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

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Introduction

Neural 3D spheroids are a rapidly developing technology with great potential for understanding brain development and neuronal diseases. They provide a more advanced and biologically relevant system for basic research and high-throughput drug discovery, including compound profiling and toxicity testing. Here, we describe methods for assembling human iPSC-derived cell types, including glutamatergic neurons, GABAergic neurons, and astrocytes into 3D neurospheres.

For disease modelling 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.

We monitored the formation, morphology, and functional activity (Ca^{2+} oscillations) of the neurospheres after 3 weeks in culture. The microtissues were also analyzed by confocal fluorescence imaging for cell organization and expression of neuronal markers (TUJ1) and astrocytes (GFAP). Cellular and spheroid morphology was characterized by using high-content imaging. The calcium assay was performed on a FLIPR Penta instrument capable of fast kinetic recordings using a calcium-sensitive dye and oscillation patterns were analyzed for peak frequency, amplitude, width, & spacing. Different baseline oscillation patterns were observed between control and disease neurospheres, however within each group calcium kinetics and patterns were highly consistent. For pharmacological characterization of the control and diseased phenotypes, we used a panel of 14 compounds, including selected molecules that affect GABA, AMPA, NMDA, sodium and potassium channels, dopamine receptors, as well as select neuroactive and neurotoxic substances. The functional responses demonstrated the predicted effects based on mode of action, consistent across control and disease model 3D neurospheres. Notably, moderately increased excitability was observed for mutated phenotypes, showing in the baseline pattern, also in the elevated responses to stimulating agents. This biological system of 3D neurospheres 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 from FUJIFILM Cellular Dynamics, 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 spheroid plates (Corning). After 2 days, cells formed compact spheroids and were then maintained until at least day 21. On the day of assay, cell spheroids were loaded with 2X concentration of Calcium 6 dye (Molecular Devices), incubated for 2 hours, then dosed with the indicated compounds. After drug exposure for 30 min and 60 min, calcium oscillations were recorded for 10 min using FLIPR Penta instrument (Molecular Devices). High content imaging was done by automated imager instrument (IXM-C, Molecular Devices).

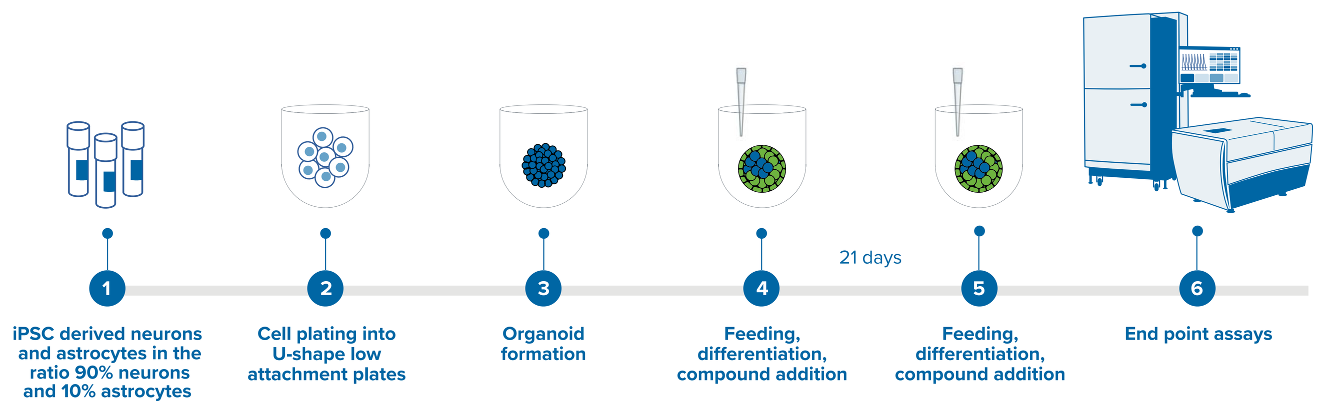


Figure 1. Schematic diagram of the process workflow.

- We used a high-speed EMCCD camera on the FLIPR Penta cellular screening system to measure the patterns and frequencies of the Ca^{2+} oscillations of 3D neurospheres through changes in intracellular Ca^{2+} levels with the Calcium 6 dye. The instrument equipped with Screen Works PeakPro2 peak analysis software allowing analysis and characterization of the primary and secondary peaks and complex oscillation patterns.
- ImageXpress Micro Confocal High-Content Imaging System was used to capture the 3D structures of the spheroids and viability evaluation.

