Functional characterization of healthy and disease-related 3D neurospheres assembled using human iPSC-derived neurons and astrocytes

Oksana Sirenko, Carole Crittenden, Krishna Macha, Jeffrey Tang, Angeline Lim I Molecular Devices, LLC
Rebecca Fiene, Scott Schachtele, Coby Carlson I FUJIFILM Cellular Dynamics, Inc

Introduction

Neural 3D organoids from human-induced pluripotent stem cells (iPSC) is a rapidly developing technology with great potential for understanding brain development and neuronal diseases. A promising parallel approach is to assemble similar structures as 3D spheroids or "neurospheres" by using defined combinations of fully differentiated human iPSC-derived cells in tri-culture, including glutamatergic neurons, GABAergic neurons, and astrocytes. We have previously demonstrated this efficient and modular approach using CRISPR genetically modified GABAergic neurons (SCN1A KO or KCNT1 P924L mutation) and their isogenic pairs as matched controls to model epilepsy. In this study, we chose to model Alzheimer's Disease (AD) by incorporating allelic variants of the ApoE gene (2/2, 3/4, and 4/4) to create disease-specific "neurospheres".

3D spheroids were formed by combining 20–25,000 cells in ultra-low attachment (ULA) plates (from various vendors). We tracked spheroid morphology over time by live cell imaging. Spheroid diameters ranged from 400–500 µm. Microtissues were also analyzed by confocal fluorescence imaging for cell organization and expression of various neural markers, including TUJ1 and GFAP. Functional performance was tested via calcium oscillation assay and was run on a FLIPR® Penta High-Throughput Cellular Screening System (Molecular Devices) capable of fast kinetic recordings. Importantly, the calcium-sensitive dye used contains a background fluorescence masking technology that enables sensitive detection of neurospheres in 384w format. The calcium oscillation patterns were analyzed for metrics like peak count, amplitude, and width. Different baseline oscillation patterns were observed between control and AD-specific neurospheres, specifically for the ApoE 4/4 mutation. Overall, within each group the calcium kinetics and patterns were highly consistent.

For pharmacological characterization, a panel of select compounds was used to show both the appropriate responses to GABA, AMPA, and NMDA, as well as changes to various neuroactive and neurotoxic substances. Some drugs previously shown to affect AD phenotypes (such as memantine) did decrease calcium peak amplitude and altered other metrics as visualized by ScreenWorks® software and analyzed by Peak Pro 2 (Molecular Devices). Taken together, this biological system of 3D neurospheres assembled from human iPSC-derived cell types paired with high-content imaging and detailed analysis of calcium oscillations demonstrates a promising tool for disease modeling and compound testing.

Methods

3D spheroids were formed using human iPSC-derived cell types (FUJIFILM CDI) including iCell® GlutaNeurons, iCell GABAneurons, and iCell Astrocytes 2.0. Briefly, cryopreserved vials were thawed and mixed in desired ratios (e.g., 90% neurons (~70:30 Gluta: GABA) and 10% astrocytes), and then 25K cells/well in complete BrainPhys™ medium were plated into 384-well ULA plates (either black Corning #4516 with clear bottom or clear Sbio #MS-9384UZ). After 2 days, cells formed compact spheroids and were then maintained with media changes every 2-3 days until at least day 21. On the day of assay, cell 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 instrument (Molecular Devices) to measure the patterns and frequencies of spontaneous calcium waveforms from 3D neurospheres. Baseline recordings were acquired for ≥10 min, and then plates were dosed with drugs for 30–90 min. Peak analysis was accomplished with ScreenWorks Peak Pro 2 software (Molecular Devices), allowing characterization of both primary and secondary peaks, as well as complex calcium oscillation patterns. High-content imaging was done by the automated ImageXpress Micro Confocal Imaging system (Molecular Devices) and was used to capture 3D structures of the spheroids and for viability evaluation.

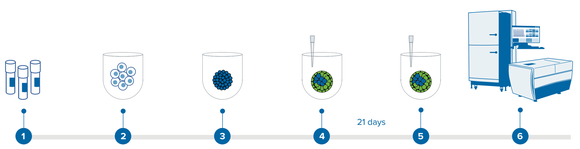


Figure 1. Schematic diagram of the process workflow. (1) iPSC-derived cells are thawed and combined in ratios of approx. 90% neurons and 10% astrocytes into (2) ULA 3D spheroid-forming plates. (3) Neurospheres form within 24-48 h and (4)-(5) are maintained in culture with regular media for >21 days. (6) Cells are assayed on the FLIPR Penta or imaging instruments.

