Neurotoxicity Assays Using iPSC-Derived Neurons and High Content Imaging

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Abstract

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. Neurotoxicity been found to be a major cause of neurodegenerative diseases such as Alzheimer's or Parkinson's. Neurotoxicity can be also caused by excessive stimulation in the brain during pathological processes such as spinal cord injury, stroke, traumatic brain injury and neurodegenerative diseases. In this process, called excitotoxicity, nerve cells are damaged and killed by neurotransmitters such as glutamate and similar substances. Accordingly, there is a great interest in developing more predictive, disease relevant cell-based models and efficient screening tools for assessing the neurotoxicity 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. Live cell assays can be automated with the ImageXpress® Micro system and MetaXpress® software to allow characterization of neurons and neuronal networks for health and extent of the network. We present here examples of the use of these systems to study inhibition of neuronal development and neurotoxicity in assays that are well suited for screening of lead compounds and potentially important for reducing animal experimentation and cost of pre-clinical development.

Introduction

Reproducible neurotoxicity assays require a consistent source of mature, high quality neurons. One such source is human iPSC-derived neurons from Cellular Dynamics International (CDI). The process for creating such cells is shown in Figure 1. First, adult cells are obtained and reprogrammed through introduction of a series of four genes. After a period of approximately four weeks, the pluripotent cells can be seen and expanded. These cells are then differentiated using proprietary media into neuronal cells or other cell types. Purity and maturity of the cells are monitored via known cell specific expression markers.
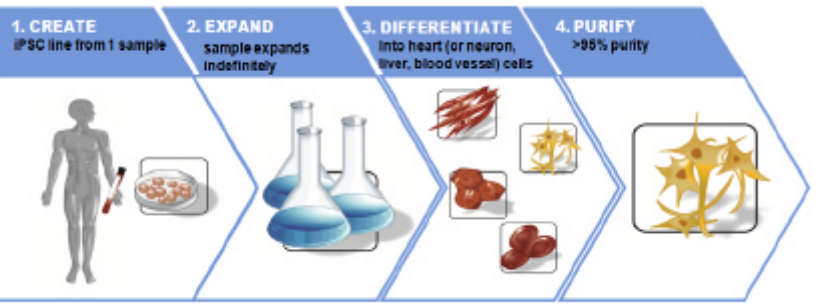

Fig. 1. Steps in creation of neuronal and other cell types from iPS cells.

We present here two models for assessment of neural toxicity. In one assay, neurons are allowed to form neurite networks in 96 or 384 well plates and then cultured in the presence of toxic compounds for 48 hours. Neurites and neural networks are visualized with antibodies against β-III tubulin, imaged with the ImageXpress Micro XL system (Figure 2), and analyzed with MetaXpress software using the Neurite Outgrowth and Cell Scoring Modules. Neuronal networks are characterized by length of neurite outgrowth, branching, and number of cells.

The second model is relevant to mitochondria integrity in neurons. Mitochondrial depolarization has been shown to be an early signal for exitotoxicity, hypoxic damage or oxidative stress. We have monitored mitochondria membrane potential using the mitochondria active dye JC-10. In healthy cells, JC-10 selectively accumulates in mitochondria as orange "J-aggregates." As the inner membrane potential is lost, the monomeric form of JC-10 is released into the cytoplasm and the cells fluoresce green. This assay can also be used for modeling hypoxic damage in brain and testing neuroprotective agents.

Experimental

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 β-tubulin III, a specific marker for neurons.
• Mitochondria were visualized with JC-10.
• Nuclei were stained with DAPI or Hoescht.

Fig. 2. ImageXpress Micro XL System

High Content Image Acquisition & Analysis
• Images were acquired with the ImageXpress Micro XL System using 10X or 20X objectives and multiple exposures.
  • AF488 anti-β-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

References:
1. Han SS at al. Constructing and deconstructing stem cell models of neurological disease. Neuron 2011
2. Chamberlain SJ at al. Induced pluripotent stem (iPS) cells as in vitro models of human neurogenetic disorders. Neurogenetics. 2008

Neuronal Cell Imaging & Analysis

High content analysis provides 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 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 and then 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 3.

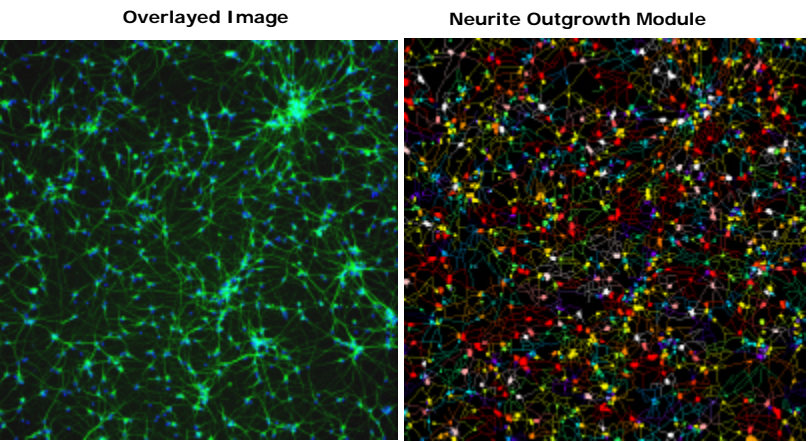**Overlayed Image**   **Neurite Outgrowth Module**

Fig. 3. Image analysis results of iCell Neurons using the Neurite Outgrowth module of MetaXpress Software. Multi-parametric outputs are generated from each image.

