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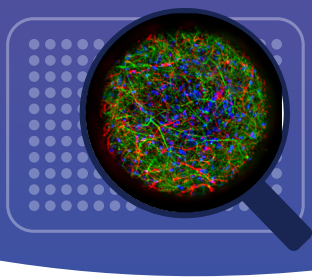
Application Note
microBrain 3D Platform



Neurotoxicity Profiling of a Diverse Chemical Library

Of the thousands of chemicals used in everything from medicine to cleaning, only a small portion have been fully studied to truly understand their neurotoxicity potential. Testing these compounds with research animals is ethically questionable, financially costly, and potentially predictively inaccurate. High throughput human, iPSC-derived neural screening platforms can provide an alternative model for assessing the toxicity potential of compounds. In this application note, we highlight results from a recent publication evaluating StemoniX's microBrain® 3D Assay Ready platform as a neurotoxicity screening platform by comparing its application in a calcium oscillation assay with traditional cell viability assays using a diverse library of compounds provided by the National Toxicology Program (NTP)[1]. Through this study, microBrain 3D was established as a biologically relevant *in vitro* tool to assess the neurotoxic potential of drugs and environmental toxicants.

The StemoniX microBrain 3D Assay Ready platform contains human iPSC-derived neural cells in a biologically relevant 3D culture format. microBrain 3D comprises a physiological mixture of human neurons and astrocytes derived from a single donor source, more accurately reflecting the biology of the human brain cortex in a high throughput compatible culture system. Coordinated spontaneous neuronal activity of the microBrain 3D spheroids results in highly rhythmic calcium oscillations that can be monitored in high throughput plate readers such as the Fluorescence Imaging Plate Reader (FLIPR®) Tetra® (Molecular Devices), thus providing a sensitive and specific phenotypic readout of neural modulation and potential hazard identification. The microBrain 3D platform has been optimized to have a highly homogeneous and consistent functional signal across wells, plates, and batches, making the system a turnkey approach for CNS drug discovery and toxicology research.

Summary

- microBrain 3D is a powerful biologically relevant tool to assess the neurotoxic potential of environmental toxins and drugs in a high throughput screening format.
- microBrain 3D identified significantly more potential neurotoxins via physiologically relevant phenotypic calcium oscillations than conventional cell viability assays, particularly for pesticides and flame retardants.
- microBrain 3D detected clear, concentration-dependent perturbations of the Ca^{2+} oscillation patterns in 57% of the library compounds compared with only 21% and 26% in cell viability and mitochondrial health assays, respectively.

Methods

A brief description of the pertinent methods for this study is provided below. Refer to Sirenko *et al.* (2019) for a detailed description of all methods used in this evaluation[1]. Of note, all assays were run in duplicate on different lots of microBrain 3D Assay Ready Plates.

Screening Model: StemoniX microBrain 3D

StemoniX microBrain 3D 384-well plates were shipped to Molecular Devices® under ambient conditions for screening studies. After receipt, media changes were performed for one week according to StemoniX's product instructions.

Compound Preparation

The chemical library was provided by the National Toxicology Program (NTP); the library reflects a diversity of chemicals from various classes including pharmaceuticals (n=19), pesticides (n=16), flame retardants (n=15), polycyclic aromatic hydrocarbons (PAHs, n=17), various industrial chemicals (n=15), and negative controls (n=5)[2]. Stock solutions of each chemical were prepared in cell-grade DMSO (Sigma) and stored at -20°C until use.

Viability Assay

To evaluate the effect of the compounds on cell viability, microBrain 3D spheroids were treated with each compound for 24 h and then were stained with Hoechst nuclear stain (total cells), Calcein AM (live cells), and MitoTracker Orange dyes (intact mitochondria). Automated confocal imaging (ImageXpress® Micro Confocal, Molecular Devices) was performed on treated spheroids, and Z-stacked 3D images were analyzed (MetaXpress® 6.2 Software, Molecular Devices) to characterize and quantify the cellular and mitochondrial toxicity in 3D neural spheroids after compound exposure[1].

