Functional And Mechanistic Neurotoxicity Profiling Using Human iPSC –Derived Neural Spheroid 3D Cultures

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INTRODUCTION

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 (microBrain 3D platform) have been developed as a high throughput screening platform that more closely resembles the constitution of native human cortical brain tissue. Neural spheroid 3D co-cultures are a physiologically relevant co-culture of iPSC-derived functionally active cortical glutamatergic and GABAergic neurons co-differentiated and matured with astrocytes from the same donor. 3D neural spheroids contain a neural network enriched in synapses, creating a highly functional neuronal circuitry and display spontaneous synchronized, readily detectable calcium oscillations.

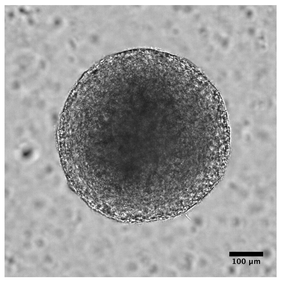
A new method for the complex analysis of calcium oscillations allows detection and multi-parametric characterization of oscillation peaks that include the oscillation rate, peak width and amplitude, characterization of secondary peaks, waveform irregularities, and several other important readouts. In addition, cellular and mitochondrial toxicity were assessed by high-content imaging.

We also include data from a neurotoxic profile of a library of 87 compounds that included pharmaceutical drugs, pesticides, flame retardants, and other chemicals. Our results show that the iPSC-derived 3D neuro-spheroid assay platform is a promising biologically-relevant tool to assess the neurotoxic potential of drugs and environmental toxicants.

iPSC-Derived microBrain® 3D

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.

StemoniX microBrain 3D spheroids:



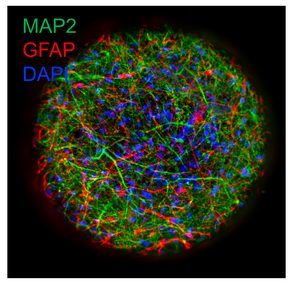


Figure 1. A. Human iPSC-derived neural spheroids composed of a co-culture of active cortical neurons (identified by MAP2; green) and astrocytes (identified by GFAP; red), approximately 600 µm diameter, imaged with ImageXpress® Micro Confocal system, 20X magnification.

INSTRUMENT

- The FLIPR® Penta System is powered by a new high-speed camera and the new ScreenWorks® Peak Pro™ 2 software.
- The system allows measuring 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 the 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.

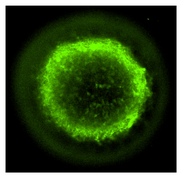
Cell Staining: To assess phenotypic changes, cells were stained live using a mixture of three dyes: the viability dye Calcein AM (1 µM), the mitochondria potential dye MitoTracker Orange (0.2 µ M), and the Hoechst nuclear dye (2 µ M) (all from Life Technologies).

RESULTS:

Calcium Oscillations Evaluated by the FLIPR 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.

3D neuro-spheroid stained with FLIPR Calcium 6 Assay Kit



Analysis of Kinetic Patterns: Measurements and Variability

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, peak raise and decay times, and irregularity. The effects of modulators of neuronal activity were evaluated by measuring changes in several measurements

Calcium oscillation traces recorded by the FLIPR Penta system:

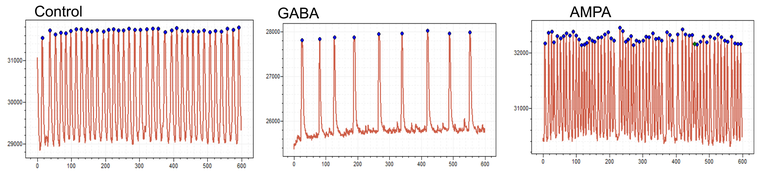


Figure 2. New ScreenWorks Peak Pro 2 software equipped with additional tools for peak analysis using >20 descriptors for observed phenotypic changes. The intracellular Ca^{2+} oscillations were assessed using the FLIPR Calcium 6 Assay Kit.

Phenotypic Effects of Neuromodulators

A set of 20+ compounds, including a number of known modulators of neuronal activity, was 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 correspondent neuromodulator.

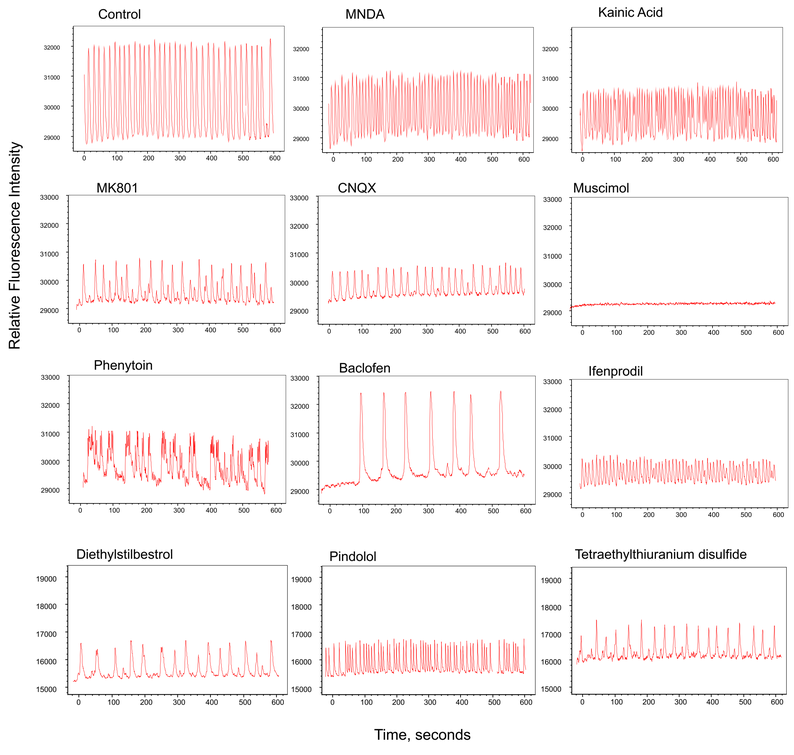
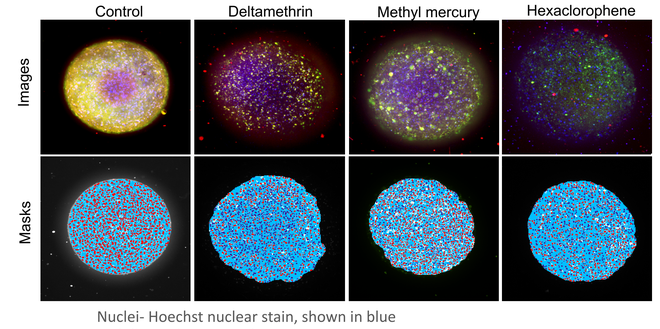


