High Content Solutions: Functionality of primary hepatocytes and adaptability to High-Content screening for in-vitro ADME/Tox applications

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ABSTRACT

Traditionally in vitro toxicology makes use of cell lines and whole well assays. These in vitro ADME/Tox models provide partial functionality, are single end-point read-outs and have modest sensitivity. Screens performed using these models may miss important indicators of toxicity. Drug-induced liver injury (DILI) is a major cause of drug market withdrawal and an ongoing challenge for new drug development efforts. To close the gap between in vitro toxicology results and in vivo outcomes, more predictable in vitro models are required. Here we demonstrate a system of cells, reagents and instruments to address these challenges and improve the usefulness of in vitro results. In this work we combined the in-depth analysis capabilities of CellInsight™ CX5 High-Content platform, the pre-developed Molecular Probes™ Cell Health Assays from and the functionality of GIBCO™ hepatocytes to showcase a model workflow for toxic response and mechanism elucidation. We used primary human and rat hepatocytes in combination with three fluorescent cytotoxicity indicators to simultaneously monitor five cell health parameters using the High-Content CX5 platform. Apoptosis was measured using CellEvent™ Green through detection of Caspase 3/7 activation, mitochondrial health and function was assessed with MitoTracker™ Orange through detection of mitochondrial membrane potential changes and nuclear number, size and condensation were quantitated with Hoechst 33342, which stains the nucleus. We collected data from treated and control groups and quantitated these parameters with the built-in algorithms for Cell Number, Nuclear Size, Nuclear condensation, caspase 3/7 activation and mitochondrial membrane potential. The results from this easy-to-use workflow demonstrate the positive contribution these integrated and multi-parametric approaches can make to in-vitro toxicology studies.

INTRODUCTION

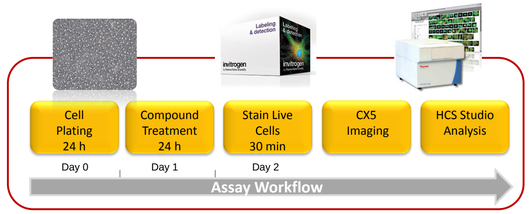
A combined effort of multiple assays is needed to detect idiosyncratic drug toxicity. The most comprehensive approach is a High-Content phenotypic screen. In this work we use GIBCO™ primary human and rat hepatocytes to show the adaptability to High-Content workflow and the capabilities to generate relevant data for ADME/Tox studies. Significantly reduced the effort of this work by using the pre-optimized Molecular Probes™ for Cell health and the user-friendly CellInsight™ CX5 platform.

MATERIALS AND METHODS

Cryopreserved hepatocytes were thawed at 37˚ C and transferred to 50 mL of Hepatocyte Thaw media (TSF, CM7500) following the Thermo Fisher Scientific (TSF) protocol with minor modification (3-step centrifugation: 3 min at 8 x g, 3 min at 32 x g, 3 min at 72 x g). Collected cells were resuspended in William's E Media (TFS, A1217601) supplemented with Cocktail A (TFS, CM4000) and plated at 36,000 cells/well (RPH) and 46,000 cells/well (HPH) in a 96w collagen I pre-coated plate (TSF, A1142803). After 4 hours, plating media was replaced with culture media, William's E Media (TFS, A1217601) supplemented with Cocktail B (TFS, CM4000). 24 hours after plating, cells were incubated with compound and CellEvent™ Caspase-3/7 Green (C10723) for an additional 24 hours. Molecular Probes used in this work are MitoTracker™ Orange (M7510), CellEvent™ Caspase-3/7 Green (C10723), and Hoechst 33342 (H3570). After 24 h compound and CellEvent™ Caspase-3/7 Green (C10723) treatment, supernatant from the cells was gently aspirated and a diluted mixture of MitoTracker™ Orange and Hoechst 33342 was added to the cells and incubated for 30 minutes at 37˚ C. Cells were washed twice with 100µL/well FluoBrite™ DMEM (A1896702). 100µL/well of FluoBrite™ DMEM was added to the cells and images were acquired. Cell images were acquired with CellInsight™ CX5 High Content Screening Platform using a 20x objective and the autofocus feature set to Hoechst channel (nuclear). The HCS Studio™ Image Analysis Cell Health Profiling bio-application was used to obtain the average signal intensities from at least six fields per well condition and values obtained were plotted with GraphPad™ Prism 7 software.

