Application Note

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Long-Term Hepatotoxicity Studies Using Cultured Human iPSC-Derived Hepatocytes

Authors

Brad Larson and Peter Banks
Agilent Technologies, Inc.

Coby Carlson and
Michael Hancock
Cellular Dynamics International

Winy Luty
Enzo Life Sciences

Abstract

Quantitative microscopy was used to determine the effect of drugs on human iPSC-derived hepatocytes aggregated into spheroids. Three phenotypes of toxicity were measured using fluorescent probes: reactive oxygen species generation, mitochondrial membrane potential decline, and plasma membrane rupture. Results were compared to hepatocytes cultured in flat-bottom microplates. Spheroid formation enabled much longer kinetic studies, extending out to 14 days. This capability allowed the measurement of full pharmacology and phenotype.

Introduction

Drug-induced liver injury (DILI), or damage to the liver caused by prescription or nonprescription medications, continues to be a growing public health problem and a challenge for drug development. Effects can be acute or chronic and are compounded by the growing market for dietary supplements and herbal or nontraditional remedies. Most DILI is the result of unexpected responses to a particular medication, or long-term chronic damage that was unseen during standard hepatotoxicity testing.

To test new drug entities for potential DILI, *in vivo* models remain the gold standard. However, these studies are costly, time-consuming, and more importantly, poor predictors of human toxicity due to the incorporation of mainly murine hepatocytes. Consequently, *in vitro* screens using primary hepatocytes are less costly, reduce animal exposure, and are more amenable to higher-throughput platforms. However, limitations such as high interindividual variability, finite batch sizes, and changes in cell morphology, as well as liver-specific functions during long-term culture are challenging this model. Human induced pluripotent stem cell (iPSC)-derived hepatocytes, by comparison, are a promising *in vitro* alternative because they demonstrate primary tissue-like phenotype, high levels of consistency, and unlimited availability.

When performing toxicity studies, hepatocytes are repeatedly dosed with varying concentrations of a potential drug over multiple days to assess any cumulative effects. This poses particular challenges when incorporating two-dimensional (2D) cell culture of primary hepatocytes due to the fact that the cells rapidly dedifferentiate and lose metabolic activity when cultured in this manner. Three-dimensional (3D) cell culture models exist that allow cells to aggregate and retain the functionality and communication networks found *in vivo*. The favorable environment created by the 3D culture model then allows long-term dosing experiments to be performed that more accurately assess the cumulative effects of a drug.

This application note demonstrates the suitability of 3D cultured human iPSC-derived hepatocytes for use in hepatotoxicity studies. Hepatocyte spheroids were exposed to multiple concentrations of three drugs with the DILI category I or III: tolcapone, acetaminophen, and mitomycin C. Direct image-based assessment of hepatocyte health based on three phenotypes, after short-term and long-term exposure to the drugs was performed. These phenotypes included generation of reactive oxygen species (ROS), which is indicative of oxidative stress, mitochondrial membrane potential (MMP) decline, which is an early trigger of the apoptotic cascade, and plasma membrane (PM) rupture, which is a sign of necrotic cell death. Comparisons were also made to iPSC-derived hepatocytes cultured in 2D.

Materials and methods

Materials

Cells

iCell Hepatocytes 2.0 (part number PHC-100-020-001) were donated by Cellular Dynamics International (Madison, WI). These cells are human iPSC-derived hepatocytes that exhibit typical hepatic functionality and phenotypic stability. Due to their human origin, native cell-like behavior, and ease of use, iCell Hepatocytes 2.0 represent an optimal test system for basic hepatic biology in all areas of drug development, disease modeling, and toxicology.

Assay and experimental components

BioCoat Collagen I-coated 24- (part number 354408) and 384-well plates (part number 354667) were donated by Corning Life Sciences (Corning, NY). GravityTRAP ULA 96-well plates (part number ISP-09-001) were purchased from PerkinElmer (Waltham, MA). Acetaminophen (part number 1706), Mitomycin C (part number 3258) and Tolcapone (part number 5864) were purchased from R&D Systems (Minneapolis, MN). ROS-ID Total ROS/Superoxide Detection Kit (part number ENZ-51010), MITO-ID Membrane Potential Detection Kit (part number ENZ-51018) and NUCLEAR-ID Blue/Red Cell Viability Reagent (GFP CERTIFIED) (part number ENZ-53005) were donated by Enzo Life Sciences (Farmingdale, NY).

Agilent BioTek Cytation 5 cell imaging multimode reader

The Cytation 5 is a modular multimode microplate reader combined with automated digital microscopy. Filter- and monochromator-based microplate reading are available, and the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield, and phase contrast. The instrument can perform fluorescence imaging in up to four channels in a single step. With special emphasis on live-cell assays, Cytation 5 features temperature control to 65 °C, CO_2/O_2 gas control, and dual injectors for kinetic assays. Z-stacking and projection can also be performed to support 3D cell biology. The integrated Agilent BioTek Gen5 microplate reader and imager software was used to control the imager and for automated dual-masking analysis.