MAP2 Expression

Additional markers can be measured that are associated with mature neurons. For example, MAP2 is visualized in neurons using an antibody labeled with Alexa647. Composite images of β-tubulin III , MAP2, and nuclear stained neurons are shown in Figure 4. Some differences can be observed in total outgrowth and with neurite growth showing higher levels of β-tubulin III than MAP2. Neurite outgrowth that is positive for either can be identified with the analysis module.

IC50 0.83
IC50 0.74
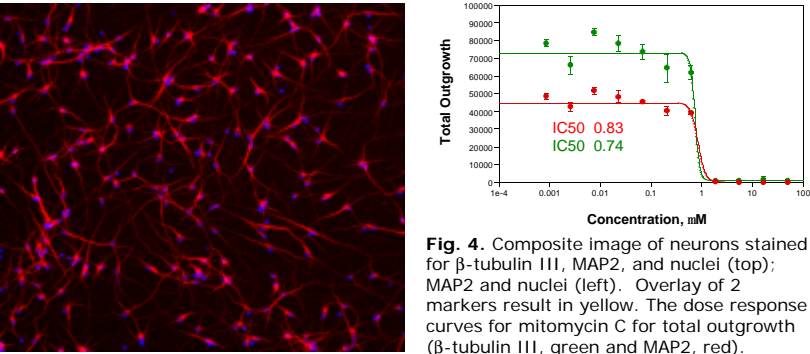
Fig. 4. Composite image of neurons stained for β-tubulin III, MAP2, and nuclei (top); MAP2 and nuclei (left). Overlay of 2 markers result in yellow. The dose response curves for mitomycin C for total outgrowth (β-tubulin III, green and MAP2, red).

Acquisition Comparison

The ImageXpress Micro XL is equipped with up to four automatically selectable objectives. The magnification of the objective is selected for a particular assay based on needs for area of analysis (e.g., total number of cells), ability to resolve fine features (e.g., neurite outgrowths), and sensitivity. The use of 10X and 20X objectives were compared for capability of imaging neurons. A single image was acquired for each well. Exposure time was optimized for peak signals at 80% of saturation. The analysis modules were also optimized for each acquisition setting. Representative portions of β-tubulin III stained neuron images acquired from the two objectives are shown in Figure 5.
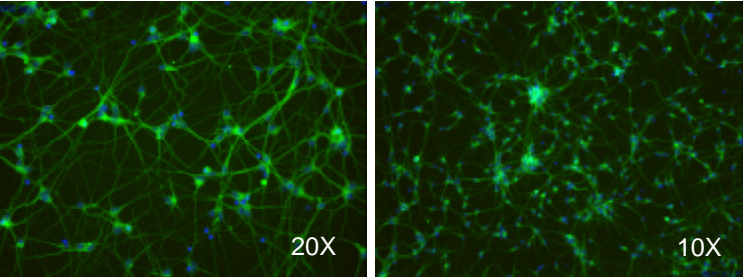

20X   10X

Fig. 5. Images acquired on the ImageXpress Micro using 20X (left) and 10X (right) objectives.

Analysis was performed with MetaXpress software using the Neurite Outgrowth modules and the images were compared for number of cells and total outgrowth (see Table 1). On average, approximately 3X as many cells were measured with the 10X objective in a single image and 2X the amount of outgrowth. It is believed that the lower magnification objective picked up relatively less outgrowth because such fine features are more difficult to automatically detect with lower resolution. Assay performance was determined on a cell-averaged basis using 5 parameters from positive and negative controls of a neurotoxicity assay. It was found that the results were very comparable for the two objectives.

	Objective Type		
Parameter	**20X**	**10X**	**% Increase**
Total Cells	55	218	296%
Total Outgrowth	13486	38920	189%
Avg. Z-Prime	0.57	0.58	

Table 1. Comparison of image processing and neurotoxicity assay results between acquisition with a 20X and 10X objective. Total Cells and Outgrowth were average amounts for a single site. Z Prime was averaged over 5 different assay parameters from a neurotoxicity assay.

