APPLICATION NOTE

Assessment of neurotoxicity and neuronal development using induced pluripotent stem cell-based neurite outgrowth assay

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Introduction

Growing concerns about the increased prevalence of untested chemicals in the environment has created a pressing need to develop reliable and efficient screening tools to identify chemicals that could potentially affect human health[1], particularly in neurological development.

We evaluated a neurite outgrowth assay as a possible screen to characterize the activity of selected compounds with the potential to adversely affect the developing nervous system. This assay was selected due to its relevance as a model of a critical process in nervous system development in which neurons extend their neurites to form a complete neural network[2]. Disruption of this process can lead to adverse effects in humans and rodents whereby studies suggest that immature, developing, and mature neurites are targets of chemical toxicity[3].

While predominantly used to assess developmental neurotoxicity, the neurite outgrowth assay can also be used to evaluate neurodegeneration as measured by neurite retraction. Furthermore, this assay might be relevant for evaluation of neuroplasticity in adult neurons[4]. For screening assays, total neurite outgrowth is typically the most common metric reported[1,5]. Utilizing automated imaging enables multi-parametric evaluation of additional features, such as the number of total branches and total processes to encompass different modes by which compounds may inhibit neurite outgrowth[6,7].

Benefits

- Identify neurotoxic effects using iPSC-derived neurons
- Quantify complex neurite networks using multi-parameter image analysis
- Evaluate and prioritize chemicals for their neurotoxicity potential

Materials

- ImageXpress® Nano Automated Imaging System with CellReporterXpress® software (Molecular Devices)
- iCell® Neurons (Cellular Dynamics International)
- Poly-d-lysine pre-coated 384-well plate (Corning Life Sciences)
- Laminin (Sigma-Aldrich)
- Paraformaldehyde (Sigma-Aldrich)
- Fetal Bovine Serum (Sigma-Aldrich)
- Beta-tubulin III (TUJ-1) (BD Biosciences)
- Hoescht (ThermoFisher Scientific)
- Anti-ß-tubulin antibodies (BD Biosciences)
- Calcein AM (ThermoFisher Scientifiic)

Identification of neurotoxic compounds using an iPSC-based neurite outgrowth assay

Previously characterized human iPSC-derived neurons (i.e. iCell Neurons), consisting of a mixture of post-mitotic GABAergic and glutamatergic neurons, and supporting media were provided by Cellular Dynamics International (CDI). Neurons used in these studies were provided by the manufacturer as a fully differentiated and purified population of cells that formed neurite networks positive for the neuronal markers beta-III tubulin and MAP2[8]. Cells were received frozen and were subsequently thawed and plated according to a protocol recommended by CDI. Cells were plated on poly-d-lysine, pre-coated 384-well plates and treated with 3.3 µg/mL laminin. Prior to compound treatment, 7,500 cells were plated per well and were maintained in iCell Neurons Maintenance Medium for 48h. Neurite networks in these cells typically start to form ~24h post plating and increase in complexity up to 10–12 days in culture. At 48h post plating, we assessed neurite outgrowth, however, neurite retraction may have also occurred concomitantly. Compounds were tested in duplicates across a 6-point concentration range (0.3, 1.0, 3.0, 10.0, 30.0, and 100 µM). Multiple DMSO controls (n = 16) and untreated controls (n = 16) were included in each plate. Up to 0.3% DMSO was used to assess solvent effects within the assay. Cells were exposed to compounds for 72h at 37°C and 5% CO_2.

