Application Note

Toxicology Research

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Cost-Effective Automated Hepatotoxicity Testing Using *in Vitro* 3D Bioprinting

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Abstract

Hepatocytes were aggregated into 3D spheroids using a magnetic particle-based bioprinting technology. Aggregation in 96-well microplates was controlled using a pin shaped magnet drive over the course of 48 hours. All cell plating and reagent additions for hepatotoxicity testing were automated using a cost-effective liquid handling system, which provided suitable dose responses from known hepatotoxins.

Introduction

Prescription medications, environmental toxins, and nonprescription herbal remedies together form the major causes of hepatic injury. When looking at drugs alone, induced hepatic injury is the most common reason cited for warnings or withdrawal of an approved drug, depending on the severity of the induced hepatotoxicity. Due to this reality, a paradigm shift has taken place in the way toxicology studies are being performed, including determination of hepatotoxicity. Early phase assessment of potential hepatotoxic effects from a wider range of drug candidates and existing drugs or chemicals is now the norm. Coupled with the change in the development phase where testing takes place, is the desire to use cell models that close the gap between *in vitro* and *in vivo* systems.

Hepatotoxicity studies historically were performed by repeatedly dosing hepatocytes cultured on the bottom of a microplate with multiple concentrations of a test drug or compound. Because hepatocytes rapidly dedifferentiate, lose metabolic activity, and lack the communication networks found *in vivo* when cultured in this manner, results may be inaccurate and yield misleading claims regarding the safety of the test agent. To combat this shortfall, three-dimensional (3D) spheroidal models, such as those using hiPSC-hepatocytes, can be incorporated. This allows cells to aggregate and retain the typical long-term viability, functionality, and communication found *in vivo*; allowing for the generation of repeatable, accurate data.

To meet the demand for increased hepatotoxicity testing, automation has been incorporated to streamline the procedure and reduce the need for large-scale manual manipulations. Typically, included liquid handling systems have been large, expensive, and many times required placement into clean rooms for sterile processing. While this type of solution is suitable for pharmaceutical, biotech, and even larger core facilities, the size and cost can be prohibitive to the typical academic research lab. Therefore, a smaller, less expensive instrumentation set, which can still provide accurate and repeatable results, is necessary.

This demonstrates the ability to combine liquid handling with a novel cell imaging multimode reader to perform automated 3D hepatotoxicity studies. hiPSC-hepatocytes were aggregated into spheroids easily and efficiently using magnetic bioprinting technology. Dose responses from multiple known hepatotoxicants were evaluated with this system to demonstrate utility.

Materials and methods

Materials

Cells

ReproHepato hiPSC-hepatocytes (part number RCDH001N) were donated by REPROCELL (Yokohama, Japan).

Assay and experimental components

The 96-well BiO assay kit (GBO part number 655846, consisting of NanoShuttle-PL, 6-well levitating magnet drive, 96-well spheroid and holding magnet drives (2), 96-well deep well mixing plate, 6-well and 96-well clear cell repellent surface microplates), and additional collagen type I coated 6-well microplates (part number 657950) and cell-repellent surface 6-well microplates (part number 657860) were donated by Nano3D Biosciences, Inc. (Houston, TX) and Greiner Bio-One (Monroe, NC). ReproHepato culture medium (part number RCDN101) and ReproHepato assay medium (part number RCDH301) were donated by ReproCELL. P450-Glo CYP3A4 Luc-IPA (part number V9002), CellTiter-Glo 3D assays (part number G9683) and CellTox Green Dye (part number G8731) were obtained from Promega Corporation (Madison, WI).

Inhibitor compounds

Daunorubicin (part number ALX-380-043-M010), Aflatoxin B1 (part number ALX-630-093-M001), Tamoxifen (part number ALX-550-095-G001), Troglitazone (part number BMLGR210-0005), Nicardipine (part number ALX-550-273-G001), Mitomycin C (part number BML-GR311-0002), Simvastatin (part number BML-G244-0050), and Phenylbutazone (part number ALX-430-112-G005) were donated by Enzo Life Sciences (Farmingdale, NY).

