Development of iPSC-derived 3D neural organoids and functional analysis by recording calcium oscillation activity

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Introduction

Neural organoids, derived from human induced pluripotent stem cells (iPSC), are a rapidly evolving technology with great potential to understand brain development, disease, and disorders within the context of diverse genetic backgrounds. In this study, we focus on the functional characterization of the spontaneous activity of neural organoids measured by calcium oscillations. Neural organoids were formed using standardized reagents and protocols from STEMCELL technologies and shipped to Molecular Devices before conducting assays to measure spontaneous activity.

Morphological characterization of 3D organoids was performed by brightfield imaging. Organoid's diameters ranged from 1800–2000 µm. Expression of neural markers including MAP2 and GAFP was detected by RT-qPCR analysis. Functional characterization of neural activity was done via calcium oscillation assay and was recorded on the FLIPR® Penta High-Throughput Cellular Screening System that measured fast kinetic changes in calcium signal. Calcium oscillations were visualized and analyzed by ScreenWorks® PeakPro2 software. In addition, kinetic imaging was recorded on the ImageXpress® Micro Confocal system. Importantly, the calcium-sensitive dye used contains a background fluorescence masking technology that enables sensitive detection of calcium oscillation without the need to wash calcium dye. The calcium oscillation patterns were analyzed for multiple parameters including peak count, and amplitude. The majority of organoids demonstrated spontaneous calcium oscillation activity with a consistent rate of oscillations. Some organoids did not show synchronous activity but such activity was induced by stimulation with 4-aminopyridine or AMPA.

Several compounds were used for pharmacological characterization to show the appropriate functional responses. AMPA and 4-AP addition resulted in a dose-dependent increase in the frequency of calcium oscillations, while GABA caused a decrease in oscillation amplitude. Taken together, this biological system of iPSC-derived 3D neural organoids paired with high-content imaging and detailed analysis of calcium oscillations demonstrates a promising tool for compound testing.

Methods

H-iPSCs maintained in mTeSR™ Plus were dissociated and seeded at a density of 3 x 10^6 cells/well in Seeding Medium (Formation Medium + 10 M rho-kinase inhibitor (ROCKi)) in AggreWell™800 plates. Cultures were fed daily with Formation Medium. After 6 days, organoids were transferred to a 6-well plate in Expansion Medium and maintained on an orbital shaker at 70 RPM (INFORS HT Celltron). Organoids were fed every 2–3 days with an Expansion Medium until day 25, at which point organoids were fed with a Differentiation Medium until day 43. From day 43 onward, organoids were fed every 2–3 days with Maintenance Medium. Neural organoids were shipped at day 60 and fed with Maintenance media with feeds every 2–3 days.

On the day of assay, cell spheroids were loaded with 2X conc. of FLIPR Calcium 6 dye indicator (Molecular Devices) and incubated for 2h. We used a high-speed EMCCD camera on the FLIPR Penta instrument (Molecular Devices) to measure the patterns and frequencies of spontaneous calcium waveforms from 3D neural organoids. Baseline recordings were acquired for ≥10 min, and then plates were dosed with drugs for 30–90 min. Peak analysis was accomplished with ScreenWorks PeakPro2 software (Molecular Devices), allowing the characterization of both primary and secondary peaks, as well as complex calcium oscillation patterns. High-content imaging was done on the ImageXpress micro confocal system (Molecular Devices) and was used to capture 3D structures of the spheroids and for viability evaluation.

Automated feeding and monitoring using CellXpress.ai

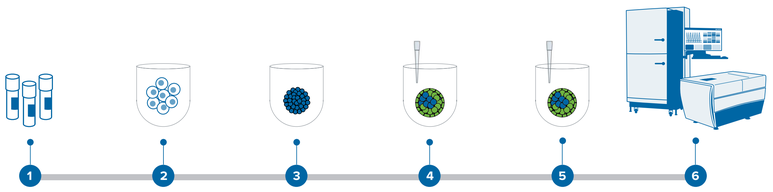


Figure 1. Schematic diagram of the process workflow. (1–4) Organoids were formed and differentiated from human pluripotent stem cells using STEMCELL Technologies products i.e., seeding, formation, expansion medium, and differentiation medium. (5) Maintenance of the organoids was done using a maintenance medium at Molecular Devices. (6) Assay and imaging were performed using the FLIPR Penta instrument (Molecular Devices) and the ImageXpress micro confocal system (Molecular Devices).