Methods

Cell culture preparation

iCell Hepatocytes 2.0 were thawed and cultured according to the manufacturer's protocol. See the CDI user guide for more details and media information. Cells intended for 3D spheroid formation were first seeded into 24-well collagen coated plates at a concentration of 600,000 cells/well. Cells kept for 2D cell culture were added to 384-well collagen-coated plates at a concentration of 30,000 cells/well. All plates were incubated at 37 °C/5% CO_2, and media was exchanged every twenty-four hours. After five days, cells in the 24-well plates were dissociated and seeded into GravityTRAP plates at a concentration of 2,000 cells/well to allow spheroid formation (typically complete after 48 hours). Media in the 384-well plates continued to be changed daily until compounds were added on day 7 post-thaw.

Hepatotoxicity assay procedure

Following spheroid formation, 10-point titrations of the known hepatotoxins acetaminophen (5,000 to 0 µM), mitomycin C (10 to 0 µM), and tolcapone (200 to 0 µM) were prepared using 1:2 serial dilutions. These concentration ranges are reflective of common dosages used for treatment. Spent media was removed from all test plate wells and replaced with fresh media and compound titrations. Wells were redosed with fresh compound every 48 hours.

Time points for assaying the health of the cells were 1 and 7 days for 2D plated hepatocytes and 1, 7, and 14 days for 3D spheroids. Assay measurements included:

- ROS generation using ROS-ID Total ROS/Superoxide detection kit, which includes two fluorescent dyes as major components: Oxidative Stress Detection Reagent (Green) for total ROS detection reagent and Superoxide Detection Reagent (Orange).
- Mitochondrial membrane potential (MMP) decline using MITO-ID, a cationic dye that fluoresces either green or orange depending upon membrane potential status. A reduction of orange fluorescence associated with MMP decline indicates early stages of apoptosis.
- PM rupture using NUCLEAR-ID, a mixture of a blue fluorescent cell-permeable nucleic acid dye for live cell imaging and a red fluorescent cell-impermeable nucleic acid dye that is suited for staining dead nuclei.

The assay workflow began with media removal followed by media replacement containing probes from either the multiplexed ROS-ID and NUCLEAR-ID, or MITO-ID and NUCLEAR-ID fluorescent microscopy kits, and incubated for 5 hours at 37 °C/5% CO_2. Wells were then washed with PBS to remove unincorporated probes, followed by image-based detection, also in PBS, using the Agilent BioTek Cytation 5. A 10x objective was used for 2D cellular imaging, and a 4x objective for 3D cellular imaging.

The signal from all multiplexed fluorescent probes was captured in a single imaging step using the following imaging channels:

- **ROS-ID/NUCLEAR-ID multiplex assay – DAPI channel:** NUCLEAR-ID live cell probe;
  Texas Red channel: NUCLEAR-ID dead cell probe;
  RFP channel: ROS-ID superoxide probe
- **MITO-ID/NUCLEAR-ID multiplex assay – DAPI channel:** NUCLEAR-ID live cell probe;
  Texas Red channel: NUCLEAR-ID dead cell probe;
  GFP channel: MITO-ID membrane potential probe cytosolic monomers;
  RFP channel: MITO-ID mitochondrial aggregates

Results and discussion

2D hepatotoxicity testing

When assessing the potential of a drug or its metabolites to cause DILI, it is common not only to examine the ability to induce overt cell death, but also to determine the cause of the observed hepatotoxicity. Two commonly measured mechanisms include induction of oxidative stress and ROS generation, in addition to loss of mitochondrial membrane potential (MMP) as an early indicator of apoptotic activity. The capacity of acetaminophen, mitomycin C, and tolcapone to induce oxidative stress and apoptosis, leading to downstream necrosis, following short-term and long-term treatment of 2D plated iCell Hepatocytes was determined using fluorescence microscopy-based probes (Figure 1).

Consistent with previously reported mechanisms of acetaminophen toxicity[1], these multiplexed fluorescent assays enabled detection of drug induced hepatotoxicity effects following exposure to high doses of acetaminophen. Increasing acetaminophen concentrations and repetitive dosing resulted in detection of ROS formation (Figures 1A to 1C), loss of mitochondrial membrane potential associated with apoptosis (Figures 1D to 1F), and eventual loss of cell membrane integrity and necrotic cell death (Figures 1G to 1I).