Figure 3. Using high speed EMCCD fluorescence imaging (FLIPR system), we have tested the impact of various compounds on the rates and patterns of intracellular Ca^{2+} oscillations using calcium-sensitive dye FLIPR Calcium 6 Assay Kit. High throughput recording and analysis of the entire 384-well plate 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 (for the last row of indicated compounds treatment was done for 24h). Concentration-dependent changes in the patterns were observed. Oscillation traces were recorded by the FLIPR system for 10min. Patterns were characterized using ScreenWorks Peak Pro 2 software.

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, 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 confocal option and 3D imaging. Then projection images were 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.

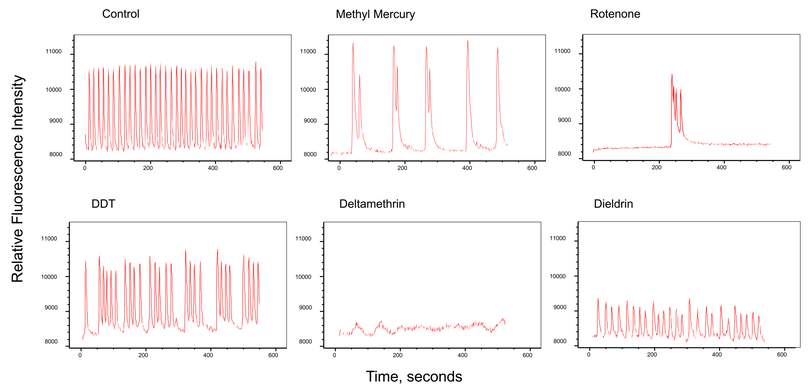


Nuclei- Hoechst nuclear stain, shown in blue
Viability- Calcein AM, shown in green
Mitochondria- MitoTracker Orange, shown in orange

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 mitochondria 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 (30 images, 15 µm apart). Maximum projection images were analyzed using 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.

Evaluation of Neurotoxicity Effects Using Selected Set of Neurotoxic Compounds

The assay shows promise for high-throughput assessment of neurotoxicity effects of chemicals *in vitro* which would help to evaluate and prioritize different substances for further testing. We have tested 87 compounds that represent different classes of toxic chemicals, including flame retardants, pesticides, poly-aromatic hydrocarbons, and demonstrated sensitivity of the assay to the number of known neurotoxicants.



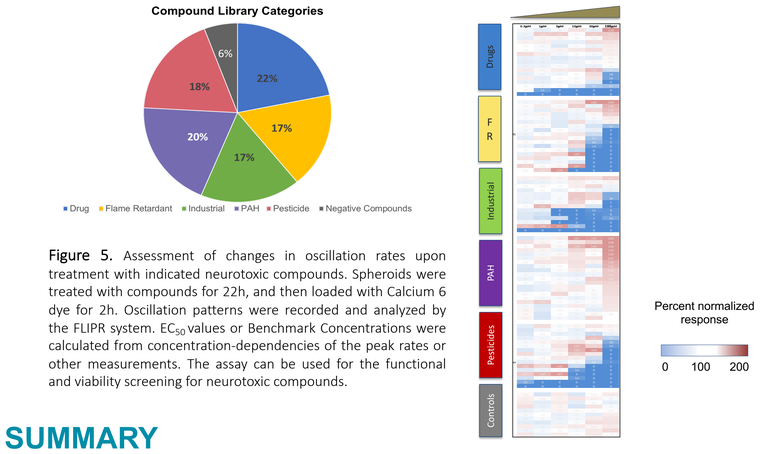


Figure 5. Assessment of changes in oscillation rates upon treatment with indicated neurotoxic compounds. Spheroids were treated with compounds for 22h, and then loaded with Calcium 6 dye for 2h. Oscillation patterns were recorded and analyzed by the FLIPR system. EC_{50} values or Benchmark Concentrations were calculated from concentration-dependencies of the peak rates or other measurements. The assay can be used for the functional and viability screening for neurotoxic compounds.

SUMMARY

- We developed the 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 expected functional responses were demonstrated using known neuromodulators and neurotoxic substances.
- The assay can be used for testing compound effects and screening for neurotoxic chemicals.

StemoniX

MOLECULAR DEVICES