Multiplexed High Content Assays for Predictive Hepatotoxicity using Induced Pluripotent Stem Cell Derived Hepatocytes

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Introduction

A large percentage of drugs fail in clinical studies,or are withdrawn from the market due to hepatic toxicity. Therefore, highly predictive in vitro assays suitable for safety and efficacy testing are extremely important for improving the drug development process and reducing drug attrition. Accordingly, there is great interest in using stem cells as tools for screening compounds during early drug development. Human hepatocytes derived from stem cells can greatly accelerate the development of new chemical entities and improve drug safety by offering more clinically relevant cell-based models than those presently available. Human induced pluripotent stem cell (iPSC) derived hepatocytes express appropriate hepatocyte markers and demonstrate intrinsic hepatocyte functions similar to primary cells. We demonstrate several models for assessing general and specific hepatotoxicity that are well-suited for automated screening environments.

Methods

Cell Preparation

• iPSC-derived hepatocytes (iCell® Hepatocytes) from Cellular Dynamics International (CDI) were plated according to their recommended protocol.

• Cells were plated at a density of 60K/well (96-well plate) or 15K/well (384-well plate) on collagen coated plates and incubated for 2-3 days. Then cells were treated with appropriate compounds for 72h.

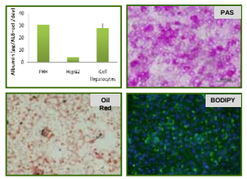


Figure 1. Characterization of iCell Hepatocytes. **Top Left:** Albumin secretion was shown to be similar to primary human hepatocytes. Cells characterized by glycogen storage using periodic acid-Schiff (PAS) staining (**Top Right**) and lipid production using Oil Red and BODIPY staining (**Bottom**)

Automated Imaging System ImageXpress® Micro XL

High Content Imaging

•Images were acquired using the ImageXpress ® Micro XL System using 20x or 10x magnification.

•The following filters were used

- Calcein AM: FITC Filter Cube
- MitoTracker Orange: TRITC Filter Cube
- Hoechst: DAPI Filter Cube.
- Cyto-ID: FITC Filter Cube

Automated Image Analysis

• Image analysis was done using **MetaXpress® Software** and image processing modules, including multi-wavelength cell scoring, live-dead and granularity.

• **AcuityXpress™ Software** contains tools for graphing, cluster and principal component analysis to provide additional insights into assay results.

• New ***Custom Module*** capabilities derived from industry leading **MetaMorph® NX** software have been developed to allow users to expand their abilities to characterize phenotypic changes. Example on right shows analysis of hepatocytes.

Multi-Mode Plate Reader SpectraMax® Paradigm

Plate Reader Acquisition & Analysis

• Data was acquired in 90 seconds/plate on the SpectraMax® Paradigm® plate reader using the TUNE tunable fluorescence cartridge in bottom read position.

• SoftMax® Pro software was used to acquire and analyze all data.

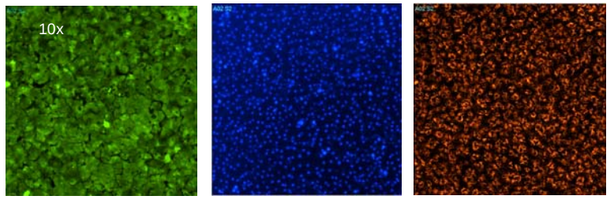
References

1. O'Brien PJ, Irwin W, Diaz D, Howard-Cofield E, Krejsa CM, Slaughter MR, Gao B, Kaludercic N, Angeline A, Bernardi P, Brain P, Hougham C. High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. Arch Toxicol. 2006 Sep;80(9):580-604.

2. Anderson N, Borlak J (2006). "Drug-induced phospholipidosis". FEBS Lett 580 (23): 5533–540.

Multi-Parameter Cytotoxicity Assay

Multi-parametric Image Analysis can be used to monitor changes in cell viability (Calcein AM), nuclear shape (Hoechst), and mitochondria potential (MitoTracker Orange) associated with different types of toxicity. The ImageXpress Micro system allows automatic analysis on a cell-by-cell basis using multi-wavelength cell scoring. The SpectraMax Paradigm plate reader can also be used to determine toxicity response on a well-by-well basis.



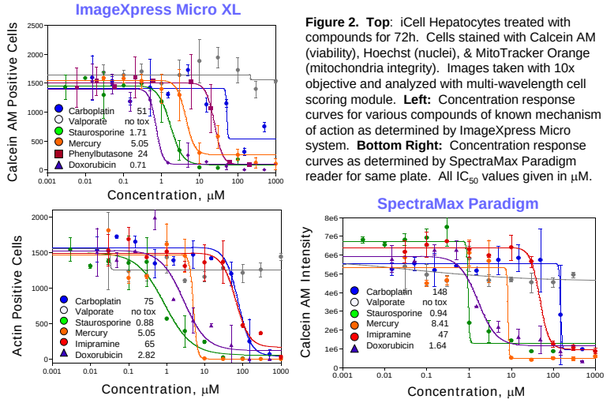
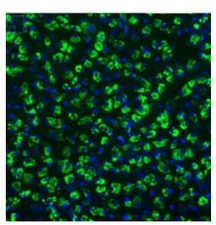


Figure 2. Top: iCell Hepatocytes treated with compounds for 72h. Cells stained with Calcein AM (viability), Hoechst (nuclei), & MitoTracker Orange (mitochondria integrity). Images taken with 10x objective and analyzed with multi-wavelength cell scoring module. **Left:** Concentration response curves for various compounds of known mechanism of action as determined by ImageXpress Micro system. **Bottom Right:** Concentration response curves as determined by SpectraMax Paradigm reader for same plate. All IC_{50} values given in µM.

