High Throughput High Content Screening for Developmental Neurotoxicity in Human iPSC-Derived Neurons

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INTRODUCTION

There is an increased need to develop reliable and efficient screening tools to identify chemicals with the potential to adversely affect neurological development. Human iPSC-derived neurons exhibit the functionality and behavior of mature neurons and are available in quantities sufficient for screening workflows. We have developed imaging and analysis methods that provide tools for the assessment developmental neurotoxicity (DNT) using iPSC-derived neuronal cells.

In an effort to develop and characterize an in vitro model system for DNT screening, we exposed human iPSC-derived neurons to a diverse set of 80 chemicals (e.g., neurotoxicants, drugs, pesticides, flame retardants (FRs), polycyclic aromatic hydrocarbons (PAHs)) across a 6-point concentration range (~0.3 to 100 µM) in 384-well plates.

Since mitochondrial function has been associated with neuronal function we have tested changes of mitochondria membrane potential. In addition, we have evaluated processes of autophagy and mitophagy, characterized by co-localization of mitochondria and autophagy granules.

Compounds were ranked by activity and selectivity (i.e., compound specific effects on neurite outgrowth and mitochondria integrity independent of cytotoxicity). This strategy is useful for ranking compounds for potential neurotoxicity effects and aids in prioritization for more comprehensive in vivo assessments.

Objectives:

- Characterize the neurite outgrowth assay as a suitable high throughput screen for a neurotoxicity
- Explore the relationship between mitochondrial dysfunction and inhibition of neurite outgrowth
- Prioritize NTP chemicals of concern (e.g., flame retardants, PAHs) for more comprehensive in vivo hazard characterization studies

MATERIALS & METHODS

Chemical Library

An 80–compound library was selected by the National Toxicology Program that included several chemicals with reported neurotoxicity or developmental neurotoxicity. Also included were chemical classes of interest based on high production volumes, environmental persistence, and lack of known neurotoxicity information. Chemicals were dissolved in dimethylsulfoxide (DMSO) and tested in replicate at each concentration (0.3-100 µM). DMSO and negative control chemicals as well as four chemicals in duplicate were included on each test plate.

In vitro Neurotoxicity Assay

Cells were received frozen from Cellular Dynamics International (CDI, Madison WI). Cells were thawed and plated according to CDI's protocol. Cells (7.5K per well) were treated for 72 hr on laminin coated 384–well plates. Cells were stained with Calcein AM & Hoescht dye (Invitrogen, Carlsbad, CA) for 30 minutes prior to imaging on an Image Xpress XL Instrument (Molecular Devices, Sunnyvale, CA). Images were acquired using 10X or 20X objectives.

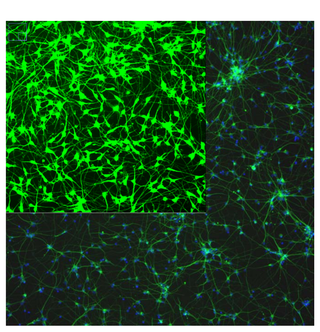
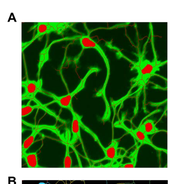
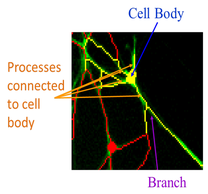


Figure 1. Representative image of iCell neurons stained with AF488 anti-b-tubulin III and DAPI or Calcein AM for visualization with MetaXpress Software.





Neuronal Bodies: Number of viable cell bodies in the image
Total Outgrowth: Total length of skeletonized outgrowth in mm (corrected for diagonal lengths)
Total Processes: Number of outgrowths in the image that are connected to cell bodies
Total Branches: Total number of branching junctions in the image

Figure 2. Image analysis process results for neurons. **A.** Identification of neuronal body with calcein AM single color image. **B.** Segmentation results showing bodies and neurite outgrowth. **C.** Definitions of endpoints.