Product	Donor	Vendor	Cat. #
iCell GlutaNeurons	01279	FUJIFILM CDI	C1033
iCell GABANeurons	01279	FUJIFILM CDI	C1008
iCell GABANeurons	01434	FUJIFILM CDI	C1012
iCell Astrocytes 2.0	01279	FUJIFILM CDI	C1249
iCell Astrocytes	01434	FUJIFILM CDI	C1037
iCell GABANeurons SCN1A KO	01279	FUJIFILM CDI	C1170
iCell GABANeurons KCNT1 P924L	01434	FUJIFILM CDI	C1173

Results

Recording and analysis of Ca^{2+} oscillation patterns

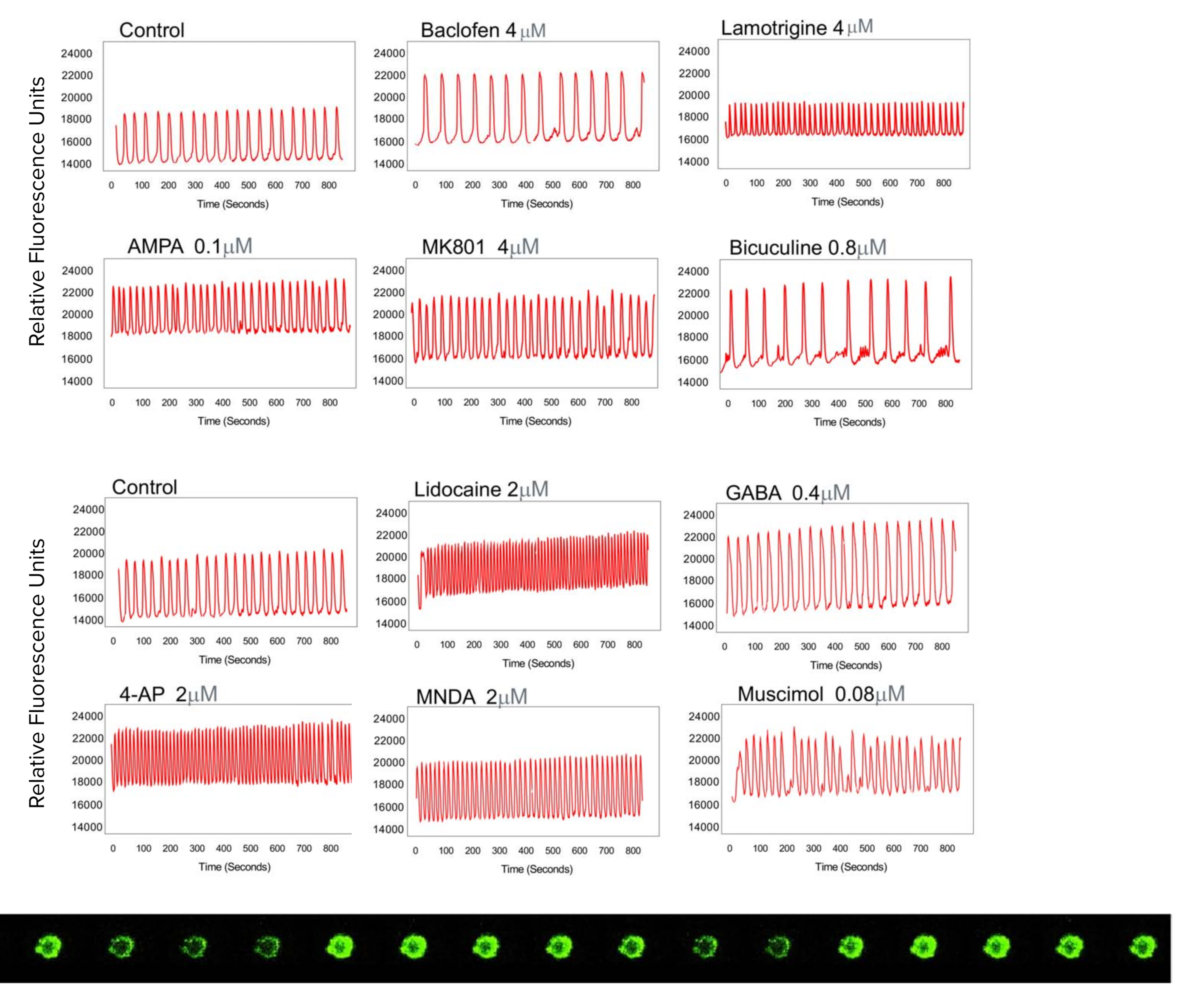


Figure 2. Calcium oscillations of 3D neurospheres were recorded by kinetic calcium imaging using FLIPR instrument. Traces represent fluctuations in fluorescent intensities recorded with calcium sensitive dye. The oscillation patterns affected by neuroactive compounds. The representative time-lapse images recorded by automated images shown below the traces. Images were taken with the interval of 0.4 sec. Images shown below (kinetic recording) were captured by using IXM-C system.

