High Content Neuronal Toxicity Assays Using iPSC Derived Neurons

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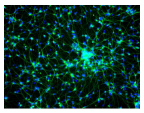
Introduction

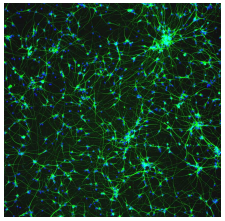
The nervous system is a target organ for the toxic effects of chemical compounds, environmental agents and some naturally occurring substances. Neurotoxicity can cause temporary or permanent damage of brain or peripheral nervous system and has been found to be a major cause of neurodegenerative diseases. Therefore, there is a great interest in developing more predictive cell-based models and efficient screening tools for qualitative and quantitative assessment of the impact of chemical compounds, drug candidates and environmental agents. Human neurons derived from induced pluripotent stem cells (iPSC) such as iCell® Neurons are very attractive for such studies because they exhibit functionality and behavior of mature neurons, and are available in large quantities.

Here we present several assays using iCell neurons and high content imaging for evaluation of impact different compounds on neurogenesis or neural toxicity. In a fixed cell assay, cells were stained with antibodies against neuro-specific markers β-tubulin III and MAP2. The cells were fluorescently imaged with an ImageXpress™ Micro XL whose large field of view and high dynamic range improves data quality and statistics. Live cell assays provide additional information for drug discovery. Positive or negative effects on development of neurite networks was assessed in real time using time-lapse transmitted light imaging, mitochondria potential, and viability dyes. We have demonstrated utility of assays showing effects of growth factors and number of neurotoxic compounds.

ImageXpress® Micro XL Automated Imaging System



"Standard" Image



"XL" Image

Fig. 1. The ImageXpress Micro comes in the Standard (bottom left image, ~120 cells) and XL (right image, ~500 cells) configurations. The XL provides a 3X increased field of view and 3 logs dyanmic range to allow collection of more cells and analysis of dim features in the presence of bright objects.

Methods

Cell Preparation

- iCell Neurons were received frozen from CDI. Cells were thawed and plated according to standard protocol.
- Cells were counted and plated 10K/well on laminin coated 96w plates in the presence of appropriate media.
- Cells were cultured for 3 days and then treated with compounds for 48h.
- Neurons were visualized with AlexaFluor 488 (AF488) conjugated antibody against b-tubulin III, a specific marker for neurons.
- Mitochondria were visualized with JC-10.
- Nuclei were stained with DAPI or Hoescht.

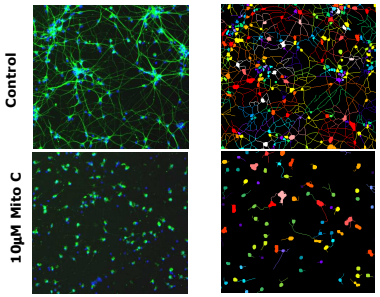
High Content Image Acquisition & Analysis

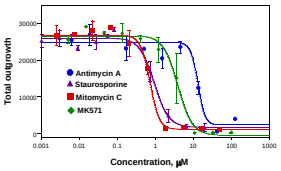
- Images were acquired with the ImageXpress Micro XL System using 10X or 20X objectives and multiple exposures.
  - AF488 anti-b-tubulin III: 488nm Ex, 520nm Em
  - JC-10: 543 Ex, 593 Em.
  - DAPI dye label for nuclei: 405nm Ex, 450nm Em
- Images were analyzed using standard algorithms from MetaXpress Software.
  - Cell Scoring – identify number of Neurons & JC-10 positive cells
  - Neurite Outgrowth – Identify number of neurons, neuron length, branching, etc.
  - Granularity – Identify cells with intact mitochondria