Agilent BioTek Cytation 5 cell imaging multimode reader

The Agilent BioTek 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, and is controlled by integrated Agilent BioTek Gen5 microplate reader and imager software. The software was also used for dual-masking and automated analysis.

Microlab NIMBUS automated multichannel pipetting workstation

The compact Microlab NIMBUS automated multichannel pipetting workstation family from Hamilton Robotics (Hamilton Company, Reno, NV) includes the NIMBUS4 Enclosed. This model features four independent 1,000 µL air-displacement pipetting channels, a locking cover set to minimize environmental contamination, and optional Clean Air Protection (CAP) system with HEPA filter for positive sterile airflow. It uses proprietary Compressed O-Ring Expansion (CO-RE) technology to attach disposable tips without mechanical stress or aerosol creation. Features include antidroplet control (ADC), monitored air displacement (MAD) for real-time tracking of aspiration performance, and a traceable digital audit trail using Total Aspirate and Dispense Monitoring (TADM) for robust, reliable operation. The highly accurate system was used for all reagent and cell aspirate, dispense, wash and transfer steps, and to perform plate-wide compound serial dilutions.

Methods

3D hepatocyte cell preparation

ReproHepato hiPSC-hepatocytes were thawed from cryopreservation, resuspended in ReproHepato culture medium, plated onto Collagen Type I coated 6-well microplates, and incubated at 37 °C/5% CO_2 for four days. After incubation, a 100 µL of NanoShuttle-PL was added to the microplate wells as part of the 96-well BiO assay kit protocol (Figure 1), and the plates were returned to the incubator for one extra day. After the combined incubation period of five days, the cells were removed from the collagen-coated microplates and added to cell-repellent surface 6-well microplates. A 6-well magnet drive was placed atop the plates to levitate the cells, where extracellular matrix (ECM) formation was induced during an eight-hour incubation at 37 °C/5% CO_2. After incubation, cells and ECM were broken up and resuspended. Using the NIMBUS4 Enclosed, a total of 5,000 cells in 100 µL of medium were added to wells in four 96-well cell repellent microplates. A magnet was placed under each plate, and the plates were incubated at 37 °C/5% CO_2 for approximately 48 hours to allow the cells to aggregate into spheroids within each well, and to allow the hepatocytes to further mature after the combined seven-day period.

