Multiplexed automated assays for neurotoxicity evaluation using induced pluripotent stem cell-derived neural 3D cell models

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Introduction

Cell-based phenotypic assays have become an increasingly attractive alternative to traditional in vitro and in vivo testing in pharmaceutical drug development and toxicological safety assessment. The effectiveness of automated imaging assays combined with the organotypic nature of human induced pluripotent stem cell (iPSC)-derived cells opens new opportunities to employ physiologically relevant in vitro model systems to improve screening for new drugs or potential chemical toxicities. In our studies, we used human iPSC-derived neural cultures to test functional and morphological end points for toxicity evaluation in a multi-parametric assay format.

For neurotoxicity assessment we employed iPSC-based 3D neural platform composed of mature cortical neurons and astrocytes (Stemonix Co.). The neuronal cells in neurospheroids generated spontaneous synchronized calcium oscillations. We used fast kinetic fluorescence imaging to measure the patterns and frequencies of the Ca2+ oscillations. Characterization of oscillation profiles in neurospheroids was performed through multi-parametric analysis presenting more than 15 read-outs including frequency, amplitude, characterized primary and secondary peaks, peak width, and waveform irregularities. In addition, cellular and mitochondrial toxicities were assessed by high-content imaging. The assay was optimized for high throughput screening in 384-well plates and displayed a highly consistent performance. We evaluated neuroactive and neurotoxic profiles of more than 120 compounds, including set of neuromodulators with known mechanisms of action, set of various pharmaceutical drugs, sample library of cannabinoids, and a library of neurotoxic chemicals including flame retardants, pesticides, and poly-aromatic hydrocarbons.

Our results show that the iPSC-derived 3D neurospheroid assay platform is a promising biologically-relevant tool to assess the neuroactive and neurotoxic potential of pharmaceutical drugs and environmental toxicants.

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 GABAergic 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.

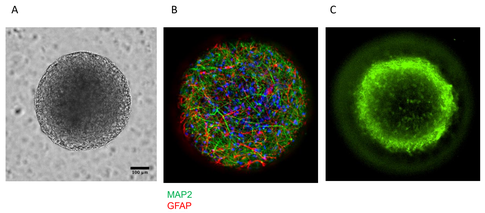

Figure 1. **A.** Human iPSC-derived neural spheroids composed of a co-culture of active cortical neurons **B.** (identified by MAP2; green) and astrocytes (identified by GFAP; red), approximately 600 µm diameter, imaged with ImageXpress® Micro Confocal system, 20X magnification. **C.** Stained with Calcium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

Instrument

- The FLIPR® Penta System is powered by a new high-speed EMCCD camera and the new ScreenWorks® Peak Pro™ 2 software.
- The system allows measurement and analysis of complex patterns of calcium oscillations in human iPSC-derived cardiomyocytes and neurons.

We used the new high speed EMCCD camera on the FLIPR Penta system to measure the fast kinetic patterns and frequencies of Ca2^{2+} oscillations of neuro-spheroids as monitored by changes in intracellular Ca^{2+} levels with FLIPR® Calcium 6 Assay Kit (Molecular Devices). The instrument equipped with the new 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.

Cell Staining

To assess phenotypic changes, cells were stained live using a mixture of three dyes: the viability dye Calcein AM (1 µM), the mitochondria potential dye MitoTracker Orange (0.2 µM), and the Hoechst nuclear dye (2 µM). Hoechst 33258 (ultra pure) (ENZ-52402)
Calcein AM (ultra pure) (ENZ-52002), MITO-ID® Membrane potential detection kit (ENZ-51018-K100)

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Results

Calcium Oscillations Evaluated by the FLIPR Penta System

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.

Analysis of Kinetic Patterns

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.

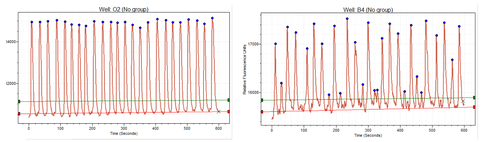


Figure 2. The assay was used for high-throughput assessment of neurotoxicity effects of chemicals in vitro which would help to evaluate and prioritize different drugs and substances for further testing. ScreenWorks PeakPro2 software allows more than 20 descriptors of waveforms. We have evaluated multi-parametric approach for description of changes in oscillations.

Effects of Neuromodulators on Calcium Oscillations

A number of known modulators of neuronal activity were assayed at different time-points and the calculated EC_{50} values for compound effects. Changes were observed as inhibitions or activations of the peak frequency or other measurements, corresponding to the expected effect of the tested neuromodulator.

