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

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INTRODUCTION

Neurological disorders affect millions of people worldwide and appear to be on the rise. While the reason for this increase remains unknown, environmental factors are a suspected contributor. Hence, there is an urgent need to develop more complex, biologically relevant and predictive in vitro assays to screen larger sets of compounds with potential for neurotoxicity. Here, we employed a human induced pluripotent stem cell (iPSC)-based 3D neural platform composed of mature cortical neurons and astrocytes as a model for this purpose. The neurospheroids present spontaneous synchronized, readily detectable calcium oscillations. This advanced neural platform was optimized for high throughput screening in 384-well plates and displays a highly consistent, functional performance across different wells and plates. Characterization of oscillation profiles in neurospheroids was performed through multi-parametric analysis that included the calcium oscillations rate and peak width, amplitude, and waveform irregularities. Cellular and mitochondrial toxicity were assessed by high-content imaging. For assay characterization, we used a set of neuromodulators with known mechanisms of action as well as set of selected known neurotoxic compounds. We then explored the neurotoxic profile of a library of 87 compounds that included pharmaceutical drugs, pesticides, flame retardants, and other chemicals. Our results demonstrated that 57% of the tested compounds exhibited effects in the assay. The compounds were then ranked according to their effective concentrations based on in vitro activity. Our results show that the iPSC-derived 3D neurospheroid 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 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: brightfield image and immunstaining

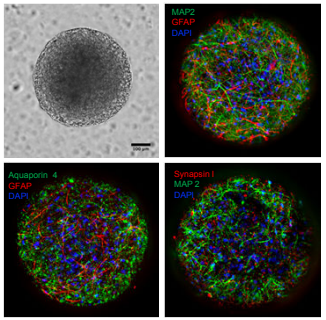


Figure 1. A. Human iPSC-derived neural spheroids, approximately 600 µm diameter, imaged with ImageXpress Micro Confocal Imaging System, 20x magnification, transmitted light. B. Fluorescent images were taken after staining cells with marker-specific antibodies as described in Materials and Methods. Images were taken using 20x objective in confocal mode. Spheroids are composed of a co-culture of active cortical neurons (identified by MAP2; green) and astrocytes (identified by GFAP; red)

INSTRUMENTS

We used fast kinetic fluorescence imaging on the FLIPR® Tetra System to measure the patterns and frequencies of the $Ca2_{2+}$ oscillations of neuro-spheroids as monitored by changes in intracellular Ca^{2+} levels with Calcium 6 dye (Molecular Devices). Cell imaging analysis was done using the ImageXpress® Micro Confocal Imaging System (IXM-C) in combination with the MetaXpress® 6.5 High-Content Imaging Software (MX 6.5). The analysis software uses algorithms for object recognition and segmentation in 3D space with a simple workflow that provide multi-parametric readouts.

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 Calcium 6 dye (Molecular Devices); 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) (all from Life Technologies). For assessment of neuro-specific markers cells were fixed with 4% formaldehyde (Sigma) and stained with anti-TuJ-1 and anti GFAP antibodies (Becton Dickinson).

RESULTS:

Calcium Oscillations Evaluated by FLIPR

The neuronal cells in the microBrain 3D spheroids generate spontaneous synchronized calcium oscillations. We used fast kinetic fluorescence imaging on the FLIPR® Tetra 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 Calcium 6 dye. A set of known neuromodulators was tested, including agonists and antagonists of NMDA, GABA and AMPA receptors; kainic acid, analgesic, and anti-epileptic drugs.

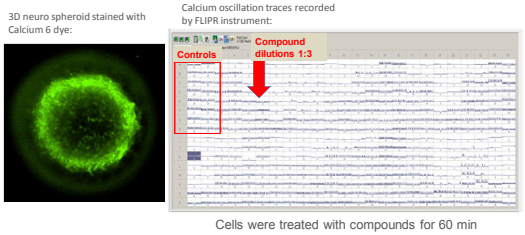


Figure 2. Using both high-content imaging and fast kinetic fluorescence imaging (FLIPR), we have tested the impact of various compounds on the rates and patterns of intracellular Ca^{2+} oscillations using calcium-sensitive dye Calcium 6. Left: Confocal image of 3D spheroid (10x) stained with Calcium 6 dye (green) taken by IXM-C instrument. Time-lapse imaging and the analysis of peak pattern can be done by using IXM-C instrument MX 6.5 software. Right: High throughput recording and analysis of the entire 384-well plate simultaneously done using FLIPR system and PeakPro software. Representative traces of calcium oscillations shown for the control and compound treated spheroids.
A screenshot shown from a 384 well plate. Spheroids were loaded with dye for 2h and then treated with compounds for 60min (recordings were performed 10min-120min). Concentration-dependent changes in the patterns were observed. Oscillation traces were recorded by FLIPR system for 10min. Patterns characterized by PeakPro software.

