Complex event analysis for neurotoxic profiling of compound effects on human iPSC-derived neural spheroid 3D cultures

Carole Crittenden[1], Oksana Sirenko, Ph.D.[1], Cassiano Carromeu, PhD.[2], Ryan Gordon, Ph.D.[2], Fabian Zanella, Ph.D.[2],
[1]Molecular Devices, LLC, San Jose, CA, [2]StemoniX, Inc., San Diego, CA

Abstract

To speed up the development of more effective and safer drugs, there is an increasing need for more complex, biologically relevant, and predictive cell-based assays for drug discovery and toxicology screening. Human iPSC-derived neural 3D co-cultures (StemoniX® microBrain® 3D platform) have been developed as a high throughput screening platform that more closely resembles the constitution of native human cortical brain tissue. 3D neural spheroids contain a neural network enriched in synapses, creating a highly functional neuronal circuitry that displays spontaneous synchronized, readily detectable calcium oscillations. Here, we describe a method for the complex analysis of calcium oscillations using the FLIPR® Penta High-Throughput Cellular Screening System that allows detection and multi-parametric characterization of oscillation peaks. ScreenWorks® Peak Pro 2 analysis software analyzes calcium oscillation rate, peak width and amplitude, characterization of secondary peaks, waveform irregularities, and several other read-outs. In addition, cellular and mitochondrial toxicity were assessed by high-content imaging using the ImageXpress® Micro Confocal High-Content Imaging System. For assay characterization, we used a set of neurotransmitters with known mechanisms of action affecting neurotransmitters such as GABA, NMDA and dopamine targets. Then we characterized the neurotoxic profile of a set of known neuroactive or seizurogenic drugs, as well as selected environmental chemicals. Our results show that neural 3D cultures when paired with complex event analysis and cytotoxicity assays form a promising, biologically-relevant system for assessing the neurotoxic potential of pharmaceutical drugs and environmental toxins.

StemoniX microBrain 3D spheroids

The StemoniX microBrain 3D Assay Ready Platform is a high-throughput 3D culture platform that more closely resembles the tissue development and constitution of native human brain tissue. In this platform, human iPSC-derived neuronal spheroids, approx. 600 µm in diameter, are composed of a physiologically relevant co-culture of functionally active cortical glutamatergic and GABAnergic neurons (identified by MAP2; green) and astrocytes (identified by GFAP; red). This balanced cellular mix allows the development of a neural network enriched in synapses, creating a highly functional neuronal circuitry. The neuronal cells in the microBrain 3D spheroids are physiologically active, with spontaneous synchronized, readily detectable calcium oscillations.

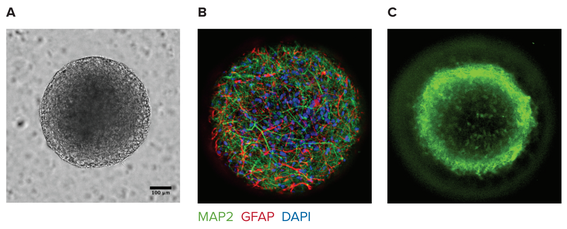


Figure 1. A. Human iPSC-derived neural spheroids composed of a co-culture of active cortical neurons B. (identified by MAP2; green) and astrocytes (identified by GFAP; red), approximately 600 µm diameter, imaged with ImageXpress Micro Confocal system, 20X magnification. C. Stained with Calcium dye.

FLIPR Penta system

- The FLIPR Penta system is powered by a new high-speed EMCCD camera that measures kinetic patterns and frequencies of Ca^{2+} oscillations as monitored by changes in intracellular Ca^{2+} levels with the FLIPR® Calcium 6 Assay Kit (Molecular Devices).
- System allows 6,000 images at up to 100 Hz.
- ScreenWorks Peak Pro 2 software allows measurement and analysis of complex patterns of calcium oscillations in human iPSC-derived cardiomyocytes and neurons. The ScreenWorks Peak Pro 2 peak analysis software module allows analysis and characterization of the primary and secondary peaks and complex oscillation patterns.

Methods

3D neural cultures

microBrain 3D Assay Ready 384-Well plates were obtained from StemoniX, Inc. Plates were shipped pre-plated under ambient conditions. Each well contained a single, uniformly sized human iPSC-derived cortical neural spheroid matured 8-9 weeks. The human spheroids were exposed to compounds for 24 hours, or as indicated in the figures.

Calcium flux assay

The intracellular Ca^{2+} oscillations were assessed using the FLIPR® Calcium 6 Assay Kit according to the regular protocol; spheroids were loaded with dye for 2 hours before measurements. Compounds were added and calcium flux was measured at intervals of 15 minutes to 24 hours on the FLIPR Penta system.