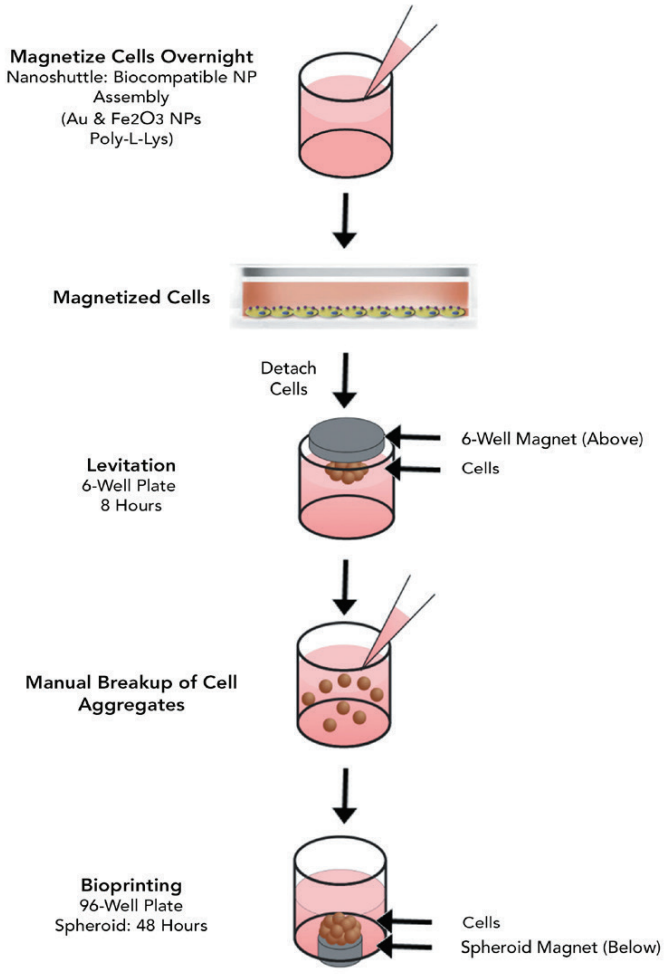

Figure 1. 96-well BiO assay kit protocol.

3D hepatocyte cell health assessment

Medium was manually removed from wells and replaced with 50 µL of prewarmed medium containing Luc-IPA substrate. The plate was then incubated at 37 °C/5% CO_2 for one (2D) or four hours (3D). Following incubation, 50 µL of supernatant was transferred to a separate white 96-well plate, and an equal volume of P450-Glo Luciferin Detection Reagent (LDR) was added to the same wells. The plate was shaken and incubated for 20 minutes at room temperature (RT) followed by luminescent signal detection. In the original cell plate, 50 µL of CellTiter-Glo 3D reagent was added to the wells, the plate was shaken at RT for 1 minute (2D) or 5 minutes (3D), followed by an additional 25-minute RT incubation. The luminescent signal was once again quantified.

Automated long-term hepatotoxicity testing

NIMBUS4 Enclosed was used to perform all steps of the automated hepatotoxicity testing procedure. Serial titrations of eight known hepatotoxins Daunorubicin, Aflatoxin B1, Tamoxifen, Troglitazone, Nicardipine, Mitomycin C, Simvastatin, and Phenylbutazone were prepared. After the titrations, culture medium was removed and replaced with medium containing compound in four separate spheroid-containing plates (Figure 2). This process was repeated every 48 hours. Following a 1, 3, 5, and 7-day incubation, culture medium containing compound was removed from the wells of a single plate and replaced with ReproHepato assay medium containing Hoechst 33342 and CellTox green dye. The plate was incubated for five hours, then fluorescence images were captured using the Agilent BioTek Cytation 5 cell imaging multimode reader.

Results and discussion

Image-based spheroid formation validation

Brightfield, label-free live cell imaging was performed throughout the hepatocyte aggregation process to ensure spheroid formation (Figure 3). As is evident in the three brightfield images, hepatocyte spheroid formation required 48 hours.

	Compound Concentrations (nM)											
	100000.0	33333.3	11111.1	3703.7	1234.6	411.5	137.2	45.7	15.2	5.1	1.7	0
Daunorubicin	1	2	3	4	5	6	7	8	9	10	11	12
Aflatoxin B1	1	2	3	4	5	6	7	8	9	10	11	12
Tamoxifen	1	2	3	4	5	6	7	8	9	10	11	12
Troglitazone	1	2	3	4	5	6	7	8	9	10	11	12
Nicardipine	1	2	3	4	5	6	7	8	9	10	11	12
Mitomycin C	1	2	3	4	5	6	7	8	9	10	11	12
Simvastatin	1	2	3	4	5	6	7	8	9	10	11	12
Phenylbutazone	1	2	3	4	5	6	7	8	9	10	11	12

Figure 2. 96-well spheroid microplate hepatotoxicant dosing scheme.

