

Modeling neuronal diseases with automatically generated iPSC-derived dorsal forebrain 3D organoids

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Introduction

Induced Pluripotent Stem Cell (iPSC)-derived 3D organoids is a rapidly developing technology with great potential for understanding brain physiology and neuronal disease modeling.

Formation of neural organoids from iPSCs is a complex and lengthy process, making it difficult for compound screening. We developed an automated workflow for the development and analysis of neural organoids using the CellXpress.ai® Automated Cell Culture System, the FLIPR® Penta High-Throughput Cellular Screening System, and the ImageXpress® HCS.ai High-Content Screening System instruments. The CellXpress.ai system has liquid handling, imager, automated incubator as well as process controlling scheduling software. The device enables automation of most repetitive tasks, including organoid culture with periodic media exchanges, agitation, and organoid development monitoring by periodic imaging. Morphological characterization of developing organoids was done by confocal imaging using neural markers TUJ1, MAP2 and GAFP.

Functional characterization of spontaneous neural activity of organoids was measured by kinetic recording and analysis of calcium ion oscillations. Calcium oscillations were recorded on the FLIPR Penta system or the ImageXpress HCS.ai system instruments that measured fast kinetic changes in calcium signal after loading organoids with Calcium 6 dye.

After 80 days in culture all organoids demonstrated either synchronous or asynchronous spontaneous calcium oscillation activity. Synchronous oscillation patterns were analyzed using the FLIPR Penta system's Peak Pro software for multiple parameters including peak count, amplitude, and peak width. Organoids that showed asynchronous activities were analyzed by imaging (the ImageXpress HCS.ai system) measuring modulations of fluorescent intensities and standard deviation from selected regions of interest covering individual cells and clusters.

For pharmacological characterization of organoids using the FLIPR Penta system we selected organoids that demonstrated synchronous oscillation activities (40–50% of organoids) because of robust analysis of well-defined oscillation patterns. Several benchmarking compounds were used to show functional responses. AMPA and 4-aminopyridine (AP) addition resulted in dose-dependent increase of frequency of calcium oscillations, while GABA caused a decrease in oscillation frequencies. Bicuculline, NMDA, memantine also caused expected changes in oscillation patterns.

We have also used neural organoids for modeling important neuronal diseases such as epilepsy and traumatic brain injury (TBI). For modeling epilepsy condition, we stimulated organoids with 4-AP and after 30 min of exposure observed increase in the frequency of oscillation. Then we applied valproic acid that attenuated oscillation activity. TBI was mimicked by applying pressure pulses (1 to 3 atm) to organoids using a PuMA System microfluidic device. We demonstrated that exposure to TBI-events significantly disrupted neuronal activities as measured by decrease in synchronous calcium ion oscillations. This activity could be recovered by addition of neuroactive compound 4-AP. In addition, TBI-events were observed to cause a modest decrease in cell viability in neural organoids after a 48hr period.

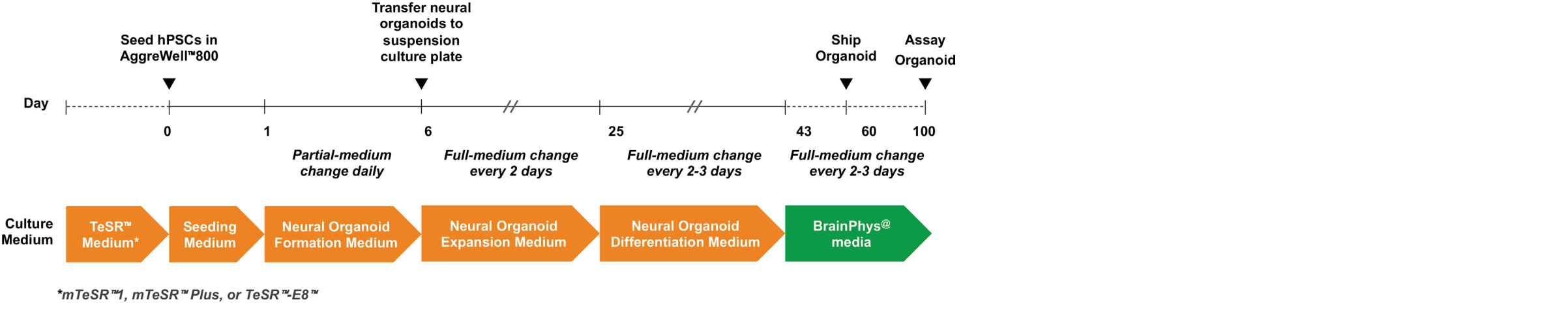
Taken together, this biological system of iPSC-derived 3D neural organoids paired with process automation and detailed analysis of calcium oscillations demonstrates a promising tool for disease modeling and compound testing.