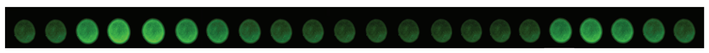
Cell staining

To assess phenotypic changes, cells were stained live after a 24 hour incubation with compounds using a mixture of three dyes: the viability dye Calcein AM (1 µM), the mitochondrial membrane potential dye MitoTracker Orange (0.2 µM), and the Hoechst nuclear dye (2 µM) (all from Life Technologies) and read on the ImageXpress Micro Confocal system.

Results

Effects of neuromodulators on calcium oscillations

The neuronal cells in the microBrain 3D spheroids generate spontaneous synchronized calcium oscillations. We used fast kinetic fluorescence imaging on the FLIPR Penta system to measure the patterns and frequencies of the Ca^{2+} oscillations of neuro-spheroids as monitored by changes in intracellular Ca^{2+} levels with FLIPR Calcium 6 Assay Kit. A set of known neuromodulators was tested, including agonists and antagonists of NMDA, GABA and AMPA receptors; kainic acid, analgesic, and anti-epileptic drugs. Changes were observed as inhibitions or activations of the peak frequency or other measurements, corresponding to the expected effect of the tested neuromodulators. MK-801, NMDA and Baclofen have been analyzed for peak count in ScreenWorks Peak Pro 2 software. Advanced analysis methods implemented in the ScreenWorks Peak Pro 2 software module provide multi-parametric characterization of the Ca^{2+} flux oscillation patterns. This phenotypic assay allows for the characterization of readouts such as oscillation frequency, amplitude, peak width, secondary peaks, and amplitude and spacing irregularity. The effects of modulators of neuronal activity were evaluated by measuring changes in multiple read-outs.



Calcium oscillations over time

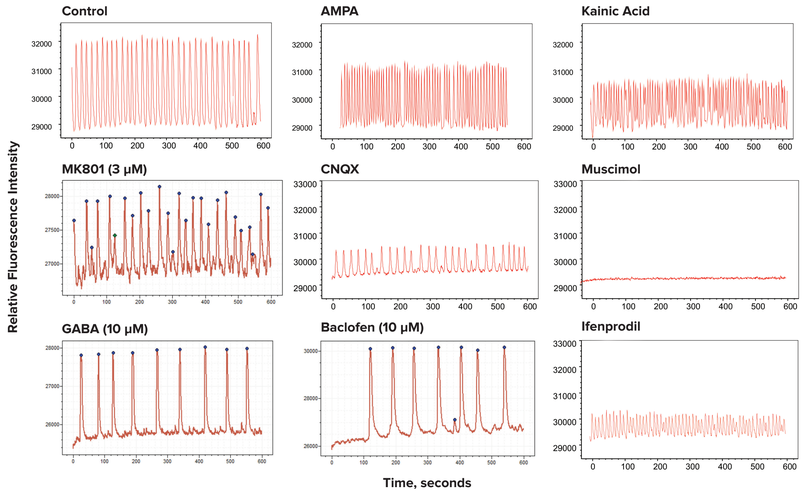


Figure 2. Using high speed EMCCD fluorescence imaging (FLIPR Penta system), we tested the impact of various compounds on the rates and patterns of intracellular Ca^{2+} oscillations using calcium-sensitive dye (FLIPR Calcium 6 Assay Kit). High throughput recording and analysis of the entire 384-well plate was performed using the FLIPR Penta system. Representative traces of calcium oscillations are shown for the control and compound treated spheroids. Spheroids were loaded with dye for 2h and then treated with compounds for 30 min. Concentration-dependent changes in the patterns were observed. Oscillation traces were recorded by the FLIPR Penta system for 10 min. Patterns were characterized using ScreenWorks Peak Pro 2 software.

Effects of neuro-active and neurotoxic compounds on calcium oscillation patterns

As a next step, we tested a selected a set of neuro-active drugs that have expected effects on the nervous system, including established drugs used for treatment of neurological disorders as well as substances known to cause psychotic effects. To test if the assay can identify chemicals for their potential neurotoxic effects, we tested a library of 23 compounds that represent different classes of drugs, known to cause peripheral neuropathy (cancer drugs), confusion (selected antibiotics), hearing loss (gentamycin), or other types of disorders. For that test, cells where treated with compounds for 24h. A number of drugs caused strong, moderate, or weak perturbations of calcium oscillation patterns.