Analysis of Kinetic Patterns: Measurements and Variability

Advanced image analysis methods were implemented to provide multi-parametric characterization of the Ca^{2+} flux oscillation patterns. This phenotypic assay allows for the characterization of read-outs 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 the read-out values. The precision and variability of the measurements was evaluated for 24 control wells for different time-points after compound addition.

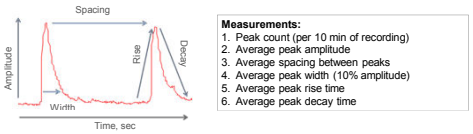


Figure 3. Descriptors for the phenotypic changes. The intracellular Ca^{2+} oscillations were assessed using the Calcium 6

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, matching the expected effect of the correspondent neuromodulator.

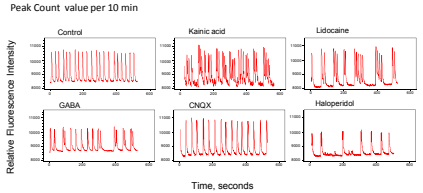


Figure 4. Assessment of changes in oscillation rates upon treatment with indicated neuromodulators. Spheroids were loaded with calcium 6 dye for 2h, treated with compounds for 60min, and then patterns were evaluated by FLIPR. Then EC_{50s} were calculated from concentration-dependencies of the peak rates, using 4-parametric curve fit.

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 IXM-C 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). Analysis methods provide efficient tools for characterization of cell and spheroid morphology.

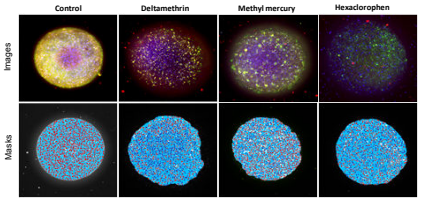
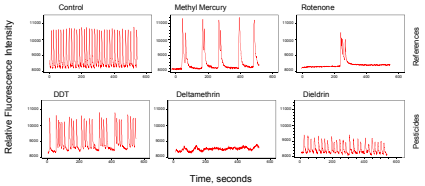


Figure 5. Composite projection images of neural spheroids. Spheroids were treated with 30 µM of indicated compounds for 24h, then stained with a nuclear stain (Hoechst 33342), viability stain (Calcein AM), and mitochondria potential dye MitoTracker Orange CMTMRos for 2 hours (2 µM, 1 µM, and 0.5 µM respectively). Spheroids were imaged with the DAPI, FITC, and TRITC, 10x Plan Fluor objective, imaged using Z-stack of confocal images (30 images, 15 um 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 spheroid. The image show nuclei (blue), Calcein AM stain (green), and mitochondria (orange). The mask showing spheroids in blue, nuclei of Calcein AM positive cells in red, negative cells in blue.

Evaluation of Neurotoxicity Effects Using Selected Set of Neurotoxic Compounds

The assay show promise for the 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 91 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.



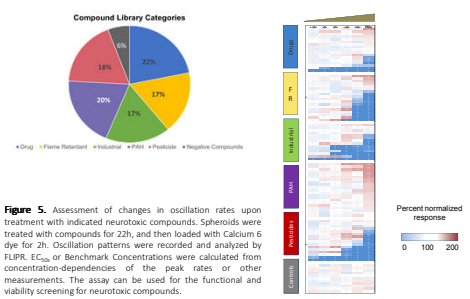


Figure 5. Assessment of changes in oscillation rates upon treatment with indicated neurotoxic compounds. Spheroids were treated with compounds for 22h, and then loaded with Calcium 6 dye for 2h. Oscillation patterns were recorded and analyzed by FLIPR. EC_{50s} or Benchmark Concentrations were calculated 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
- Expected functional responses were demonstrated using known neuromodulators
- The assay can be used for testing compound effects and screening for neurotoxic chemicals

StemoniX MOLECULAR DEVICES