Functional Testing of Human iPSC-derived 3D Neural Spheroids Against a Panel of Neurotoxic Compounds

Ouissame Filali[1], Rebecca Fiene[1], Kimihiko Tomotoshi[1], Zhisong Tong[2], Carole Crittenden[2], Oksana Sirenko[2], Scott Schachtele[1], and Coby Carlson[1]

[1]FUJIFILM Cellular Dynamics, Inc., Madison, WI USA; [2]Molecular Devices, LLC., San Jose, CA USA

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Abstract

Objective: 3D neural spheroids are a rapidly developing technology that has great potential for understanding neuronal diseases and testing the effects of seizurogenic compounds or neurotoxic agents. There has been significant progress in developing reproducible methods for 3D cell culture of neural cell types, specifically those derived from induced pluripotent stem cells (iPSC).

Methods: Here we describe the generation of "neurospheres" that are formed by combining fully differentiated iPSC-derived neurons and astrocytes in ultra low attachment (ULA) plates at defined ratios and cell numbers to mimic specific regions of the brain. Tracking size and morphology during spheroid formation was accomplished using live-cell imaging (Incucyte SX5). Spontaneous functional activity was measured by recording calcium wave oscillations (FLIPR), and perturbations in these waveforms were used for detection of neurotoxic effects *in vitro*.

Results: 3D spheroid formation was consistent and uniform (diameter ~400 µm). Immunostaining for cell-specific markers (TUJ1 and GFAP) was performed and cells were further analyzed using confocal imaging. The functional impact of different ratios of glutamatergic to GABAergic neurons (70:30 vs. 50:50 vs. 20:80) and incorporation of astrocytes was demonstrated via calcium oscillation assays. Baseline neuronal activity focused on simple metrics, including peak count, peak amplitude, and peak width. To validate the use of neurospheres in functional cell-based assays, we tested a panel of >20 compounds, including neuromodulators with known seizurogenic properties (such as 4-aminopyridine, bicuculline, GABAzine, picrotoxin, strychnine, and chlorpromazine) and neurotoxic substances (such as rotenone, baclofen, deltamethrin, amiodarone, and kainic acid). Acetaminophen and DMSO were used as negative and vehicle controls, respectively. The observed changes in oscillation patterns were consistent with expected mechanism of actions of respective compounds.

Conclusion: Advanced systems of 3D neural spheroids composed of various human iPSC-derived cell types, paired with high-content imaging and complex analysis of calcium oscillations demonstrates a promising, biologically relevant system for testing the effect of pharmaceutical drug candidates or toxic compounds.

Overview of Assay Workflow for iCell® NeuroSpheres

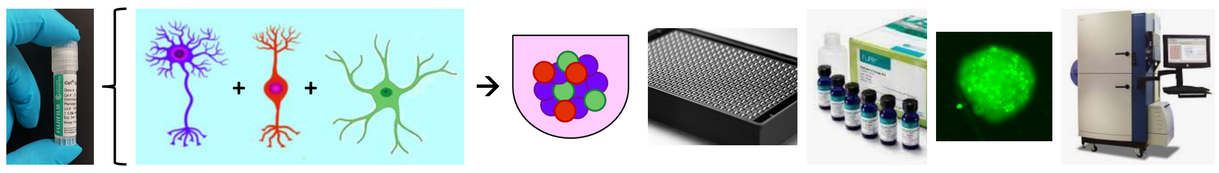
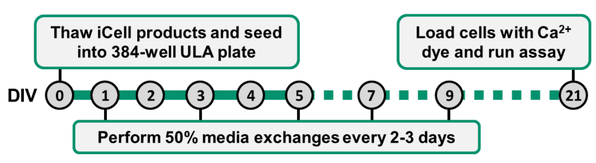


Figure 1. Materials and Methods. iCell NeuroSpheres can be generated by thawing cryopreserved iCell neural products and combining them in defined ratios and total cell numbers. Approx. 10 million total cells in 10 mL are needed to fill a 384-well plate (25K in 50 uL per well). Cells are maintained in complete BrainPhys medium until DIV 21 for calcium assay. Cells are loaded with Calcium 6 dye and recorded on an HTS-compatible instrument.

