**Functional and Mechanistic Neurotoxicity Profiling Using Human iPSC-Derived Neural Spheron 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 neurospheres 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 neurospheres 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 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 neurosphero assay platform is a promising biologically relevant tool to assess the neurotoxic potential of drugs and environmental toxins.

**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 neural spheroids, approx. 600 µm in diameter, are composed of a physiologically relevant coculture 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 circuit. The neuronal cells in the microbrain 3D spheroids are physiologically active, spontaneously synchronized, readily detectable calcium oscillations.

StemoniX microbrain 3D spheroids: brightfield image and immunostaining

![Image](Image 74x282 to 132x340)

**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 Ca2+ oscillations of neuro-spheroids as monitored by changes in intracellular Ca2+ levels with Calcium 6 dye. A set of known neuromodulators was tested, including agonists and antagonists of NMDA, GABA and AMPA receptors, kainic acid, anaglogs, and anti-epileptic drugs. 3D neuro spheroids stained with Calcium 6 dye: 3D micro spheroids stained with Calcium 6 dye: 3D neuro spheroids stained with Calcium 6 dye:

![Image](Image 313x425 to 447x493)

**Analysis of Kinetic Patterns:**

Advanced image analysis methods were implemented to provide multi-parametric characterization of the Ca2+ 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 the read-out values. The precision and variability of the measurements was evaluated for 24 control wells for different time-points after compound addition.

**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 EC50 values for compound effects. Changes were observed as changes in activities of the peak frequency, or other measurements, matching the expected effect of the correspondent neuromodulator.

**Assessment of Sphered 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, Calcium AM, and Mitotracker Orange dyes. Images were acquired using the In Cell System, using confocal option and 3D imaging. Then project images were analyzed using the Custom Module Editor and Cell Scoring algorithms for detection of cells for all numbers, live cells (Calcium AM positive cells), and cells with intact mitochondria (Mitotracker positive cells). Analysis methods provide efficient tools for characterization of cell and spheroid morphology.

**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.

**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 measured 8-9mm. The human spheroids were exposed to compounds for 24 hours, or as indicated in the figures. Calcium Flux Assay: The intracellular Ca2+ oscillations were assessed using the Calcium 6 dye (Molecular Devices); spheroids were loaded 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 (10 µM), and the Hoechst nuclear dye (40 µM) (all from Life Technologies). For assessment of neuro-specific markers, cells were fixed with 4% formaldehyde (Sigma) and stained with anti-Tu-1 and anti-GFAP antibodies (Becton Dickinson).

**Analysis:** Assessment of calcium oscillation rates upon treatment with indicated neuromodulators. Spheroids were loaded with calcium 6 dye 2h, treated with compounds for 60min, and then patterns were evaluated by FLIPR. Then EC50s were calculated from concentration-dependencies of the peak rates, using 4-parametric curve fit.

**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 in known neurotoxicants.
- The assay can be used for testing compound effects and screening for neurotoxic chemicals.