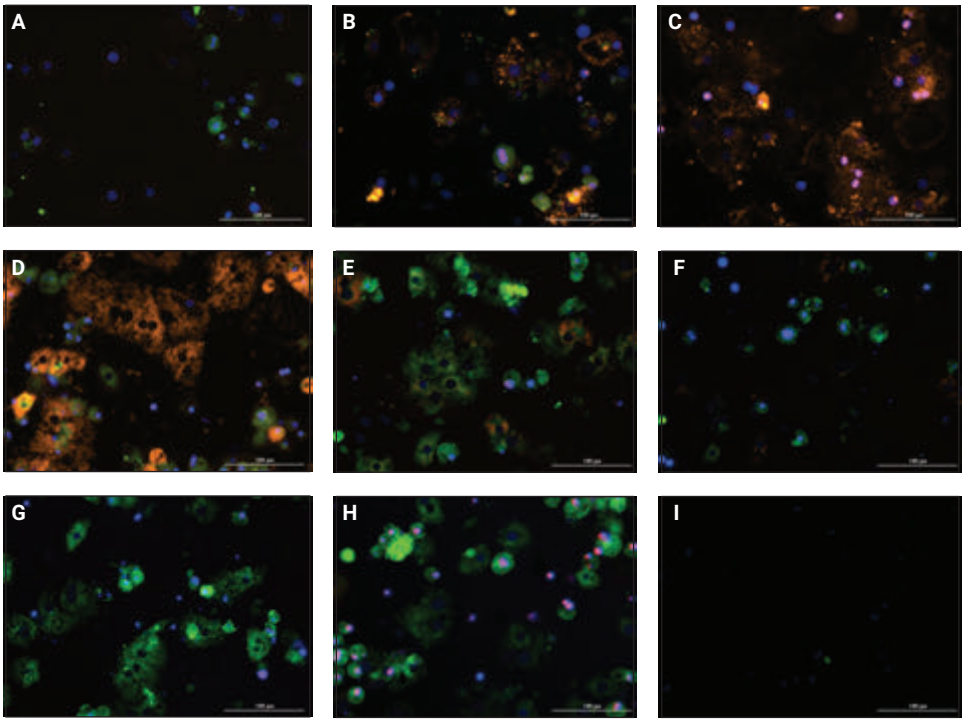


Figure 1. Images of 2D plated iCell Hepatocytes 2.0 expressing phenotypes of cellular distress after treatment with acetaminophen. Images captured using a 10x objective. Top row: ROS generation detected as an increase in orange puncta using the ROS-ID probe, whereas NUCLEAR-ID stained live cells blue or dead cells pink/red. (A) Low; and (B) intermediate ROS generation after one day of acetaminophen exposure; and (C) high ROS levels following seven days of acetaminophen treatment (625 µM). Middle row: MMP decline visualized as a loss of orange aggregates and a simultaneous increase in smaller, rounded-up green stained cells due to compromised MMP as detected by the MITO-ID reagent. Cells were also stained with NUCLEAR-ID probes to stain live cells blue and dead cells pink/red. (D) Control cell population exhibiting stable mitochondrial membrane potentials. (E) Partial and (F) complete loss of orange mitochondrial aggregates indicative of all cells having compromised MMP following one and seven days of acetaminophen exposure (625 µM). Bottom row: PM rupture indicated by a loss of green cytosolic staining using the green-fluorescent MITO-ID probe along with an increase in pink/red stained dead cells versus blue live cells via NUCLEAR-ID. (G) Low amount of PM rupture following one-day exposure versus (H) high amount of cells with PM rupture following seven days of acetaminophen exposure (625 µM). (I) Loss of cell attachment after seven days of treatment with 5 mM acetaminophen.

2D Hepatocyte quantitative image analysis

Quantification of superoxide expression, induction of apoptosis, and induction of necrotic activity, was performed for all compound treatments and incubation periods using the cellular analysis features of Gen5 and the parameters found in Tables 1 to 3.

Table 1. 2D Superoxide expression cellular analysis parameters.

Primary Cellular Analysis Parameters	
Channel	DAPI
Threshold	10,000
Background	Dark
Split Touching Objects	Checked
Fill Holes in Masks	Checked
Minimium Object Size	5 µm
Maximum Object Size	100 µm
Include Primary Edge Objects	Unchecked
Analyze the Entire Image	Checked
Advanced Detection Options	
Background Flattening Size	50 µm (rolling ball diameter)
Image Smoothing Strength	0 Cycles of 3 × 3 average filter
Evaluate Background On	5% of lowest pixels
Primary Mask	Use threshold mask
Secondary Cellular Analysis Parameters	
Channel	RFP
Measure Within a Secondary Mask	Include secondary area only in analysis
Distance from Primary Mask	0 µm
Ring Width	75 µm
Threshold	5,000
Smoothing	0
Method	Threshold in mask
Fill Holes in the Mask	Unchecked

Table 2. 2D Apoptotic activity cellular analysis parameters.