Instruments



Methods

Organoid culture. KYOU-DXR0109B Human Induced Pluripotent Stem (IPS) Cells [2018] (ATCC ACS-1023) were maintained in mTeSR™ Plus, dissociated, and seeded at a density of 3×10^6 cells/well in Seeding Medium (Formation Medium supplemented with $10 \mu\text{M}$ rho-kinase inhibitor (ROCKi)) in AggreWell®800 plates. Dorsal forebrain organoids were generated using the STEMdiff Dorsal Forebrain Organoid Kit (STEMCELL Technologies, Catalog #08620) that includes several media components. Cell aggregates were first fed daily with Formation Medium. After 6 days, organoids were transferred to 6-well plates in Expansion Medium and maintained in the CellXpress.ai system incubator with periodic feeding and imaging. Organoids were fed every 2 days with Expansion Medium until day 25, followed by Differentiation Medium until day 43. From day 43 onward, Maintenance Medium was used every 2–3 days. After 50 days in culture, organoids were maintained in BrainPhys media. The flowchart below represents the STEMdiff Dorsal Forebrain Organoid protocol.



Automation of brain organoid culture. We have automated repetitive steps for organoid culture including media exchanges and periodic imaging, using the CellXpress.ai automated cell culture system. The CellXpress.ai system contains a liquid handling component, an automated incubator, an embedded imager, also automated tools providing transport plates from the incubator to the imager or liquid handler and back. The system can perform the tasks for cell culture, including cell plating, media exchanges, passaging, and monitoring by imaging.

Functional characterization of neural activity by calcium imaging. On the day of assay, spheroids were loaded with 2X conc. of FLIPR Calcium 6 dye indicator (Molecular Devices) and incubated for 2 h. We used a high-speed EMCCD camera on the FLIPR Penta system (Mol Dev) 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 (Mol Dev). Organoids exhibiting asynchronous activity were analyzed using imaging to measure fluorescence intensity modulation, intensity standard deviation of intensity, and synchronicity at the level of individual active cells.

Morphological characterization was done by high-content imaging on the ImageXpress HCS.ai system (Molecular Devices) and was used to capture 3D structures of organoids stained with fluorescently labeled antibodies against SOX2, TuJ1, MAP2, and GAFP. GFAP (BD Biosciences, Cat# 560298, AF488) at $1:500$ ($0.4 \mu\text{L}/200 \mu\text{L}$, $\sim 0.02 \mu\text{M}$). TuJ1 (Bll-Tubulin) (BD Biosciences, Cat# 560339, AF555) at $1:500$ ($\sim 0.02 \mu\text{M}$) labeled neuronal cytoskeleton. MAP2 (Abcam, Cat# ab303465, AF568) at $1:500$ ($\sim 0.02 \mu\text{M}$) marked dendrites. SOX2 (Abcam, Cat# ab279687, AF647, red in this image) at $1:200$ ($1.0 \mu\text{L}/200 \mu\text{L}$, $\sim 0.05 \mu\text{M}$) identified progenitor cells and was predominantly expressed in the undifferentiated organoids. Hoechst 33342 – A nuclear marker (here shown in blue). Antibodies were diluted in PBS with 0.1% Triton X-100 and 1% BSA, incubated overnight at 4°C , and washed twice with PBS before imaging.