Product	Donor	Cat. #
iCell GlutaNeurons	01279	C1033
iCell GABANeurons	01279	C1008
iCell GABANeurons	01434	C1012
iCell Astrocytes 2.0	01279	C1249
iCell Astrocytes	01434	C1037
iCell GABANeurons ApoE 2/2	01434	C1176
iCell GABANeurons ApoE 4/4	01434	C1175

iPSC cell type	Gluta	GABA	ASC 2.0	Total
Cell ratios	90%		10%	100%
Total # of cells	22,500		2,500	25,000
Ratio of neurons	70%	30%	XX	XX
Based on # of cells	15,750	6,750	2,500	25,000
Corrected for % of Gluta	21,000	1,500	2,500	25,000

Genetic background of Alzheimer's disease

The estimate of developing AD by age 85 is ~65% in people with two copies of the ApoE4 allele as compared to only 10% in people with both wild type ApoE3 alleles. This striking difference identifies apolipoprotein E4 (ApoE4) as a major genetic risk factor for AD and highlights its importance in disease pathogenesis. ApoE is a lipid transport protein and the human ApoE3 and ApoE4 isoforms differ only at one amino acid residue (position 112). It is unclear how ApoE4 perturbs the intracellular lipid state, but there are reports that ApoE 4/4 can disrupt the cellular lipidomes of human iPSC-derived astrocytes. ApoE mutations can also be engineered into iPSC-derived neurons.

Results

Formation and characterization of 3D spheroids

In this panel "iCell Neurospheres" were composed of both glutamatergic and GABAergic neurons together with iPSC-derived astrocytes. Cells were maintained in co-culture with complete BrainPhys™ medium until day of assay as indicated.

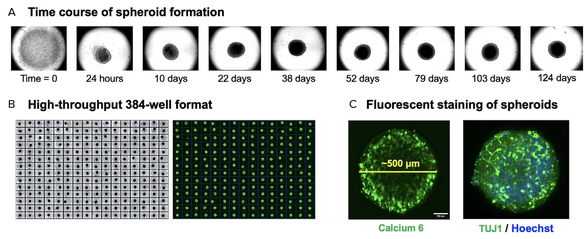
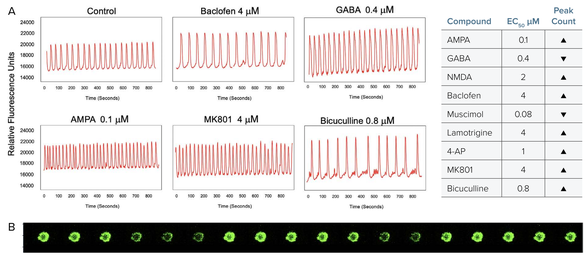


Figure 2. Formation of iCell Neurospheres. (A) 3D spheroids can be formed in various ULA plate types, incl. the faCellitate BIOFLOAT™ 384-well option shown here. Cell culture is straightforward, and cells can survive for many days. (B) Self assembly of iPSC-derived neurospheres is done in high-throughput format (Corning 4516 384w B/C plate) and results in uniform spheroid formation (only one 3D structure per well); (C) Spheroids can be stained with calcium indicator dyes for function testing or with antibodies for neural-specific markers, such as TUJ1.



Compound	EC_{50} µM	Peak Count
AMPA	0.1	▲
GABA	0.4	▼
NMDA	2	▲
Baclofen	4	▲
Muscimol	0.08	▼
Lamotrigine	4	▲
4-AP	1	▲
MK801	4	▲
Bicuculline	0.8	▲

Figure 3. Functional testing of iCell Neurospheres. (A) Ca^{2+} oscillations of 3D neurospheres were recorded by kinetic imaging using a FLIPR Penta instrument. Traces (in red) represent fluctuations in fluorescent intensities recorded with the calcium-sensitive dye Calcium 6 (60 min post-dose). Waveform patterns affected by neuroactive compounds are listed in the table to the right. (B) Representative time-lapse images of 3D spheroids loaded with Cal 6 dye were captured with an interval of 0.4 sec using the ImageXpress Micro Confocal Imaging system.

ApoE GABA neurons for AD disease modeling

Here, we analyzed AD-related phenotypes through FLIPR Penta measurement of calcium oscillations from 3D neurospheres assembled with GABAergic neurons from four different ApoE allelic variants, including 2/2, 3/3 (control), 3/4, and 4/4 (in addition to 70% glutaneurons and 10% astrocytes). Analysis of baseline data is presented below:

