal subtypes. At day 30 of differentiation, expression of a radial glial progenitor marker (Pax6) and an intermediate progenitor cell marker (TBR2) were detected in and above the ventricular-like zone (Figure 7A). Expression of preplate markers TBR1 and CTIP2 was observed in organoids at day 50 of differentiation, indicating the generation of deep-layer neurons (Figure 7B). These data prove early multi-layer progenitor zone organization recapitulating human cortical development.

Conclusion & Outlook

In summary, we have successfully developed a novel workflow for generation and characterization of human PSC-derived cerebral organoids, developing well defined ventricular-like zones and early cortical layering. The workflow includes a matrix-free differentiation protocol enabling for highly efficient and standardized generation of human PSC-derived brain organoids. In addition, new methods for automated dissociation, single cell analysis, *in vitro* expansion and in-depth characterization by 2D and 3D microscopy were established. Transfer of 48 fixed organoids to a multi-sample holder enables for parallelized, non-toxic tissue clearing as well as labeling using optimized antibody conjugates. Thereby, fast and high throughput sample preparation as well as detailed 3D imaging of respective cellular compositions is feasible. This workflow will allow for in-depth characterization of patient-derived brain organoids and *in vitro* evaluation of cellular therapeutics.

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