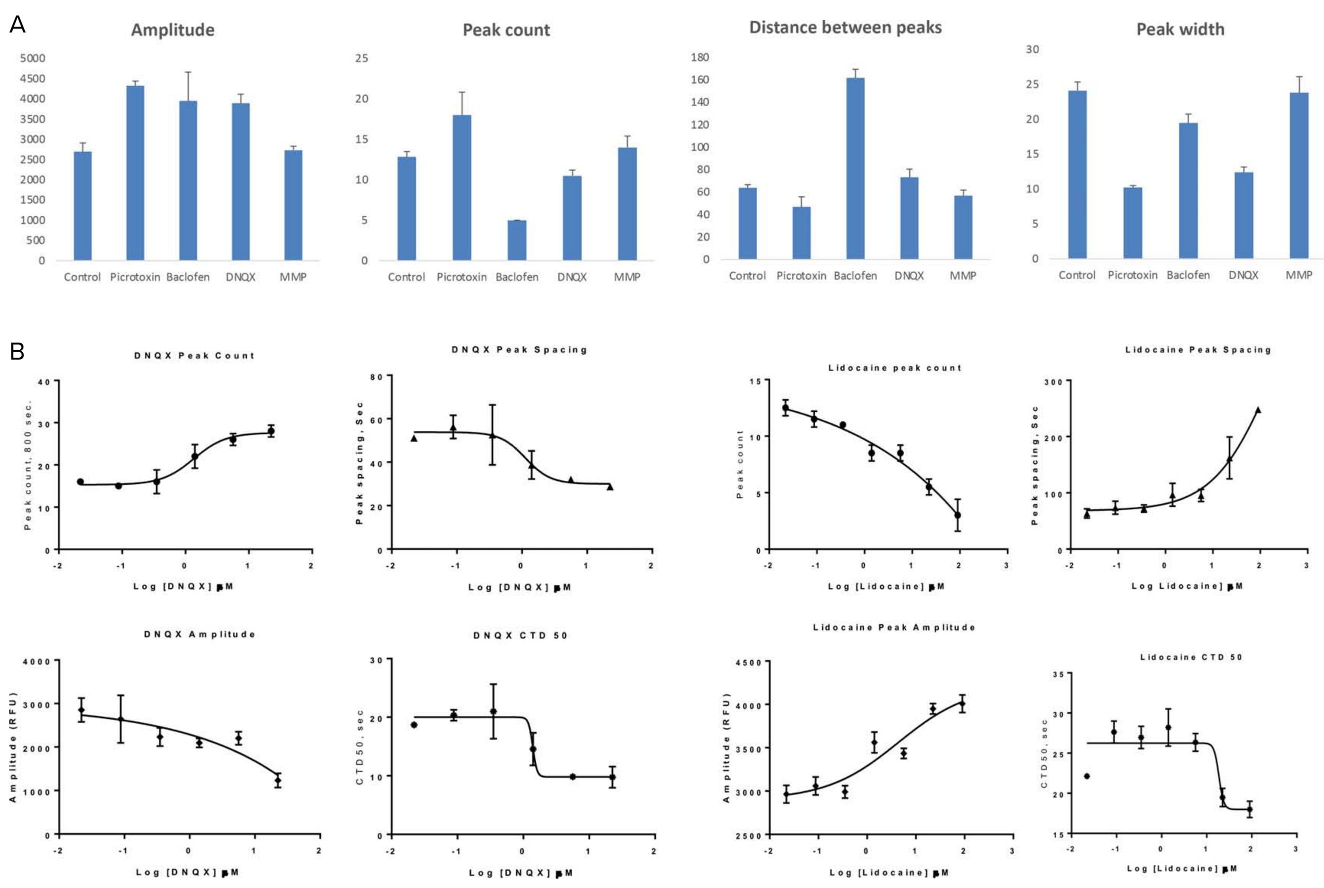


Figure 3. Ca^{2+} oscillations of neural spheroids determined by kinetic calcium imaging using FLIPR instrument and analyzed using PeakPro2 software. Multiple measurement were derived from recordings from samples treated with different compounds and different concentrations. Peak count, peak amplitude, peak width, distance between peaks, and several other metrics were derived from the analysis. Concentration-response curves were generated via GraphPad Prism and concentration dependencies were evaluated. Interestingly, an increase in peak frequency was observed with increasing concentration, which then halted oscillations and activities at the highest concentrations tested. A. Bar graphs represent changes in selected readouts for several compounds tested (20 μM). B. Concentration curves shown for DNQX and Lidocaine.

