APPLICATION NOTE

Use of 3D Cultured Human iPSC-Derived Hepatocytes for Long-Term Hepatotoxicity Studies

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INTRODUCTION

Drug-induced liver injury (DILI) or injury to the liver caused by prescription or non-prescription medications continues to be a growing public health problem and a challenge for drug development. Effects can be acute or chronic and are compounded not only by the number of new drug entities but also by the growing market for herbal and other non-traditional 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, rather 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 inter-individual 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 cells (iPSC)-derived hepatocytes, by comparison, are a promising *in vitro* alternative to *in vivo* models by demonstrating primary tissue-like phenotype, high levels of consistency and unlimited availability.

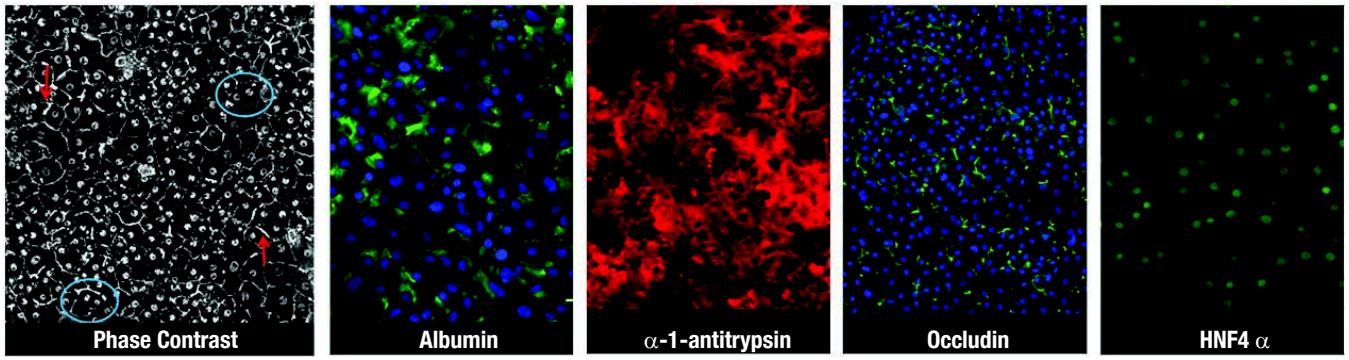
When performing toxicity studies, hepatocytes are repeatedly dosed with varying concentrations of a potential drug over multiple days to assess any cumulative effects. This poses particular challenges when incorporating two-dimensional (2D) cell culture of hepatocytes due to the fact that the cells rapidly 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 accurately analyze a potential drug's cumulative effects.

Here we demonstrate the suitability of 3D cultured human iPSC-derived hepatocytes for use in hepatotoxicity studies. Hepatocyte spheroids were exposed to multiple concentrations of the DILI category I and III drugs tolcapone, acetaminophen, and mitomycin C. Direct image-based assessment of hepatocyte mitochondrial health, after short-term and long-term exposure to the drugs, was performed. Comparisons were also made to iPSC-derived hepatocytes cultured in 2D.

BIOTEK INSTRUMENTATION

Cytation™ 5 Cell Imaging Multi-Mode Reader: Cytation 5 from BioTek Instruments 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 Gen5™ Microplate Reader and Imager Software was used to control the imager and for automated dual-masking analysis.

ICELL® HEPATOCYTES 2.0



Bi-nucleation & Bile Canaliculi	Mature Hepatocyte Marker	Liver Secreted Protease Inhibitor	Tight Junction Formation	Liver Specific Transcription Factor

iCell® Hepatocytes 2.0, human induced pluripotent stem cell-derived hepatocytes, from Cellular Dynamics, 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.

