Multiplexed Hepatotoxicity Assays using iCell® Hepatocytes

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Abstract

Predictive *in vitro* assays suitable for safety testing are extremely important for reducing the incidence of late-stage drug attrition. We have paired human iPSC-derived hepatocytes with the Screen-Well™ Hepatotoxicity library in order to demonstrate several models for assessing general and specific hepatotoxicity using the power of high content imaging. We used multi-parametric image analysis to monitor cell viability, apoptosis, and mitochondria membrane potential. Additionally, intuitive software modules were used to quantitate phospholipidosis, steatosis, and autophagy associated with different types of toxicity. The ImageXpress® Micro XL system and MetaXpress® software provided automatic image acquisition in 384 well plates and analysis on a cell-by-cell basis.

Introduction

Since Drug Induced Liver Injury (DILI) causes 33% of acute liver failure in the United States and ~1000 drugs have been implicated in causing liver toxicity, pharmaceutical companies would benefit greatly from identifying compounds that are hepatotoxic to humans early in the drug discovery process. Since *in vivo* animal testing is labor-intensive and expensive and engineered liver cell lines often lack key metabolic enzyme functions, iPSC-derived human liver cells, which exhibit typical characteristics and metabolism, are an ideal solution for *in vitro* toxicity screening.

Cell-based cytotoxicity methods utilizing high content and high-throughput imaging are well-established. Cellular responses to drug treatment in hepatocytes can be measured by:

• Nuclear count • Nuclear morphology • Live Cell count • Whole cell morphology
• Mitochondria count • Loss of mitochondria membrane potential • Apoptosis
• Necrosis • Autophagy • Glycogen levels • Lipidosis • Steatosis • Vacuole formation

These assays can be conducted in microplates up to 384 wells and multiple assays can be run in each well. Each assay will yield multiparametric results which can be evaluated to determine the most significant responses to study.

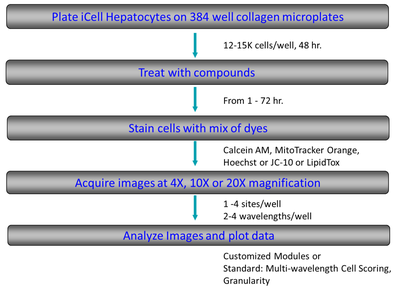
Materials

- **iCell Hepatocytes** – Cellular Dynamics Intl. P/N HCC-100-010-005-PC
- **Assay Reagents**
  HCS LipidTox™ Reagent – Life Technologies (Invitrogen) P/N H34157
  Calcein, AM cell viability reagent – Life Technologies (Invitrogen) P/N C3100MP
  MitoTracker® Orange CMTMRos - Life Technologies (Invitrogen) P/N M7510
  Screen-Well™ Hepatotoxicity Library – Enzo P/N BML-2851-0100
  CellMeter™ JC-10 Mitochondrial Membrane Potential Kit – AAT Bioquest P/N 22801
- **High content Imaging** – Molecular Devices, LLC
  ImageXpress Micro XL High Throughput Imaging System with standard DAPI, FITC, TRITC filter cubes and 4X, 10X, 20X objectives

- **Automated Analysis**
  MetaXpress Image Analysis Software processes images using either pre-configured software modules or Custom Module Editor to characterize phenotypic changes and allow specific outputs

  AcuityXpress™ High Content Data Analysis Software contains tools for statistics, curve fitting, hierarchical clustering, and principal component analysis to provide additional insights into assay results

Method



Viability Assessment

The Hepatotoxicity Library of 240 compounds was tested in duplicate with a 72 hour compound treatment and the cells' viability was measured by different methods. Quantitation of the nuclei stained with Hoechst shows the number of cells remaining in the well at the end of the treatment period but hepatocytes with cytoplasm positive for Calcein AM staining indicate the metabolic activity of living cells. Figure 1 shows example images.

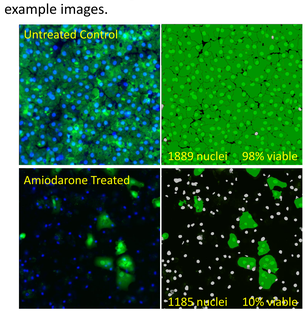


Figure 1. iCell Hepatocytes treated with amiodarone for 72 hours. Cells stained with Calcein AM (viability) and Hoechst (nuclei) and imaged with 10X objective (Left). Resulting mask after analysis with Multi-wavelength Cell Scoring module (Right) shows cells stained with viability dye in green, nuclei belonging to live cells are light green and nuclei not associated with live cells are grey. Measurement of viable cell area is more sensitive than total nuclear count (bottom).

Many compounds cause nuclear condensation, indicating apoptosis. Decreased nuclear area and/or increased average intensity can be used to determine an IC50.

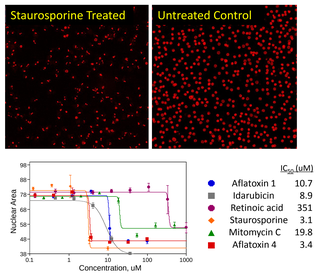


Figure 2. Untreated nuclei (right) compared to staurosporine treated nuclei (left). Hepatocytes were treated with compounds for 72 hours then fixed and counter-stained with Hoechst (red nuclei). Average nuclear areas or nuclear intensity (not shown) are reported and can be used to determine compound IC50s.

Cell Area Measurements

Although not significantly affecting the total number of cells in a well, some compounds cause morphological changes to the hepatocytes such as an increase or decrease in cell size. The area of each cell can be determined using the Cell Scoring module or as part of a custom module. The average area of each cell in the well is one of the parameters reported.