Primary Cellular Analysis Parameters	
Channel	DAPI
Threshold	10,000
Background	Dark
Split Touching Objects	Checked
Fill Holes in Masks	Checked
Minimium Object Size	5 µm
Maximum Object Size	100 µm
Include Primary Edge Objects	Unchecked
Analyze the Entire Image	Checked
Advanced Detection Options	
Background Flattening Size	50 µm (rolling ball diameter)
Image Smoothing Strength	0 Cycles of 3 × 3 average filter
Evaluate Background On	5% of lowest pixels
Primary Mask	Use threshold mask
Secondary Cellular Analysis Parameters	
Channel	RFP
Measure Within a Secondary Mask	Include secondary area only in analysis
Distance from Primary Mask	0 µm
Ring Width	100 µm
Threshold	1,000
Smoothing	0
Method	Threshold in mask
Fill Holes in the Mask	Unchecked

Table 3. 2D Necrotic activity cellular analysis parameters.

Primary Cellular Analysis Parameters	
Channel	DAPI
Threshold	10,000
Background	Dark
Split Touching Objects	Checked
Fill Holes in Masks	Checked
Minimium Object Size	5 µm
Maximum Object Size	100 µm
Include Primary Edge Objects	Unchecked
Analyze the Entire Image	Checked
Advanced Detection Options	
Background Flattening Size	50 µm (rolling ball diameter)
Image Smoothing Strength	0 Cycles of 3 × 3 average filter
Evaluate Background On	5% of lowest pixels
Primary Mask	Use threshold mask

For superoxide expression and apoptotic activity, primary masks were placed around Hoechst 33342-stained nuclei (Figure 2A). Secondary masks were then placed around areas of the target probe exceeding set threshold values (Figure 2B).

For necrotic activity, primary masks again were placed around nuclei stained with the live cell probe from the NUCLEAR-ID Blue/Red Cell Viability Reagent (Figure 2C). As both the live and dead cell probes were localized to the nucleus, the fluorescence from the dead cell probe was also analyzed within the primary mask (Figure 2D).

Finally, subpopulation criteria were set to identify cells statistically responding to compound treatments (positive responder cells). Table 4 describes the specific calculated cellular analysis metric and subpopulation criteria used to identify the cells exhibiting the three different phenotypic effects from the compound treatment. The fraction of responding to total cells, expressed as a percentage, indicated the effect each compound treatment had on the hepatocytes in the well (Figure 3).

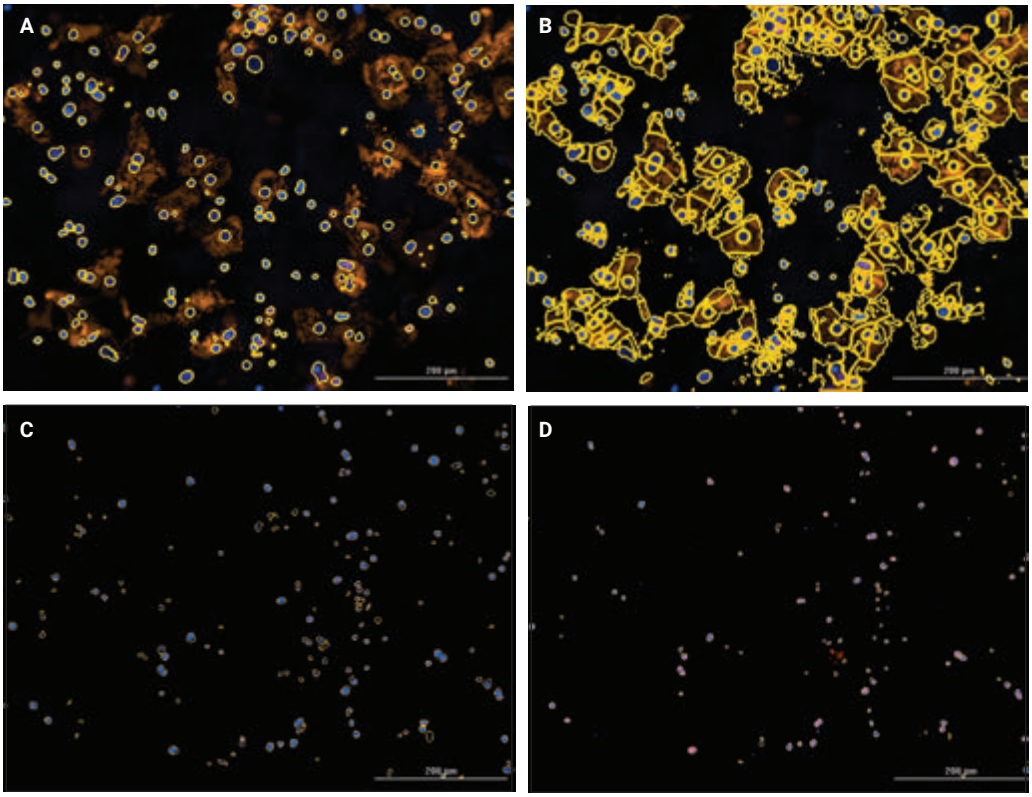


Figure 2. 2D hepatotoxicity primary and secondary object mask placement. Untreated hepatocytes following addition of MITO-ID superoxide probe showing (A) Primary nuclear object mask and (B) secondary object masks around target probe signal. Hepatocytes following seven day acetaminophen exposure (625 µM) showing (C) primary mask placement using signal from NUCLEAR-ID live cell probe, and (D) dead cell probe signal within primary masks.