Benchmark Concentration Analysis

Dose-response information is critical for ranking chemicals for toxicity or safety. However, robust calculation of half-maximum activity responses (i.e., AC_{50}) is limited by the need for two asymptotes. High concentration response plateaus are often not present, leading to a need for an improved analysis method. To overcome this limitation, we applied the Benchmark Concentration (BMC) analysis method developed by the U.S. Environmental Protection Agency for analysis of toxicity test data[1,2] to the multi-parametric neurite outgrowth assay. Response data were normalized by dividing by the mean of the DMSO controls. Concentration-response was modeled using a Hill model. The model parameters were optimized by least-squares fitting using MATLAB. The lower confidence interval of the BMC (BMCL) was calculated using Hill model parameters.

Model equation: $\mathbf{f(x)=v_0 + (v_{max}-v_0)* x^n/(k^n + x^n);}$ v_0 is fixed at 1 (mean value of DMSO control data)

Mitochondria Potential and Autophagy Assays

Effects were monitored by the addition of the mitochondria active dye JC-10 (ATT BioQuest Inc., Sunnyvale, CA) for 30 mins prior to imaging on an Image Xpress XL Instrument (Molecular Devices, Sunnyvale, CA). Images were acquired using 20X objectives. Autophagy was detected by using CytoID Autophagy Dye (Enzo) and co-staining with mitotracker orange reagent (Life Technologies). We have evaluated processes of autophagy and mitophagy (recycling of mitochondria), by characterizing co-localization of mitochondria and autophagy granules

RESULTS:

Compound-Specific Effects on Neurite Outgrowth

Effects on neurite outgrowth, complexity of networks and viable cell number were assessed following a 72 hr of exposure. Dose-dependent responses to selected toxic compounds shown below. Phenotypic read-outs included characterization of neurite outgrowth, branching, number of processes, and cell viability.

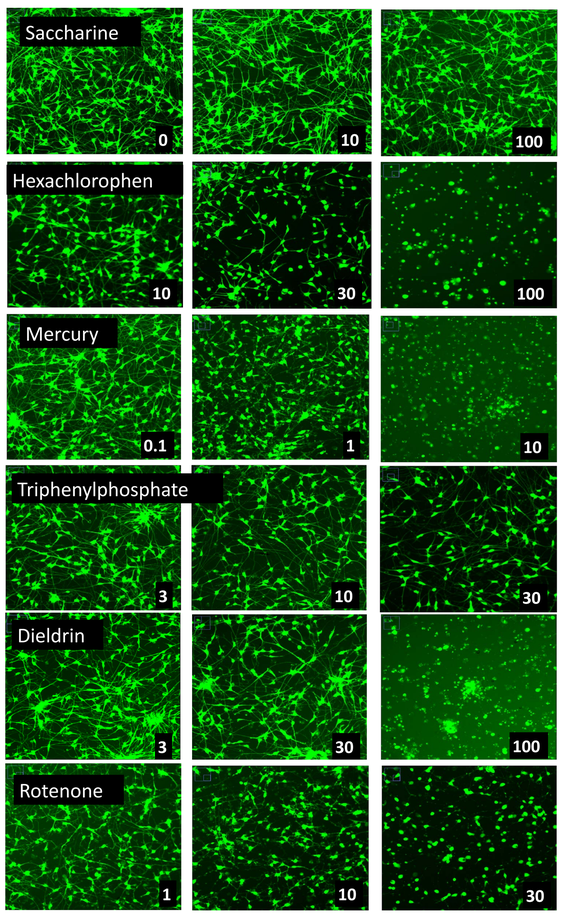
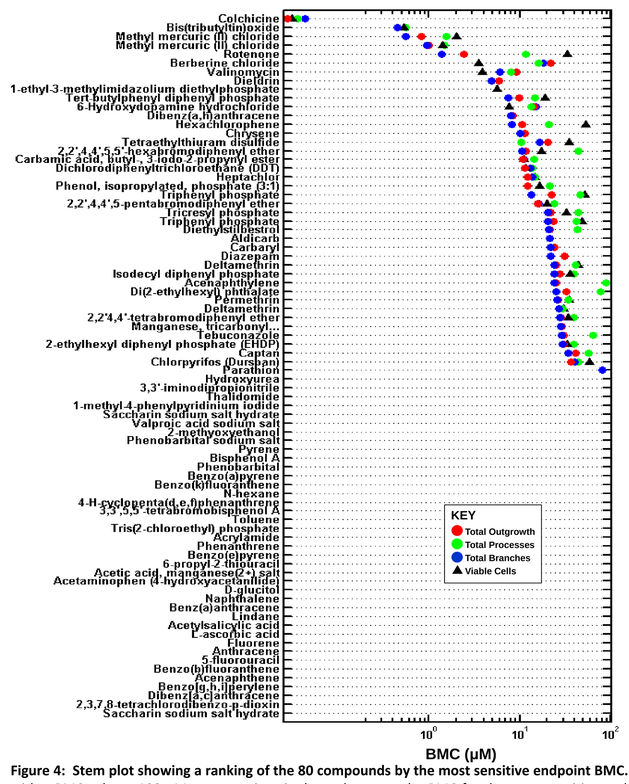