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Results

Development iPSC-derived 3D Dorsal forebrain organoids and morphological characterization

Dorsal forebrain organoids were developed from iPSC cells (ATCC ACS-1023 A*) using the STEMdiff Dorsal Forebrain Organoid Kit Catalog #08620 from STEMCELL Technologies Inc. The process contains several development steps: iPSC maintenance, seeding cells into AggreWell V bottom plates, formation of spheroids (days 0–6), then transfer spheroids into 6-well plates and continuous culture with periodic automated media exchanges during the expansion period (days 6–25), differentiation period (days 26–43), then maintenance period (days 43–60+). After 60 days in culture, organoids were maintained in BrainPhys media. We used CX.ai for automation of media exchanges and monitoring organoids starting from differentiation culture period.

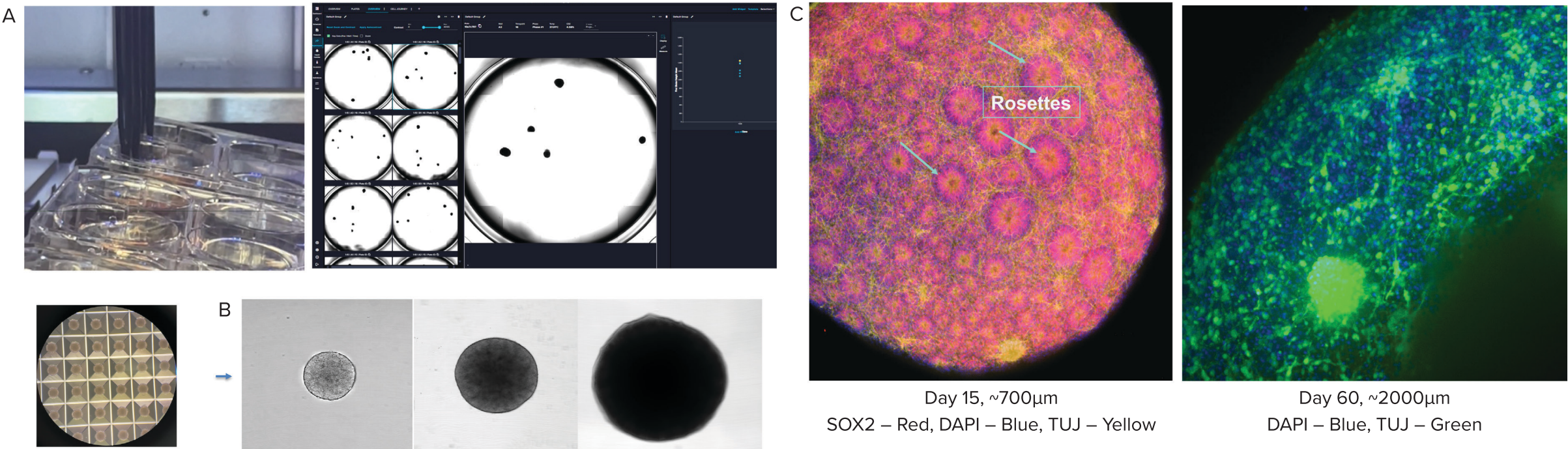


Figure 1. The STEMdiff Dorsal Forebrain Organoid Kit (STEMCELL Technologies) was used to generate dorsal forebrain organoids starting from the iPSCs line from ATCC, ACS-1023. iPSCs maintained in mTeSR™ Plus were dissociated and seeded manually into AggreWell®800 plates and cultured for 6 days with Formation Medium. After 6 days, organoids were transferred to a 6-well plates in Expansion Medium and maintained in the CellXpress.ai system incubator with periodic media exchanges and imaging (A, B). C. Imaging was performed using the ImageXpress HCS.ai system at various magnifications. Undifferentiated organoids (15 days) were showing rosettes with predominantly Sox-2 signal. SOX2 marker identified progenitor cells and was predominantly expressed in the undifferentiated organoid. Differentiated organoids, 60+ days old, showed a very minimal SOX2 signal, but were positive for neuron and astrocyte markers. Dorsal forebrain organoids were stained using four markers. GFAP marked astrocytes and was absent in the undifferentiated organoid (left) but present in the differentiated organoid (right). TuJ1 (Bll-Tubulin) and MAP2 labeled neurons were observed in both immature and mature organoids.

