Predictive High-Content/High-Throughput Assays for Hepatotoxicity Using Induced Pluripotent Stem Cell (iPSC)-Derived Hepatocytes

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Introduction

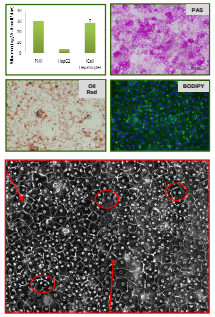
Human iPSC-derived hepatocytes have been developed as a replacement for primary cells and show promise with respect to liver-like phenotype, unlimited availability, and a potential to establish cells from individuals who are prone/resistant to adverse drug reactions. Accordingly, there is great interest in using iPSC-derived hepatocytes as tools for screening in drug development. While unlimited supply of such cells from multiple donors addresses one common bottleneck (i.e., availability of cells), it is yet to be shown that iPSC-derived hepatocytes are amenable to high-throughput and high-content screening analyses. In this project we tested several automated screening approaches for assessing general and mechanism-specific hepatotoxicity using iPSC-derived hepatocytes. We found that multi-parametric automated image analysis greatly increases assay sensitivity while also providing important information about possible toxicity mechanisms. Specifically, we found for testing a library of 240 compounds an assay sensitivity of 60% with a specificity of 91% and predictivity of 75%. This was superior to evaluation of cell viability endpoint only. We conclude that the high-throughput and high-content automated screening assays using iPSC-derived hepatocytes is feasible and can facilitate safety assessment or drugs and chemicals.

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 72 hr.

Figure 1. Characterization of iCell Hepatocytes.



Top: 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**)

Bottom: Transmitted light image of iCell Hepatocytes after 4 days in culture. Bi-nucleation is indicated by circles and bile canaliculi by arrows.

High Content Imaging

- Images were acquired using the ImageXpress® Micro XL System using 20x, 10x, or 4x objectives.
- The following filters were used
  - Calcein AM, Cyto-ID, Neutral Lipids: FITC Filter Cube
  - MitoTracker Orange, Phospholipids: TRITC Filter Cube
  - Hoechst: DAPI Filter Cube.

Automated Image Analysis

- Image analysis was done using MetaXpress® 5 Software and image processing modules, including Multi-Wavelength Cell Scoring, Live-Dead and Granularity.
- New Custom Module Editor (CME) capabilities derived from industry leading MetaXpress 5 software have been developed to allow users to expand their abilities to characterize phenotypic changes.

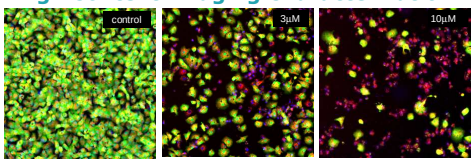
References

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.

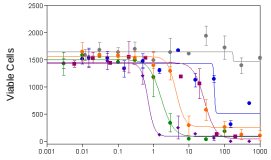
Multi-Parameter Cytotoxicity Assay

Multi-parametric Image Analysis can be used to monitor changes in cell viability (Calcein AM), nuclear shape (Hoechst), and mitochondria integrity (MitoTracker Orange) associated with different types of toxicity. The ImageXpress Micro system allows automatic analysis on a cell-by-cell basis using the MetaXpress 5 software Multi-Wavelength Cell Scoring (MWSC) module.

High Content Imaging Characterization



Calcein AM, MitoTracker Orange, Hoechst



	IC_{50} (µM)
Carboplatin	51
Valproate	no tox
Staurosporine	1.71
Mercury	5.05
Phenylbutasone	24
Doxorubicin	0.71

Figure 2. Top: iCell Hepatocytes treated with Amitriptyline for 48 hours. Images taken with 10x objective and analyzed with MWSC module. Response for various compounds of known mechanism of action using analysis for viable cells. All IC_{50} values given in µM.

Hepatotoxicity Phenotypes

Phenotypic characterization of hepatotoxicity by quantitation of the average and total stained cell areas.

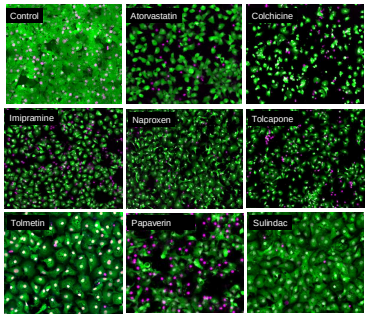
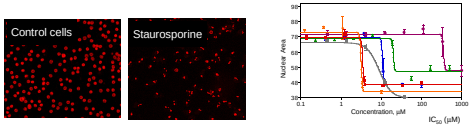


Figure 3. Images of iCell Hepatocytes treated with 100 µM of indicated compounds for 72 hr, then stained with Calcein AM and Hoechst 33258. Examples presented show impact of different compounds on total positive cell area and/or average positive cell area.

Nuclear Characterization

Nuclear condensation is characterized by: "decreased nuclear area" + "increased average intensity". This can provide additional sensitivity to toxicity.



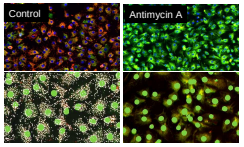
	IC_{50} (µM)
Aflatoxin 1	10.7
Idarubicin	8.9
Retinoic acid	351
Staurosporine	3.1
Mitomycin C	19.8
Aflatoxin 4	3.4

Figure 4. Top: Images are of iCell Hepatocytes untreated and treated with 10 µM of staurosporine, stained with Hoechst 33258. **Right:** Concentration-dependent responses for several compounds using average nuclear area as a read-out.