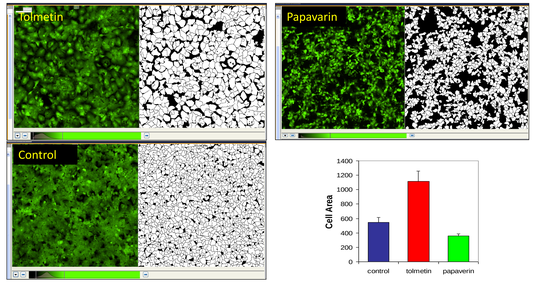


Figure 3. iCell Hepatocytes treated with compound for 48 hours were stained with Calcein AM and imaged with a 20X objective. Analysis of average cell area with MetaXpress software shows that Tolmetin causes cells to enlarge, while Papavarin causes them to contract (images above and graph on the right).

Mitochondrial Membrane Integrity

Mitochondrial depolarization is an early signal of hypoxic damage or oxidative stress. Hepatocytes were treated for 60 minutes with compounds and the mitochondria membrane potential was monitored with the dye JC-10 using the ImageXpress Micro system. Images were analyzed using the Granularity software module to quantitate the healthy mitochondria which retain the red stain. This assay can be used either as an end-point or live-cell real time assay.

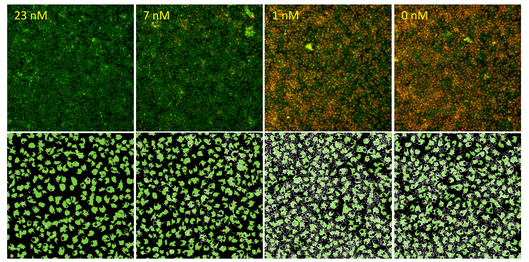
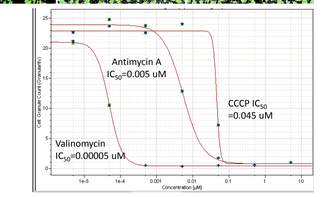


Figure 4. iCell Hepatocytes treated with Antimycin A for 60 minutes. Live cells were stained with JC-10 and imaged with a 10X objective. Overlay of green cytoplasm and red mitochondria with the resulting mask (zoomed) after analysis with Granularity module (top) shows mitochondria identified (white) in a dose response to the compound. IC50 curves were plotted using AcuityXpress software (right).



Phospholipidosis and Steatosis

Some drugs have been found to adversely affect lipid metabolism, causing an excess accumulation of lipids in the tissues. The amount and distribution of both phospholipids and neutral lipids can be detected with imaging methods and quantitated on a per cell basis even when compound cytotoxicity affects total cell number (Figure 5).

Phospholipidosis and Steatosis Assays

Cells were plated and incubated for 4 days in 384 well plates then treated with select compounds from the Screen-Well Hepatotoxicity Library for 24 hours.

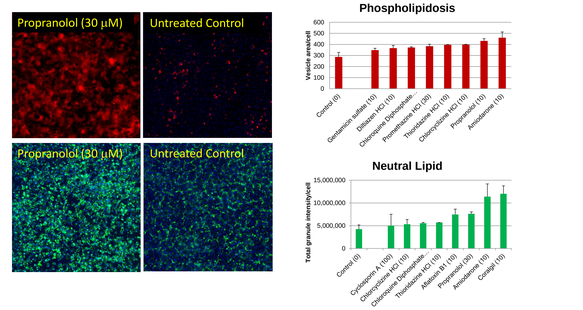


Figure 5. Hepatocytes showing phospholipid (top) and neutral lipid (bottom) staining in images acquired with a 10X objective. Quantitation of the red or green lipid staining (graphs on right) gives insight into the mechanism of compound toxicity.

Multi-parametric Toxicity Assay

The previously described toxicity assays may be multi-plexed within a well and the scientist can specify which parameters to analyze using MetaXpress Custom Module Editor.

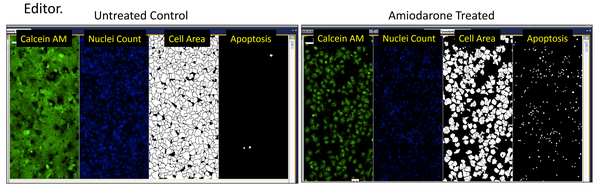
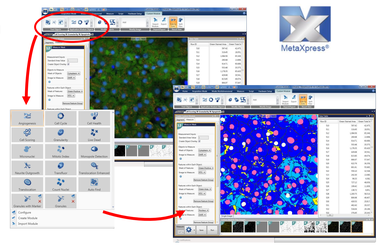


Figure 6. The Custom Module Editor in MetaXpress Image Analysis software allows measurement of cell area as well as incidence of apoptosis in hepatocytes after compound treatment (top). Hepatocytes (right) stained with Hoechst (blue nuclei), MitoTracker (red mitochondria), and Calcein AM viability stain (green cytoplasm) can be analyzed for multiple outputs such as total nuclei (pink), apoptotic nuclei (white), and number or area of cells containing live-cell stain (dark blue) and healthy mitochondria (light blue).



Summary

- We have demonstrated several high-content imaging assays for assessing general and specific hepatotoxicity that are well-suited for automated screening environments.
- iCell® hepatocytes derived from human induced pluripotent stem cells provide relevant information about hepatotoxicity and are readily scalable to 384 well plates.
- Multi-parametric image analysis greatly increases assay sensitivity and provides valuable information about mechanisms of compound toxicity.

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