Calcium Oscillation Assay

To determine whether Ca^{2+} regulation in microBrain 3D could be measurably and quantifiably perturbed by the addition of chemicals, we employed a multiparametric approach examining six quantitative parameters of intracellular Ca^{2+} oscillations: peak count; peak rise and decay times; and average peak amplitude, width, and spacing over a 10-minute recording. microBrain 3D neural spheroids were exposed to six different concentrations of each compound for 22 h in duplicate[1]. Vehicle and untreated controls were included on each assay plate for normalization of plate-specific phenotypic readouts. After 22 h, Calcium 6 dye (Molecular Devices) was added to each well according to the manufacturer's recommendations, and spheroids were incubated for an additional 2 h. Patterns and frequencies of intracellular Ca^{2+} oscillations were measured using the FLIPR Tetra High Throughput Cellular Screening System (Molecular Devices).

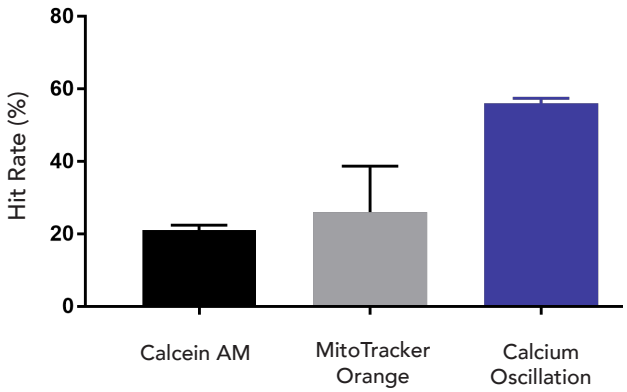


Figure 1. Overall, the calcium oscillation assay identified significantly more neurotoxic compounds than either of the viability assays. Hit rate is based on multiparametric Bench Mark Concentration analysis described by Sirenko *et al.*[1]. Error bars indicate the high level of consistency between replicate experiments

Results and Discussion

As originally reported[1], we tested a library of 87 compounds with known and unknown neurotoxicity potential. Of the diverse and unique compounds tested, 47 (57%) were identified as neuroactive/neurotoxic, showing clear, concentration-dependent perturbations of the Ca^{2+} oscillation patterns compared with 22 (21%) from Calcein AM and 27 (26%) from MitoTracker Orange (**Figure 1**). This result suggests that the physiologically relevant down-stream calcium oscillations of microBrain 3D afford greater sensitivity to compounds that impact cell function than more classic terminal endpoints such as cell viability. Of note, this result is more than twice that observed in a zebrafish embryo model[3] used to screen the same NTP library.

The screening results were further compared by chemical subclass (**Figure 2**). Modulation of calcium oscillations in microBrain 3D was significantly more sensitive than cell viability studies, which have been

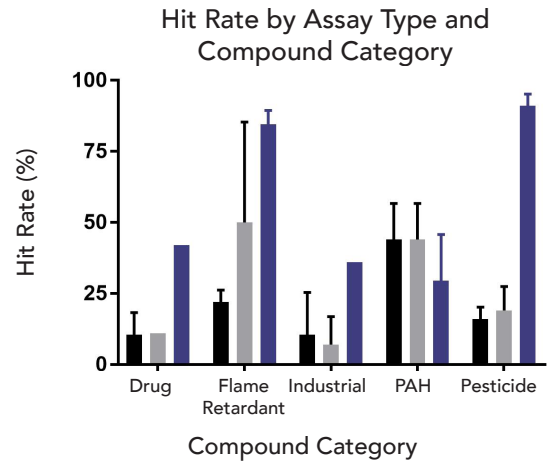


Figure 2. Comparison of results between the calcium oscillation assay and viability assays for the five different classes of compounds in the NTP library. The calcium oscillation assay demonstrated greater sensitivity for 4 of the 5 compound subclasses.

conventionally used for *in vitro* toxicity testing, for both flame retardants (84% vs. 36%) and pesticides (91% vs. 17%, respectively). Similarly, industrial chemicals (36% vs. 9%) and pharmaceuticals (42% vs. 11%) effected greater impact on functional neural cell calcium handling than on viability. Of the five chemical classes, only PAHs showed marginally higher sensitivity in viability than in calcium handling (44% vs. 29%, respectively).