MATERIALS AND METHODS

Cells, Assay and Experimental Components: iCell Hepatocytes 2.0 Kits (Catalog No. PHC-100-020-001) were donated by Cellular Dynamics International (Madison, WI). BioCoat™ Collagen I coated 24- (Catalog No. 354408) and 384-well plates (Catalog No. 354667) were donated by Corning Life Sciences (Corning, NY). GravityTRAP® ULA 96-well plates (Catalog No. ISP-09-001) were purchased from PerkinElmer (Waltham, MA). Acetaminophen (Catalog No. 1706), Mitomycin C (Catalog No. 3258) and Tolcapone (Catalog No. 5864) were purchased from R&D Systems (Minneapolis, MN). ROS-ID® Total ROS/Superoxide Detection Kit (Catalog No. ENZ-51010), MITO-ID® Membrane Potential Detection Kit (Catalog No. ENZ-51018) and NUCLEAR-ID® Blue/Red Cell Viability Reagent (GFP-CERTIFIED®) (Catalog No. ENZ-53005) were donated by Enzo Life Sciences (Farmingdale, NY).

Assay Procedure: iCell Hepatocytes 2.0 were thawed and prepared according to the manufacturer's protocol. Following preparation, cells were added to either 24- or 384-well collagen coated plates, 600,000 and 30,000 cells/well, respectively, and incubated at 37° C/5% CO_2. Media was exchanged every twenty-four hours. After five days, cells in the 24-well plates were removed, added to wells of the 96-well GravityTRAP plates at a concentration of 2,000 cells/well, and incubated an additional forty-eight hours to allow for spheroid formation. Media in the 384-well plates continued to be changed on a daily basis until compounds were added on culture day seven.

Following spheroid formation, 10-point titrations of the known hepatotoxins acetaminophen (5000-0 µM), mitomycin C (10-0 µM), and tolcapone (200-0 µM) were prepared containing 1:2 serial diluted compound. Media was removed from all test plate

wells and replaced with media and compound. Wells were re-dosed with fresh compound every forty-eight hours.

After compound incubations of one and seven days for 2D plated hepatocytes, and one, seven, and fourteen days for 3D hepatocyte spheroids, cells were assayed to determine the extent of induced oxidative stress, and apoptotic and necrotic activity. Media was again removed, replaced with media containing probes from either the multiplexed ROS-ID and NUCLEAR-ID, or MITO-ID and NUCLEAR-ID fluorescent microscopy kits, and incubated for five hours at 37 ºC/5% CO_2. Wells were then washed to remove unincorporated probes, followed by image-based detection using the 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 could be 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.

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 superoxide formation, 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 fluorescent microscopy-based probes (Figure 2).

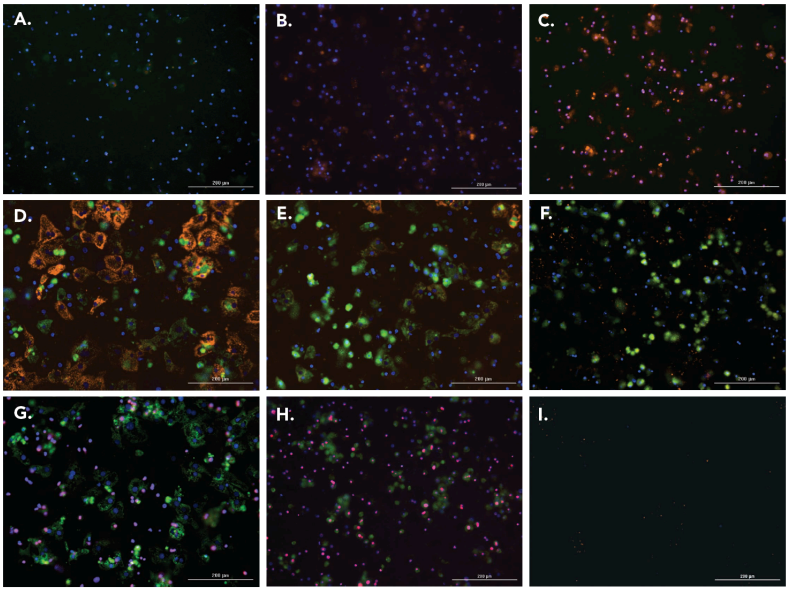


Figure 2. 2D images of induced superoxide formation, apoptosis, and cellular necrosis. Images captured using a 10x objective.

Top panel: Superoxide formation was 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 superoxide formation after one day of acetaminophen exposure; and (C) high superoxide levels following seven days of acetaminophen treatment (625 µM).