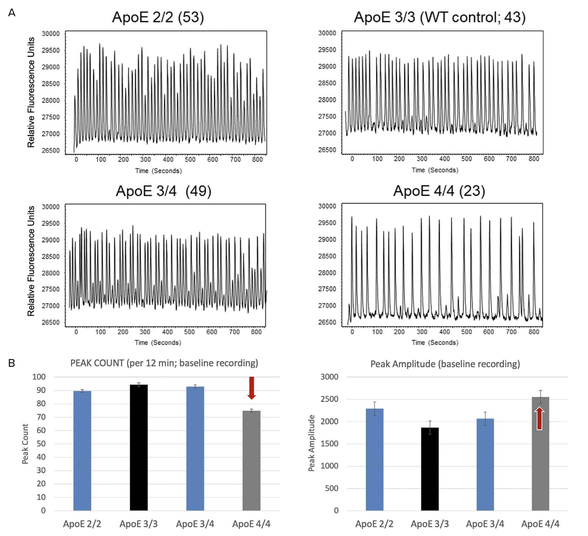


Figure 4. Baseline calcium oscillations of 3D neurospheres are impacted by ApoE status. (A) Ca^{2+} waveforms were measured by kinetic calcium imaging using the FLIPR Penta instrument and analyzed using Peak Pro 2 software. (B) Bar graphs represent changes in peak count readouts for cultures created with appropriate mutations of GABA-neurons.

Results

Compound testing using AD 3D neurospheres

With the ApoE 4/4-containing disease model showing the strongest difference from control cells (ApoE 3/3), we compared the treatment of neurospheres to a select group of neuroactive compounds. This experiment demonstrated that the expected responses were observed with AMPA, baclofen, and GABA.

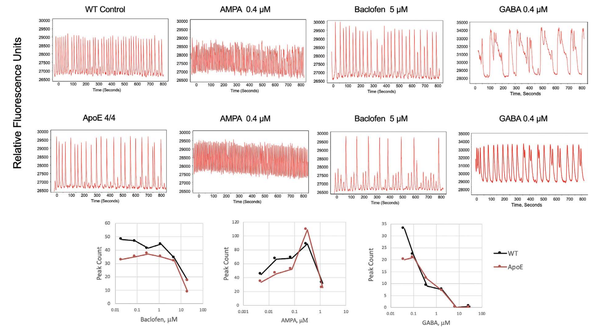


Figure 5. Ca^{2+} oscillations of WT and genetically modified (Gaba-neurons containing ApoE 4/4 mutation) neurospheres were determined by kinetic calcium imaging using the FLIPR Penta instrument and analyzed using Peak Pro 2 software. Neurospheres were treated with compounds for 60 min at different concentrations. Responses to the key neuro-active compounds (AMPA, GABA, Baclofen) were consistent with expected changes in the activity and consistent in the expected concentration-response.

Compounds used for the treatment of AD disorder

Previous work published by Strong et. al. (DOI: 10.1101/2022.05.04.490442) had similarly illustrated the functional deficits caused by the ApoE4 mutation in GABA neurons could be measured in 3D spheroids using the same iPSC-derived cell types. Importantly, they demonstrated that various clinically-approved compounds used to treat the symptoms of AD could reverse the calcium waveform phenotypes generated in vitro.

Memantine is a clinically useful treatment and is believed to be the blockade of current flow through channels of NMDA receptors, which are broadly involved in brain function. In rodents, memantine antagonizes native NMDA receptors with a micromolar potency.

Donepezil is a clinically-approved acetylcholinesterase inhibitor used to increase cortical acetylcholine levels. Symptoms of AD are believed to be related to cholinergic deficit, particularly in the cerebral cortex.

EUK-134 is a synthetic superoxide dismutase/catalase mimetic that is thought to prevent excitotoxic neuronal injury. This compound and other mimetics have also been shown to inhibit beta amyloid plaque production in animal models.

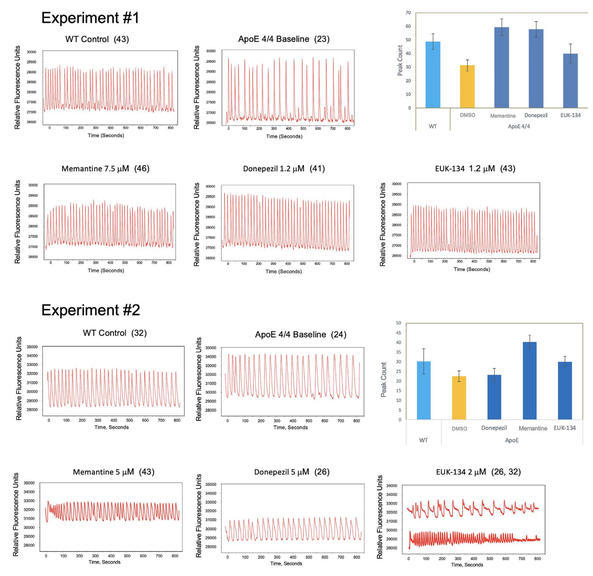


Figure 6. Ca^{2+} oscillations of WT and genetically modified (GABA-neurons contain ApoE 4/4 mutation) neurospheres determined by kinetic calcium imaging using the FLIPR Penta instrument and analyzed using Peak Pro 2 software. Neurospheres were treated with compounds for 45 min and 90 min for two experiments, respectively. Modulations of the activity phenotypes were observed in two independent experiments.

