3D High Content Screening of Liver Spheroids

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Abstract

Drug induced liver injury (DILI) is one of the most cited causes of clinical drug attrition, and is often the reason for withdrawal of approved drugs from the market[1]. More accurate *in vitro* toxicity assays are required to detect adverse drug effects in earlier phases of drug development. Three-dimensional (3D) cell models have been shown to demonstrate the formation of functional bile canaliculi and have exhibited increased albumin secretion and CYP expression, as well as stability for longer periods in culture compared with two dimensional (2D) cultures[2,3]. Corning® HepatoCells are immortalized cells derived from primary human hepatocytes (PHH) that retain metabolic and enzymatic functionality of PHH but without the lot-to-lot variability and reliance on human donors of PHH. Here we demonstrate how Corning HepatoCells in conjunction with Corning Spheroid microplates can be utilized for a 3D drug screen to discover potential hepatotoxins by way of multiparameter high content screening (HCS) analyses. Specifically, lipid accumulation and mitochondrial membrane potential loss were examined after exposure to a variety of known hepatotoxic compounds. These results indicate that Corning HepatoCells, together with Corning spheroid microplates, are powerful tools for reliable and reproducible *in vitro* 3D hepatotoxicity screening to predict liver injury.

Methods

Spheroid Formation

1. Cryopreserved HepatoCells (Corning Cat. No. 354881) were thawed into culture medium for HepatoCells (Corning Cat. No. 354882) supplemented with 10% fetal bovine serum (FBS; Corning Cat. No. 35-010-CV) following vendor's protocol. HepG2 cells (ATCC® HB-8065™) were cultured following vendor's protocol in DMEM (Corning Cat. No. 10-016-CM) supplemented with 10% FBS.
2. HepatoCells and HepG2 cells were filtered through a 40 µm cell strainer (Corning Cat. No. 431750) to ensure single cell suspension and were seeded at 1,000 cells/well in 100 µL into 96-well spheroid microplates (Corning Cat. No. 4520)
3. Cells were incubated in a humidified 37˚C, 5% CO_2 incubator for the formation of a single spheroid per well, which took 96 hours for HepatoCells and 48 hours for HepG2 cells.
4. For HepatoCells, an additional 100 µL of fresh HepatoCells medium was added to each well 72 hours after cell seeding and spheroids were incubated another 24 hours.
5. Ninety-six hours after cell seeding, serum was removed from culture medium of HepatoCells spheroids via 5 half media changes with HepatoCells medium, leaving 100 µL/well.

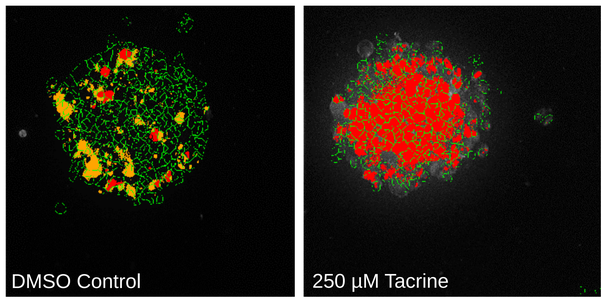
Compound Addition and Staining

1. HepG2 cells were used for toxicity staining optimization with spheroids because of their short culture time and ability to be expanded in culture. Compounds known to affect mitochondrial health, or induce phospholipidosis or neutral lipid accumulation were added in dose series in 0.5% dimethyl sulfoxide (DMSO) to HepG2 spheroids 48 hours after cell seeding.
2. HepatoCells were used for a sample study with Tacrine because they retain the metabolic and enzymatic functionality of PHH. Tacrine was added in a dose series in 0.5% DMSO to HepatoCells spheroids 96 hours after cell seeding.
3. Spheroids were incubated for an additional 48 hours prior to fixation and imaging.
4. For phospholipid staining, HCS LipidTOX™ Green Phospholipidosis Detection Reagent (Thermo Fisher Cat. No. H34350) was added at 1X final concentration concurrently with compound addition.
5. To monitor mitochondrial health, 200 nM MitoTracker™ Orange CMTMRos (Thermo Fisher Cat. No. M7510) was added to culture media and incubated for 1 hour in a humidified 37˚C, 5% CO_2 incubator.
6. All spheroids were fixed in 4% paraformaldehyde (PFA; Boston BioProducts Cat. No. BM1552) with 15 µM Hoechst 33342 (Thermo Fisher Cat. No. H3570) for 45 minutes at room temperature and were rinsed 3 times with DPBS (Corning Cat. No. 21-031-CM).
7. For neutral lipid staining, HCS LipidTOX™ Green Neutral Lipid Stain (Thermo Fisher Cat. No. H34475) was added at 1X final concentration in DPBS and spheroids were incubated for ≥1 hour prior to imaging.