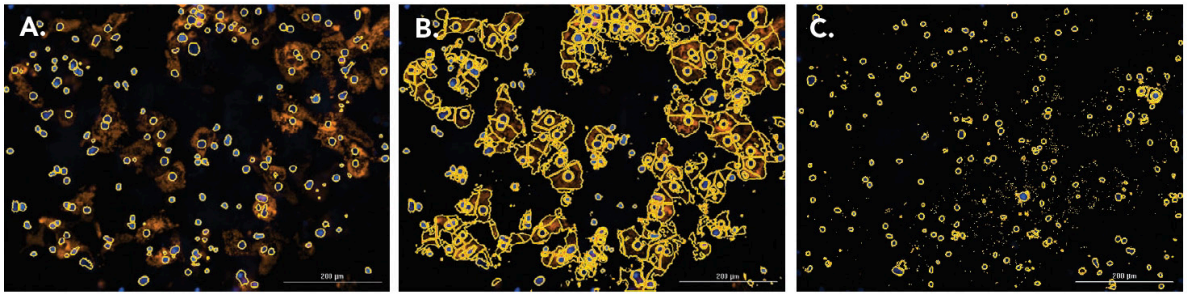
Middle panel: Apoptosis induction was visualized as a loss of orange aggregates and a simultaneous increase in smaller, rounded-up green stained cells due to compromised mitochondrial membrane potential as detected by the MITO-ID reagent. Cells were also stained with NUCLEAR-ID probes to stain live cells blue and dead cells pink/red. (D) Control cell population exhibiting a minimal number of apoptotic cells. (E) Partial and (F) complete loss of orange mitochondrial aggregates indicative of apoptosis induction following one and seven days of acetaminophen exposure (625 µM).

Bottom panel: Necrosis induction was indicated by a loss of green cytosolic staining using the greenfluorescent MITO-ID probe along with an increase in pink/red stained dead cells versus blue live cells via NUCLEAR-ID. (G) Low amount of necrotic cells following one day exposure versus (H) high amounts of necrotic cells following seven days of acetaminophen exposure (625 µM). (I) Loss of cell attachment after seven days of treatment with 5 mM acetaminophen.

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 superoxide formation (Figures 2A-C), loss of mitochondrial membrane potential associated with apoptosis (Figures 2D-F), and eventual loss of cell membrane integrity and necrotic cell death (Figures 2G-I).

2D HEPATOCYTE IMAGE ANALYSIS

Quantification of superoxide expression, and induction of apoptotic and necrotic activity, was performed for all compound treatments and incubation periods using the cellular analysis features of the Gen5 software. Primary masks are placed around nuclei using the stained nuclei (Figure 3A), then secondary masks are added if the stained cell signal from the target probe exceeds the threshold values established from control well values. Finally, subpopulation criteria are set to identify cells statistically responding to compound treatments. The fraction of responding to total cells, expressed as a percentage, indicates the effect each compound treatment has on the hepatocytes in the well.



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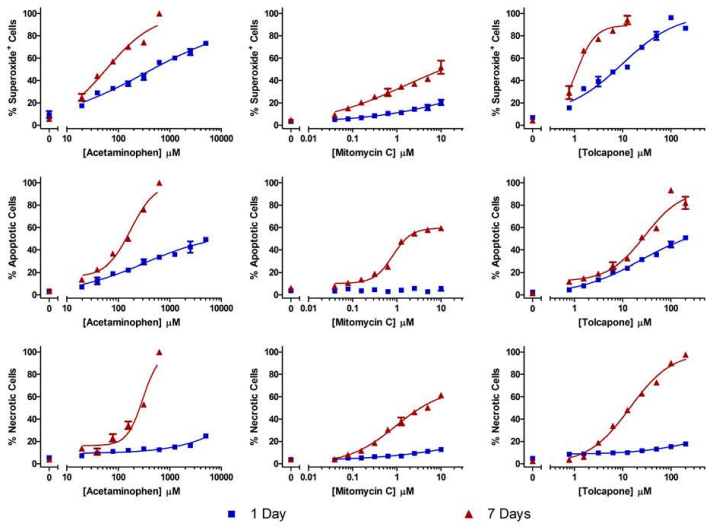


E.