Results

Comparison of ULA plate type

With the increased availability of biologically relevant cell types (i.e., human iPSC-derived) that can be incorporated into 3D neurospheres, there is a concurrent rise in innovation around cell culture technologies (i.e., ULA plates) for the formation of such 3D structures. We compared two popular plates: Corning 4516 (Figure 7. A) which is black with clear bottom vs. S-bio PrimeSurface U-bottom (Figure 7. B) which is clear. Both are user friendly and high performing (Figure 7. C). Here are our observations.

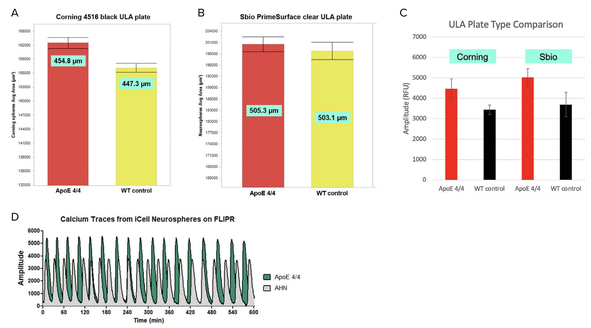
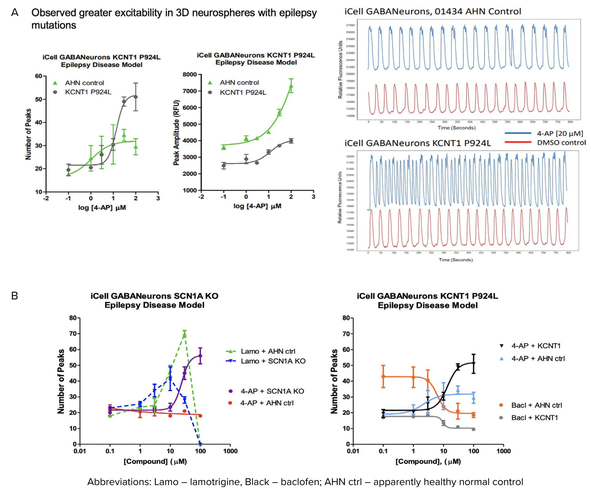


Figure 7. Spheroid sizes (areas from the transmitted light [TL] image) were determined from images taken in TL and compared across different plates. (D) Ca^{2+} oscillations of WT and genetically modified (Gaba-neurons containing ApoE 4/4 mutation) neurospheres were recorded by kinetic calcium imaging using the FLIPR Penta instrument.

Additional disease modeling possible

We can also do epilepsy modeling with this approach. For disease modeling of epilepsy phenotypes, we used two different genetically modified GABAergic neurons (SCN1A KO or KCNT1 P924L mutation) and their isogenic pairs as matched controls. The *SCN1A* gene encodes the alpha subunit of the sodium channel NaV1.1 and it is the major gene implicated in Dravet Syndrome, a severe childhood *epilepsy*. The *KCNT1* gene encodes a potassium channel and the P924L mutation is linked to an early-onset epileptic encephalopathy.



Abbreviations: Lamo – lamotrigine, Black – baclofen; AHN ctrl – apparently healthy normal control

Figure 8A. Increased sensitivity to compounds that cause hyperexcitability (4-AP) was observed in 3D spheroids made with GABAergic neurons that carry the indicated epilepsy mutations (KCNT1 P924L) as compared to the AHN isogenic matched controls. **8B.** Ca^{2+} oscillations of WT and genetically modified (GABA-neurons containing mutations) neuro spheres were determined by kinetic calcium imaging using FLIPR Penta instrument.

Summary

- In vitro 3D neural organoids, generated using terminally differentiated iPSC-derived neural cells, present a useful cell–based assay for assessment of neurotoxicity, neuro-active effects of various neuromodulators, and disease modeling.
- This assay platform shows promise for the evaluation of compound effects and early detection of neurotoxicity in vitro due to its ease of use, consistency across wells and assay plates, simplicity of data analysis, and ultimate biological relevance.
- Analysis of kinetic calcium imaging provides reliable and accurate read-outs for the functional neural activity and can be used for evaluation of phenotypic changes and compound effects.
- Decreased excitability was observed for organoids formed with ApoE 4/4 mutants of GABA-neurons which was reversed with drugs used for treatment of AD disorder.
- Moderately increased excitability was observed for mutated phenotypes, showing elevated responses to stimulating agents.
- This biological system of 3D neurospheres paired with a detailed analysis of calcium oscillations demonstrates a promising and informative tool for disease modeling and phenotypic functional evaluation that could be used for compound testing.