Recording and analysis calcium oscillations with imaging

Observed patterns of calcium oscillation activity: Functional characterization was done at different time points after 75 days of culture. Forebrain organoids exhibit spontaneous calcium oscillations after 60+ days in culture. Calcium oscillations can be asynchronous, more often observed earlier in development, or synchronous, extending oscillations for an entire organoid. Synchronous oscillation activities were readily detectable using the FLIPR Penta system and peaks were counted using PeakPro software. Asynchronous calcium oscillations were not detectable by the FLIPR Penta system but can be recorded by imaging (time-lapse recording).

We describe below how imaging and image analysis can be used for evaluation of calcium oscillation activity with either asynchronous or synchronous oscillations. While synchronous activities can be visualized by fluctuations of fluorescent intensity (A,B), standard deviations of fluorescent intensity, from entire organoid region or smaller regions defining cell clusters or individual cells. Modulations of standard deviations of fluorescent intensity versus time was used for evaluation of asynchronous oscillations. Synchronicity score was used for additional evaluation.

Analysis of Calcium Oscillation by Imaging methods: Segmentation – Neurite outgrowth was detected in the calcium dye channel (FITC) using the built-in analysis algorithm. Cell bodies were better detected using CellPose algorithm. During segmentation of both soma and neurite outgrowth, features related to morphology and fluorescent intensity were extracted.

To calculate the synchronization score of neuronal firing (Figure 2C), image registration was performed to correct for minor positional shifts in time-lapse images.

After correcting shifts, masks within $3 \mu\text{m}$ were grouped as the same cell body. The mean intensity of each cell body across time points was computed and fitted using the HuberRegressor model from the scikit-learn package—a robust linear regression method that effectively handles outliers. This procedure was intended to define a baseline threshold, beyond which signals will be labeled as firing events. Intensity values exceeding 6% above the fitted baseline were annotated as spikes. The discrete spike times from all neurons were processed in Spike_sync_profile function in the PySpike package to calculate the synchronization scores among the firing events.