Summary of parametric effects for select compounds

Compound	Peak Count / 800 Sec	Peak Spacing (Sec)	Amplitude RFU	Rise Time Sec	CTD 90 Sec	CTSD 50 Sec	MOA
	EC_{50} values μM						
DNQX	1.35 ↑	1.13 ↓	10* ↓	3.74 ↓	~1.36 ↓	~1.39 ↓	Non-NMDA iGluR Antagonist
Haloperidol	22* ↑	22* ↓	5.63 ↓	~3.27 ↓	20.2* ↓	~85.5↓	Dopamine Antagonist
Lidocaine	20* ↓	187.3 ↓	4.37	~27	~19.9	~18.6	Sodium channel blocker
Picrotoxin	20* ↑	20* ↓	~1.49	76.1	7.28	4.05	GABA A antagonist, CNS stimulant
Lamotrigine	20* ↑	20* ↓	22 ↓	no change	1.4* ↓	1.4* ↓	Inhibits glutamate release possibly thru ion channels

~ denotes ambiguous results in Prism. * denotes the concentration that shows a definite change in trend.

Table 1. Summary chart of EC_{50} values (μM) calculated with Prism.

Results

Phenotypic profiling of functional activity and viability

A

	Measurement: Peak Count	Effective Concentration (μm)
AMPA	increase	0.1
GABA	decrease	0.4
NMDA	increase	2
GABA	decrease	2
Baclofen	decrease	4
Muscimol	increase	0.08
Lamotrigin	increase	4
4-AP	increase	1
MK801	increase	4
Bicuculin	decrease	0.8

