CHARACTERIZATION OF CEREBRAL ORGANOIDS GENERATED UNDER MATRIX-FREE CONDITIONS USING HIGH-THROUGHPUT 3D LIGHT-SHEET IMAGING

Poster T1094

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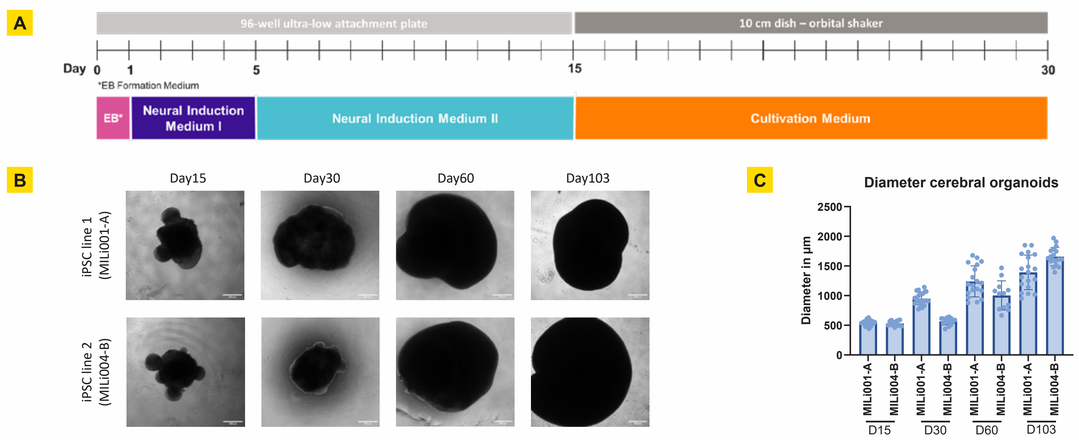
Introduction

Human brain development is highly complex and remains incompletely understood. New *in vitro* models and implementation of advanced analytical workflows, as part of New Approach Methodologies (NAMs), are urgently required to improve our understanding of neurological diseases and to support therapeutic development. To overcome current limitations, we established a simple, standardized workflow for matrix-free generation of human pluripotent stem cell-derived cerebral organoids followed by detailed characterization of the cellular composition using cyclic immunofluorescence imaging, 3D light-sheet microscopy, flow cytometry, and single-nuclei sequencing. By combining multiple complementary techniques, organoids were analyzed at structural, cellular, and transcriptional levels.