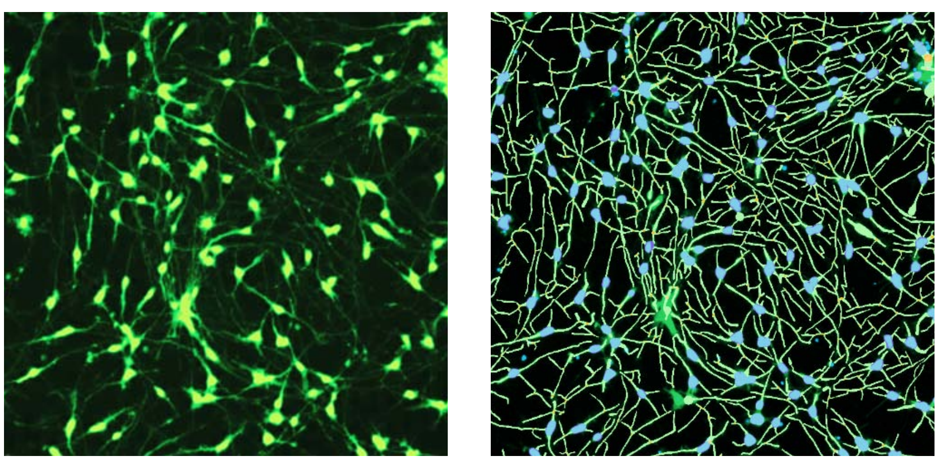

Figure 1. *Images of ß-tubulin (green) stain and the software analysis traces shown for the control cells. iCell Neurons were plated for five days, then fixed and stained with AF488-conjugated anti-ß-tubulin (TUJ-1) antibodies (1:100). Images were taken by the ImageXpress Nano system using a 10X Plan Fluor objective and FITC channel. Images were processed using the Neurite Tracing analysis algorithm in CellReporterXpress software. Analysis masks on the right show the outgrowth (green) and cell bodies (blue).*

Next, media was removed and cells were fixed with 4% paraformaldehyde for 2h. This was followed by permeabilization with 0.01% saponin in PBS with 1% Fetal Bovine Serum. Then, the cells were incubated with AF488-conjugated mouse anti-human antibodies against beta-tubulin III (TUJ-1) (1:100 dilution) and 2 µg/mL Hoechst for 3h. Beta-tubulin was used as a marker for neurite outgrowth and also for counts of the intact neuronal cell bodies. After incubation, the staining solution was replaced with phosphate buffered saline (PBS). Alternatively, the neurotoxicity assay can be done using live cells, stained for 30 min with Calcein AM and Hoechst dyes (0.5 µM and 2 µM respectively). Details regarding optimization of plating density and the protocol for the 384-well format assay are described in detail in Sirenko et al.[6]

Images from individual wells were acquired with the ImageXpress Nano Automated Imaging System using a 10X Plan Fluor objective. One 10X image was captured at a single site per well in a 384-well plate. The 10X objective provided sufficient resolution to distinguish neurite networks and sub-cellular structures in a relatively large number of cells (500–1,000) per image, which represented about 1/4 of the total well area. Following image capture, all image analysis was accomplished using the CellReporterXpress software, which contained image processing application modules for neurite outgrowth and viability assessment. As an example of image processing, Figure 1 shows zoomed in representative images from neurite images and corresponding analysis masks. Figure 2 shows images from DMSO-treated neurons and compound treated neurons with software tracing overlays.

