A Combined, Affordable Solution for the Performance of Automated 3D *in vitro* Hepatotoxicity Testing

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Introduction

Prescription medications, environmental toxins, and non-prescription 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 new 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 de-differentiate, 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 that allow cells to aggregate and retain 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.

Here we demonstrate the ability to combine liquid handling with a novel cell imaging multi-mode reader to perform 3D hepatotoxicity studies. hiPSC-hepatocytes were aggregated into spheroids easily and efficiently using magnetic bioprinting technology. Multiple known hepatotoxicants were tested with the automation and cell model to validate the ability of the combined solution to be used to perform automated, walk-away hepatotoxicity studies in an academic setting.

Instrumentation

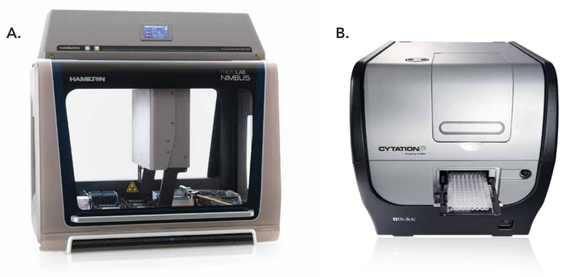

Figure 1. Automated liquid handling and imaging instrumentation. (A) Microlab NIMBUS4 Enclosed, **(B)** Cytation 5.

Cytation™ 5 Cell Imaging Multi-Mode Reader: Cytation 5 from BioTek Instruments is a modular multi-mode 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 Gen5™ Microplate Reader and Imager Software. The software was also used for dual-masking and automated analysis.

Microlab® NIMBUS®: The Microlab NIMBUS from Hamilton Company is a compact, automated multi-channel pipetting workstation. The NIMBUS4 Enclosed features four independent 1000 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 anti-droplet 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.

3D Hepatocyte Cell Preparation

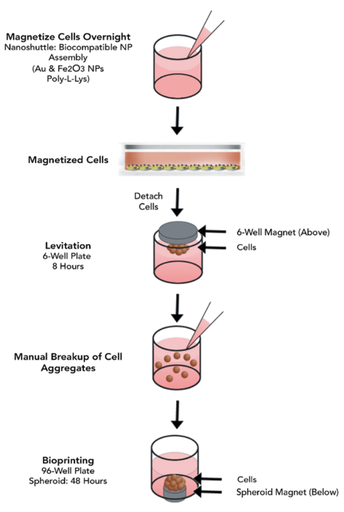

Figure 2. 96-Well Spheroid Magnetic Bioprinting Kit Protocol

ReproHepato™ hiPSC-Hepatocytes (Cat. No. RCDH001N) from ReproCELL® were thawed from cryopreservation, resuspended in ReproHepato Culture Medium (ReproCELL Cat. No. RCDN101), plated onto Collagen Type I coated 6-well microplates (Cat. No. 657950) from Greiner Bio-One (Monroe, NC), and incubated at 37 °C/5% CO_2 for four days. After which, a 100 µL volume of NanoShuttle-PL was added to the microplate wells, and the plates were returned to the incubator for one additional 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 (GBO Catalog No. 657860) microplates. A 6-well magnet drive was placed atop the plates to levitate the cells, where extra-cellular 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 incubation period.

Image-Based Spheroid Formation Validation

Brightfield, label-free live cell imaging was performed prior to, and throughout the hepatocyte aggregation process to ensure spheroid formation. 2D hepatocyte images were captured following addition of the NanoShuttle-PL particles. Spheroid formation was tracked throughout the 48 hour incubation period of the 96-well cell repellent microplates being on top of the 96-well spheroid magnet drives.

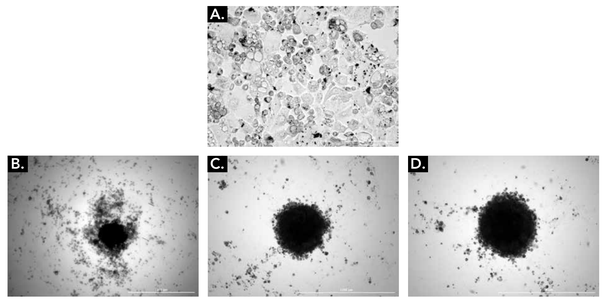

Figure 3. Spheroid formation conformational imaging. Brightfield images captured of hepatocyte spheroid formation.

Top panel: 2D cultured hepatocyte images captured using 20x objective in 6-well collagen coated plate prior to removal **(A)**.

Bottom panel: Images also captured using 2.5x objective following 5000 hepatocyte addition to 96-well cell repellent plate and **(B)** 24, **(C)** 36, and **(D)** 48 hour incubation.

As seen by the images in Figure 3, the NanoShuttle-PL particles, ECM induction prior to cell plating, and placement of the cell repellent plates on the spheroid magnet drive allow hepatocytes to form 3D spheroids. Partial aggregation is seen within 24 hours (Figure 3B), and is completed after a total 48 hour incubation period (Figure 3D).