RESULTS

Figure 1. High-Content Workflow



Simplified *in-vitro* toxicity studies

Figure 2. GIBCO® Functional Hepatocytes

Main Characteristics

- **Human & Animal**
- **Transporter-qualified**
- **Induction-qualified**
- **Metabolism-qualified**
- **Multi-donor pools: suspension and plateable**
- **Cryopreserved**

Viability and yield
- Cell viability
- Viability stability after 2 hours
- Cell yield per vial
- Seeding density

Cell health markers
- Membrane integrity
- Cytosolic clarity
- Organelle size and shape
- Debris; excretion products
- Cell-cell contacts (plateable)
- Bile canalicular networks (plateable)

Donor characteristics
- Genotyping
- Donor demographics

Metabolic activity testing
- ECOD, 7-HCG, 7-HCS
- CYP1A2, CYP2B6, CYP2C8,
- CYP2C9, CYP2C19, CYP2D6,
- CYP2E1, CYP3A, FMO,
- Others upon request

Application qualification tests
- Attachment efficiency (4—6 hours)
- Monolayer confluency (5 days)
- Photomicrographs (through day 5)
- 96-well suitability
- In situ intrinsic clearance
- CYP induction profiling
- Transporter uptake activity
- Transporter efflux activity
- Biliary excretion index (Americas)

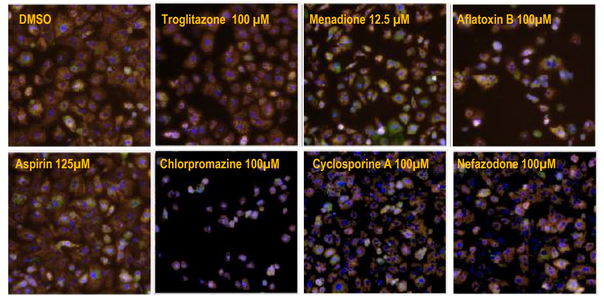
Figure 3. Molecular Probes Cell Health Assays for High-Content

Relevant Cell Health Indicators

- **MitoTracker® Orange** brightly stains healthy mitochondria and diffuses as membrane potential changes and becomes permeable due to drug effect.
- **CellEvent™ Caspase-3/7 Green** serves as substrate for activated caspases and the highly fluorogenic cleaved dye is free to bind DNA.
- **Hoechst 33342** stains brightly the nucleus and serves to obtain cell number, nuclear size and condensation measurements.

Ex/Em (nm)
554/576
502/530
350/461

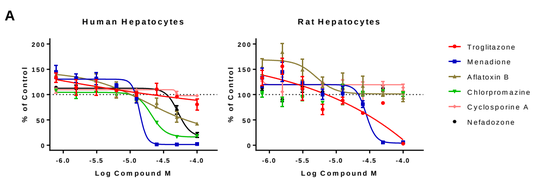
Figure 4. Toxic phenotype of drug targets



Representative images of human primary hepatocytes treated with compounds for 24 hours followed by staining with CellEvent™ Caspase-3/7 Green, MitoTracker® Orange and Hoechst 33342 (blue). The *Cell Health Bio-application* was used to create a primary mask based on the nucleus to identify cell number per field. Key drug-induced toxic phenotypes are cell loss, reduced cytoplasmic region, diffuse mitochondrial stain and small bright nuclei. Co-localization of Caspase 3/7 activation and Hoechst 33342 stain in the nucleus indicates the nuclear membrane is permeable as shown in chlorpromazine treated cells. Bright Mitochondria (orange) cells and basal levels of Caspase 3/7 (green) occur in the cytoplasmic region of DMSO treated cells.