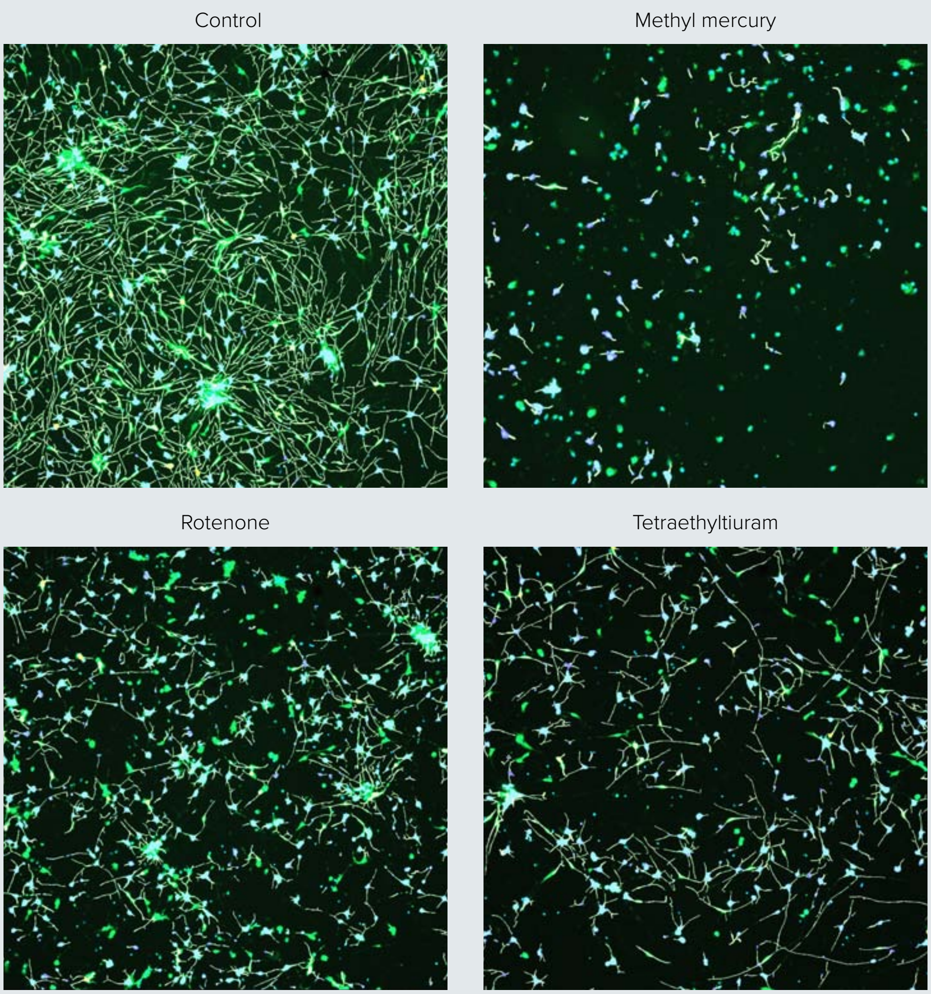

Figure 2. *Composite images of ß-tubulin (green) and Hoechst (blue), with the analysis traces shown for the control cells and the cells treated with selected compounds. iCell Neurons were plated for 48h, treated with compounds for 72h, and then fixed and stained with a combination of Hoechst (2 µM) and AF488-conjugated anti-TUJ-1 antibodies (1:100). Images were taken by the ImageXpress Nano system using a 10X Plan Fluor objective and DAPI and FITC channels. Images were processed using the Neurite Tracing analysis algorithm in CellReporterXpress software. Disruption of neurite networks and cell death was observed for neurons treated with indicated compounds.*

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Quantifying the complexity of neurite networks using multi-parameter image analysis

We observed a dose-dependent inhibition of neuronal network formation due to compound treatment effects (Figure 3). Quantitative analysis of the images captured in these experiments included the derivation of multiple parameters allowing for the assessment of both the morphological features of cultured neurons, and the extent and degree of complexity of the neuronal networks. Specifically, neurite outgrowth was characterized by the extent of the outgrowth (e.g. length of total outgrowth or mean outgrowth per cell), the number of neurite processes (e.g. total number of processes and mean number of processes per cell), and the extent of branching (e.g. total number of branches and mean number of branches per cell). Cell plating and neurite outgrowth were uniform across the experiment, so we used total numbers of features (branches and processes) per image for statistical analysis. In addition, the number of ß-tubulin (TUJ-1 positive), or Calcein AM positive cell bodies in each image was quantitated to assess compound-induced cell death. The length of outgrowth per cell and the numbers of processes and branches per individual cell were also measured but were not used for statistical analysis due to redundancy.

The toxic effects of compounds can be compared by EC_{50} values (concentration of compound for 50% inhibition of neurite outgrowth). EC_{50} values were derived from the 4-parametric curve fits by the values for neurite outgrowth, number of branches, number of processes, and number of viable cell bodies. Figure 3 shows concentration dependent curves for the total length of neurite networks (total outgrowth) and total numbers of branches. These measurements allowed us to define effective toxic concentrations, compare compounds for their potential neurotoxic effects, and prioritize for further toxicity evaluation.