Table 4. 2D Positive responder cell criteria.

Positive Responder Cell Identification Parameters		
Phenotypic Response	Identification Metric	Subpopulation criteria
Superoxide Expression	RFP Secondary Mask Peak RFU (Peak_2)	>12,000 RFU
Apoptotic Induction	RFP Secondary Mask Peak RFU (Peak_2)	<25,000 RFU
Necrotic Induction	Texas Red Primary Mask Peak RFU (Peak)	<40,000 RFU

A

2D Hepatocyte EC_{50} Values (µM)		
Superoxide Expression		
	1 Day	**7 Days**
Acetaminophen	350	58
Mitomycin C	>10	1.6
Tolcapone	9.7	1.0
Apoptosis		
	1 Day	**7 Days**
Acetaminophen	320	170
Mitomycin C	>10	0.8
Tolcapone	24	34
Necrosis		
	1 Day	**7 Days**
Acetaminophen	>5,000	300
Mitomycin C	>10	0.9
Tolcapone	>200	14

The over-the-counter pain reliever acetaminophen and the Parkinson's disease drug tolcapone demonstrated the phenotypes of ROS generation and MMP decline after only 24 hours treatment in a similar fashion. The expression of these phenotypes was increased and evident at lower doses after 7 days treatment. PM rupture, however, was only significantly evident after 7 days treatment. The chemotherapeutic agent mitomycin C demonstrated relatively muted phenotypic responses compared to the other drugs, especially after only one day of treatment. However, still induces a long-term toxic response on the 2D plated hepatocytes.

B

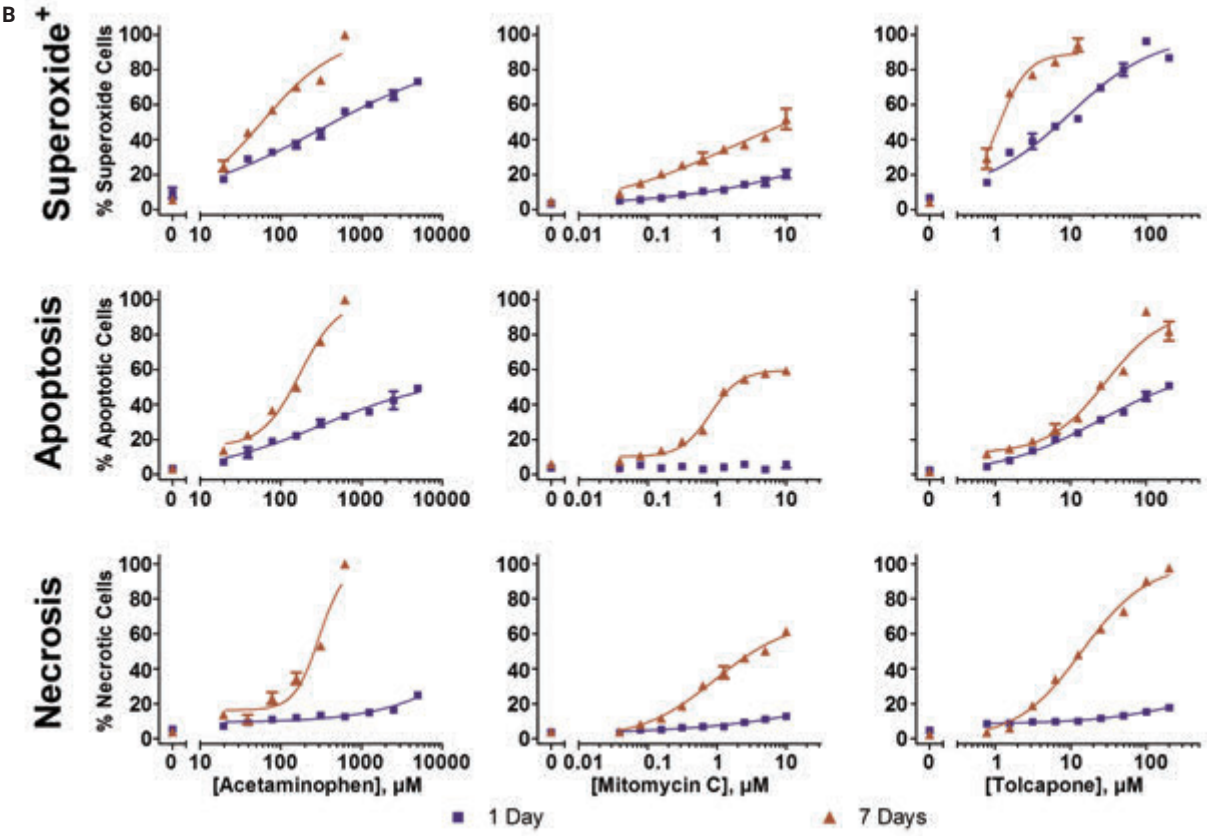


Figure 3. 2D hepatotoxicity results. (A) Calculated EC_{50} values. (B) Compound dose response curves.