Product	Vendor	Cat. #
iCell GlutaNeurons, 01279	FCDI	C1033
iCell GABANeurons, 01279	FCDI	C1008
iCell Astrocytes 2.0, 01279 (†)	FCDI	C1249
BrainPhys™ Neuronal Medium	STEMCELL Tech	05790
iCell Neural Supplement B	FCDI	M1029
iCell Nervous System Supplement	FCDI	M1031
N-2 Supplement	Thermo Fisher	17502-048
Laminin	Sigma	L2020
PrimeSurface® 384w ULA Plate (‡)	Sbio	MS-9384UZ
FLIPR Calcium 6 Assay Kit	Molecular Devices	R8194

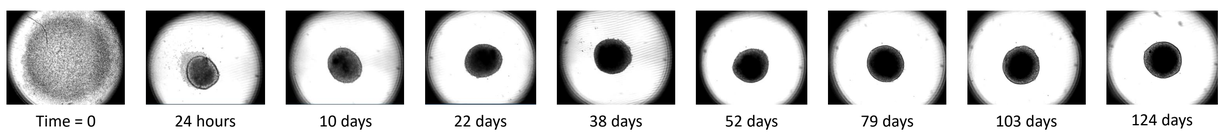


Generation of brain region-specific 3D neural spheroids was also demonstrated in a 2022 publication Strong et. al. featuring various iCell products. DOI: 10.1101/2022.05.04.490442

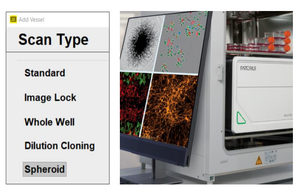
† iCell Astrocytes, 01434 (Cat. # C1037) can also be used
‡ Other ULA plates for 3D cell culture are also compatible (Corning, InSphero, FaCellitate)