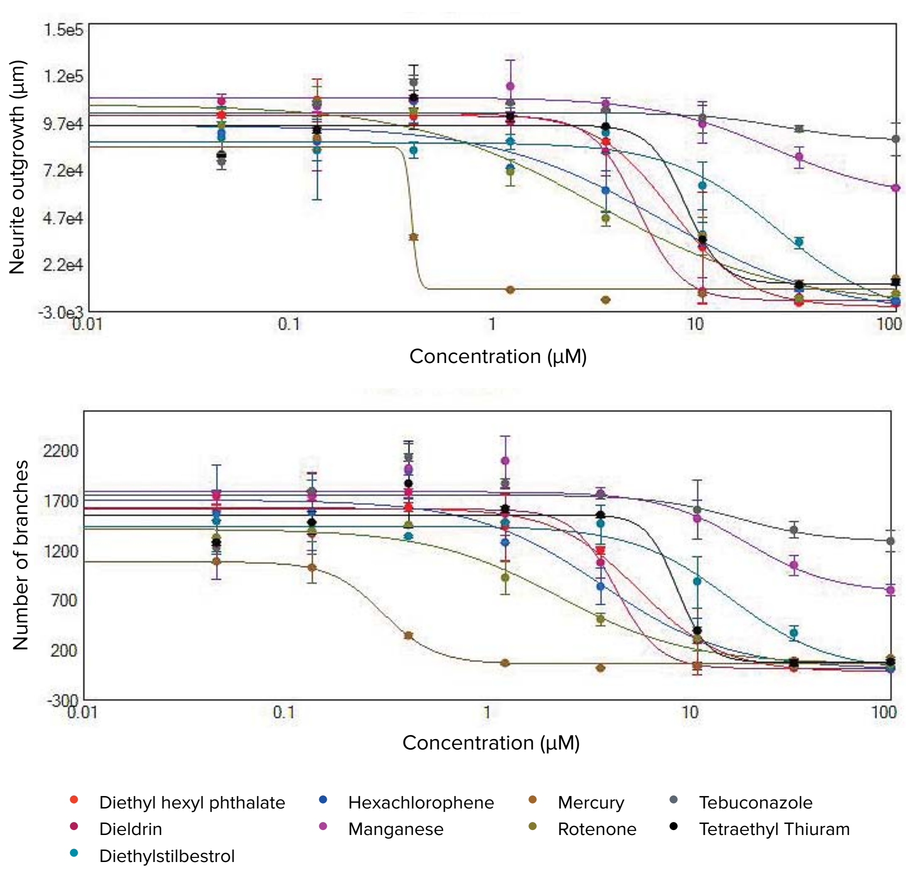

Figure 3. *Concentration response curves for total neurite outgrowth lengths and total numbers of branches. Disruption of neurite networks and cell death observed after compound treatments were quantitated using the Neurite Outgrowth analysis algorithm in CellReporterXpress software. Dose-dependent effects are indicated by decreased total outgrowth lengths and numbers of branches. The 4-parameter curve fit is shown here for the selected compounds using these two readouts.* EC_{50} *values are included in Table 1.*

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Evaluating dose-response for multiple parameters

The concentration response curves were evaluated using the Hill model to derive EC_{50} concentration values. The EC_{50} values for different readouts are presented in Table 1. Among the 16 tested compounds, treatment with 11 of these resulted in decreased neurite outgrowths as well as reduced numbers of branches and processes. Of these 11 compounds, treatment with six of these also resulted in a decreased number of cell bodies. The other five tested compounds had a relatively minor effect, therefore EC_{50} values were not determined. The EC_{50} values for inhibition of total branches and total outgrowth endpoints exhibited high concordance. The disruption of neuronal networks was evident at lower concentrations versus the cytotoxicity effect for selected treatments. Therefore, the EC_{50} values measured for the decrease in the number of cell bodies were typically higher than EC_{50} values for inhibition of neurite outgrowth due to treatment with these compounds.

Compounds EC_{50} value (µM)	Total outgrowth	Branches	Processes	Cell bodies
Methyl mercuric chloride	0.403**	0.307±0.029	0.569±0.183	1.19
Rotenone	3.31±1.56	2.28±0.616	9.77±6.94	11.5
Dieldrin	5.31±1.19	4.38±1.05	6.53±2.33	10.4
Hexachlorophene	7.08±3.84	3.72±1.46	19.2±11.9	32.6±21.8
Di(2-ethylhexyl) phthalate	7.79±0.63	5.72±0.73	9.48±0.49	12.6±1.07
Tetraethylthiram disulfide	9.133±4.28	8.82±7.94	13.2±5.92	12.3±5.08
Carbaryl	15.5±30.4	2.5±0.673	>100	102
Manganese tricarbonyl	24.9±12.6	19.38±17.7	2.19±4.16	>100
Diethylbbestrol	26.5±7.73	16.2±3.54	35.2	>100
Tebuconazole	27.7±82.5	17.7±35.3	22.5±151	>100
Cryzene	31.6±8.28	23.5±30.9	>100	>100
Aldicarb	>100***	>100	>100	no effect****
Acenaphthylene	>100	>100	>100	no effect
Triphenyl phosphate	>100	>100	>100	>100
Diazepam	>100	>100	>100	no effect
Dibenz(a,c) anthracene	>100	>100	>100	no effect

* EC_{50} values (in µM) measured for tested compounds using the length of total neurite outgrowth and number of branches as readout. Error limits are Standard Error of the parameter estimate defined from the curve fit.

** The undefined standard errors for some parameters indicate that although the curve fits have converged, the uncertainty in the parameter estimates could not be determined.

*** ">100" means toxic effects (decrease of neurite outgrowth, numbers of branches, etc.) were observed at the highest concentration tested (100 µM), but EC_{50} values were not determined.

**** "No effect" means no apparent effects were observed at the highest concentrations tested (100 µM).

Table 1. *EC_{50} values for neurotoxic effects measured for tested compounds using different readouts. Concentration response curves were calculated for total neurite outgrowth, total branches, total processes, and total cell bodies. EC_{50} values were derived from the 4-parameter curve fits for each compound treatment using the Hill model.*

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Conclusion

The neurite outgrowth assay is an efficient and effective high-throughput screening experiment suitable for evaluation of neurotoxicity across large libraries of chemical compounds. This screen allows for rapid identification, evaluation, and prioritization of compounds for their potential to induce neurotoxicity in humans. This assay can also be used to evaluate compounds that may have a neuroprotective or stimulative effect on neuronal development. The ImageXpress Nano system is an efficient tool for performing advanced phenotypic assays suitable for a high-throughput screening campaign.

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