Neuronal Cell Imaging & Analysis

High content analysis provides a quantitative method to determine effects of positive and negative factors on neurite outgrowth. An aliquot of iCell Neurons was plated into 96-well plates at 10K/well. The cells were cultured for 3 days and inspected for cell health and maturity of neural networks. Neurons were visualized using ImageXpress Micro XL System and markers for nuclei and β-tubulin III. Image analysis was done using the Neurite Outgrowth module in MetaXpress Software, and data visualization and analysis was done using AcuityXpress™ Software. The Neurite Outgrowth module finds nuclei, determines a "positive" neuron cell by presence of both nuclear and β-tubulin III stains, and then characterizes β-tubulin III labeled neurites extending from those cells. Output parameters, in addition to number of neurons, include number of neurites, length of outgrowths, number of branches, etc. per cell or per field. Statistics on number and phenotype of cells in each well are then calculated. Examples of image analysis results are shown in Figure 2.

Integrity of Neuronal Networks



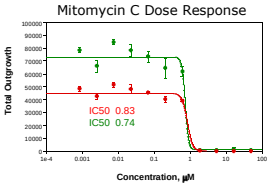


Compound	Total neurite outgrowth IC50 (μM)	% cells with outgrowth IC50 (μM)
Antimycin A	13	14
Staurosporine	0.9	1.7
MK571	4.1	3.9
Mitomycin C	0.7	0.7

NMDA	n/d	n/d
SNAP	68	84
PD98059	31	46

Fig. 2. Top: Image analysis results of iCell Neurons using the Neurite Outgrowth module of MetaXpress Software for Control and High Dose wells. Multi-parametric outputs are generated from each image. Bottom: Dose response curves to cytotoxic compounds for total outgrowth. IC50 values for total outgrowth and % of cells significant growth (β-tubulin III) are listed in the table.

In a second study, neurons were prepared as described previously and then cultured in the presence of mitomycin C for 48 hours. Neurites and neural networks were visualized with antibodies against β-tubulin III or MAP2 and imaged with the ImageXpress Micro XL system. Several parameters characterizing neuronal networks were analyzed. Both marker show the dose-dependent disintegration of networks and neuronal toxicity.



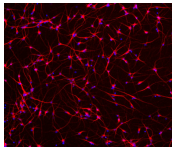
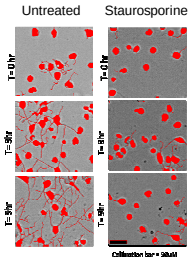


Fig. 3. Top: Image of neurons stained for MAP2 and nuclei. Left: Comparison of β-tubulin III (Green) and MAP2 (Red) markers for determining dose response of neurite outgrowth to mitomycin C.

Transmitted Light Time Lapse Assay

Live non-stained neurons were imaged using transmitted light. By using transmitted light instead of fluorescent light, data is obtained without risk of disruption to the naturally occurring physiology via addition of foreign molecules. In addition, cell health is not impacted during acquisition as cultures are not subjected to the harsh exposure or free-radical production during fluorescent excitation. Cells were maintained inside the ImageXpress Micro XL instrument with environmental (CO_2, humidity, and temperature) control. iCell neurons were plated in the presence of the growth stimulators Nerve Growth Factor (NGF) and Brain-Derived Neurotrophic Factor (BDNF) and the kinase inhibitor Staurosporine. Effects of the compounds on neurite outgrowth was measured every 20 minutes for 12 hours time period using a 10X objective.



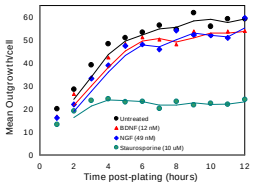
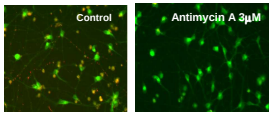


Fig. 4. The Untreated control cells (left) rapidly sent out neurite outgrowths while the cells treated with 10 uM Staurosporine (right) grew fewer outgrowths over the 12 hour sampling period. The resultant images were analyzed for mean outgrowth per cell (n=4).

Integrity of Mitochondria

Mitochondrial depolarization is an early signal for exitotoxicity, hypoxic damage or oxidative stress. We have monitored mitochondria membrane potential using the mitochondria active dye JC-10.