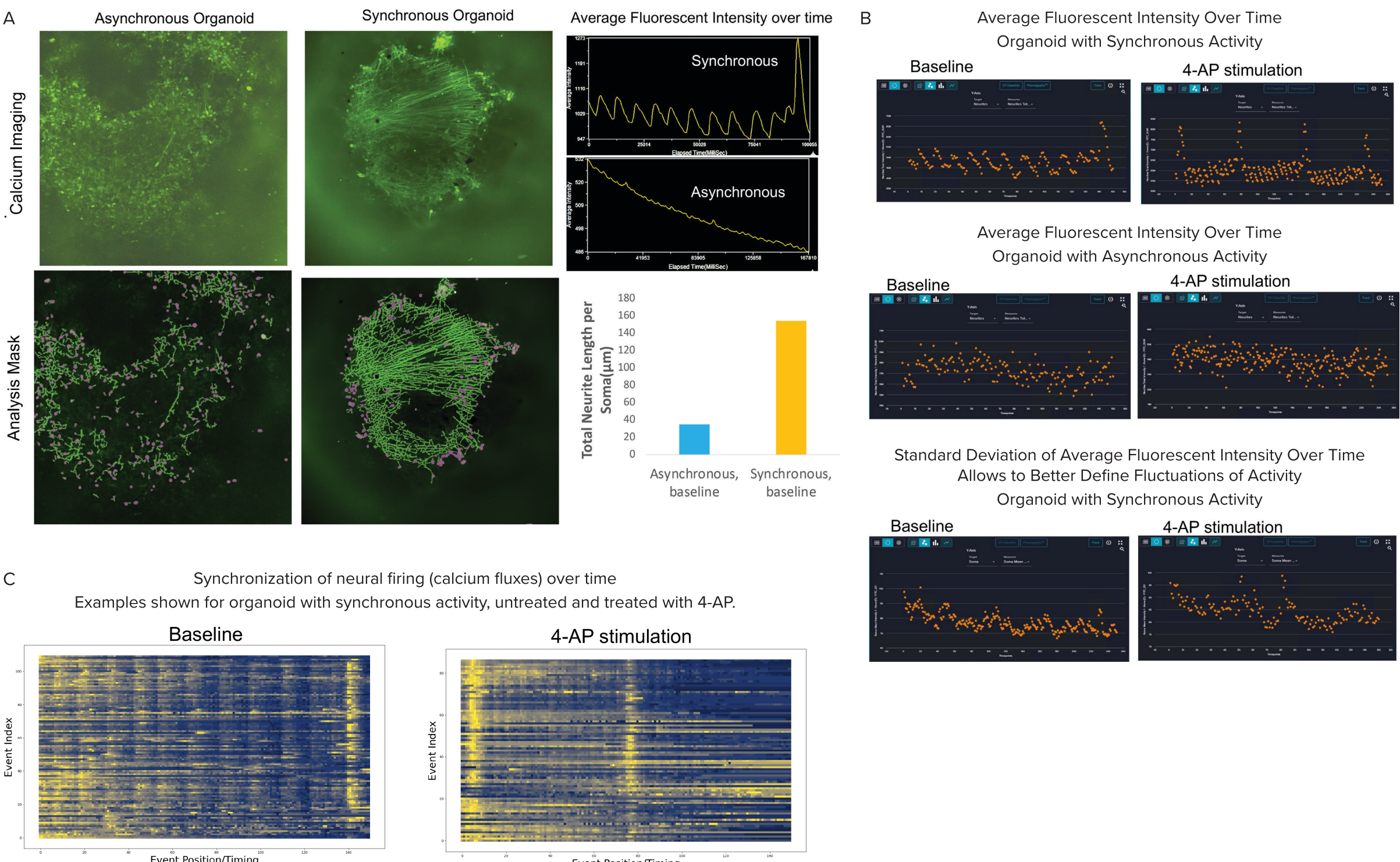


Figure 2. Calcium oscillations were recorded after loading organoids with Calcium 6 dye for 1.5h. A, B. Images were acquired at 10x magnification, with the confocal option and time-lapses every 0.4 seconds. The graphs show the time course of the fluorescence intensity oscillations over time within the selected region. Alternatively, fluctuations of standard deviations of fluorescent intensity versus time was used for evaluation of asynchronous oscillations. This method allows the detection and visualization of asynchronous neuronal firing. Calcium imaging recording: time-lapse, exposure 100msec, 0.4 sec interval, focus on first image only, 150–200 seconds recording.

Notably, synchronous oscillation activity apparently correlated with the appearance of long strings of structures that going across organoid, that likely represent extended neurites or nerve fibers. The neurite length in presented images was calculated using neurite outgrowth analysis. Images and analysis masks presented in Figure 2A.

Characterization of brain organoid activity and compound responses with the FLIPR Penta system

Synchronous calcium oscillations were recorded with the FLIPR Penta system. After day 75, approximately 40–50% of cultured organoids exhibit spontaneous calcium oscillation activity. Synchronous activity was typically correlated with the presence of long neurite extensions, visible with Calcium 6 dye staining (Figure 2 & 3). Oscillation patterns from different organoids show variations in peak frequencies and amplitudes, as well as patterns and regularities. However, expected compound responses were observed for benchmarking neuro-active compounds, made feasible using iPSC-derived organoids for compound testing and disease modeling.