Results

Formation and characterization of 3D neural organoids

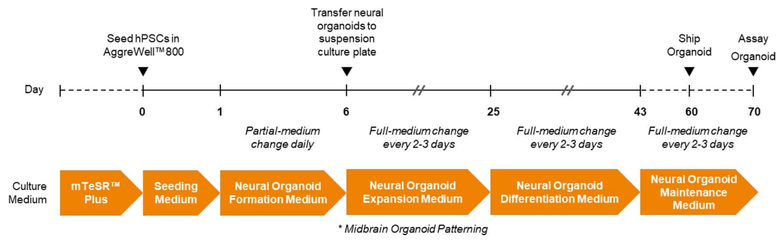


Figure 2. The STEMdiff Dorsal Forebrain Organoid Kit (STEMCELL Technologies Catalog # 08620) and STEMdiff™ Midbrain Organoid Differentiation Kit (STEMCELL Technologies Catalog #100-1096) were used to generate dorsal forebrain organoids or midbrain organoids, respectively, according to manufacturer protocols using the human pluripotent stem cell (hPSCs) line SCTi003-A (Catalog # 200-0511). hPSCs maintained in mTeSR™ Plus were dissociated and seeded at a density of 3 x 10^6 cells/well in Seeding Medium (Formation Medium + 10 M rho-kinase inhibitor (ROCKi)) in AggreWell™800 plates. Cultures were fed daily with Formation Medium. After 6 days, organoids were transferred to a 6-well plate in Expansion Medium and maintained on an orbital shaker at 70 RPM (INFORS HT Celltron). Organoids were fed every 2–3 days with an Expansion Medium until day 25, at which point organoids were fed with a Differentiation Medium until day 43. From day 43 onward, organoids were fed every 2–3 days with Maintenance Medium. Neural Organoids were shipped at day 60 and fed with Maintenance media with feeds every 2–3 days.

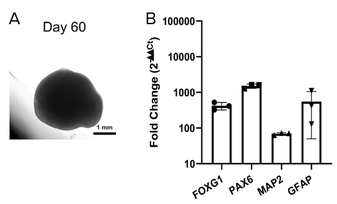


Figure 3. (A) Representative brightfield morphology of a day 60 dorsal forebrain organoids generated using the STEMdiff Dorsal Forebrain Organoid Kit (B) RT-qPCR analysis of day 60 dorsal forebrain organoids. Results show upregulation of forebrain specification marker FOXG1, progenitor marker PAX6, neuronal marker MAP2, and glial marker GFAP. Results displayed as Log10(Fold Change $2^{-\Delta\Delta Ct}$ Method) (Average ± SEM n = 3 organoids) Data is normalized to TBP and compared to undifferentiated hPSC control.

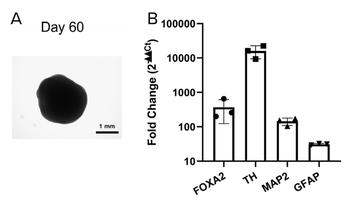


Figure 4. (A) Representative brightfield morphology of a day 60 midbrain organoids generated using the STEMdiff Midbrain Organoid Kit (B) RT-qPCR analysis of day 60 midbrain organoids. Results show upregulation of midbrain progenitor marker FOXA2, dopaminergic neuron marker TH, neuronal marker MAP2, and glial marker GFAP. Results displayed as Log10(Fold Change $2^{-\Delta\Delta Ct}$ Method) (Average ± SEM n = 3 organoids) Data is normalized to TBP and compared to undifferentiated hPSC control.