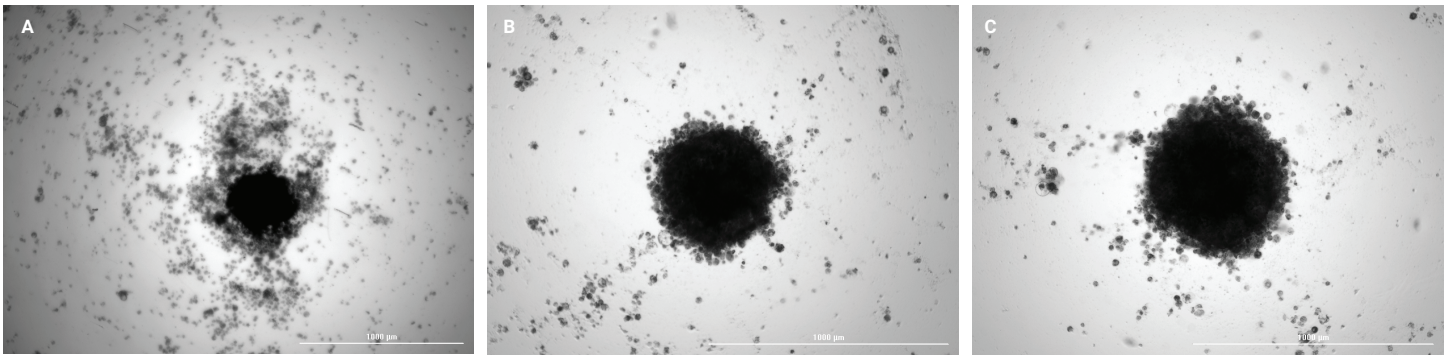

Figure 3. Spheroid formation conformational imaging. Brightfield images captured of hepatocyte spheroid formation using 2.5x objective following 5,000 hepatocyte addition to 96-well cell repellent plate and (A) 24-, (B) 36-, and (C) 48-hour incubation.

Confirmation of long-term 3D hepatocyte cell health

To ensure that the observed results of long-term hepatotoxicity testing are truly from the interaction of test molecules with 3D hepatocyte spheroids, it is essential to confirm that untreated aggregated cells remain healthy during the dosing period. This was carried out using the P450-Glo CYP3A4 Luc-IPA and CellTiter-Glo 3D assays to assess CYP3A4 activity and cell viability, respectively. Assays were performed immediately following spheroid formation and for 1, 3, 5, and 7 days subsequent. Media exchanges were performed every 48 hours on nonassayed wells.

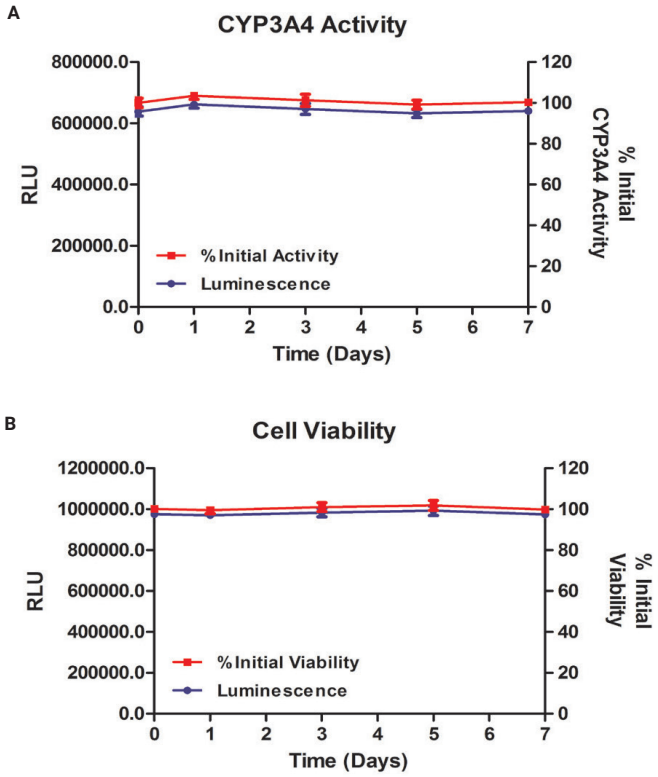

Figure 4. Cell health assessment of long-term 3D hepatocyte cultures. Raw RLU and normalized percent of initial (A) P450-Glo CYP3A4; and (B) CellTiter-Glo 3D values plotted after 0, 1, 3, 5, and 7 day incubations at 37 °C/5% CO_2. n = 6 for each condition and time point tested.

