Automated monitoring of development and activity analysis of iPSC-derived 3D cerebral organoids

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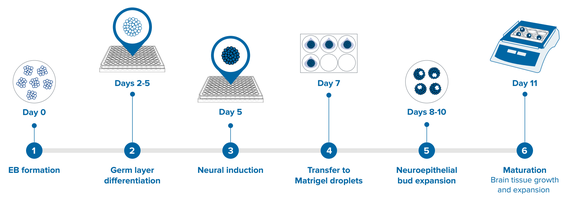
Summary

The culture of complex organoids such as the cerebral organoid is a rapidly developing technology that has immense potential in areas of developmental neurobiology and neurodegeneration. Recent progress has made it possible to culture brain organoids from iPSCs which allows for the study of cortical development and the diseases associated with it. Further work is required before cerebral organoids can be used at scale for functional genomics studies, drug discovery, and the study of compound-induced toxicity effects. Here, we describe a method for the semi-automated culture and monitoring of cerebral organoids, as well as testing functional neuronal activity by means of recording Ca^{2+} oscillations.

Cerebral organoids were developed from iPSC using previously published methods (Lancaster 2014). The method was adopted for a partially automated workflow and culture monitoring using the ImageXpress® Confocal HT.ai High-Content Imaging System. We monitored size and morphology of developing brain microtissues in transmitted light over 20 weeks of development using deep learning-based image analysis (IN Carta® Image Analysis Software) for defining the size, shape, and density of the tissues. Selected microtissues were analyzed during the different phases of development using confocal imaging by expression of Sox2, TuJ1, and GAFP markers. For detection of functional activities, calcium oscillations from selected clusters of the microtissues were recorded by imaging and analyzed using MetaXpress® High-Content Image Acquisition and Analysis Software for kinetic peak analysis. Oscillations were observed after day 50 in culture and were sporadic through the volume of micro-tissue, while significantly activated by 4-aminopyridine. Cellular viabilities and organoid morphologies were analyzed using viability markers and 3D image analysis. For assay characterization, we used several neuromodulators including 4-aminopyridine, GABA, AMPA, and muscimol. In addition, we tested known neurotoxins rotenone and mercury. The method demonstrates a promise for evaluation of effects of pharmaceutical drugs, toxins, and genetic mutations, while further method development is needed for screening applications.

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 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 the 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 mTeSRTM 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 STEMdiffTM Cerebral Organoid Kit (STEMCELL Technologies #08570). The kit was 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.

Methods

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 4oC 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. using reagents from STEMCELL Technologies. In this method, the cerebral organoids are grown in culture media that promotes self-organization and patterning. We used automated imaging and analysis of organoid cultures during the process of development. Figure 1 illustrates the main steps used to generate iPSC-derived cerebral organoids.