Figure 3: Concentration-dependent des-integration of the neuronal networks as a result of compound treatment (um). Cells stained with Calcein AM and imaged using 10x magnification.

Ranking of Chemicals by BMC per endpoint

The assay-specific noise threshold was calculated based on DMSO control variability and concentration-response profiles were evaluated using a Hill model to derive benchmark concentrations (BMC). Chemicals were ranked by toxicity and selectivity. Neurite total outgrowth and branching were the most sensitive endpoints; 16 chemicals had an effect on neurite outgrowth independent of cytotoxicity.

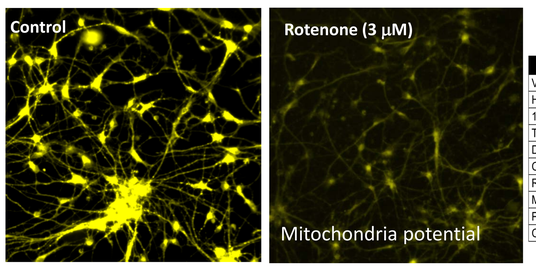


41/80 (51%) of the compounds had a effect on at least 1 endpoint in the neurite outgrowth assay

Figure 4: Stem plot showing a ranking of the 80 compounds by the most sensitive endpoint BMC. For all compounds with a BMC value < 100 µM, a comparison is shown between the BMC for the most sensitive endpoint and the BMC for viability as a marker of cytotoxicity. For several compounds, the BMC was not estimated because no effect was observed over the concentration range tested (0.3 – 100 µM). BMC = benchmark concentration.

Compound Effects on Mitochondrial Membrane Potential

Since toxic effects of some chemicals (e.g. rotenone) was associated with mitochondria damage, we have tested effects of compounds on mitochondria membrane potential. Of the 76 compounds, 39 decreased MMP and 9 were active in both assays.



Compound	BMC (µM)
Valinomycin	0.04
Hexachlorophene	0.06
1-ethyl-3-methylimidazolium diethylphosphate	0.51
Triphenyl phosphate	0.76
Dieldrin	0.88
Carbaryl	1.29
Rotenone	1.60
Methyl mercuric (II) chloride	1.86
Phenol, isopropylated, phosphate (3:1)	2.65
Carbamic acid, butyl-, 3-iodo-2-propynyl ester	2.81

Figure 5. Images from mitochondria potential assay for control and treated samples. Calculated BMC values are given in the table on the right.

- 39/80 (49%) of the compounds had a effect on mitochondrial membrane potential (MMP)

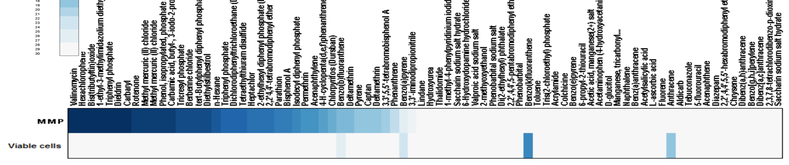


Figure 6. Heatmap of BMC values for all compounds tested in the 1 hr MMP assay. Blue = decreasing responses in MMP or cell viability. White = no effect in the concentration range tested (0.1 – 30 µM).