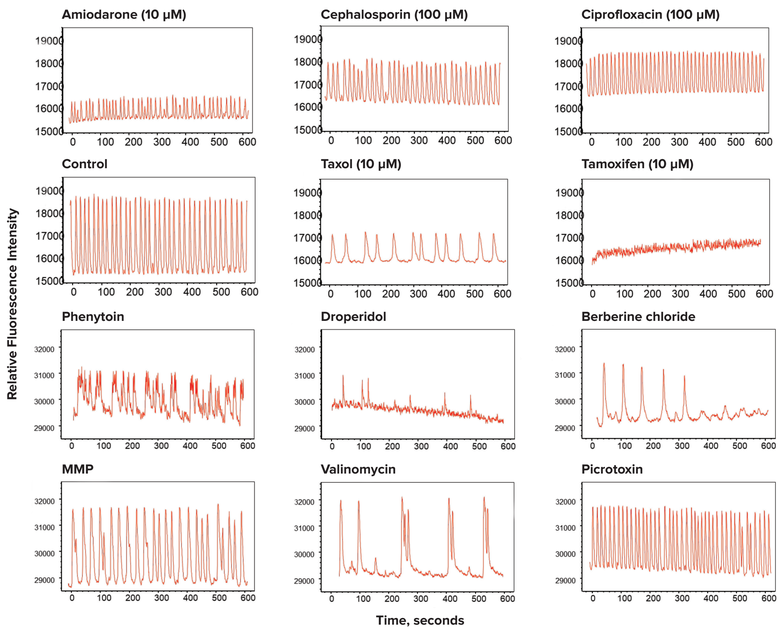
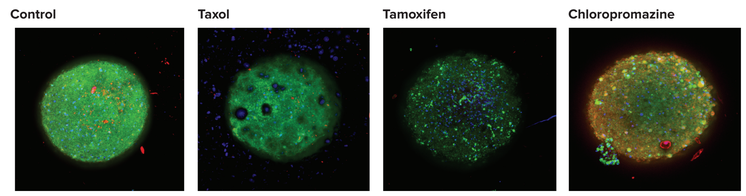


Figure 3. Using the High Speed EMCCD FLIPR Penta system we tested the impact of compounds after a 2 hour incubation with Calcium 6 Assay Kit and 30 minutes to 24 hours exposure to drugs.

Results, continued

Assessment of spheroid morphology and viability by high content imaging

Confocal imaging and 3D image analysis methods were used to characterize compound effects on the morphology and viability of 3D neural spheroids. To evaluate cytotoxicity effects, cells were treated with various compounds for 24h, then stained with Hoechst nuclear stain, Calcein AM, and MitoTracker Orange dyes. Images were acquired using the ImageXpress Micro Confocal system, using the confocal option and 3D imaging. Projection images were then analyzed using the Custom Module Editor and Cell Scoring algorithms for detection of cell numbers for all live cells. To evaluate cytotoxicity effects, cells were treated with various compounds for 24h (Calcein AM positive cells), and cells with intact mitochondria (MitoTracker positive cells). The analysis methods provide efficient tools for characterization of cell and spheroid morphology.



Nuclei – Hoechst nuclear stain, shown in blue; Viability – Calcein AM, show in green; Mitochondria – MitoTracker Orange, shown in orange

Figure 4. Composite projection images of neural spheroids. Spheroids were treated with 30 µM of indicated compounds for 24h, then stained with a nuclear stain (Hoechst 33342), viability stain (Calcein AM), and mitochondrial membrane potential dye MitoTracker Orange CMTMRos for 2 hours (2 µM, 1 µM, and 0.5 µM, respectively). Spheroids were imaged with the DAPI, FITC, and TRITC, 10X Plan Fluor objective, imaged using Z-stack of confocal images (20 images, 10 µm apart). Maximum projection images were analyzed using the custom module editor for detection of spheroid size and shape, and also count of positive and negative cells in a spheroid. The image shows nuclei (blue), Calcein AM stain (green), and mitochondria (orange). The mask shows spheroids in blue, nuclei of Calcein AM positive cells in red, and negative cells in blue.

Multi-parametric evaluation of neurotoxicity effects

The assay shows promise for high-throughput assessment of neurotoxicity effects of chemicals in vitro which would help to evaluate and prioritize different drugs and substances for further testing. ScreenWorks PeakPro2 software allows more than 20 descriptors of waveforms. We have evaluated a multi-parametric approach for description of changes in oscillations.

Neurotransmitters

	Compounds	Peak Count[1]	Amplitude (decrease)	Oscillation Frequency	Fibrillations*	Oscillation Stop*	Amplitude Irregularity*	Peak spacing Irregularity*	Secondary Peaks*	Cytotoxicity*	Mitochondria toxicity*	Max concentration	Description
1	AMPA	~0.2	0.48	up		1	0.3	0.3	0.3			10	AMPA agonist
2	Kainic acid	3.2	2.2	up down	3	10	1	1	0.3			10	Kainate receptor agonist
3	MK-801	10.2	0.28	down			0.03	0.03	0.03			10	NMDA antagonist, Channel Blocker
4	CNQX		10.2	down			15	15	15			50	AMPA/Kainate antagonist
5	Muscimol	0.53	0.33	down	0.3	0.94	0.3	0.3	0.3			25	GABA A agonist
6	Baclofen	~2		down			3		3			10	GABA B agonist
7	GABA	~15	3.56	down			3	3	3			30	Endogenous agonist
8	L-glutamic acid	120		up								1000	Non specific agonist
9	Ifenprodil	~2	~1	up	3	10	3		3			10	NMDA 2B agoinist
10	NMDA	3.36	~5	up	10		3					10	NMDA agoinsit, Non Specific
11	DNQX	~1										50	Non NMDA antagonist, Kainate
12	DMSO 0.1%	no effect	no effect	no effect	no effect	no effect	no effect	no effect	no effect	no effect	no effect	100	Control