Autophagy

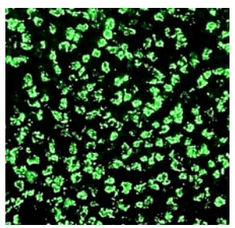
Selective degradation of intracellular targets, such as misfolded proteins and damaged organelles is an important homeostatic function of the cell. In disease, autophagy may function as a survival mechanism by removing damaged organelles and toxic metabolites (e.g. protein aggregates, intracellular pathogens) to maintain viability during periods of stress. Several drugs that regulate autophagy have been reported recently, suggesting that the autophagic machinery can be manipulated to treat human diseases.



Overlay of Cyto-ID and Hoechst images

Figure 3. iCell Hepatocytes treated with compounds for 24h. Cyto-ID™ Autophagy detection dye (Enzo) becomes brightly fluorescent in vesicles produced during conditions known to modulate the autophagy pathway. Images taken with 10x objective. Granularity module used for analysis.

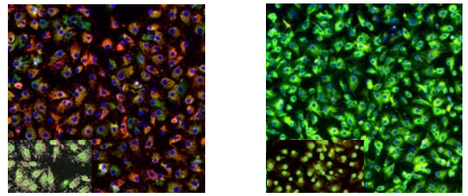
Compound	IC_{50}, µM
Chloroquine	1.3
Verapamil	33.5
Rapamycin	3.1



Overlay of Cyto-ID image and Granularity Masks

Mitochondria Integrity Assay

Mitochondrial depolarization is an early signal for hypoxic damage or oxidative stress. Mitochondria membrane potential was monitored with the mitochondria active dye JC-10 on the ImageXpress Micro system. Data analyzed using the Granularity module. This assay can be used either as an end-point or live-cell real time assay.



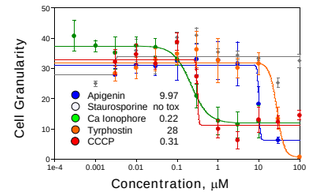


Figure 4. iCell Hepatocytes treated with compounds for 72h. Cells stained with Hoechst (nuclei), and JC-10 (mitochondria integrity). Images taken with 10x or 4x objective and analyzed with MetaXpress software Granularity module. IC_{50} values given in µM.

Phospholipidolysis

Phospholipidosis is a lysosomal storage disorder characterized by the excess accumulation of phospholipids in tissues. Many cationic amphiphilic drugs, including anti-depressants, antianginal, antimalarial, and cholesterol-lowering agents, are reported to cause drug-induced phospholipidosis (DIPL) in humans. The mechanisms of DIPL involve trapping or selective uptake of DIPL drugs within the lysosomes and acidic vesicles of affected cells. Drug trapping is followed by a gradual accumulation of drug-phospholipid complexes within the internal lysosomal membranes. The increase in undigested materials results in the abnormal accumulation of multi-lamellar bodies (myeloid bodies) in tissues. Drug-induced phospholipidosis represents a concern in safety risk assessment.

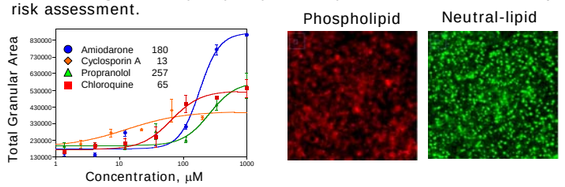


Figure 5. iCell Hepatocytes treated with compounds for 48h followed by phospholipidosis and steatosis detection using LipidTOX reagent. Images taken with 10x objective. Phospholipid (Red, TRITC) and neutral-lipid staining (Green, FITC) in cells treated with 30 µM propranolol. A dramatic increase in phospholipid levels was observed in response to drug treatment.. IC_{50} values given in µM.

Summary

•Live-cell assays using the ImageXpress Micro XL High Content Imaging System or SpectraMax Paradigm plate reader with human iCell Hepatocytes can measure the impact of pharmacological compounds on hepatocyte viability and intrinsic hepatocyte functions.

• Multi-parametric read-outs allow simultaneous assessment of viability, membrane permeability, lipid accumulation, cytoskeleton integrity, and mitochondrial depolarization in live cells.

• We demonstrate applications of these assays for prospective toxicity screening using iPSC-derived hepatocytes by measuring the impact of potential hepatotoxic effects and for development of cell-based models of diseases.