To further highlight the value of the functional assay compared with viability assays, we examined the drug subclass of chemicals where the modes of action of the compounds are better understood. microBrain 3D elicited functional responses in 8 of the 19 compounds (42%) compared with 5% and 10% in the Calcein AM and MitoTracker Orange viability assays, respectively (Table 1). Again, functional Ca^{2+} oscillation proved to be a more sensitive detector of neuromodulation. While significantly more drug compounds elicited neuromodulatory responses on the StemoniX microBrain 3D platform, the library contains several compounds that were clearly not expected to show results in our model as they are expected to impact non-terminally differentiated neurons or biological processes, such as cell proliferation, that are not present in mature neuronal structures (Table 1). Examples include Thalidomide[a] (developmental toxin), 5-FU[b], colchicine[b], and hydroxyurea[b] (cell proliferation), and 6-hydroxydopamine[c] (adrenergic and dopaminergic targets not present in microBrain 3D).

Conclusions

The significant advantages of microBrain 3D for drug screening and toxicology studies are two-fold: 1) the consistent structure of the preparation provides a robust foundation for interrogation, and 2) the highly homogeneous and reproducible synchronous calcium signal affords a robust and reliable platform to analyze physiological modulation and hazard identification. Furthermore, the compatible format of microBrain 3D enables more granular investigation, such as cell viability and mitochondrial health, to further dissect and provide mechanism of action for the phenotypic alert. This study, the first reported in the literature to characterize calcium oscillations in a 3D neural spheroid culture as a screening tool for neurotoxicity, successfully demonstrated that the calcium oscillations of microBrain 3D are more sensitive to perturbation than traditional downstream assays. Thus, microBrain 3D is emerging as a powerful tool to assess neurotoxicity hazard identification in high throughput investigations.

References

1. Sirenko et al., Tox. Sci. 167(1), 58-76 (2019)
2. Frank et al., Tox. Sci. 160(1), 121-135 (2017)
3. Quevedo et al. Biobide.com
4. Vargesson. Birth Defects Res. C: Embryo Today. 105, 140–156 (2015)

This work done in collaboration with scientists at MOLECULAR DEVICES

Table 1. Assay results for the compounds in the drug subclass. "No Hit" means that the compound did not elicit a statistically significant response in the assay across the dosage range for any of the parameters analyzed, whereas "Hit" indicates that the compound did elicit a response.

Compounds	Functional	Viability	
	Calcium Oscillation	Calcein AM	MitoTracker Orange
1-Methyl-4-phenylpyridinium iodide	**Hit**	No Hit	No Hit
Berberine chloride	**Hit**	No Hit	No Hit
Diazepam	**Hit**	No Hit	No Hit
Diethylstilbestrol	**Hit**	No Hit	No Hit
Estradiol	**Hit**	No Hit	No Hit
Tetraethylthiuram disulfide	**Hit**	No Hit	No Hit
Valinomycin	**Hit**	No Hit	**Hit**
Hexachlorophene	**Hit**	**Hit**	**Hit**
5-Fluorouracil[b]	No Hit	No Hit	No Hit
5-Nitro-2-furaldehyde semicarbazone	No Hit	No Hit	No Hit
6-Hydroxydopamine hydrochloride[c]	No Hit	No Hit	No Hit
6-Propyl-2-thiouracil	No Hit	No Hit	No Hit
Amoxicillin	No Hit	No Hit	No Hit
Colchicine[b]	No Hit	No Hit	No Hit
Hexachlorophene	No Hit	No Hit	No Hit
Hydroxyurea[b]	No Hit	No Hit	No Hit
Phenobarbital	No Hit	No Hit	No Hit
Phenobarbital sodium salt	No Hit	No Hit	No Hit
Thalidomide[c]	No Hit	No Hit	No Hit
Valproic acid sodium salt	No Hit	No Hit	No Hit

[a]Inhibition of pro-angiogenic factors
[b]Inhibition of cell proliferation
[c]Selective to adrenergic & dopaminergic neurons

Product Information

Product Name	Catalog #
microBrain® 3D Assay Ready 96-Well Plate	BSARX-AA-0096
microBrain® 3D Assay Ready 384-Well Plate	BSARX-AA-0384

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