Monitoring organoid development and characterization of calcium oscillation activities in iPSC-derived 3D cerebral organoids

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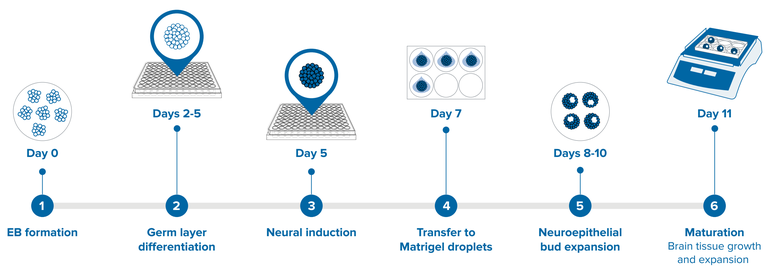
Introduction

Cerebral organoids are a rapidly developing technology that has great potential for understanding brain development and neuronal diseases. They can also be used for testing effects of compounds and genetic disorders. The model allows characterization of later events in cortical development, which provides an advanced and more biologically relevant system for research and drug discovery. Further method development would include adoption of the model for compound screening and testing the functional neuronal activities. We describe the methods for monitoring cerebral organoids and testing their functional activity by recording and analyzing calcium oscillations. Cerebral organoids were developed from iPSC using established methods[1]. We monitored size and morphology of developing brain microtissues over 4–12 weeks of development using AI-based analysis tools for defining size and shape of the tissues. Selected microtissues were analyzed by confocal imaging during different phases of development for cell organization and expression of neuronal markers. For detection of functional activities, organoids were loaded with calcium sensitive dye and then Ca^{2+} oscillations were recorded with the ImageXpress® Confocal HT.ai High-Content Imaging System. We show that high-content imaging paired with AI-based analysis used with 3D cerebral organoids is a promising tool for compound screens and toxicity evaluations.

Methods



Main steps used to generate iPSC derived cerebral organoids. The protocol used is based on Lancaster & Knoblich, 2014 using media from STEMCELL Technologies.

Image acquisition and analysis

All images were acquired on the ImageXpress Confocal HT.ai system (Molecular Devices) using the MetaXpress® High-Content Image Acquisition and Analysis Software. IN Carta™ Image Analysis Software was used for all analysis. SINAP was used to carry out segmentation of images. Each model was trained and verified before being used in the analysis protocol. For calcium imaging, images were acquired with camera binning set to 2. To increase acquisition speed, the 50 µm confocal slit module was used.

Cell culture

iPSC culture: Human iPSC cells adapted to feeder-free conditions (SC102A-1, System Biosciences) were thawed and cultured in Complete mTeSR™ Plus culture medium (STEMCELL Technologies) in Matrigel coated plates (cat. #354277, Corning). Media was changed every day except once per week when a double volume of media was added to skip changing media on one day. Cells were passaged using the enzyme-free reagent ReLeSRTM (STEMCELL Technologies) at a 1:6–1:10 split ratio.

Cerebral organoid culture

All reagents used to generate cerebral organoids were from the STEMdiff™ Cerebral Organoid Kit (STEMCELL Technologies #08570). The kit has been optimized for cerebral organoid formation based on Lancaster et al[1]. Briefly, iPSC cells were seeded at 9000 cells/well in the EB (embryoid body) seeding media in a 96-well ultra low attachment plate (Corning). The media was replaced with EB formation media. On day 5, EBs were imaged to ensure they were ready for neural induction. EBs were transferred to a 24-well plate containing the induction medium. On day 7, EBs were transferred into Matrigel (Corning) droplets. About 6-8 droplets were placed in each well of a 6-well plate containing Maturation medium. The plate containing organoids were allowed to mature in a 37°C incubator on an orbital shaker. Fresh media was replaced every 3–4 days.

Calcium flux assay

Calcium flux was assessed using the FLIPR® Calcium 6 Assay dye (Molecular Devices) according to the manufacturer protocol. Starting from day 17, one cerebral organoid was loaded with dye for 2 hrs and then assessed for activity.

Cell staining

Whole organoids were fixed in 4% PFA overnight at 4°C followed quick wash with PBS. Permeabilization was done with 0.5% triton-X in PBS. Organoids were incubated with the following antibodies and stains for 48 hours: Hoechst (33 µM, Invitrogen cat. #33342), Alexa Fluor 555 mouse anti-ß-tubulin (1:100, Becton Dickinson cat. #560339), Alexa Fluor 647 mouse anti-Sox2 (1:100, Becton Dickinson cat. #562139), Alexa Fluor Plus 750 Phalloidin (ThermoFisher Scientific cat. #A30105).