Confirmation of Long-Term 3D Hepatocyte Cell Health

In order 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 (Catalog No. V9002) and CellTiter-Glo® 3D assays (Catalog No. G9683) from Promega Corporation (Madison, WI) to assess CYP3A4 activity and cell viability, respectively. Assays were performed immediately following spheroid formation and 1, 3, 5 and 7 days subsequent. Media exchanges were performed every 48 hours on non-assayed wells.

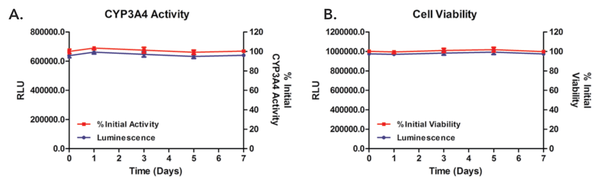

Figure 4. Cell health assessment of long-term 3D hepatocyte cultures. Raw RLU and normalized percent of initial **(A)** P450-Glo CYP3A4; and **(B)** CellTiter-Glo 3D values plotted after 0, 1, 3, 5, and 7 day incubations at 37 °C/5% CO_2. n=6 for each condition and timepoint tested.

As seen in Figure 4, ReproHepato hiPSC-Hepatocytes aggregated into 3D spheroids maintain consistent levels of CYP3A4 activity and cell viability throughout the complete 7 day hepatotoxicity testing period that are equivalent to levels seen immediately following spheroid formation, agreeing with previously published findings regarding 3D hepatocyte cultures[1].

Automated Long-Term HepatotoxicityTesting

NIMBUS4 Enclosed was used to perform all steps of the automated hepatotoxicity testing procedure. Serial titrations of eight known hepatotoxins: Daunorubicin (Cat. No. ALX-380-043-M010), Aflatoxin B1 (Cat. No. ALX-630-093-M001), Tamoxifen (Cat. No. ALX-550-095-G001), Troglitazone (Cat. No. BML-GR210-0005), Nicardipine (Cat. No. ALX-550-273-G001), Mitomycin C (Cat. No. BML-GR311-0002), Simvastatin (Cat. No. BML-G244-0050), and Phenylbutazone (Cat. No. ALX-430-112-G005), generously donated by Enzo Life Sciences (Farmingdale, NY) were prepared. After which, culture medium was removed and replaced with medium containing compound in four separate spheroid containing plates (Figure 5). 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 (Cat. No. RCDH301), containing Hoechst 33342 and CellTox™ Green Dye (Promega Corp. Cat. No. G8731). The plate was incubated for five hours, then fluorescently imaged using Cytation 5 Cell Imaging Multi-Mode Reader.

	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 5. 96-well spheroid microplate hepatotoxicant dosing scheme.

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 capabilities in the Gen5 software (Figure 6A-C). This ensured that the most in focus portions of the cells within each imaging channel were captured. A final projected image was then created by Gen5 to increase the clarity of the blue Hoechst 33342 stained nuclei and green necrotic CellTox Green stained nuclei within each spheroid (Figure 6D).

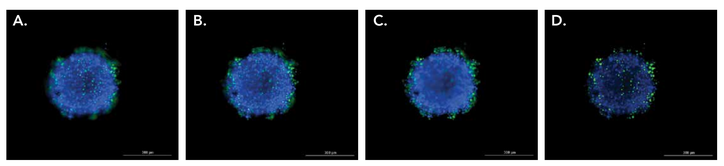

Figure 6. Z-stacking and projection of 3D spheroid images captured with a 10x objective. (A-C) Images captured at individual z-planes. **(D)** Final z-projected image of hepatocyte spheroid. DAPI: Hoechst 33342 stained nuclei, GFP: CellTox Green stained dead cells.

Analysis of 3D Captured Images

The ability of the Cytation 5 and Gen5 imaging software to accurately detect the increasing number of CellTox Green labeled necrotic nuclei due to induced hepatotoxicity was initially examined.

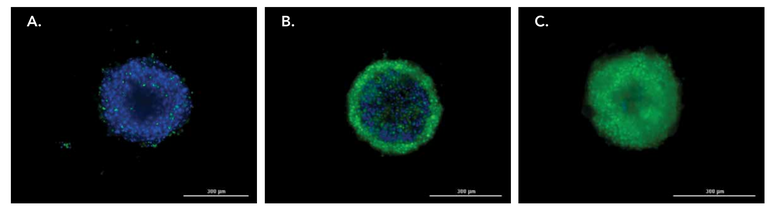

Figure 7. 3D hepatocyte spheroid hepatotoxicity imaging. Z-projected images captured with a 10x objective following 24 hour spheroid treatments with **(A)** 0; **(B)** 1.2; and **(C)** 100 µM troglitazone. DAPI: Hoechst 33342 stained nuclei, GFP: CellTox Green stained dead cells.

As seen in Figure 7, untreated hepatocyte spheroids exhibit a low level of cellular necrosis (Figure 7A). 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 (Figure 7B and C), agreeing with previously published results regarding the compound's toxic effect on 3D cultured hepatocytes[2, 3]. This observation validates the capability of the NIMBUS4 to accurately perform the steps of the hepatotoxicity testing procedure, as well as the Cytation 5 to detect compound induced hepatotoxicity.