As seen in Figure 4, the raw luminescent units (RLU) from both the CYP3A4 activity and viability assays remain consistent at each assessed incubation period. By normalizing the raw values from subsequent time points to those from the initial readings taken at time 0, it is evident that ReproHepato hiPSC-hepatocytes aggregated into 3D spheroids maintain levels of CYP3A4 activity and cell health throughout the complete 7-day hepatotoxicity testing period that are equivalent to levels seen immediately following spheroid formation. This finding agrees with previously published findings regarding 3D hepatocyte cultures.[1]

Automated long-term hepatotoxicity testing

To aid in the discernment of the hepatotoxic effect of each test compound on the 3D aggregated hepatocytes, spheroid images were taken on multiple z-planes using the z-stacking and z-projection capabilities in the Agilent BioTek Gen5 software (Table 1 and Figures 5A to 5C).

Table 1. Agilent BioTek Gen5 z-stacking and projection criteria.

Z-Stack Image Capture Parameters	
Number of Slices	11
Step Size	9.9 µm (Default for 10x objective)
Images Below Focus Point	0
Image Focusing Method	Fixed focus on well bottom
Z-Projection Parameters	
Channel 1	DAPI
Channel 2	GFP
Projection Method	Focus stacking
Size of Maximum Filter	11 pixels
Top Slice	11
Bottom Slice	1
Channel 2 Settings	Use settings of channel 1

The imaging bottom of the 96-well plate used was optimized so that focusing took place at the most bottom location of the spheroids closest to the well bottom. This allowed for a fixed focus setting to be used which served to increase the speed of image capture. The number of slices chosen allowed focusing on the cells within the spheroid ranging from those at the bottom middle of the spheroid to those at the widest portion of the spheroid. The step size incorporated was the depth of field for a 10x objective, ensuring that all cells within the total included span of z-heights would be imaged.

Following image capture, a final z-projected image was created using the Focus Stacking projection method (Figure 5D). This algorithm allows only the most in focus portions of each image within the stack to be included in the final projection. The combination of the image capture and projection method enables a primary object mask to be placed around the widest portion of the spheroid, which serves to create the most accurate size and area measurements possible.