Neuro-active compounds

	Compounds	Peak Count[1]	Amplitude (decrease)[1]	Oscillation Frequency	Fibrillations*	Oscillation Stop*	Amplitude Irregularity*	Peak spacing Irregularity*	Secondary Peaks*	Cytotoxicity*	Mitochondria toxicity*	Max concentration	Description
1	Haloperidol	2.5	0.71	down		3	1	1	1			30	Anti-psycotic
2	Lidocaine	6.8	5.7	down		30						30	Anti-pain
3	Chloropromazine	30.7	10.1	down	10	30	10		10	30	30	30	Anti-psycotic
4	Phenytoin	122.6	~100	down		100	10	30	10			100	Anti-epilepsy
5	Lamotrigin	~50	no effect	up	30				30			30	Anti-epilepsy
6	4-AP	3.4	no effect	up	30		3	3	3			30	Stimulant
7	Valinomycin	0.128	~1	down		1	0.03	0.03	0.03	3	3	10	K^+ ionophore, antibiotic neurotox
8	MPP+	59.5	no effect	down			10	10	10			30	Stimulant
9	Valproic Acid Na Salt						100	100	100			100	Anti-epilepsy
10	Picrotoxin	29.5	1.28	up			none					30	Stimulant, antidote
11	Droperidole	40.2	4.6	up			10		10			30	Anti-psycotic
12	Acetaminophen	no effect	no effect	no effect	no effect	no effect	no effect	no effect	no effect	no effect	no effect	100	Negative control

Neurotoxic drugs

	Compounds	Peak Count[1]	Amplitude (decrease)[1]	Oscillation Frequency	Fibrillations*	Oscillation Stop*	Amplitude Irregularity*	Peak spacing Irregularity*	Secondary Peaks*	Cytotoxicity*	Mitochondria toxicity*	Max concentration	Description
1	Pindolol	57.3	30	up/down		100	3	3	3			100	Beta-blocker
2	Diethylstilbestrol	>100	39.3	down		100	10	10	10		100	100	Estrogen agonist
3	Tamoxifen	6.43	3.59	down		100	30	30		100	100	100	Estrogen receptor modulator
4	Taxol	9.15	>30	down		30	10	3	3	100	100	100	Anti cancer
5	Cefepime HCl											100	Antibiotic
6	Ciprofloxacin											100	Antibiotic
7	Cephalosporine		~100				100	30	30			100	Antibiotic
8	Gentamycin Sulfate											100	Antibiotic
9	Isoniazid							100			100	100	Antibiotic
10	Berberine (HCl)	~30	no effect	down		30	10	10	10		30	30	Alkaloid, antibiotic
11	ß estradiol	202		down			30	30	30			100	Hormone, osteoporosis
12	Amiodarone	8	1.48	up/down		30	3	10				100	Anti-arrhythmic drug
13	Oxotremorine M						10				30	30	Anti tremor
14	Pentylenetetrazole									1000	1000	1000	Respiratory stimulant
15	Pilocarpine (HCl)											31	Glaucoma drug
16	6-hydroxydopamine hydrobromide	>100		down						30	30	30	Neurotoxin induces a reduction of dopamine levels in the brain
21	Linopiridine (HCl)		100				3	10	10			100	Cognition-enhancing drug
22	Tetraethylthiuram disulfide	200	34	down			100	30		100	100	100	Anti-alcohol abuse
23	Carbamazepine	101	>30	up/down		100	30	10	30			100	Anti-epileptic drug
24	Amoxicillin	no effect	no effect	no effect		no effect	no effect	no effect	no effect	no effect	no effect	100	Negative control

Table 1. EC_{50} values (1) or lowest concentrations (µM) that cause specific changes (*) are indicated for different read-outs. No changes indicated with blank cells.

Summary

- We developed methods and demonstrated feasibility of the iPSC-derived StemoniX microBrain 3D Assay Ready neural cultures for evaluation of compound effects on the FLIPR Penta system.
- The functional responses from known neuromodulators, neuro-active, and neurotoxic drugs were measured using ScreenWorks Peak Pro 2 analysis software.
- The assay can be used for testing compound effects and early detection of neurotoxicity.

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