Quantification of Observed Hepatotoxicity

Using the z-projected images, Gen5 software automatically pre-processed the samples to remove excess background signal and prepare the image for quantitative analysis. Primary cellular analysis criteria were optimized to consistently place object masks around the entire spheroid (Figure 8A). Secondary analysis criteria were then used to automatically mask areas within the spheroid where the GFP signal from CellTox Green labeled nuclei was statistically greater than background threshold levels determined from untreated spheroids, representing true necrotic spheroidal cells. The signal from the secondary masked areas was then used to compute fold induction of necrotic activity due to compound treatment, such that the total signal from treated spheroid masked areas (Figure 8C) divided by the total signal from untreated negative control spheroids (Figure 8B) determined fold induction.

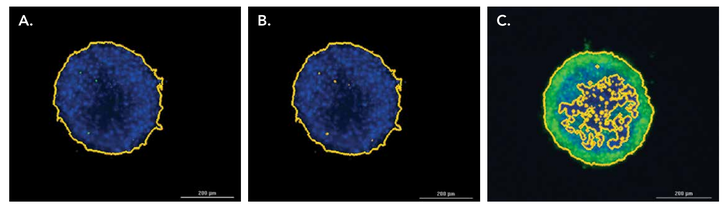

Figure 8. Automated dual-mask analysis. (A) Primary mask placed around the entire spheroid, and secondary mask placed around discontinuous areas of statistically increased GFP signal in **(B)** untreated; and **(C)** compound treated spheroids.

Calculated fold induction values can then be plotted in terms of the concentration of each compound treatment to determine the total effect of each hepatotoxin on the 3D hepatocyte spheroids (Figure 9A). Results for individual compounds can also be graphed to examine the effect of short- and long-term exposures (Figures 9B and C).

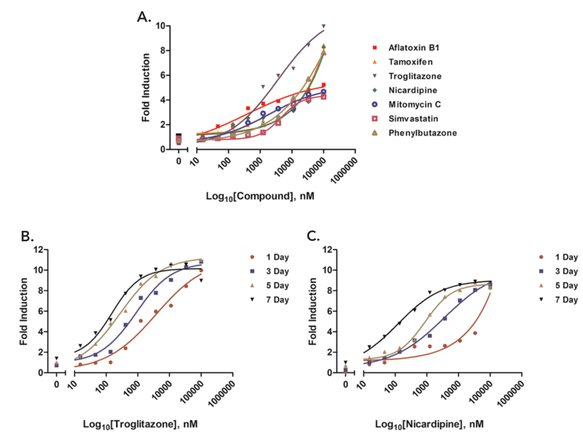

Figure 9. Quantification of hepatotoxic effect on 3D hepatocyte spheroids. (A) Graph of hepatotoxic fold induction following 24-hour treatment with previously described hepatotoxins. (Daunorubicin curve not shown due to excessive toxic effect at highest tested concentrations not consistent with non-linear curve fit). Multi-day fold induction graphs also shown for 1, 3, 5, and 7 day treatments with **(B)** troglitazone; and **(C)** nicardipine.

Conclusions

1. Use of ReproHepato™ hiPSC-Hepatocyte 3D spheroids provides a robust cell model for the performance of long-term hepatotoxicity studies.
2. The magnetic bioprinting kit and NanoShuttle-PL particles manufactured by nano3D Biosciences, in addition to cell repellent plates from Greiner Bio-One, provide a simple, repeatable method to create biomimetic hepatocyte spheroids.
3. 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.
4. Spheroid aggregation and image-based analysis of induced hepatotoxicity can be automatically performed using brightfield and fluorescent imaging with the Cytation 5 and Gen5 cellular analysis.
5. The combination of appropriate 3D cell models, assay methodology, and automated liquid handling, imaging and analysis creates a robust method to determine the potential hepatotoxic effect of test molecules.

1. Bell, CC.; Hendriks, DF.; Moro, SM.; Ellis, E.; Walsh, J.; Renblom, A.; Fredriksson Puigvert, L.; Dankers, AC.; Jacobs, F.; Snoeys, J.; Sison-Young, RL.; Jenkins, RE.; Nordling, A.; Mkrtchian, S.; Park, BK.; Kitteringham, NR.; Goldring, CE.; Lauschke, VM.; Ingelman-Sundberg, M. Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease. *Sci Rep.* **2016,** 6:25187. doi: 10.1038/srep25187. 2. Jaeschke, H. Troglitazone hepatotoxicity: are we getting closer to understanding idiosyncratic liver injury? *Toxicol Sci.* **2007,** 97(1), 1-3. 3. Letzsch, S.; Hänel, F.; Böttcher, K.; Kelm, J. *Cytotoxicity Studies on 3D Primary Liver Microtissues;* Application Note 011591A_01; PerkinElmer, Inc., Waltham, MA, 2014.