Methods and Results

1 A Matrix-Free Protocol for the Generation of Cerebral Organoids from Pluripotent Stem Cells

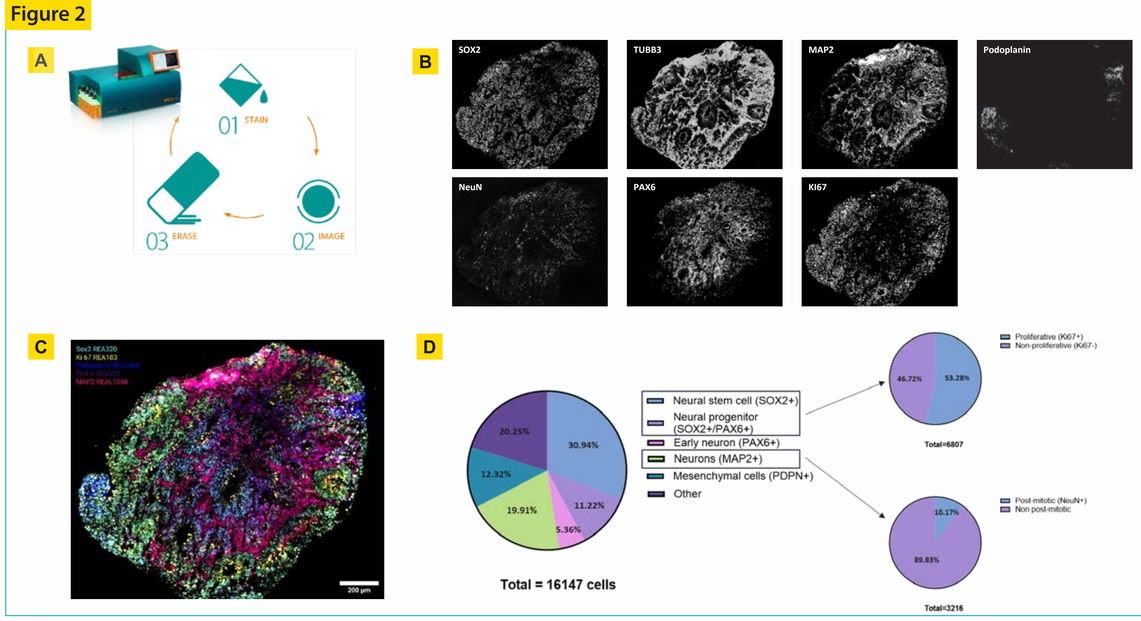
Figure 1



Cerebral organoids were generated from two independent iPSC lines using the StemMACS™ Cerebral Organoid Differentiation Kit XF under matrix-free conditions. The directed differentiation workflow over 30 days is outlined in (A). Brightfield images (B) show the development of organoids from early aggregates to structurally complex three-dimensional structures during subsequent culture and maturation up to day 103. Organoids formed robustly and exhibited stable development over time. Quantitative analysis of organoid diameter (C) indicates consistent size progression throughout the culture period, with both lines displaying comparable growth dynamics, highlighting the reproducibility of the system. Overall, these data demonstrate reliable and scalable generation of cerebral organoids using a matrix-free approach.

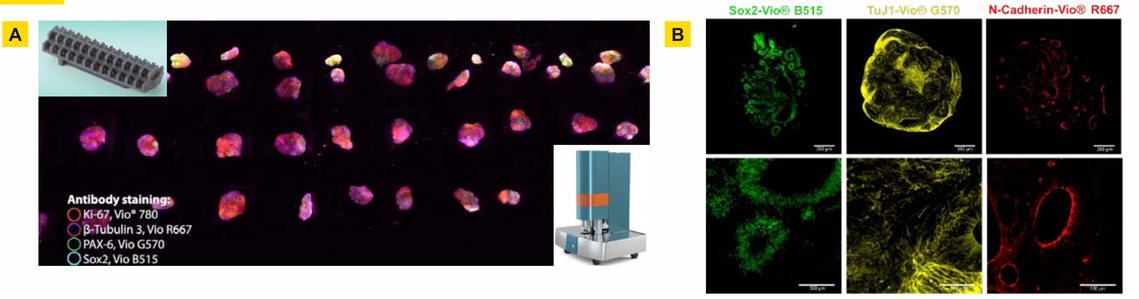
2 Multidimensional Imaging of Cerebral Organoids by Cyclic Immunofluorescence and 3D Lightsheet MIcroscopy

Figure 2



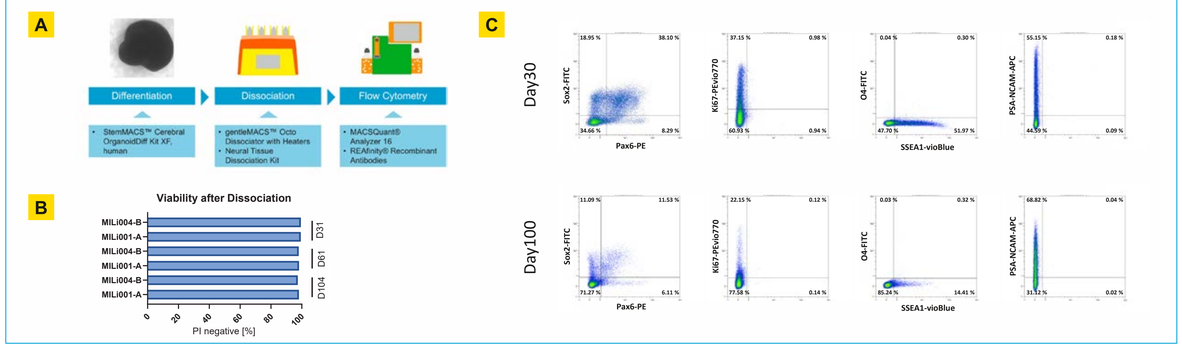
Two-dimensional and three-dimensional immunofluorescence approaches were used to characterize cerebral organoids. For 2D analysis (Figure 2), cyclic immunofluorescence imaging was performed on the MACSima platform, with data derived from a section of a day 60 organoid. The principle of iterative staining and imaging is illustrated (Fig.2 A), and representative marker expression patterns are shown (Fig.2 B), including neural progenitor markers (SOX2, PAX6), proliferation marker (Ki67), neuronal markers (TUBB3, MAP2, NeuN), and Podoplanin highlighting non-neuronal populations with mesenchymal characteristics. The overlay image (Fig.2 C) demonstrates spatial co-localization of these markers, and quantitative analysis (Fig.2 D) enabled phenotyping of proliferative and non-proliferative neural progenitors as well as mitotic and post-mitotic MAP2-positive neurons. For 3D analysis (Figure 3), whole organoids were processed using the MACS® Clearing Kit, stained with 3D-validated antibodies, and imaged on the UltraMicroscope Blaze. Imaging in the MACS® UltraMount 48 multi-sample holder (Fig.3 A) enabled parallel analysis of up to 48 samples. Higher magnification views (Fig.3 B) reveal structural details, including ventricular-like zones with dorsal forebrain progenitor cells ($SOX2^+$), apical membrane organization ($N\text{-cadherin}^+$), and immature neurons ($\beta\text{-tubulin III}^+$), highlighting the spatial organization of cerebral organoids.