B

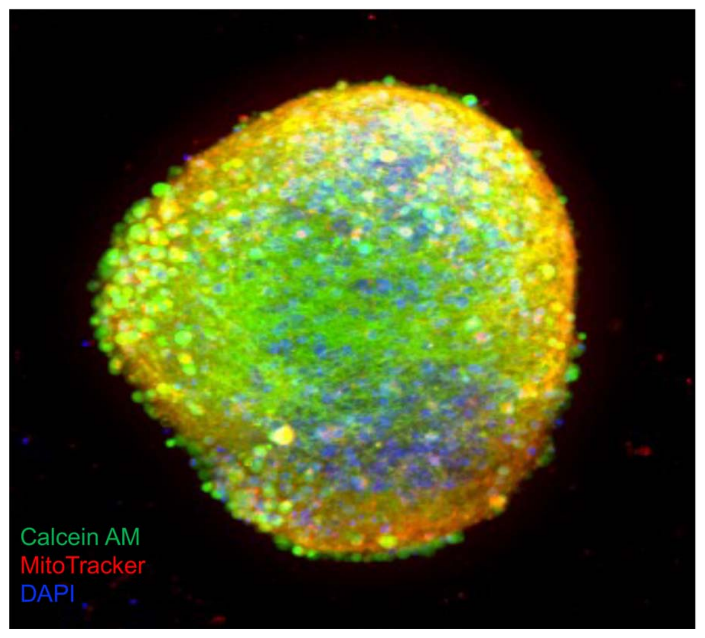
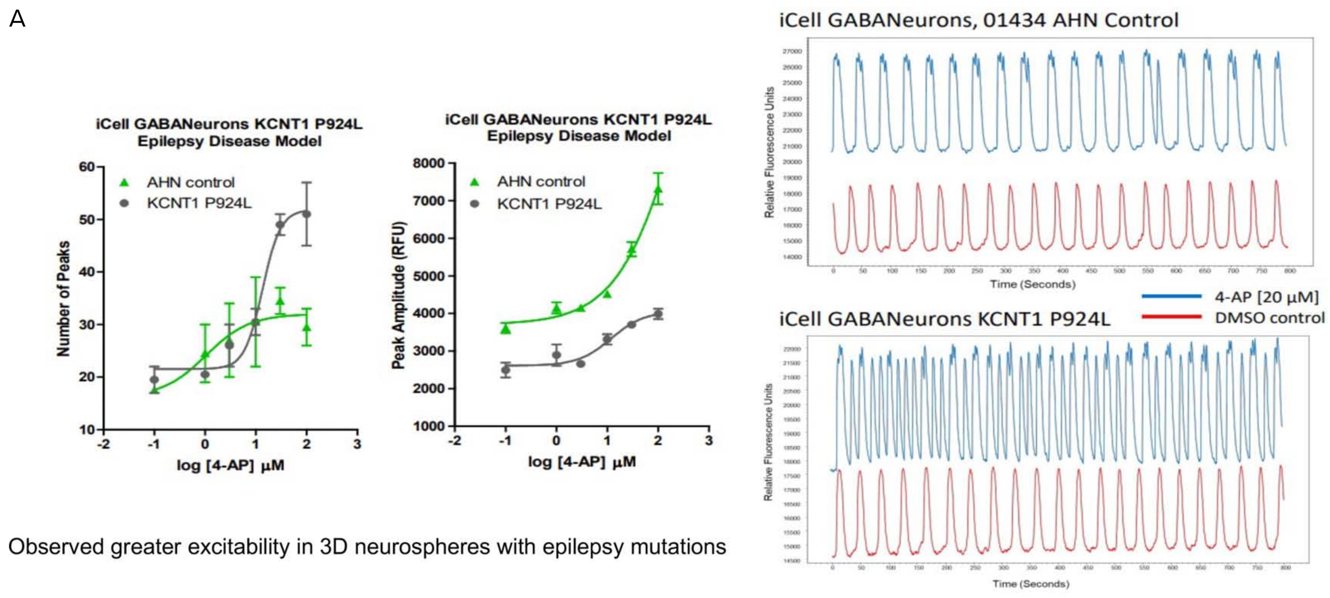
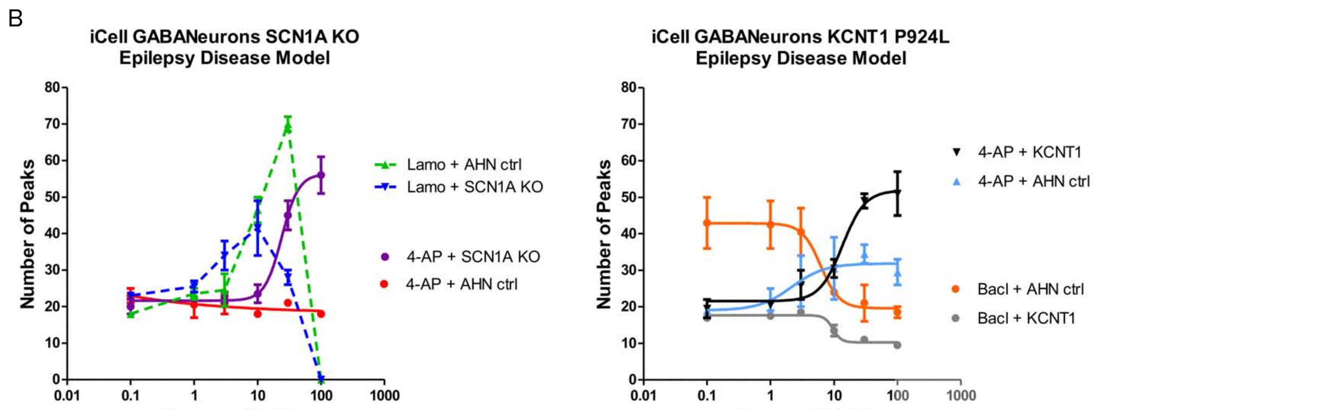


Figure 5. A. Table presenting effective concentrations for indicated compounds. B. Composite images of neural spheroid microtissues. Live cells were stained with Calcein AM, MitoTracker and Hoechst nuclear stain. Cells were imaged with the DAPI, FITC and Texas Red channels, 10X Plan Fluor objective. Red: MitoTracker; Green: Calcein AM; Blue: DAPI

Disease modeling: epilepsy

For disease modelling 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 - lamotrigin, Back - baclofen; AHN ctrl – apparently healthy normal control

Figure 6. Increased sensitivity to compounds that cause hyperexcitability was observed in 3D spheroids made with GABAergic neurons that carry the indicated epilepsy mutations (SCN1A KO and KCNT1 P924L) as compared to the AHN isogenic matched controls.

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 modelling.
- This assay platform shows promise for evaluation of compound effects and early detection of neurotoxicity *in vitro* due to its easy 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 pf phenotypic changes and compound effects.
- Moderately increased excitability was observed for mutated phenotypes, showing the elevated responses to stimulating agents.
- This biological system of 3D neurospheres paired with high-content imaging and detailed analysis of calcium oscillations demonstrates a promising and easily implemented tool for disease modeling and compound testing.

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