Culture and differentiation of iPSC-derived cerebral organoid

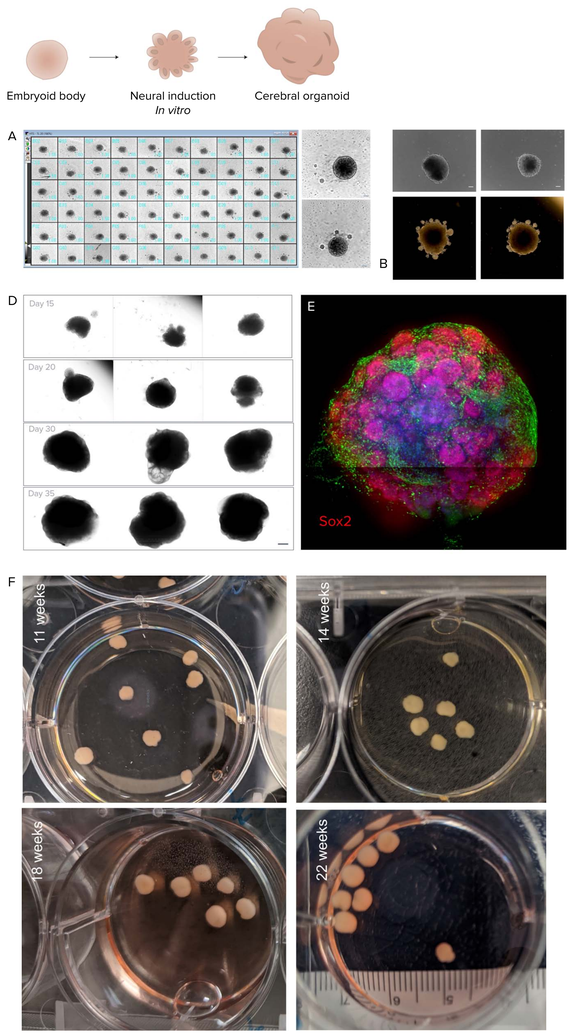


Figure 1. iPSC-derived cerebral organoids generated using the intrinsic patterning approach. iPSC cells were used to create Embryoid bodies (EBs). EBs are further differentiated in an ECM to improve polarization of the neuroepithelia as well as support the growth of large neuroepithelia buds. These buds extend from the EBs and contain cavities resembling brain ventricles. A. By day 5, the 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 = 100µm. C. 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. E. Organoids were fixed and stained with Hoechst (blue), TuJ (green), and SOX2 (radial glia, in red). F. Digital camera imaged cerebral organoids.

Results

Automated monitoring of cerebral organoids' development 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 and imaging artifacts.

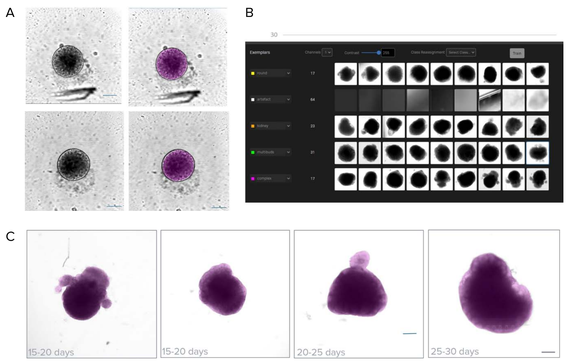


Figure 2. Analysis of brightfield images using deep-learning based segmentation (SINAP). A) EBs were monitored for their sizes before proceeding to the neural induction step. Shown are examples of images and their corresponding segmentation masks using the SINAP tool in IN Carta software. B) Histogram 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. Scale bar = 100 µm.

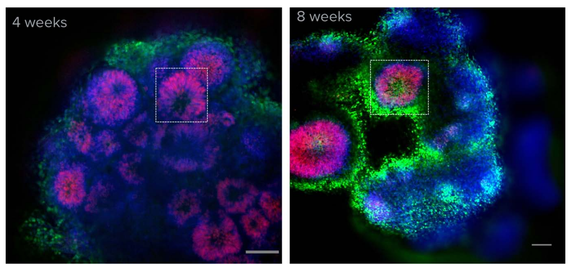


Figure 3. Cerebral organoids show organization reminiscent of developing brain. Organoids were fixed and stained with Hoechst (blue), TuJ (green), and SOX2 (radial glia, in red). Shown here is one optical section. Ventricles (boxed) can be observed in both 4- and 8-weeks old organoid. SOX2 staining is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

Calcium kinetic imaging

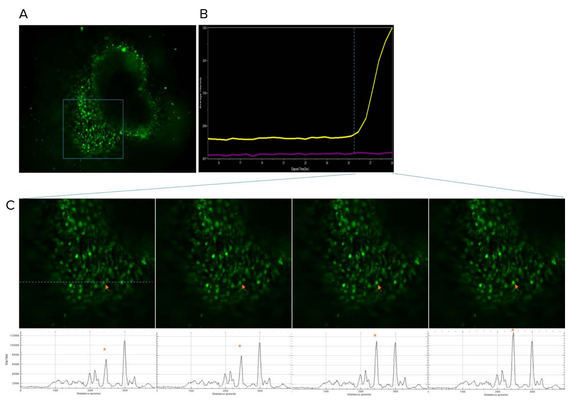


Figure 4. Calcium activity present as early as week 4. A) Organoid was loaded with FLIPR Calcium 6 dye and calcium flux activity monitored on the ImageXpress Confocal HT.ai system. B) Calcium intensity plot over time shown on the right. The yellow trace corresponds to the region indicated by arrow in (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.

