IN VITRO CARDIOTOXICITY AND NEUROTOXICITY ASSESSMENT OF ENVIRONMENTAL CHEMICALS USING ORGANOTYPIC HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED MODELS

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INTRODUCTION

Due to the increasing prevalence of neurological and cardiovascular disorders possibly related to exposure to environmental toxicants, there is an increasing need to develop reliable and efficient screening tools to identify environmental chemicals that could potentially affect human health. There is a great interest in using stem cell derived cell models for *in vitro* high-throughput quantitative assays that would allow for detecting the potential hazard of chemicals and prioritizing them for further testing.

We developed several phenotypic assays testing neuronal and cardiac toxicity using automated imaging methods and induced pluripotent stem cell (iPSC)-derived cardiomyocytes and neurons. Human iPSC-derived cardiomyocytes or neurons were exposed to a number of known toxic compounds using concentration-responses and various lengths of time. We have tested a representative set of compounds that have been known to be associated with neurotoxicity or cardiotoxicity including pesticides, polycyclic aromatic hydrocarbons and flame retardants. Effects of compounds were assessed by automated imaging and image analysis. Effects on the development of neuronal networks were assessed by quantifying total neurite outgrowth, number of branches and processes, as well as cell viability. Effects on cardiomyocyte cellular and mitochondrial toxicity were assessed by using viability read-outs and a mitochondrial depolarization probe. We have characterized a number of phenotypic read-outs that can be used for determining effective toxic concentrations and comparing the effects of different compounds. In addition we evaluated the feasibility of a combinatorial screening approach for functional and mechanistic toxicity profiling of environmental hazards. These studies demonstrate the utility of stem cell-derived systems in identifying, ranking, and prioritizing compounds with cardiotoxic and neurotoxic potential for further *in vivo* testing.

GOAL

The focus of the present study was to develop high-throughput assays using iPSC-derived neurons and cardiomyocytes with the goal of establishing automation-compatible complex models for cardiotoxicity, neurodegenerative and neurotoxicity assessment.

INSTRUMENT

- ImageXpress® Nano Automated Imaging System
- CellReporterXpress™ Automated Image Acquisition and Analysis Software

ASSAY DEVELOPMENT

Cells: Previously characterized human iPSC-derived neurons and cardiomyocytes (iCell Neurons and iCell Cardiomyocytes) and supporting media were provided by Cellular Dynamics International (CDI, Madison, WI, USA). Neurons and cardiomyocytes were provided as a fully differentiated and highly pure population of cells (http://www.cellulardynamics.com/products/neurons.html). Cells were received frozen, and were subsequently thawed and plated according to a protocol recommended by Cellular Dynamics International.

Toxicity assays: To evaluate cardiac and neuronal toxicity, cells were treated with various compounds for 24-72h, and then live cells were stained with Calcein AM viability dye, Hoechst nuclear stain, and MitoTracker orange dye for detection of intact mitochondria (final concentrations 0.5 µM, 2 µM and 1 µM respectively). Compounds were tested in duplicates at 0.3, 1.0, 3.0, 10, 30, and 100 µM. DMSO controls (n = 12) and untreated controls (n = 4) were included in each plate.

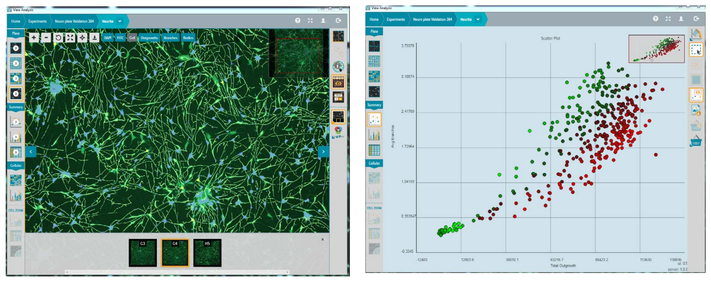
Imaging: Images of cells were acquired using the ImageXpress® Nano Automated Imaging System (Molecular Devices) using a 10x Plan Fluor objective. One 10x image was captured per well in a 384-well plate. A 10x objective image provides sufficient resolution to distinguish neurites, cytoskeletal organization, cellular junctions, and sub-cellular structures in a relatively large number of cells per image. After imaging live cells, they were fixed with 4% formaldehyde and re-stained with other markers in order to perform additional imaging.