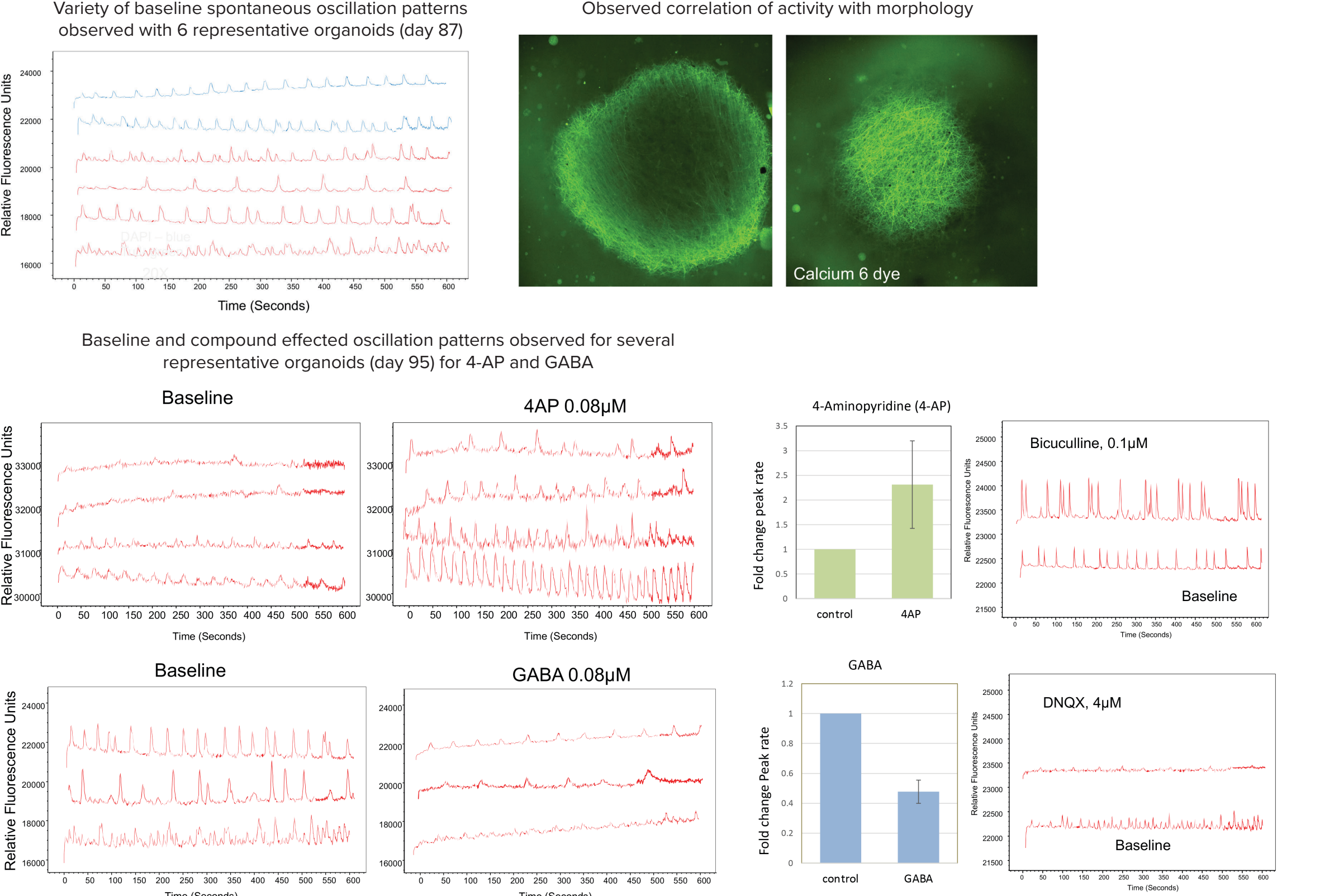


Figure 3. Calcium oscillations were recorded after loading organoids with Calcium 6 dye for 1.5h. Recordings of Calcium oscillations were done on the FLIPR Penta instrument. Kinetic readings were taken every 0.4 seconds, for 10min. Readings were taken after treatment with indicated compounds for 30 min. The graphs above show responses to the indicated benchmarking drugs.