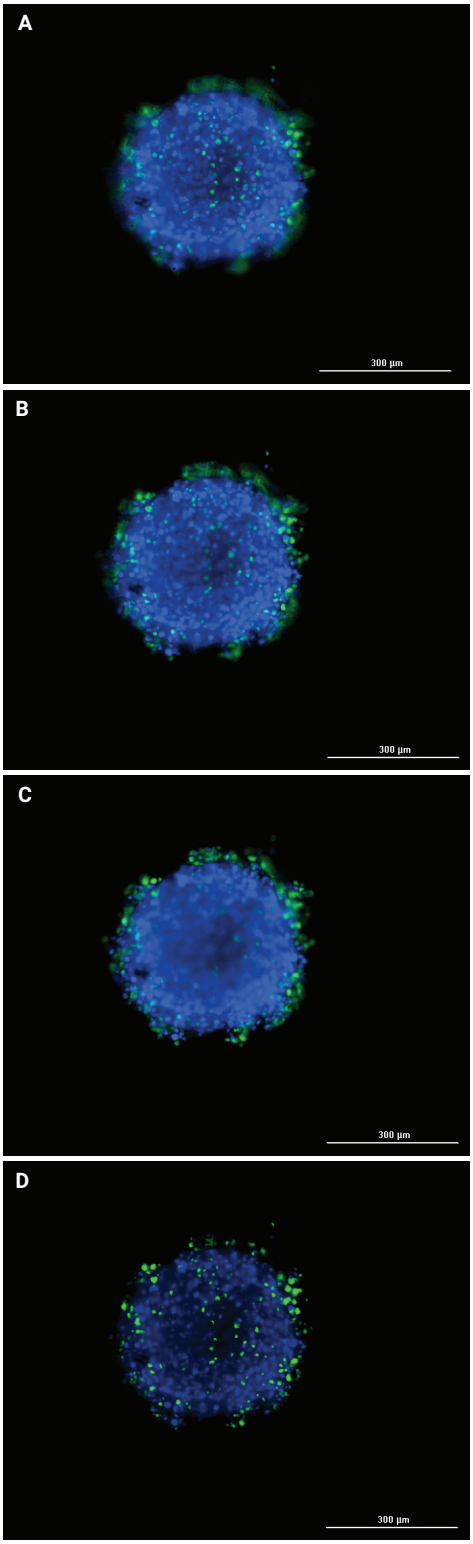

Figure 5. Z-stacking and projection of 3D spheroid images. (A to C) Images captured at individual z-planes. (D) Final z-projected image of hepatocyte spheroid. DAPI: Hoechst 33342 stained nuclei, GFP: CellTox Green stained dead cells.

Analysis of 3D captured images

The ability of the Agilent BioTek Cytation 5 and Agilent BioTek Gen5 software to accurately detect the increasing number of CellTox Green labeled necrotic nuclei due to induced hepatotoxicity was initially examined. As seen in Figure 6, untreated hepatocyte spheroids exhibit a low level of cellular necrosis (Figure 6A). The number of green labeled necrotic cell nuclei within each z-projected image then increases following a 24-hour exposure to increasing doses of troglitazone (Figures 6B and 6C), agreeing with previously published results regarding the compound's toxic effect on 3D cultured hepatocytes.[2,3] This validates the ability of the NIMBUS4 to accurately perform the steps of the hepatotoxicity testing procedure, as well as the Agilent BioTek Cytation 5 to detect compound induced hepatotoxicity.

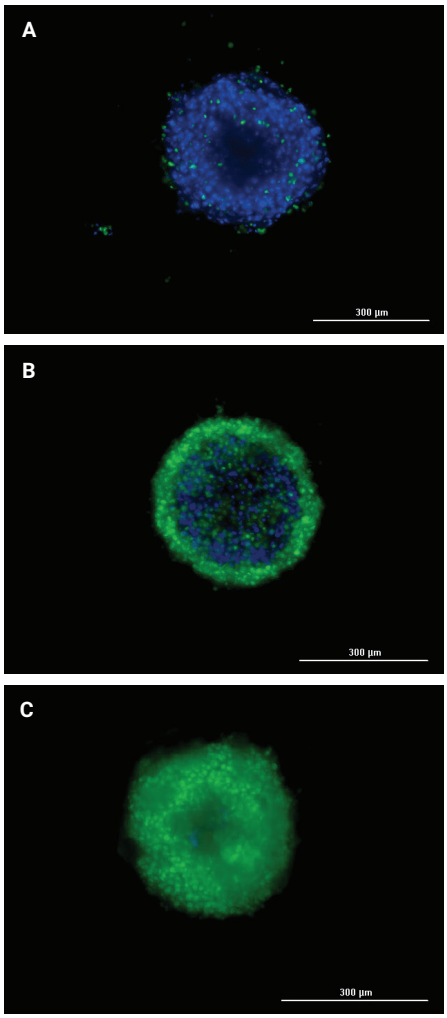

Figure 6. 3D hepatocyte spheroid hepatotoxicity imaging. Z-projected images captured with a 10x objective following 24-hour spheroid treatments with (A) 0; (B) 1.2; and (C) 100 µM troglitazone. DAPI: Hoechst 33342 stained nuclei, GFP: CellTox Green stained dead cells.

Quantification of observed hepatotoxicity

Image preprocessing was then carried out by the Gen5 software using the z-projected images (Table 2).

Table 2. Agilent BioTek Gen5 microplate reader and imager software image preprocessing criteria.

Image Preprocessing Parameters	
Background	Dark
Background Flattening Size	1,000
Image Smoothing	0
Channel 2 Settings	Use same options as channel 1

This was done to remove excess background signal, increasing the ability of the software to detect subtle changes in fluorescent probe signal, creating a more sensitive analysis of the hepatotoxic effect caused by each test compound.