Image Analysis: Images were processed and quantified using the Neurite Tracing, Cell Scoring, or Cell Viability application modules with CellReporterXpress™ Automated Image Acquisition and Analysis Software (Molecular Devices).

RESULTS

Automated Analysis of Phenotypic Effects

CellReporterXpress Automated Imaging Acquisition and Analysis Software provides an improved workflow for optimization of imaging protocols, complex image analysis and processing of numeric data to obtain multi-parametric information about biological changes and compound effects.



Neurotoxicity Assay

We evaluated the neurite tracing assay as a potential screening assay to characterize the activity of selected compounds with potential to adversely affect the developing nervous system. Automated imaging and analysis was used for evaluation of complexity of neuronal networks. We observed a dose-dependent inhibition of neuronal network formation due to compound treatment effects. 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).

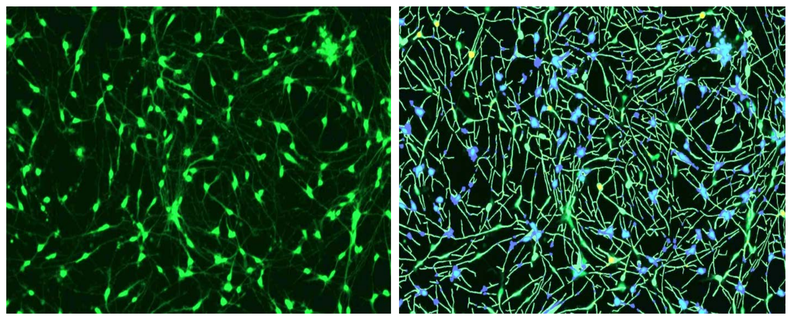


Figure 1. Images of β-tubulin (green) stain and the software analysis traces shown for the control cells. iCell Neurons were plated for 5 days, and were then fixed and stained with AF488-conjugated anti-β-tubulin (TUJ-1) antibodies (1:100, Becton Dickinson). Images were taken by the ImageXpress Nano System, using a 10x Plan Fluor objective and FITC channel. Images were processed using the Neurite Tracing analysis algorithm. Analysis masks on the right show the outgrowth (green), as well as cell bodies (blue), and branching points (purple).

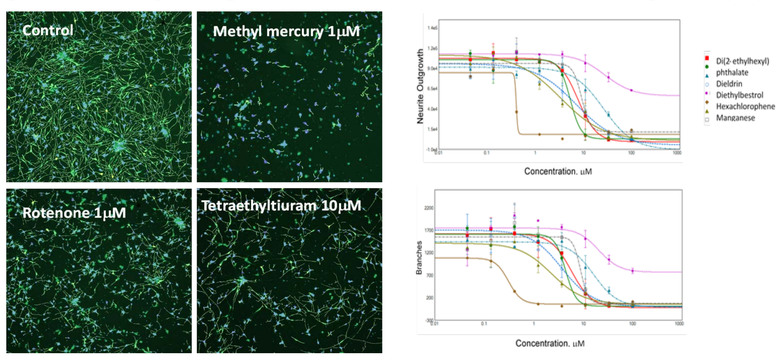


Figure 2. Composite images of β-tubulin (green) and Hoechst (blue) with the analysis traces shown for the control cells and the cells treated with selected compounds. iCell Neurons were plated for 48h, treated with compounds for 72h, and then were fixed and stained with Hoechst and AF488-conjugated anti-TUJ-1 antibodies. Disruption of neurite networks and cell death was observed for neurons treated with indicated compounds. Dose–dependent effects are indicated by decreased total outgrowth lengths and numbers of branches.