3D hepatotoxicity testing

The toxic effects of acetaminophen, mitomycin C, and tolcapone were also examined in 3D cultured iCell Hepatocytes 2.0 spheroids (Figure 4). The same three phenotypes were assessed following 1, 7, and 14 day exposures to each compound.

Similar to that seen with imaging of 2D plated hepatocytes, the signal from target probes was also detected from hepatocytes aggregated into 3D spheroids. By collecting images at multiple z-planes and then projecting a final image for each imaging channel containing the most in focus portions of the stack, accurate analysis of the impact of compound treatment was performed.

3D hepatocyte spheroid Z-projected image analysis

Levels of ROS generation, MMP decline, and PM rupture were calculated in a similar manner as with the 2D plated hepatocytes. For optimal calculations when using images of the 3D spheroids, the Gen5 cellular analysis parameters were adjusted appropriately (Tables 5 to 7).

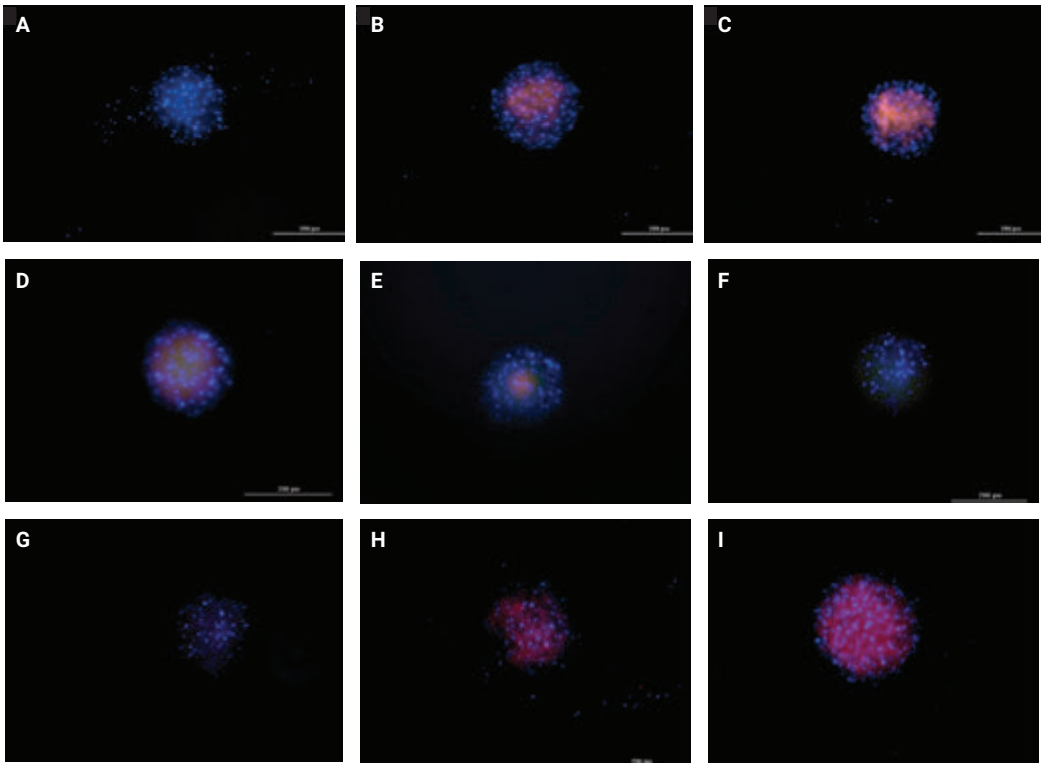


Figure 4. Images of iCell Hepatocytes 2.0 spheroids expressing phenotypes of cellular distress after treatment with acetaminophen. Images captured using a 4x objective. Top panel: ROS generation was detected as an increase in orange signal within the spheroid using the ROS-ID probe. (A) Low; (B) intermediate; and (C) high ROS levels following fourteen days of acetaminophen treatment. Middle panel: MMP decline was visualized as a loss of orange signal within the spheroid while maintaining consistent green signal as detected by the MITO-ID reagent. (D) Control cell population exhibiting stable MMP. (E) Partial; and (F) complete loss of orange mitochondrial aggregates following fourteen days of acetaminophen treatment. Bottom panel: PM rupture was indicated by an increase in pink/red stained dead cells within the spheroid versus blue live cells via NUCLEAR-ID. (G) Untreated hepatocyte spheroid; (H) intermediate; (I) high amounts of PM rupture following fourteen-day acetaminophen treatment.