Selective Compounds in the Neurite Outgrowth Assay[a]	Category	Neurite Outgrowth BMC (µM)	MMP BMC (µM)
Rotenone	NT/DNT	1.37	1.60
Dieldrin	NT/DNT	4.91	0.88
Hexachlorophene	NT/DNT	8.12	0.06
Dibenz(a,h)anthracene	PAH	7.91	30.00
Diethylstilbestrol	NT/DNT	20.84	3.97
Aldicarb	NT/DNT	21.14	30.00
Chrysene	PAH	10.11	30.00
Carbaryl	NT/DNT	21.65	1.29
Diazepam	NT/DNT	21.82	30.00
Acenaphthylene	PAH	24.14	12.59
Di(2-ethylhexyl) phthalate	NT/DNT	25.06	30.00
Triphenyl phosphate	FR	13.33	6.24
Methyl mercuric (II) chloride	NT/DNT	0.55	1.86
Manganese, tricarbonyl[(1,2,3,4,5-.eta.)-1-methyl-2,4-cyclopentadien-1-yl]-	Other	28.52	30.00
Tebuconazole	NT/DNT	29.14	30.00
Tetraethylthiuram disulfide	NT/DNT	10.29	6.61

[a] compounds listed in order of selectivity scores (SS) (high to low) for neurite outgrowth by the most sensitive endpoint. Gray colored box: compounds affected both neurite outgrowth and MMP < 30 µM.
NT/DNT = neurotoxicant/developmental neurotoxicant; FR = flame retardant; PAH = polycyclic aromatic phosphate; BMC = benchmark concentration value

Autophagy

Neuronal damage has been associated with disruption of the normal process of mitochondria recycling (mitophagy). Processes of autophagy and mitophagy can be evaluated in the future studies by characterizing co-localization of mitochondria and autophagy granules (shown in PC12 neuroblastoma cell line and iPSC-derived neurons).

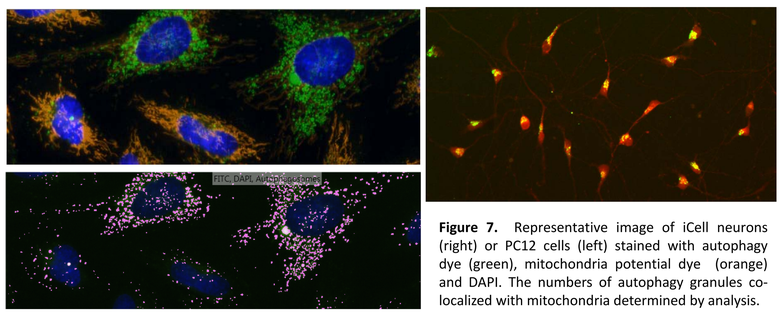


Figure 7. Representative image of iCell neurons (right) or PC12 cells (left) stained with autophagy dye (green), mitochondria potential dye (orange) and DAPI. The numbers of autophagy granules co-localized with mitochondria determined by analysis.

CONCLUSIONS

- Automated high throughput, high content imaging can be utilized to evaluate the effects of chemicals on neurite outgrowth in human iPSCs.
- The endpoint with the least noise in the solvent control data was viable cells (measure of cytotoxicity), followed by neurite total outgrowth, total processes, and total branches.
- The majority of actives in this assay were identified by effects on total branching > total outgrowth > total processes.
- Duplicate chemicals show similar concentration responses & calculated BMC values indicating good internal assay reproducibility.
- 9/76 unique compounds inhibited both MMP (1 hr) and neurite outgrowth (72 hr) and belong to diverse structural classes such as pesticides, drugs, metal-containing compounds, FRs, and PAHs, suggesting that mitochondrial dysfunction may be an upstream signaling event of neurite outgrowth inhibition.

References

1. US EPA (2012) Benchmark Dose Technical Guidance http://www.epa.gov/raf/publications/pdfs/benchmark_dose_guidance.pdf
2. Robin X et al. (2011) BMC Bioinformatics 12: 77
3. Radio N et al. (2008) Tox Sci 105(1): 106-118
4. Harrill et al. (2011) Toxicol and Appl Pharmcol 256: 268-280
5. Bal-Price et al. (2011) ALTEX 29(2): 202-15

Disclaimer

This poster does not necessarily represent any final NTP determination or policy.

NTP National Toxicology Program U.S. Department of Health and Human Services

MOLECULAR DEVICES