Phenotypic Characterization of Cardiotoxicity

To evaluate cytotoxicity effects, cells were treated with various cardiotoxic compounds for 24h, and then live cells were stained with Calcein AM viability dye, Hoechst nuclear stain, and MitoTracker Orange dye for detection of intact mitochondria (final concentrations 0.5 µM, 2 µM and 1 µM respectively). Images were acquired using the ImageXpress Nano Automated Imaging System and were processed and quantified using the Cell Scoring or Cell Viability application modules in CellReporterXpress software (Molecular Devices).

Read-outs included nuclei count and intensity for Hoechst 33342, percent live cells and total area of live cells positive for Calcein AM staining, and the number of cells positive for MitoTracker Orange dye. After imaging live cells, they were fixed with 4% formaldehyde and re-stained with AF-488 Phalloidin (1:100 dilutions) in order to perform additional imaging for detection of cytoskeleton integrity of cells. Images were analyzed using the Cell Scoring algorithm for detection of total and average areas of cardiac cells, as well as the number and percentage of cells with intact cytoskeleton.

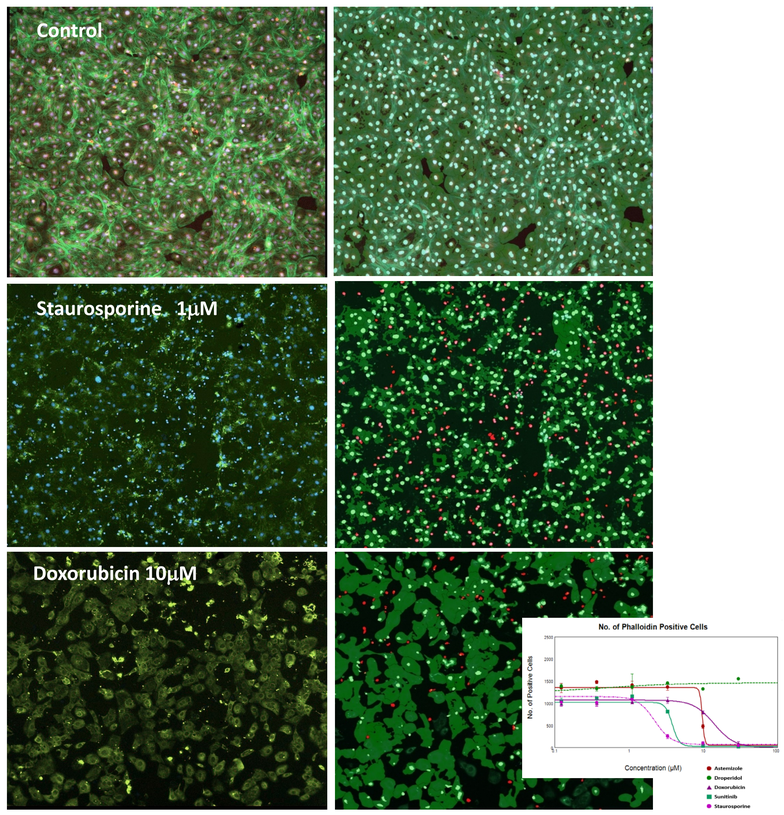


Figure 3. Cytoskeleton staining of cardiomyocytes for visualization of cardiotoxicity. iCell Cardiomyocytes were plated and treated with compounds for 24h, and stained with a combination of Hoechst (2 µM), MitoTracker Orange (1 µM), and then fixed and stained with AF488-conjugated Phalloidin (1:100). Images were taken by ImageXpress Nano system, using a 10x Plan Fluor objective, and DAPI, TRITC, and FITC channels. Images were processed using the Cell Scoring analysis algorithm. Composite images of actin, nuclei, and mitochondria (Left) and the analysis masks (Right) are shown for the control and compound treated cells. Disruption of the cytoskeleton and cell death was observed for cardiomyocytes treated with indicated compounds.

SUMMARY

The assay demonstrates the efficiency and reliability of the iPSC-derived cells and ImageXpress Nano Automated Imaging System for the automated imaging and analysis of chemicals for cardiotoxicity and neurotoxicity assessment. The method would allow for the identification of toxic drugs early in drug development and the testing of chemicals for their potential to induce neurotoxicity and cardiac toxicity in humans.

MOLECULAR DEVICES