Table 5. 3D Superoxide expression cellular analysis parameters.

Primary Cellular Analysis Parameters	
Channel	DAPI
Threshold	8,000
Background	Dark
Split Touching Objects	Unhecked
Fill Holes in Masks	Checked
Minimium Object Size	100 µm
Maximum Object Size	1,000 µm
Include Primary Edge Objects	Unchecked
Analyze the Entire Image	Checked
Advanced Detection Options	
Background Flattening Size	Auto (rolling ball diameter)
Image Smoothing Strength	0 Cycles of 3 × 3 average filter
Evaluate Background On	5% of lowest pixels
Primary Mask	Use threshold mask
Secondary Cellular Analysis Parameters	
Channel	RFP
Measure Within a Secondary Mask	Include primary and secondary area in analysis
Expand Primary Mask	1 µm
Threshold	8,000
Smoothing	0
Method	Propagate mask
Fill Holes in the Mask	Unchecked

By adjusting primary analysis criteria, such as minimum and maximum object size, primary masks were placed around the entire spheroid as a single object (Figure 5A). Secondary masks were then placed around the target fluorescent probe signal emanating from all cells within the spheroid meeting threshold criteria indicative of responding cells to compound treatment (Figure 5B). The percentage of area covered by the secondary masks divided by the entire spheroid area represented the portion of responding cells (Figures 5C and 5D).

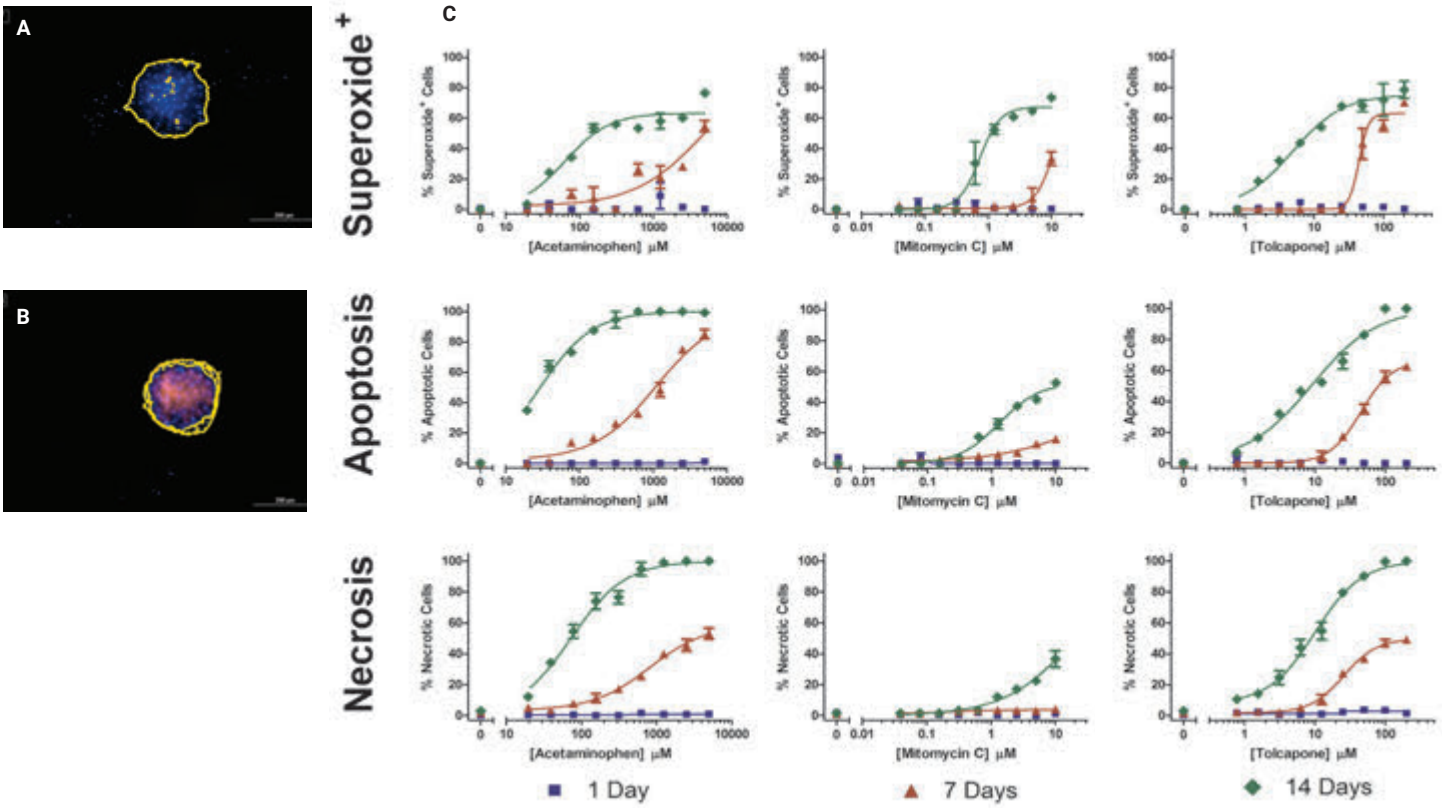
Analysis of the projected 3D images, dose-response curves, and generated EC_{50} values (Figures 5A, 5B, 5C, and 5D, respectively) indicates that the hepatotoxins have a minimal short-term effect and right-shifted pharmacology in 3D compared to 2D plated hepatocytes. The images also demonstrate that induced necrosis in spheroidal hepatocytes does not cause large-scale cell loss. This feature of 3D cultures can allow long-term hepatotoxicity results to be generated with greater accuracy. Finally, the ability to perform extended compound treatments with the 3D hepatocyte cell model allow the elucidation of potential mechanisms of action not possible in 2D. This is seen by the induced ROS generation following a prolonged 14-day mitomycin C treatment (Figure 5C).