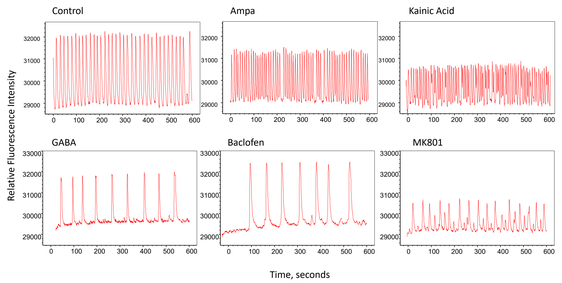


Figure 3. Using high speed EMCCD fluorescence imaging (FLIPR Penta system), we have tested the impact of various compounds on the rates and patterns of intracellular Ca^{2+} oscillations using calcium-sensitive dye (FLIPR Calcium 6 Assay Kit). High throughput recording and analysis of the entire 384-well plate was simultaneously 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 30min. Concentration-dependent changes in the patterns were observed. Oscillation traces were recorded by the FLIPR system for 10 min. Patterns were characterized using ScreenWorks Peak Pro 2 software. Representative traces of calcium oscillations are shown for 1uM concentrations, 30min treatment. [1]

Effects of Environmental Neurotoxicants

A library of environmental compounds containing known and unknown neurotoxicants was tested in the assay. Large number of compounds (57 out of 80) demonstrated deviations from the control pattern and were flagged in the assay. Effective concentrations for compounds effect were determined for various read-outs demonstrating sensitivity of the method for HTS in vitro assay for potential neurotoxicity.

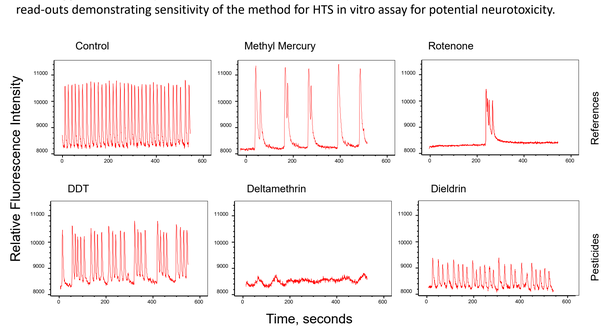


Figure 4. Modulations of calcium oscillation patterns for selected neurotoxic compounds. Detailed information about screen and effective concentrations can be found at ToxSci. [1]

Sirenko et al., Toxicol Sci 2019 Jan 1;167(1):58-76.

Results, continued

Effects of Neuro-active compounds

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.

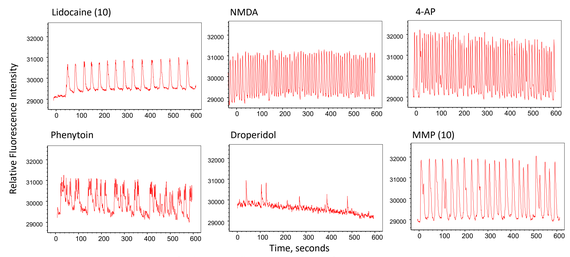


Figure 5. Representative traces of calcium oscillations are shown for 3µM concentrations, 30min treatment.

Effects of Neurotoxic Drugs

To test if the assay can identify chemicals for their potential neurotoxic effects, we tested a panel 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 neurotoxicity test neurospheroids where treated with compounds for 24h. Selected drugs caused mostly moderate perturbations of calcium oscillation patterns.

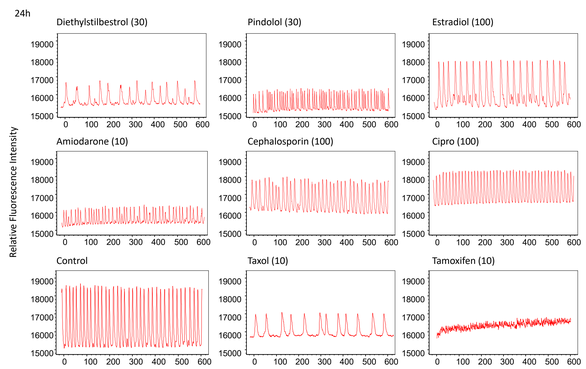


Figure 6. Representative traces of calcium oscillations are shown for selected anti-cancer drugs and antibiotics. Concentrations (µM), 24h treatment.

Multi-parametric assessment of compound effects

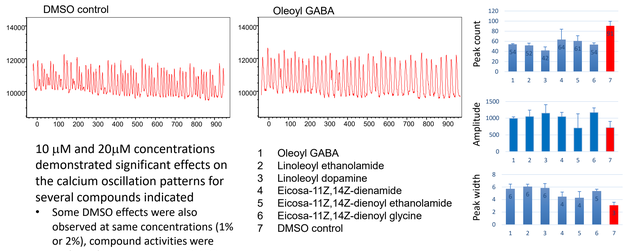
The assay shows promise for high-throughput assessment of neurotoxicity effects of chemicals in vitro. ScreenWorks PeakPro2 software allows more than 20 descriptors of waveforms. We have evaluated multi-parametric approach for description of changes in oscillations.