Figure 5. Dose dependent toxicity using relevant primary hepatocytes

A



B

Drug	Human IC_{50} (µM)	Drug-Induced Liver Injury [DILI]
Troglitazone	ND	Bile acid transport
Menadione	13.9	Oxidative Stress
Aflatoxin B	18	Cytoskeleton integrity
Chlorpromazine	20.8	Cytotoxic, Lipid metabolism
Cyclosporine A	ND	Oxidative Stress, Lipid metabolism
Nefazodone	52.3	Oxidative Stress

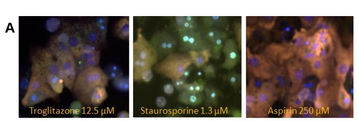
A. Dose-dependent responses from human and rat hepatocytes to known-toxicants using cell number as read-out.

B. The table shows the cytotoxicity IC_{50} of the compounds in human primary hepatocytes.

Figure 6. Validation of hepatocyte functionality – LDH Release

A) Rat primary hepatocytes treated with troglitazone, staurosporine or aspirin for 24 h and stained with CellEvent™ Caspase-3/7 , MitoTracker® Orange and Hoechst .

A



B) Lactate dehydrogenase (LDH) release is a common method to determine compound toxicity. Supernatants of compound treated cells were tested for LDH levels. Staurosporine treated cells released LDH, an indication of toxicity response. Troglitazone and aspirin did not show detectable levels of LDH.

B

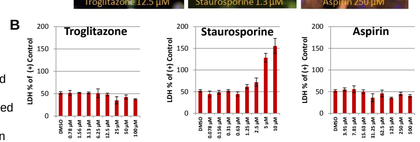
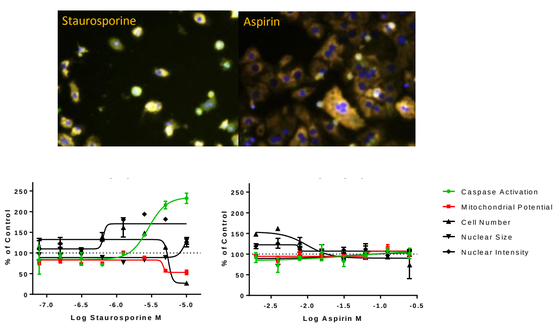


Figure 7. Multiparametric phenotypic Assay for safety compound profiling



Graphs show the measurements obtained from five cell health parameters from rat primary hepatocytes treated with a dose-response of staurosporine and aspirin for 24 h, followed by staining with CellEvent™ Caspase-3/7 Green, MitoTracker® Orange and Hoechst 33342.

Parameter	Staurosporine IC_{50} (µM)	Aspirin IC_{50} (µM)
Cell Number	5.52	non-toxic
Mitochondrial membrane Potential	4.65	non-toxic
Caspase 3/7 Activation	2.83	non-toxic
Nuclear Size	9.7	non-toxic
Nuclear Intensity	0.65	non-toxic

The table summarizes the IC_{50} obtained for each of the five phenotypic parameters shown as example of parallel monitoring of toxicity.

CONCLUSIONS

GIBCO™ hepatocytes allowed parallel measurements of important cell health indicators in a High-Content assay.

- GIBCO™ hepatocytes underwent phenotypic changes in response to hepato-toxicant treatments *in vitro*
- Minimum adaptation needed for Molecular Probes™ Cell Health Assays
- Automatically collected images with CX5 platform
- Performed live cell measurements of biomarkers with user-friendly HCS Studio™ software
  - Generated cytotoxicity curves
  - Measured nuclear size and condensation
  - Parallel monitoring of Caspase 3/7 activation and mitochondrial disruption

REFERENCES

1. Xu JJ,Diaz D, O'Brien PJ (2004) Applications of cytotoxicity assays and pre-lethal mechanistic assays for assesment of human hepatotoxicity potential. Chem Biol Interact 150(1):115-128