Modeling traumatic brain injury (TBI) with Pu-MA System

Brain organoids were incubated with Calcium 6 dye for 2 hours and then transferred into Pu-MA System flowchips for TBI experiments. Results are shown in Figure 4. Neuronal firing activity of three brain organoids in flowchips was measured on the FLIPR Penta system for baseline behavior (Pre-TBI). They were then subjected to simulated TBI-events using Pu-MA System and re-measured on the FLIPR Penta (1st TBI). A clear decrease in synchronous neuronal firing was found in the organoids. The organoids were then treated with 0.4 mM 4-aminopyridine (4-AP) for 15 min and measured again on the FLIPR Penta system (4-AP Treat). The 4-AP compound was able to recover synchronous activity in all three organoids. The organoids were then subjected to a second round of simulated TBI-events (2nd TBI) which once again disrupted the neuronal firing activity. The neuronal firing peaks were manually counted and the average and standard deviation of these results for the three organoids are shown in Figure 4 Left. These results show the possibility of using brain organoids as an ex vivo model to understand TBI pathology and assay for new therapeutics in a physiologically relevant manner.

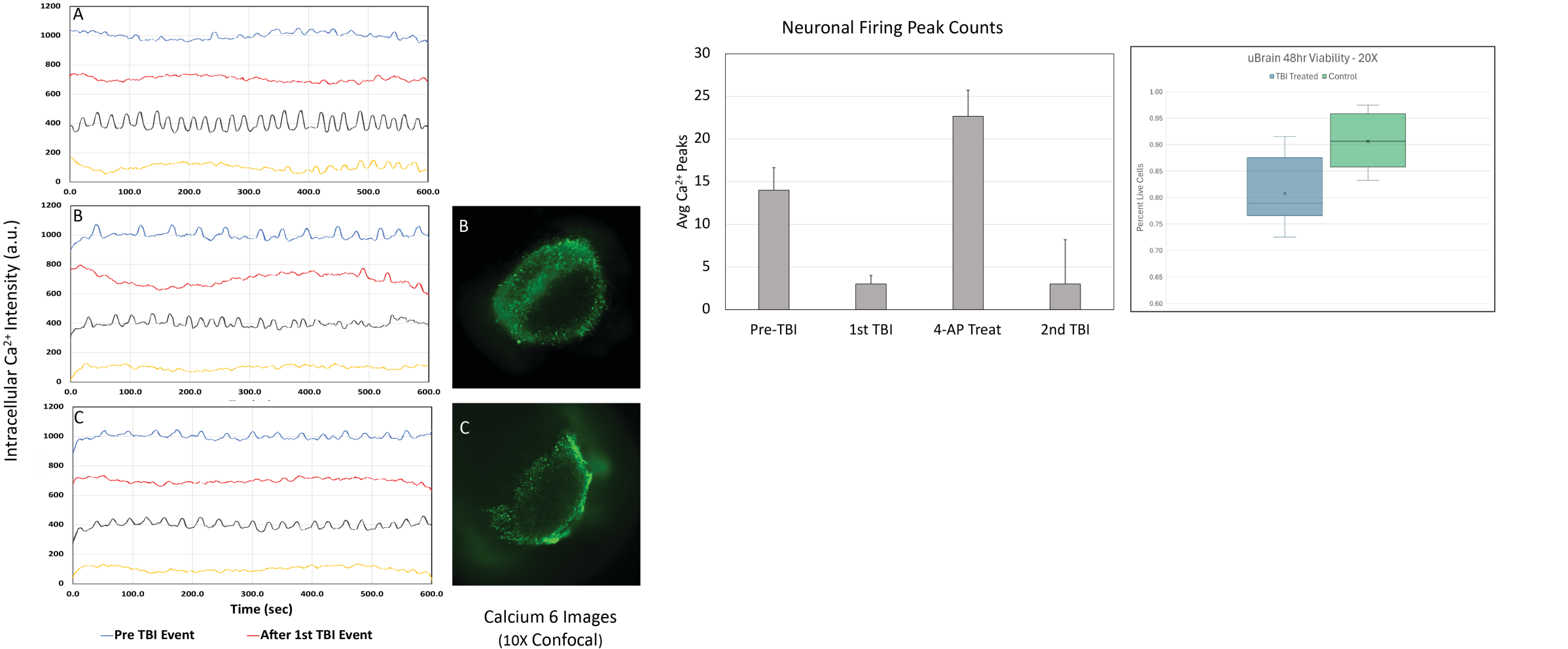


Figure 4. Left: the FLIPR Penta system Ca^{2+} flux measurements and the corresponding fluorescence images of three different brain organoids in Pu-MA System flowchips incubated with Calcium 6 dye (A,B,C). The FLIPR Penta system measurements were done repeatedly 4 times over a "1 hour period under different conditions as described above. The FLIPR Penta system data was smoothed & flattened, and traces were