Results

Calcium imaging of 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 organoids loaded with FLIPR Calcium 6 dye. (Figure 5). 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 6). Calcium activity varied in intensity and spatial location.

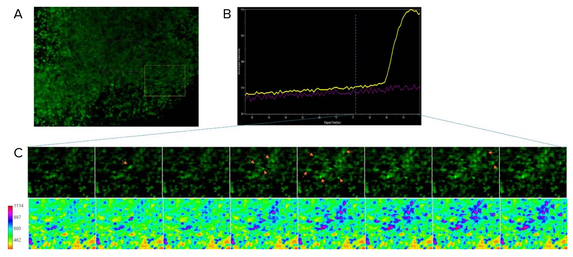


Figure 5. Synchronous calcium activity by week 13 . A) Image shows an optical section of calcium 6 loaded organoid. B) Calcium intensity from boxed region represented as average intensity over time. C) Close up view of box region from (A) shown over time. Arrows indicate elevated intensity. Bottom images represent calcium intensity as a heat map. Note the intensity spreading from the initial spike suggesting presence of neuronal network.

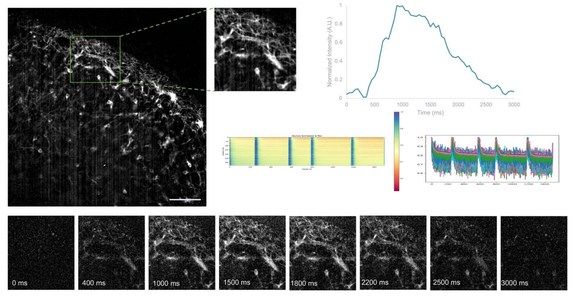


Figure 6. Synchronous calcium activity >week 20. A) Image shows an optical section of organoid. B) FL intensity from boxed region represented as average intensity over time. C) Boxed region from (B) shown over time. Note the intensities for different cells synchronized (observed in selected sample from a:head lab).

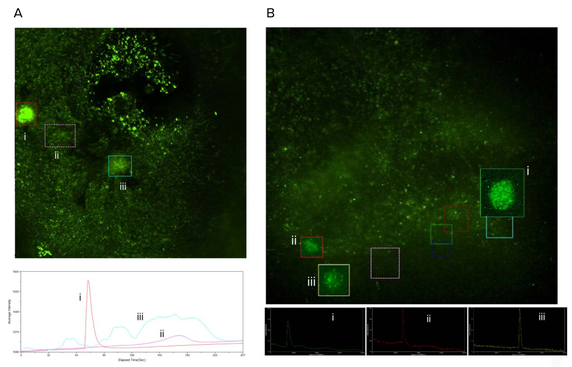


Figure 7. Calcium activity spikes triggered by neuro-stimulants. A) CO was incubated with 100nM of 4-AP for 30 mins and then imaged. Numerous calcium signaling events were observed. Boxed regions indicate areas where calcium spikes were observed. B) CO treated with muscimol. Inhibitory effect of GABA was inconclusive due to the transient/inconsistent activity of organoids (not shown).

Conclusions

- Here we demonstrate the workflow and tools needed to automate the imaging and monitoring of cerebral organoid development.
- The presence of calcium flux and the organization of the cells within the organoid is consistent with *in vivo* cortical development.
- Results indicate the potential use of cerebral organoids for future compound screening assays; however, the culture and analysis methods need to be further optimized to achieve more robust and consistent performance.

References

1. Lancaster MA, Knoblich JA. Generation of cerebral organoids from human pluripotent stem cells. *Nat Protoc.* 2014 Oct;9(10):2329–40.
2. Grienberger C, Konnerth A. Imaging calcium in neurons. *Neuron.* 2012; 73:862–885.
3. Lancaster MA, Renner M, Martin CA, et al. Cerebral organoids model human brain development and microcephaly. *Nature.* 2013;501(7467):373–379.

MOLECULAR DEVICES