Formation and Characterization of iCell NeuroSpheres

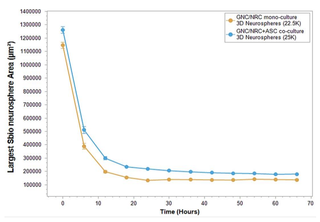
A Time course of spheroid formation



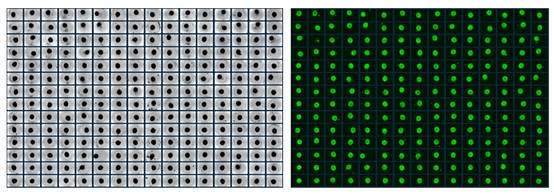
B Incucyte SX5 spheroid module



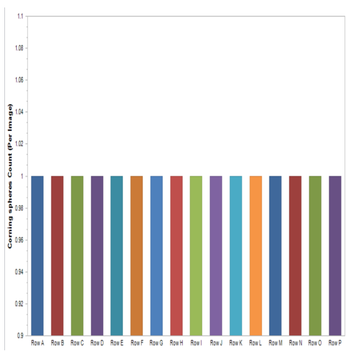
C Spheroids form in 24 hours



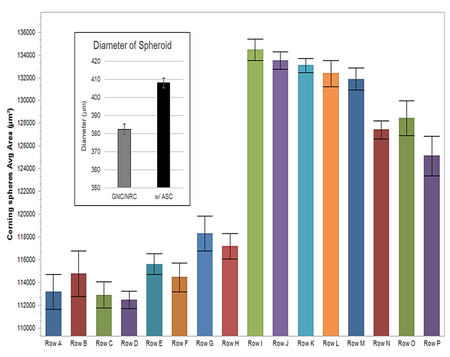
D High-throughput 384-well format



E Uniform spheroid formation



F Tracking spheroid size



G Imaging of iCell NeuroSpheres

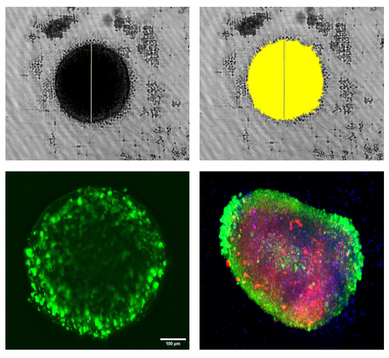
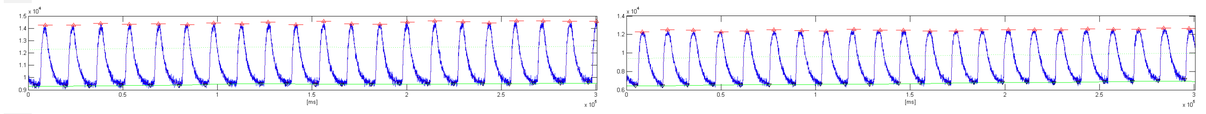


Figure 2. Formation and Characterization of iCell NeuroSpheres. (A) iCell Neurospheres can been formed and maintained in various ULA plate types, including the faCellitate BIOFLOAT™ 384-well plate (shown here) where we have kept cells out to 124 days in culture. (B) Tracking the formation of 3D spheroids over time is easy to do with an Incucyte SX5 live-cell imager with the spheroid module. (C) Self assembly occurs within 24 hours after seeding and there is little difference between neurons only (Gluta+GABA) vs. tri-culture (Gluta+GABA+ASC). (D) Brightfield and fluorescent images of neurons in a 384-well plate illustrates their compatibility with HTS formats. (E) Quantifying the number of spheres per image row-by-row on the Incucyte demonstrates uniform spheroid formation (1 spheroid per well). (F) Incucyte analysis of 3D spheroids containing Gluta+GABA (neurons only) or with astrocytes (tri-culture) showed that the spheroid diameters were ~380 µm and ~410 µm, respectively. (G) Multiple characterization images of iCell NeuroSpheres, including brightfield, Incucyte mask (yellow), cells with Calcium 6 dye (green), and ICC staining on high content imager: TUJ1 (green), GFAP (red), nuclei (blue).

Optimization of Calcium Oscillation Assay

A Cell ratio corrected for % of GlutaNeurons



B Composition based on number of each cell type

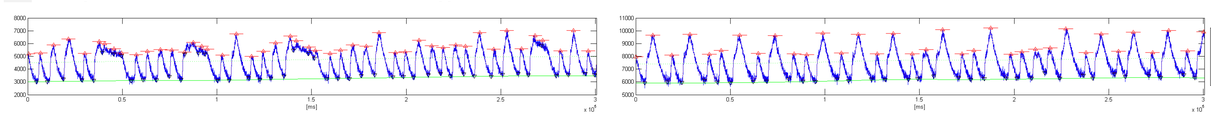


Figure 3. Ratio of glutamatergic to GABAergic neurons. iCell GlutaNeurons are human iPSC-derived neurons that are predominantly excitatory (≥70% glutamatergic). When making iCell NeuroSpheres with GlutaNeurons, we observed that assay performance is more consistent when the ratio of neurons is calculated using (A) a number of cells that is corrected for the lot-specific % of GlutaNeurons listed on the CoA (Certificate of Analysis) in blue vs. (B) a spheroid composition based strictly on the number of cells (70% Gluta and 30% GABA) in yellow.