offset for display. Brain organoid images are a single confocal slice of organoids in flowchips. Middle: Analysis results showing average and standard deviation of neuronal firing peaks. Right: Viability staining was done with EtHD that marks damaged cells, and viability was assessed by confocal imaging. Modest decrease in viability (normalized to control) was observed 48 hours after TBI simulation event.

Modeling epilepsy by stimulation with 4-AP

Inhibition of stimulated oscillation activity with Valproic acid

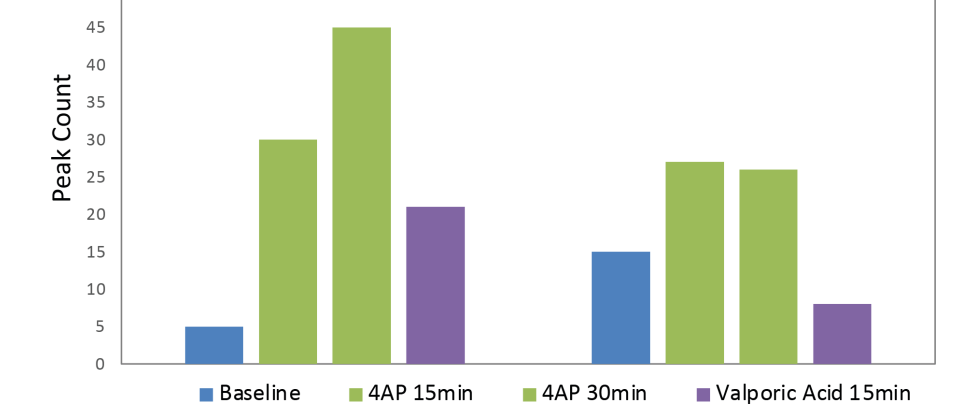


Figure 5. Modeling epilepsy. Baseline organoids activity was recorded by the FLIPR Penta system (blue bars), then organoids were stimulated with $0.08 \mu\text{M}$ 4-AP and recorded after 15 and 30 min after stimulation (green bars). After 30 min $1 \mu\text{M}$ of Valproic acid was added which attenuated oscillation activity (purple bars).

Summary

- We present here a partially automated method for growing forebrain organoids and the morphological and functional characterization of iPSC-derived forebrain organoids.
- Characterization by imaging demonstrated the presence of neurons and astrocytes, as shown by TuJ1 and GFAP staining, and progenitor cells positive for SOX2, which decreased during continuous culture.
- Functional characterization of forebrain organoids was done by recording calcium oscillation activities using the ImageXpress HCS.ai system or the FLIPR Penta system. Expected responses to benchmarking compounds were shown.
- iPSC-derived organoids offer a feasible system for disease modeling as demonstrated by the presence of functional responses to perturbations mimicking TBI or epilepsy conditions.