Compound testing using organoids

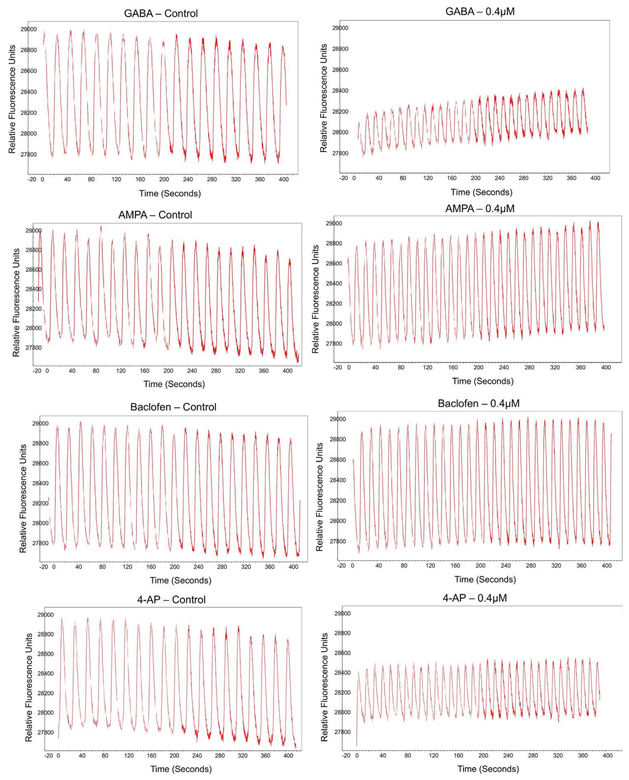
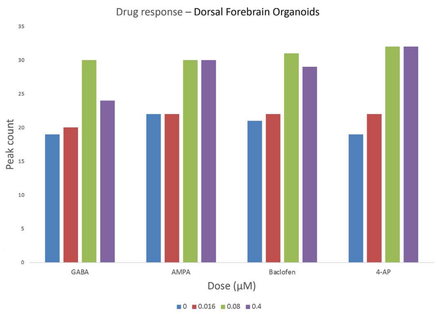
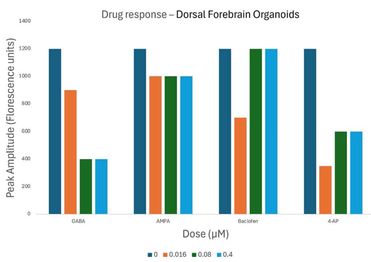


Figure 5. Baseline calcium oscillations vs. drug response of dorsal forebrain organoids. Ca^{2+} waveforms were recorded by kinetic calcium imaging using the FLIPR instrument and analyzed using PeakPro2 software. Organoids had consistent baseline activity in the absence of compound additions (left panel). After the addition of indicated compounds (4 dose concentrations, 0.4 µM presented here), the pattern was modified as indicated on the right panel.

Results

Compound testing using organoids





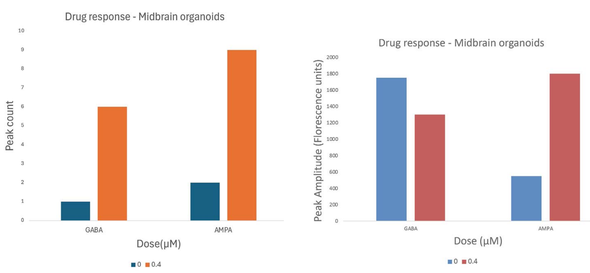


Figure 6. Bar graphs represent peak counts and amplitudes in controls and after the addition of indicated concentrations of compounds. We were able to observe expected phenotypic responses to 4-AP and AMPA causing an increase in the frequency of calcium oscillations. Interestingly, GABA and Baclofen were causing some frequency increase, while the amplitude of oscillations decreased.

Automated monitoring and media exchanges with CellXpress.ai Automated Cell Culture System

Since the continuous culture of neural organoids is labor-intensive, we developed protocols for automated imaging and media exchanges using the CellXpress.ai™ Automated Cell Culture System. A subset of the organoids was placed into the CellXpress.ai system and cultured in 96-well format for 10 days with imaging and media exchange every 24h. Images were taken with transmitted light (see below) and media exchanges were done by a liquid handler replacing 2/3 media volumes.

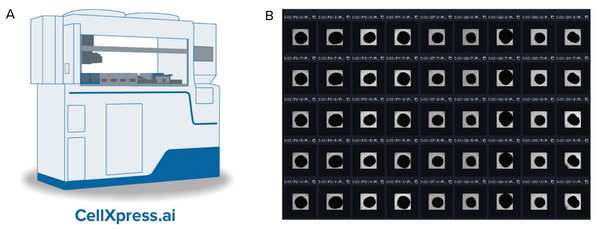


Figure 7. A. Schematic diagram of CellXpress.ai Automated Cell Culture system. B. Images of the subset of 96-well plates with cultured neural organoids.

Summary

- In vitro 3D neural organoids, generated using human pluripotent stem cells, present a useful cell-based assay for assessment of neurotoxicity and neuro-active effects of various neuromodulators.
- This assay platform shows promise for the evaluation of compound effects and early detection of neurotoxicity in vitro.
- Analysis of kinetic calcium imaging demonstrates spontaneous activity of neural organoids and provides read-outs for the functional neural activity and can be used for the evaluation of phenotypic changes and compound effects.
- Organoid culture is amenable to automation which would allow scaling up and increased throughput of the assay.