Cellular analysis was then performed using the dual masking capabilities of the Gen5 software to quantify the level of induced hepatotoxicity by each of the test compounds over the entire incubation period.

Table 3. Agilent BioTek Gen5 microplate reader and imager software hepatosphere cellular analysis criteria.

Primary Cellular Analysis Parameters	
Channel	DAPI
Threshold	Auto (78)
Background	Dark
Split Touching Objects	Unchecked
Fill Holes in Masks	Checked
Minimum 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	500 (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	GFP
Measure Within a Secondary Mask	Include primary and secondary area in analysis
Expand Primary Mask	1 µm
Threshold	15,000
Smoothing	3
Method	Propagate mask
Calculated Metrics	
Object Int_2	Total intensity within GFP secondary mask

Primary cellular analysis criteria were optimized to accurately and consistently place object masks around the entire spheroid (Table 3). By increasing the minimum and maximum object size values, in addition to the larger rolling ball diameter, the analysis algorithm sees the spheroids as a single object versus numerous small individual nuclei (Figure 7A). To accurately determine necrotic cells within each spheroid, the fluorescence from the CellTox Green probe in untreated cells was analyzed. By determining the average fluorescence in the GFP channel plus two standard deviations, a threshold value could be set so that secondary masks would only be placed around areas within the spheroid containing cells exhibiting actual necrosis (Figures 7B to 7C).

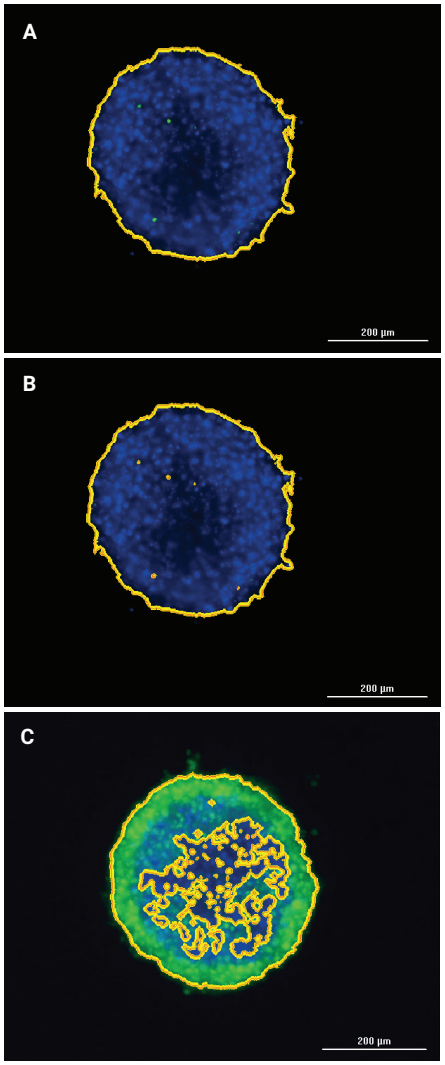

Figure 7. Automated dual-mask analysis. (A) Primary mask placed around the entire spheroid, and secondary mask placed around discontinuous areas of statistically increased GFP signal in (B) untreated and (C) compound-treated spheroids.

The signal from the secondary masked areas was then used to compute fold induction of necrotic activity due to compound treatment, so that the total signal from treated spheroid masked areas (Figure 7C) divided by the total signal from untreated negative control spheroids (Figure 7B) determined fold response.

Finally, dose-response curves were created for each of the hepatotoxins using calculated fold response values (Figure 8A). Hepatotoxicity was differentiated by both an EC_{50} type measurement or the magnitude of the fold response. Troglitazone and Nicardipine demonstrated the greatest fold response at approximately 10-fold. Results for individual compounds were also graphed to examine the effect of short- and long-term exposures (Figures 8B and 8C). It was apparent that for both Troglitazone and Nicardipine the extent of the fold response was unchanged over time, but the EC_{50} measurement became progressively shifted to lower concentrations.