Results

Cerebral organoids were grown according to the protocol adapted from Lancaster et al. In this method, the cerebral organoids are grown in culture media that promotes self organization and patterning. Embryoid bodies (EBs) are further differentiated in an ECM to improve polarization of the neuroepithelia as well as support the growth of large neuroepithelia buds. These buds extend from the EBs and contain cavities similar to brain ventricles. Figure 1 illustrates the main steps used to generate iPSC-derived cerebral organoids.

Culture and differentiation of iPSC-derived cerebral organoids

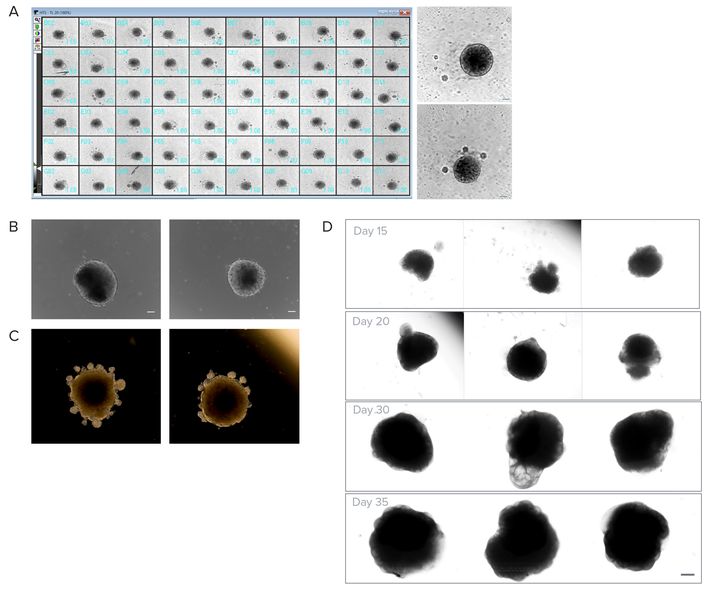


Figure 1. iPSC-derived cerebral organoids generated using the intrinsic patterning approach. iPSC cells were used to create EBs. A) By day 5, majority of EBs were at least 400 µm in diameter. The EBs show smooth outlines and regions near the surface were optically brighter (in brightfield microscopy). Example of EBs from plate overview enlarged on right. Scale bar = 100 µm. B) Images of EBs after they were transferred to neural induction medium. Optically translucent edges suggests formation of neuroepithelia. Scale bar = 100 µm. From day 8–10, organoids were embedded in Matrigel to support expansion of neuroepithelia. Note the formation of numerous epithelial buds. D) Organoids were transferred to an orbital shaker and allowed to mature. Scale bar = 500 µm.

Monitor developing cerebral organoids using AI-based analysis tools

To monitor the quality of developing organoids, we used AI-based segmentation to analyze images acquired with brightfield imaging. Growth of EBs and organoids can be monitored by measuring their diameters over time. One of the advantages of using deep learning-based methods to identify EBs include more robust segmentation. The model is trained to exclude most debris, dust particles, and imaging artifacts.

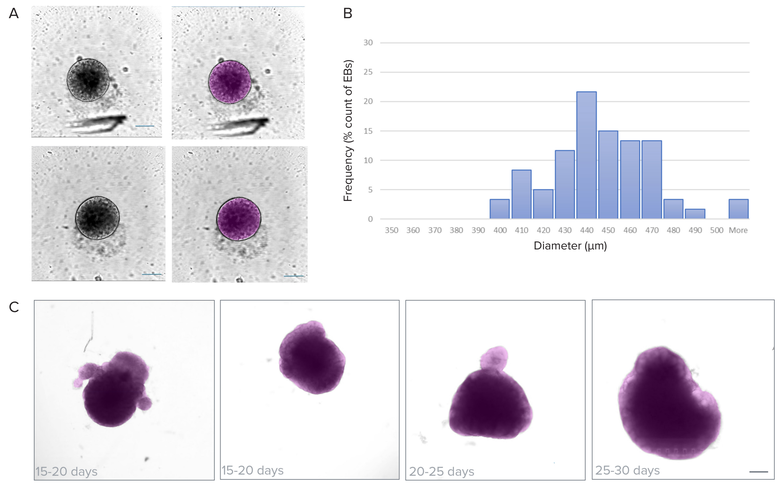


Figure 2. Analysis of brightfield images using deep-learning based segmentation (SINAP). A) EBs were monitored for their sizes before proceeding to the neural induction step. Shown are examples of images and their corresponding segmentation masks using the SINAP tool in IN Carta software. B) Histogram shows distribution of EB diameter. Binned size = 10 µm. C) Maturation of organoids can be monitored using brightfield imaging and analyzed using IN Carta SINAP tool. Custom deep learning model was trained and then applied to the dataset. Examples of organoids with segmentation mask (magenta) shown. Note that the SINAP model allows for segmentation of organoids with different shapes and sizes. Scale bar = 100 µm.