2D Hepatocyte EC_{50} Values (µM)		
Superoxide Expression		
	1 Day	7 Day
Acetaminophen	350	58
Mitomycin C	>10	1.6
Tolcapone	9.7	1.0
Apoptosis		
	1 Day	7 Day
Acetaminophen	320	170
Mitomycin C	>10	0.8
Tolcapone	24	34
Necrosis		
	1 Day	7 Day
Acetaminophen	>5000	300
Mitomycin C	>10	0.9
Tolcapone	>200	14

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

1. Hinson, JA.; Roberts, DW.; James, LP. Mechanisms of Acetaminophen-Induced Liver Necrosis. Handb Exp Pharmacol. 2010, (196), 369-405.

2D hepatocytes exhibit a rapid increase in oxidative stress leading to apoptotic and eventual necrotic activities following acetaminophen and tolcapone treatment. Mitomycin C elicits a less pronounced level of superoxide expression, however it still induces a long-term toxic response on the plated cells.

3D HEPATOTOXICITY TESTING

In the same manner, the toxic effects of acetaminophen, mitomycin C and tolcapone were also examined in 3D cultured iCell Hepatocyte spheroids (Figure 4). Superoxide induction, and apoptotic and necrotic activities were assessed following 1, 7, and 14 day exposures to each compound.

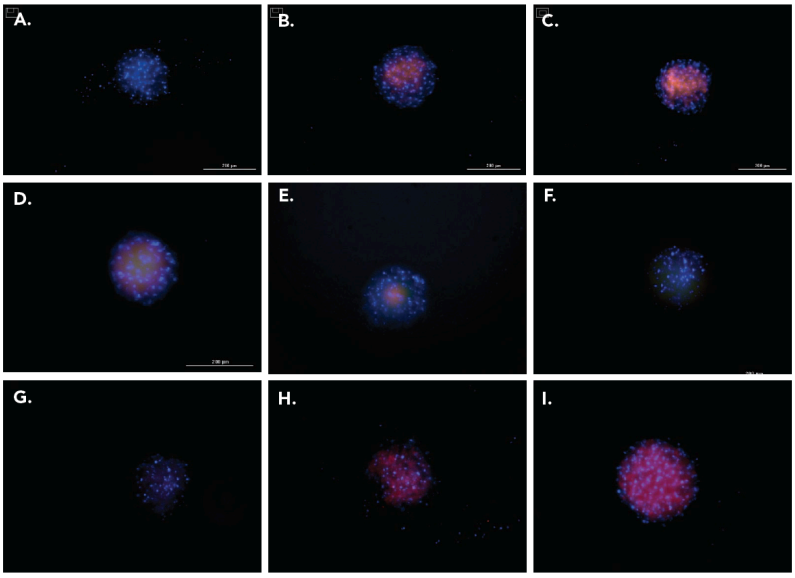


Figure 4. 3D images of induced superoxide formation, apoptosis, and cellular necrosis. Images captured using a 4x objective.

Top panel: Superoxide formation was detected as an increase in orange signal within the spheroid using the ROS-ID probe. (A) Low; (B) intermediate; and (C) high superoxide levels following fourteen days of acetaminophen treatment.