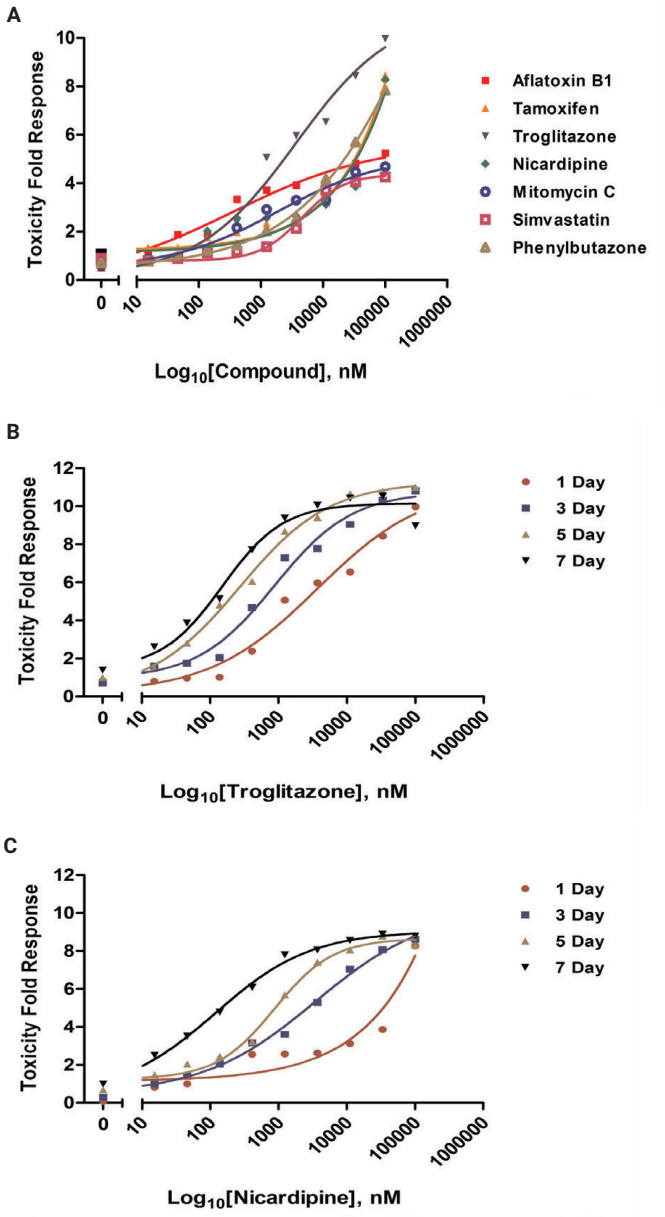

Figure 8. Quantification of hepatotoxic effect on 3D hepatocyte spheroids. (A) Graph of hepatotoxic fold induction following 24-hour treatment with previously described hepatotoxins. (Daunorubicin curve not shown due to excessive toxic effect at highest tested concentrations not consistent with nonlinear curve fit). Multiday fold induction graphs also shown for 1, 3, 5, and 7 day treatments with (B) troglitazone and (C) nicardipine.

Conclusion

ReproHepato hiPSC-hepatocytes 3D spheroids provide a robust cell model for the performance of long-term hepatotoxicity studies, and the 384-well BiO assay kit and NanoShuttle-PL particles, in addition to cell repellent plates, provide a simple, repeatable method to create biomimetic hepatocyte spheroids. Through incorporation of the NIMBUS4, the hepatotoxicity testing process can be fully automated to simplify and increase the repeatability of included procedures, without sacrificing sample integrity. Finally, spheroid aggregation and image-based analysis of induced hepatotoxicity can be automatically performed using brightfield and fluorescent imaging with the Agilent BioTek Cytation 5 cell imaging multimode reader and Agilent BioTek Gen5 microplate reader and imager software for cellular analysis. The combination of appropriate 3D cell models, assay methodology, and automated liquid handling, imaging, and analysis create a robust method to determine the potential hepatotoxic effect of test molecules.

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