Toxicity Assay I: Integrity of Neuronal Network

In this assay, iCell Neurons were allowed to form neurite networks in 96 or 384 well plates and then cultured in the presence of toxic compounds for 48 hours. Neurites and neural networks were visualized with antibodies against β-tubulin III and imaged with the ImageXpress Micro XL system. Several parameters characterizing neuronal networks were analyzed. We have shown dose-dependent disintegration of networks and neuronal toxicity of kinase inhibitors staurosporine, MEK1 kinase inhibitor PD98059, and other cytotoxic compounds including Antimycin A, MK 571, and Mitomycin C. However at present studies we were not able to detect sensitivity of iPSC derived neurons to excitotoxic substances as glutamate or glutamate receptor agonist NMDA. Dose response curves and IC50s are shown in Figure 6.
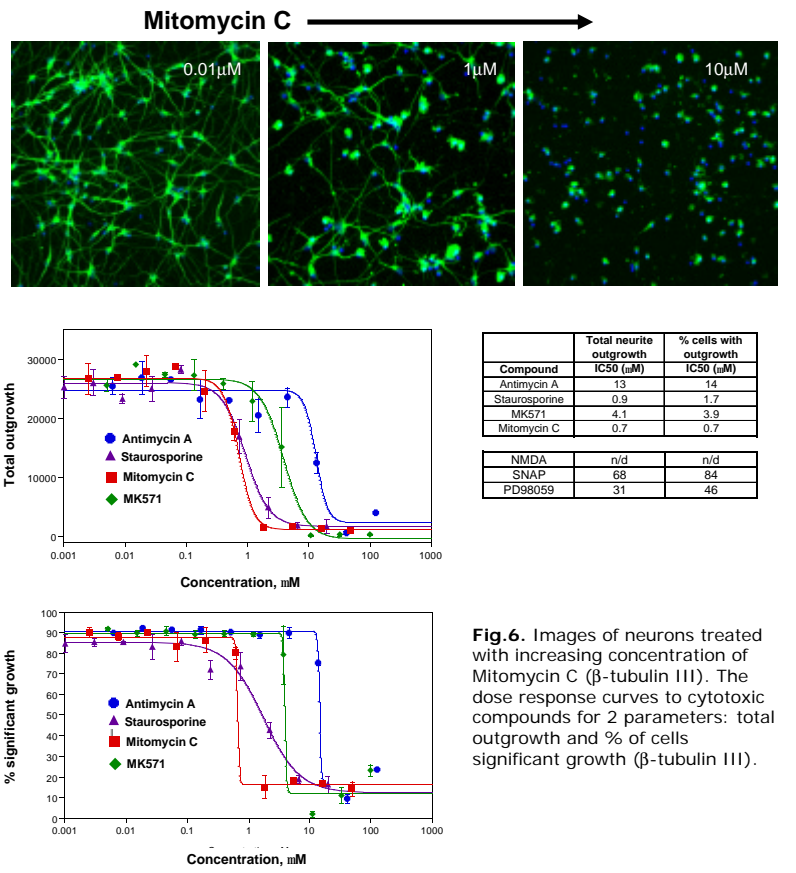

Mitomycin C

0.01µM   1µM   10µM

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.6. Images of neurons treated with increasing concentration of Mitomycin C (β-tubulin III). The dose response curves to cytotoxic compounds for 2 parameters: total outgrowth and % cells with significant growth (β-tubulin III).

Toxicity Assay II: Integrity of Mitochondria

Mitochondrial depolarization has been shown to be an early signal for exitotoxicity, hypoxic damage or oxidative stress. We have monitored mitochondrial membrane potential using the mitochondria active dye JC-10. In healthy cells, JC-10 selectively accumulates in mitochondria as orange "J-aggregates." As the inner membrane potential is lost, the monomeric form of JC-10 is released into the cytoplasm and the cells fluoresce green. 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) and imaged on the ImageXpress Micro system. Images were analyzed using the Granularity module of MetaXpress software.

Control   **Antimycin A 3µM**

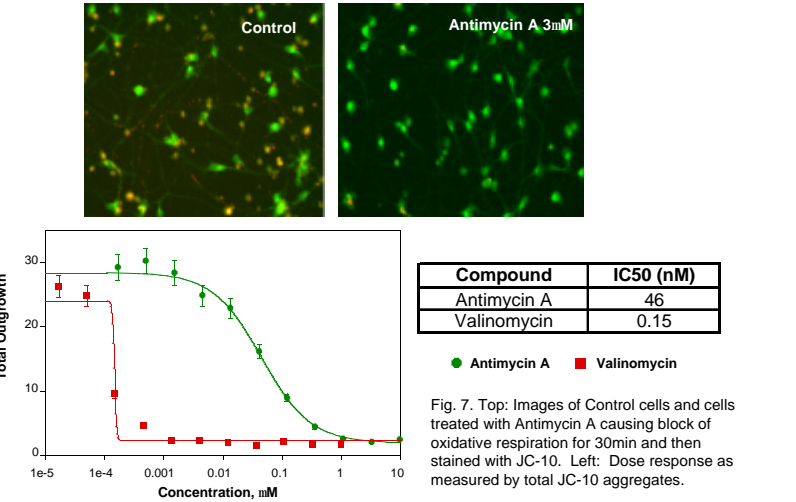| Compound	IC50 (nM)
Antimycin A	46
Valinomycin	0.15

● Antimycin A   ■ Valinomycin

Fig. 7. Top: Images of Control cells and cells treated with Antimycin A causing block of oxidative respiration for 30min and then stained with JC-10. Left: Dose response as measured by total JC-10 aggregates.

Summary

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

We have demonstrated two automated neurotoxicity assays using iPSC-derived neurons that are suitable for screening environments:
- Neural network integrity
- Mitochondrial integrity

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

Molecular Devices