Table 6. 3D Apoptotic activity cellular analysis parameters.

Primary Cellular Analysis Parameters	
Channel	DAPI
Threshold	7,000
Background	Dark
Split Touching Objects	Unhecked
Fill Holes in Masks	Checked
Minimium Object Size	100 µm
Maximum Object Size	500 µm
Include Primary Edge Objects	Unchecked
Analyze the Entire Image	Checked
Advanced Detection Options	
Background Flattening Size	Auto (rolling ball diameter)
Image Smoothing Strength	0 Cycles of 3 × 3 average filter
Evaluate Background On	5% of lowest pixels
Primary Mask	Use threshold mask
Secondary Cellular Analysis Parameters	
Channel	RFP
Measure Within a Secondary Mask	Include primary and secondary area in analysis
Expand Primary Mask	1 µm
Threshold	40,000
Smoothing	0
Method	Propagate mask
Fill Holes in the Mask	Unchecked

Table 7. 3D Necrotic activity cellular analysis parameters.

Primary Cellular Analysis Parameters	
Channel	DAPI
Threshold	5,000
Background	Dark
Split Touching Objects	Unhecked
Fill Holes in Masks	Checked
Minimium Object Size	100 µm
Maximum Object Size	1,000 µm
Include Primary Edge Objects	Unchecked
Analyze the Entire Image	Checked
Advanced Detection Options	
Background Flattening Size	1,000 (rolling ball diameter)
Image Smoothing Strength	5 Cycles of 3 × 3 average filter
Evaluate Background On	10% of lowest pixels
Primary Mask	Use threshold mask



D

2D and 3D Hepatocyte EC_{50} Values (µM)						
Acetaminophen						
	1 Day		7 Days		14 Days	
	2D	3D	2D	3D	2D	3D
Superoxide	350	>5,000	58	>5,000	n.d.	64
Apoptosis	320	>5,000	170	1100	n.d.	29
Necrosis	>5,000	>5,000	300	810	n.d.	73
Mitomycin C						
	1 Day		7 Days		14 Days	
2D	3D	2D	3D	2D	3D	
Superoxide	>10	>10	1.6	13	n.d.	0.7
Apoptosis	>10	>10	0.8	>10	n.d.	1.3
Necrosis	>10	>10	0.9	>10	n.d.	>10
Necrosis						
	1 Day		7 Days		14 Days	
2D	3D	2D	3D	2D	3D	
Superoxide	9.7	>200	1.0	44	n.d.	4.8
Apoptosis	24	>200	34	45	n.d.	9
Necrosis	>200	>200	14	26	n.d.	10

Figure 5. 3D hepatotoxicity results. Primary nuclear object masks, and secondary object masks around (A) low; and (B) high target probe signal. (C) Compound dose response curves. (D) Calculated EC_{50} values for 2D and 3D hepatocytes.

Conclusion

3D spheroid cultures of iPSC-derived human hepatocytes, such as iCell Hepatocytes 2.0, provide a relevant cell model to perform long-term *in vitro* hepatotoxicity testing, while incorporation of fluorescent probes allow quantification of cell toxicity phenotypes in 2D and 3D cell models. Additionally, the optimized capabilities of the Agilent BioTek Cytation 5 cell imaging multimode reader and Agilent BioTek Gen5 microplate reader and imager software provide automated, dependable imaging and analysis of incorporated cell models and fluorescent probes. The combination of appropriate cell models, assay methodology, and imaging and analysis create an optimal method to determine the potential chronic hepatotoxic effects of test molecules.

Reference

1. Hinson, J. A.; Roberts, D. W.; James, L. P. Mechanisms of Acetaminophen-Induced Liver Necrosis. *Handb. Exp. Pharmacol.* **2010**, *(196)*, 369–405.

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