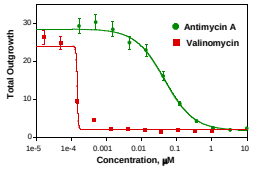
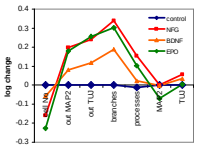


Fig. 5. Top: Images of Control cells and cells treated with Antimycin A causing blockage of oxidative respiration for 30min and then stained with JC-10. Bottom: Dose responses as measured by total JC-10 aggregates. iCell Neurons were treated with JC-10 and exposed to antimycin A and valinomycin for 30 minutes (compounds cause interruption of oxidative respiration and Ca overload).
Images were analyzed using the Granularity module of MetaXpress software.

Compound	IC50 (nM)
Antimycin A	46
Valinomycin	0.15

Multi-Parametric Profiling

Multi-parameter analysis allows comparison of compound "signatures" and determination of differential responses. This information can be used to give insight into mechanisms of action.



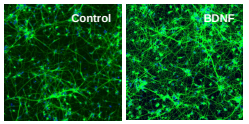


Fig. 6. iCell neurons were maintained in the presence of growth factors for 14 days and were fixed and stained with β-tubulin III and Hoechst. Cells were imaged with 10X magnification and analyzed for different characteristics of neurite outgrowth. Data presented as a log of fold change for each parameter.

Live Cell Toxicity Assay

End point live cell toxicity assay was done using combination of viability dye Calcein AM and nuclear stain Hoechst. This method is more suitable for screening then β-tubulin III staining as it has a more simple and cost efficient protocol. Neurite networks analyzed using neurite outgrowth module, while content of live and dead neurons determined by nuclear size (cell scoring module). We have validated method testing number of known neurotoxic compounds as well as several "safe" compounds. Observed neurotoxic activity of compounds in this model correlated with in vivo or clinical data showing that the assay can potentially be used for predicative neurotoxicology.

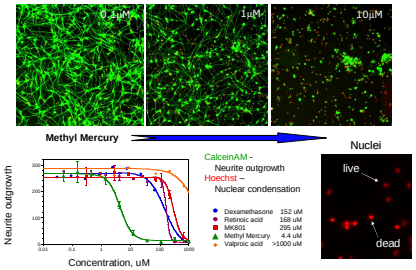


Fig. 7. Top: Images showing dose-dependent toxic effect of methyl mercury (Calcein AM + Hoechst) on neurons. **Left:** IC50 curves of cytotoxic compounds (neurite outgrowth). **Right:** Evaluation of cytotoxicity by nuclear size.

		IC50, uM	*in vivo* Neurotoxicity	Description
Known Neurotoxins	Methyl Mercury	4	+	metal
	Dexamethasone	77	+	anti-inflammatory (steroid)
	Retinoic acid	197	+	vitamin A, anti-cancer
	MK801	270	+	anti-convulsant, NMDA rec.ant.
	Kainic acid	723	+	neurostimulant, receptor agonist
	Valproic acid	>1000	+	anti-convulsant
Anti-Proliferation	Cytosine arabinoside	151	+	anti-cancer
	Hydroxyurea	n/d	-	anti-cancer
	5-Fluorouracil	n/d	-	anti-cancer
	Ochratoxin A	n/d	-	mycotoxin
Safe	Ascorbic acid	n/d	-	vitamin C
	Aspirin	n/d	-	anti-inflammatory

Summary

We have developed high content imaging methods that allow automatic evaluation of neuronal development & toxicity

We have demonstrated several automated neurotoxicity assays using iPSC-derived neurons that are suitable for screening environments:
- Neural network integrity
- Mitochondrial integrity and viability markers
- Live cell time-lapse assay in transmitted light

These assays can be used for:
- Testing biologics or chemical compounds on neuronal development
- Screening and validation of drug candidates
- Evaluating potential neurotoxic or neuroprotectant effects of different agents