Figure 3



3 Gentle Dissociation and Flow Cytometric Characterization of Cerebral Organoids

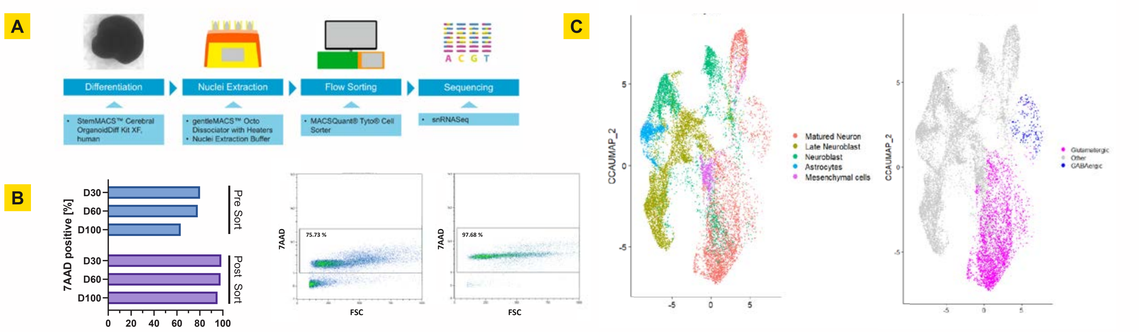
Figure 4



Organoids were dissociated at day 30, 60 and 100 using the Neural Tissue Dissociation Kit in combination with the gentleMACS Dissociator, followed by flow cytometry analysis to assess cell viability and composition. The workflow for dissociation and analysis is illustrated in (A). Cell viability after dissociation is shown in (B) and remained high across both iPSC lines and time points, demonstrating robust sample processing. Representative marker expression at day 30 and day 100 is shown in (C). Early-stage organoids were characterized by a high proportion of $SOX2^+/PAX6^+$ neural progenitor cells, which decreased over time, reflecting progression toward more differentiated cell types. Ki67 expression declined, indicating reduced cellular proliferation during maturation. SSEA-1 (CD15), associated with early neural progenitors and neuroepithelial cells, was reduced at later stages, consistent with decreased progenitor abundance. In contrast, PSA-NCAM expression increased, indicating enhanced neuronal differentiation.

4 Nuclei Isolation and Single-Nuclei RNA Sequencing for Cellular Profiling of Cerebral Organoids

Figure 5



Nuclei were isolated from cerebral organoids using the Nuclei Extraction Buffer in combination with the gentleMACS Dissociator, followed by enrichment of intact nuclei via 7-AAD staining and sorting on the MACSQuant® Tyto® Cell Sorter prior to single-nuclei RNA sequencing. The workflow is illustrated in (A). The purity of isolated nuclei is shown (B), demonstrating enrichment of 7AAD--positive nuclei after sorting compared to the pre-sort fraction on different days of isolation, including exemplary flow plots from D60 organoids. Single-nuclei sequencing results are shown (C), revealing the cellular composition of an exemplary Day 100 organoid, including mesenchymal cells, astrocytes, neuroblasts, late neuroblasts, and mature neurons (left). In addition, neuronal subtypes are resolved into GABAergic and glutamatergic populations (right), representing inhibitory and excitatory neurons, respectively. The use of nuclei, rather than whole cells, enables robust transcriptional profiling of complex and mature organoids while minimizing dissociation-induced bias and improving recovery of diverse cell types.

Conclusion

A robust and standardized workflow for the matrix-free generation of cerebral organoids was established, enabling reliable formation and long-term maturation of complex three-dimensional structures. The combination of complementary analytical approaches, including imaging, flow cytometry, and single-nuclei sequencing, allows detailed characterization of organoid structure and cellular composition. This integrated strategy supports comprehensive analysis of organoid systems and provides a scalable platform to study human brain development and neurological disease.

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