	Compounds	Peak Count*	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	Neuromodulators
2	Kainic acid	3.2	2.2	up-down	3	10	1	1	0.3			10	Kainate receptor agonist	
3	MK801	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	Ifenoprodil	^2	*1	up	3	10	3		3			10	NMDA 2B agonist	
10	NMDA	3.36	*5	up	10		3					10	NMDA agonist, 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	
	Compounds	Peak Count*	Amplitude (decrease)*	Oscillation Frequency*	Fibrillations*	Oscillation Stop*	Amplitude Irregularity*	Peak spacing Irregularity*	Secondary Peaks*	Cytotoxicity*	Mitochondria toxicity*	Max concentration	Drug Action	
1	Haloperidol	2.5	0.71	down		3	1	1	1			30	Anti-psycotic	Neuro-active
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-psychotic	
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	
	Compounds	Peak Count*	Amplitude (decrease)*	Oscillation Frequency*	Fibrillations*	Oscillation Stop*	Amplitude Irregularity*	Peak spacing Irregularity*	Secondary Peaks*	Cytotoxicity*	Mitochondria toxicity*	Max concentration	Drug Action	
1	Pindolol	57.3	30	up/down		100	3	3	3			100	Beta-blocker	Neuro-toxic
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	>80	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)	^90	no effect	down		30	10	10	10		30	30	Alkaloid, antibiotic	
11	8 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-tremmor	
14	Pentylenetetrazole									1000	1000	1000	Respiratory stimulant	
15	Fiscarpine (HCl)											31	Glaucoma drug	
16	6-hydroxydopamine hydrobromide	>100		down						30	30	30	Neurotoxin	
21	Linopirdine (HCl)		100				3	10	10			100	Cognition-enhancing drug	
22	tetraethylthiuram disulfide	200	34	down			100	30		100	100	100	Anti-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 or lowest effective concentrations (µM) that cause specific changes (*) are indicated for different read-outs. No changes indicated with blank cells.

Effects of Cannabinoids

The assay was used for testing neuroactive effects of the panel of cannabinoid substances (from Enzo Life Sciences). Several substances were active in the assay.



10 µM and 20µM concentrations demonstrated significant effects on the calcium oscillation patterns for several compounds indicated

- Some DMSO effects were also observed at same concentrations (1% or 2%), compound activities were evaluated in comparison to DMSO

1 Oleoyl GABA
2 Linoleoyl ethanolamide
3 Linoleoyl dopamine
4 Eicosa-11Z,14Z-dienamide
5 Eicosa-11Z,14Z-dienoyl ethanolamide
6 Eicosa-11Z,14Z-dienoyl glycine
7 DMSO control

Figure 7. Calcium oscillations traces and measurements are shown for 10µM concentrations, 30min treatment. SCREEN-WELL® Endocannabinoid library (BML-2801-0100).

Assessment of 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, and then, live cells were 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 cells, live cells (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.

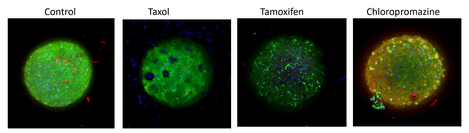
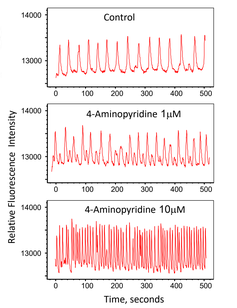


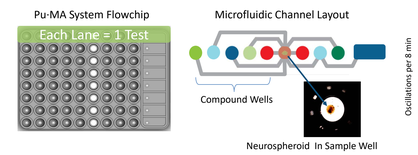
Figure 8. Composite projection images of neural spheroids. ImageXpress Micro Confocal system. Color balance reflects decreases in Calcein AM or MitoTracker signal intensities.

Automated Assay with Pu·MA System

The Pu.MA System performs automated media and compound exchanges within an incubator. 3D neurospheroids are located in a protected chamber connected to 8 compound reservoirs. An optically clear bottom allows fluorescence imaging

- Neurospheroids were loaded with Ca^{2+} sensitive dye and transferred to flowchips
- Multiple concentrations of compounds were added sequentially to each neurospheroid using microfluidics
- Calcium oscillations were recorded in the flowchips using ImageXpress or FLIPR instruments





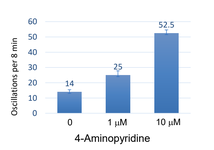


Figure 9. Assay workflow using automated microfluidics with Pu.MA system. Effect of compounds on Ca^{2+} oscillations shown after automated additions of compounds. Oscillations recorded by FLIPR and analyzed by PeakPro2.

Summary

- We described methods and demonstrated multiple examples for using 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 evaluated using FLIPR instrument and ScreenWorks PeakPro2 analysis software.
- The assays show promise for testing compound effects and early detection of neurotoxicity.