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	2500	25,000
Corrected for % of Gluta	21,000	1,500	2500	25,000

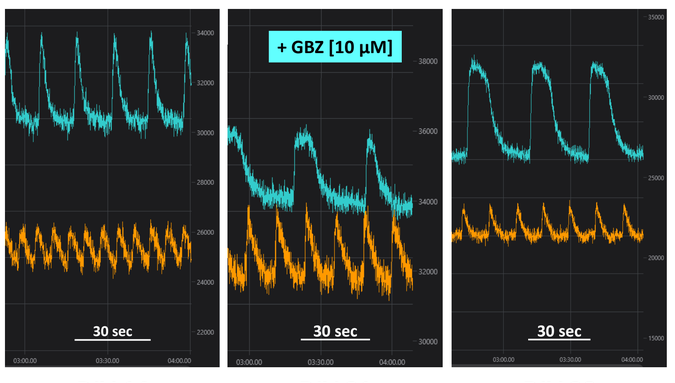
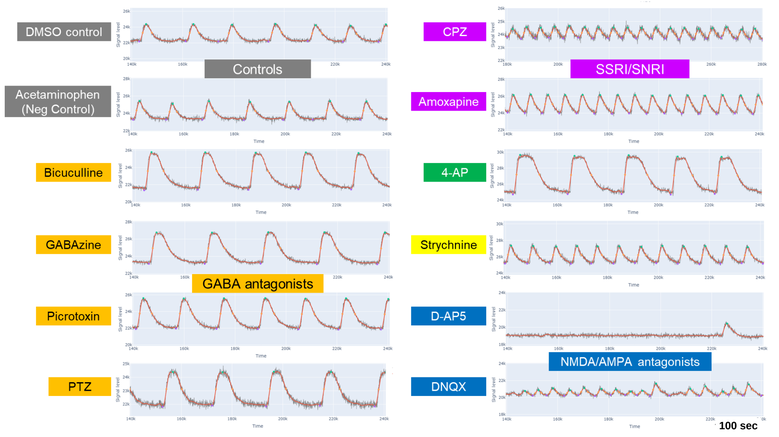


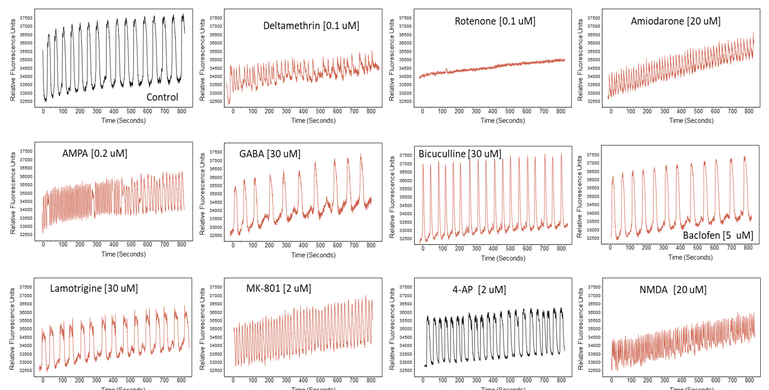
Figure 4. Day of assay. While 3D neurospheres form rapidly, it was necessary to determine the optimal day in vitro (DIV) for spontaneous calcium oscillations to be recorded and if an expected response from drug treatment with GABAzine (GBZ) could be observed. Cells displayed spontaneous waveform activity and responded to GBZ at all time points tested, but DIV 21 is recommended based on consistency of baseline assay signal (orange) and robustness of drug response (blue). Functional Ca^{2+} assays can also be performed at least until DIV 28 with good results, if desired.

Assay Validation with Neuromodulatory Compounds



Compound	MOA	Peak Amplitude	Peak counts	FWHM
Acetaminophen	Neg. control	-	-	-
D-AP5	NMDA-R antag	block	Inc / Dec	block
DNQX	AMPA-R antag	Block	Inc / Dec	block
Bicuculline	GABAA-R antag	Increase	-	-
GABAzine	GABAA-R antag	Increase	-	-
Picrotoxin	GABAA-R antag	Increase	-	Increase
PTZ	GABAA-R antag	Increase	-	Increase
Amoxapine	SSRI/SNRI	Decrease	Increase	Decrease
CPZ	SSRI/SNRI	Decrease	Increase	Decrease
4-AP	K^+ Ch inhibitor	Increase	-	Increase
Strychnine	Glycine-R antag	Decrease	Increase	Inc / Dec