Results

Calcium imaging of whole brain organoids

Calcium imaging can be used as a functional assay to determine neuronal activity[2]. Starting from week 4, we observed calcium activity in cerebral organoid loaded with FLIPR Calcium 6 dye. (Figure 3). Frequency of calcium activity was low, suggesting that the neurons in the organoid at that time were still immature. By week 13, the calcium activity appeared more synchronous, suggesting that neurons are interconnected in a functional network (Figure 4).

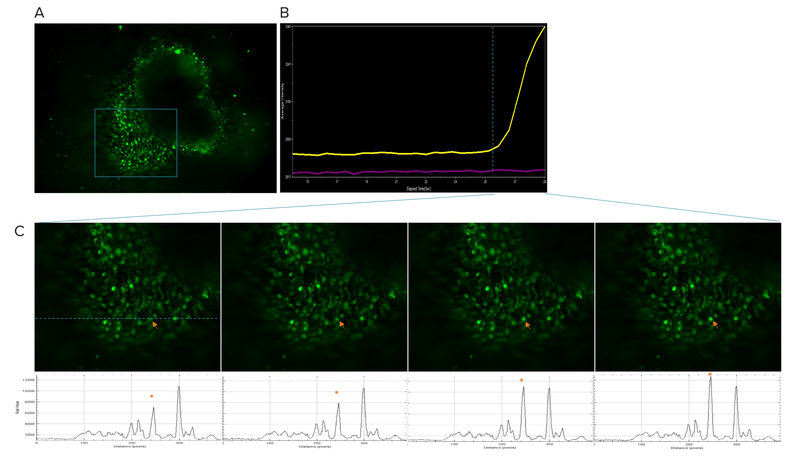


Figure 3. Calcium activity present as early as week 4. A) Organoid was loaded with FLIPR Calcium 6 dye and calcium flux activity monitored on the ImageXpress Confocal HT.ai system. B) Calcium intensity plot over time shown on the right. The yellow trace corresponds to the region indicated by arrow in (C). Purple trace represents a neighboring region. C) Close up view of boxed region from (A). Intensity profile along the dotted line shown at the bottom. Arrow corresponds to the asterisk peak in the intensity profile.

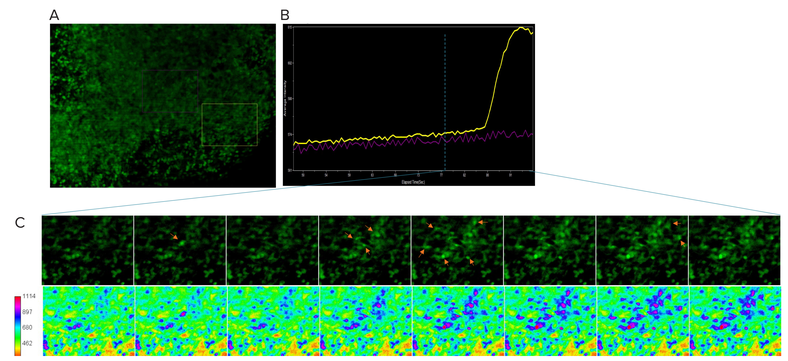


Figure 4. Synchronous calcium activity by week 13 . A) Image shows an optical section of calcium 6 loaded organoid. B) Calcium intensity from boxed region represented as average intensity over time. C) Close up view of box region from (A) shown over time. Arrows indicate elevated intensity. Bottom images represent calcium intensity as a heat map. Note the intensity spreading from the initial spike suggesting presence of neuronal network.

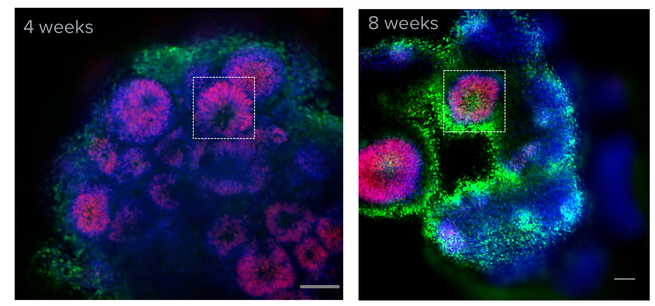


Figure 5. Cerebral organoids show organization reminiscent of developing brain. After calcium imaging, organoids were fixed and stained with Hoechst (blue) and SOX2 (radial glia, in red). Shown here is one optical section. Ventricles (boxed) can be observed in both 4- and 8-weeks old organoid. SOX2 staining is localized near the ventricles similar to the developing cortex[3]. Scale bar = 100 µm.

Conclusions

- We show that monitoring of cerebral organoids can be achieved with AI-based analysis tools which enables robust high-throughput analysis.
- The presence of calcium flux and the organization of the cells within the organoid is consistent with *in vivo* cortical development.
- Our preliminary results highlight the potential use of cerebral organoids for future compound screening assays.

References

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