Specific Toxicity Assays

Mitochondria Potential 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. Data was analyzed using the MetaXpress 5 software Granularity module. This assay can be used either as an end-point or live-cell real time assay.



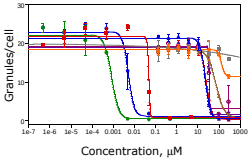
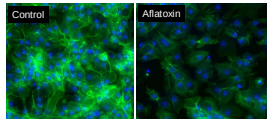


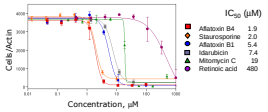
Figure 5. iCell Hepatocytes treated with compounds for 72h. Cells stained with Hoechst (nuclei), and JC-10 (mitochondria integrity). Images taken with 10x objective and analyzed with MetaXpress software Granularity module. Analysis results are shown in the bottom images. Concentration response curves and IC50 values are shown on right.

	IC50, µM		IC50, nM
Tolcapone	25.7	Antimycin A	5
Nefazodone	33.6	CCCP	47
Leflunomide	34.3	Valinomycin	1
Ketoconazole	71.4		
Isoniazid	-		
Fusariotoxin	132		
Flutamide	22.2		

Cytoskeleton Integrity

Cytoskeleton integrity was assessed by phalloidin staining. The parameters measured in this assay were count of cells positive for actin staining, total positive cell area, or integrated fluorescence intensity. These measurements can be used for the characterization of concentration dependent hepatotoxicity response. The cytoskeleton integrity assay had an excellent assay window (W>50) and low variance (Z'-value >0.8).





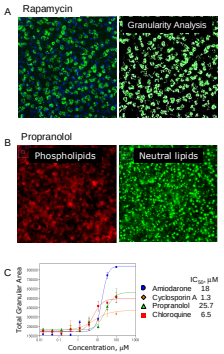
	IC_{50} (µM)
Aflatoxin B4	1.9
Staurosporine	2.0
Aflatoxin B1	5.4
Idarubicin	7.4
Mitomycin C	19
Retinoic acid	480

Figure 6. Top: Images of iCell Hepatocytes treated with 30 µM of Aflatoxin B1 for 72 hr, then fixed and stained with AlexaFluor-488 - Phalloidin and Hoechst 33258. **Bottom:** Concentration-dependent responses for several compounds using number of actin positive cells as a read-out.

Autophagy & Phospholipidosis

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 to maintain viability during periods of stress. Autophagic machinery can be manipulated to treat human diseases.

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.



	IC_{50}, µM
Amiodarone	18
Cyclosporin A	1.3
Propranolol	25.7
Chloroquine	6.5

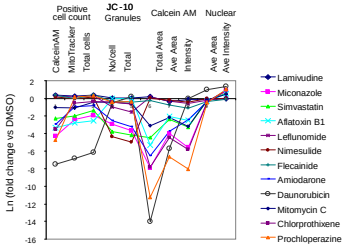
Figure 7. A: Autophagy Assay. Images of iCell Hepatocytes treated with Rapamycin for 24 hr and stained with Cyto-ID Autophagy detection kit. Analysis with Granularity module is shown on the right. **B:** Phospholipidosis, lipid accumulation assay. Images of iCell Hepatocytes treated with propranolol for 48 hr. Phospholipidosis and steatosis detected using LipidTOX reagent showing phospholipids and neutral lipids as indicated. **C:** Concentration dependent responses using Total Granular Area output and corresponding IC_{50} values for phospholipidosis.

Compound Library Screening

Screen-Well™ Hepatotoxicity Library (ENZO) contains 240 compounds including anti-cancer , anti-inflammatory, neuroleptic, antibiotics, and other classes. Compounds represent different mechanisms of hepatotoxicity: ALT elevation, steatosis, phospholipidosis, mitochondria damage, etc. A multi-parameter hepatotoxicity assay (Calcein AM, MitoTracker, Hoechst) 72 hr, 5 concentrations was used to asses toxicity of compounds in the library. In addition, mitochondria potential/oxidative stress assay (JC-10) 60min was also used to increase overall predictivity.

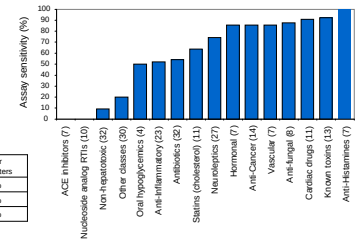
Output parameters:

- Number of Calcein positive cells
- Number of MitoTracker positive
- Total cell number
- Calcein AM intensity
- Calcein AM cell area
- MitoTracker intensity
- MitoTracker cell area
- Nuclear area
- Nuclear average intensity
- JC-10 Granules/Cell
- JC-10 Total Granules



Assay Sensitivity by Compound Class

- Assay Sensitivity "hits" based on output parameters deviating more than ±3σ from DMSO controls.
- Number of compounds in group shown in parentheses



Toxicity	Cell Count	Other Parameters
Sensitivity	40%	60%
Specificity	97%	91%
Predictive value	69%	75%

- High predictivity was found for many classes of compounds, e.g. neuroleptic, anti-cancer, cardiac drugs, toxins.
- Lower predictivity was observed for anti-inflammatory, antibiotics, and anti-viral drugs.

Summary

- Live-cell assays using the ImageXpress Micro XL High Content Imaging System 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 and increased assay sensitivity.
- We demonstrate utility of these in vitro assay models for toxicity screening and understanding potential hepatotoxic effects early in the drug development process.

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