Figure 5. Assay validation with known compounds. A primary application for iCell NeuroSpheres is safety/toxicity testing and seizurogenic risk assessment. Calcium assay data analysis is simpler than MEA platforms, with fewer endpoint metrics calculated, including peak count, peak amplitude, and peak width or FWHM (full width at half maximum). GABA-A receptor antagonists (orange) and NMDA/AMPA receptor antagonists (blue), demonstrate that the cells respond to drug treatment as expected on DIV 21 relative to DMSO control. SSRI/SNRI, K^+ channel inhibitors and glycine receptor antagonists also modified calcium waveforms. Data analysis was performed using Wavechecker software (Hamamatsu) and internally developed scripts.



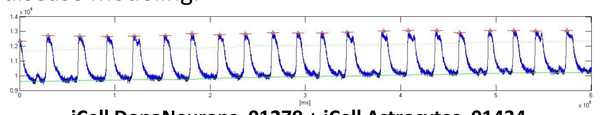
Compound	Conc (uM)	Peak Count (Frequency)	Peak Height (Amplitude)
DMSO control	0.10%	0	0
Deltamethrin	0.1	↑	↓
Rotenone	0.1	X	↓
Amiodarone	20	↑	↓
AMPA	0.2	↑	↓
GABA	30	↓	0
Bicuculline	30	↑	↑
Baclofen	5	↓	0
Lamotrigin	30	0	↓
MK-801	2	↑	↓
AP-4	2	↑	↓
NMDA	20	↑	↓

MOLECULAR DEVICES

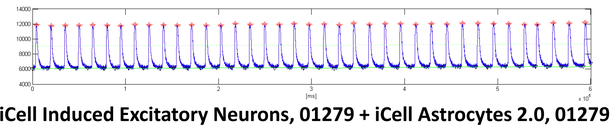
Figure 6. Neuromodulatory Pharmacology. iCell NeuroSpheres were formed in Corning 384-well ULA plates and tested on DIV 21 in culture. Compounds were incubated with cells for 60 minutes and then recorded on a FLIPR Penta instrument. Testing of all 11 compounds shown here was performed in collaboration with Molecular Devices. Data was analyzed with PeakPro2 software.

Summary and Future Directions

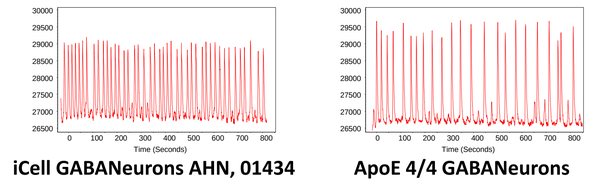
iCell NeuroSpheres can be formed from other available iCell Products for pharmacological testing as well as for disease modeling.



iCell DopaNeurons, 01279 + iCell Astrocytes, 01434

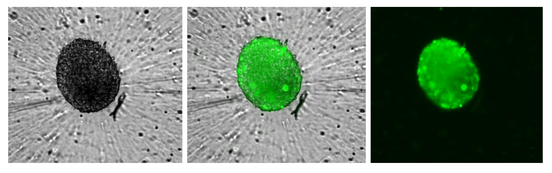


iCell Induced Excitatory Neurons, 01279 + iCell Astrocytes 2.0, 01279



iCell GABANeurons AHN, 01434

ApoE 4/4 GABANeurons



"iCell NeuroSpheres" provide a modular approach to 3D spheroid formation, enabling control over cell type ratios and spheroid composition. Using Gluta+ GABA neurons, the resulting cell cultures have been shown to respond appropriately to neurotoxic compounds. Importantly, this advances "brain-in-a-dish" studies and sets the stage for potential disease modeling applications. This model offers a logical alternative to StemoniX microBrain technology and has been extensively developed via influential studies carried out at NCATS (see Strong et. al.)