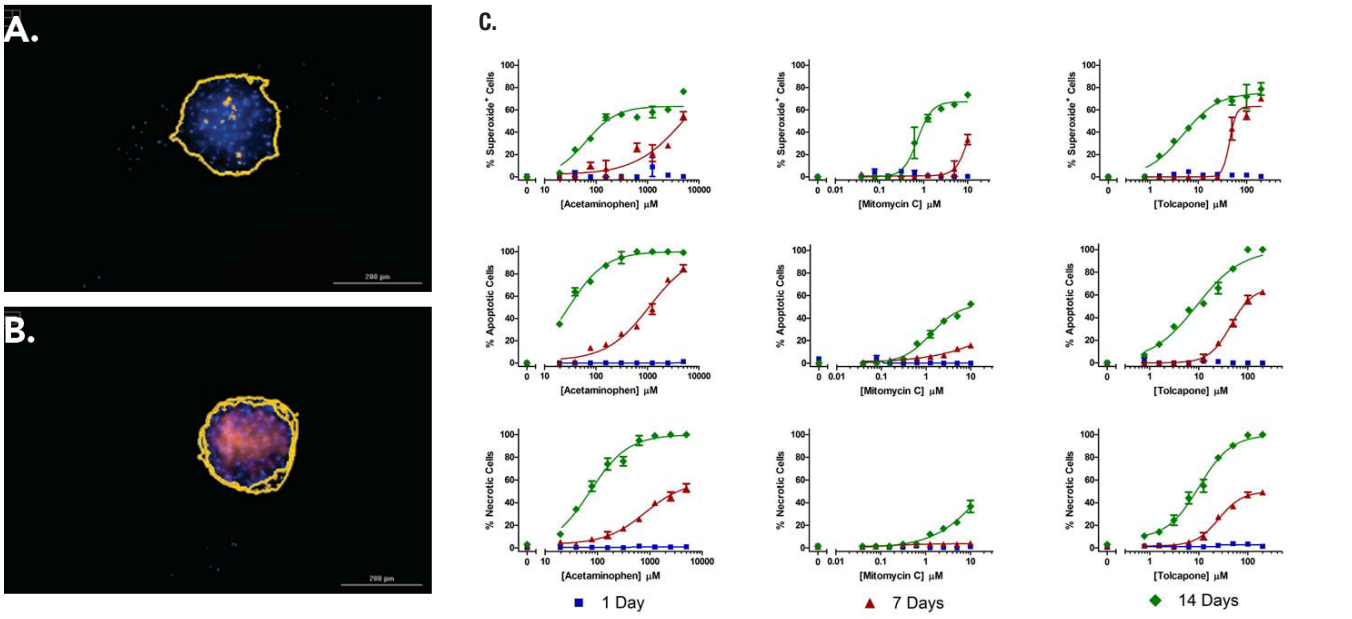
Middle panel: Apoptosis induction was visualized as a loss of orange signal within the spheroid while maintaining consistent green signal as detected by the MITO-ID reagent. (D) Control cell population exhibiting a minimal number of apoptotic cells. (E) Partial; and (F) complete loss of orange mitochondrial aggregates following fourteen days of acetaminophen treatment.

Bottom panel: Necrosis induction 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 cellular necrosis following fourteen days of acetaminophen treatment.

Similar to that seen with imaging of 2D plated hepatocytes, the signal from target probes can also be 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 can be performed.

3D HEPATOCYTE SPHEROID Z-PROJECTED IMAGE ANALYSIS

Levels of superoxide formation, and apoptosis and necrosis induction can also be calculated using the 3D spheroidal images. By adjusting primary analysis criteria, such as minimum and maximum object size, primary masks are placed around the entire spheroid as a single object (Figure 5A). Secondary masks are 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 (Figures 5A and B). The percentage of area covered by the secondary masks divided by the entire spheroid area represents the portion of responding cells.



D.

2D and 3D Hepatocyte EC_{50} Values (µM)						
Acetaminophen						
	1 Day		7 Days		14 Days	
	2D	3D	2D	3D	2D	3D
Superoxide	350	>5000	58	>5000	n.d.	64
Apoptosis	320	>5000	170	1100	n.d.	29
Necrosis	>5000	>5000	300	810	n.d.	73
MitomcyinC						
	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
Tocapone						
	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.

Analysis of the projected 3D images, dose response curves, and generated EC_{50} values indicates that the hepatotoxins have a minimal short-term effect 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 for the elucidation of potential mechanisms of action not possible in 2D. This is seen by the induced superoxide expression following a prolonged fourteen day mitomycin C treatment (Figure 5C).

CONCLUSIONS

1. 3D spheroid cultures of iPSC-derived human hepatocytes (iCell Hepatocytes 2.0) provide a relevant cell model to perform long-term *in vitro* hepatotoxicity testing.
2. Incorporation of fluorescent probes from Enzo Life Sciences allows for image-based detection of induced toxicity in 2D and 3D cell models.
3. The optimized capabilities of the Cytation 5 and Gen5 software from BioTek Instruments provide automated, dependable imaging and analysis of incorporated cell models and fluorescent probes.
4. The combination of appropriate cell models, assay methodology, imaging, and analysis creates an optimal method to determine the potential chronic hepatotoxic effects of test molecules.

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