High Content 3D Analysis

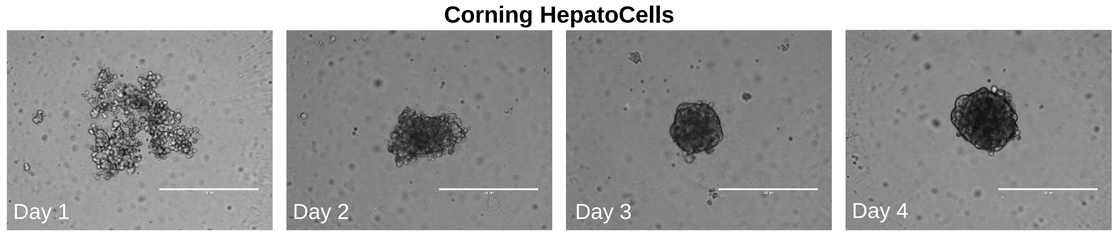
Imaging/Analysis

1. Spheroids were imaged and analyzed using a Thermo Fisher CellInsight™ CX7 High-Content Screening (HCS) Platform with a 20X objective in confocal mode. Images were taken in 2 channels: (1) for nuclei staining, (2) for toxicity stain. For each channel, 12 images were taken at Z height steps of 20 µm in the center 4 of 16 fields.
2. For analysis, a spot detector method was used. Nuclei (channel 1) were used to define individual cells as objects, shown in green in the representative composite images of spheroids stained for phospholipidosis. The level of staining was identified as spots (channel 2) shown in red. The spots were selected only if they met an intensity threshold. Spots below this threshold that were excluded are shown in orange.

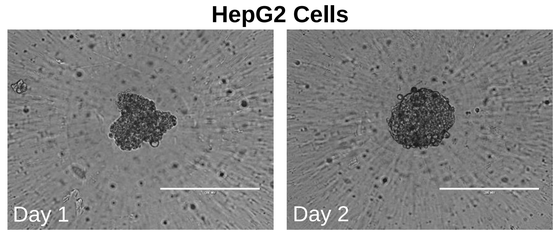


Spheroid Formation

Corning HepatoCells



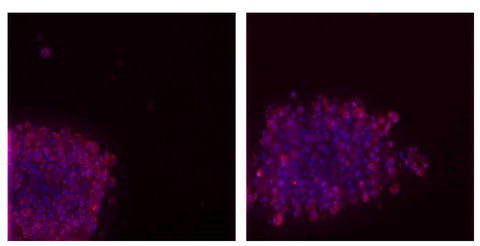
HepG2 Cells



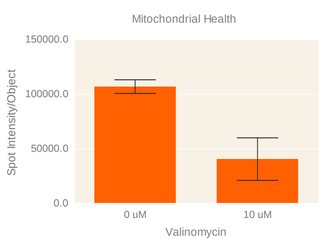
Corning HepatoCells and HepG2 spheroids form in spheroid microplates. HepatoCells and HepG2 cells were seeded at 1,000 cells/well into 96-well spheroid microplates and were cultured for 4 or 2 days, respectively, with fresh media addition of the HepatoCells after 3 days. Representative brightfield images were captured at 10X using an EVOS™ FL Imaging System. Scale bar = 400 µm.

High Content Imaging of HepG2 Spheroids Stain Optimization for 3D

Mitochondrial Health

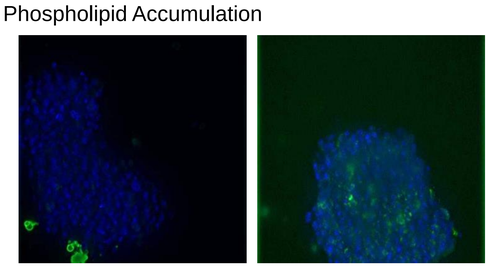


DMSO Control 10 µM Valinomycin

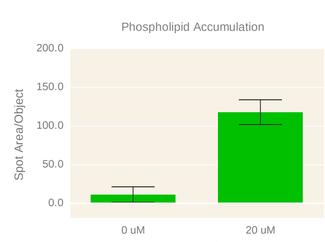


HepG2 spheroids demonstrated a decrease in mitochondrial health as monitored by 200 nM stain MitoTracker Orange after 48-hour exposure to Valinomycin. Representative composite images were captured using a CellInsight CX7 HCS platform.

Phospholipid Accumulation

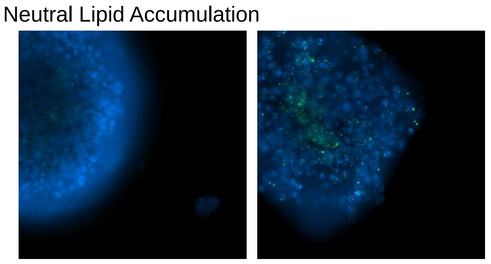


DMSO Control 20 µM Propranolol

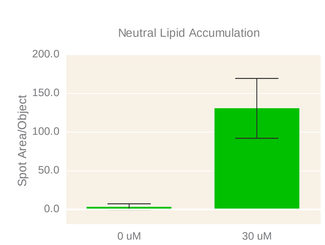


HepG2 spheroids displayed an increase in 1X HCS LipidTOX Green Phospholipid stain after 48-hour exposure to Propranolol. Representative composite images were captured using a CellInsight CX7 HCS platform.

Neutral Lipid Accumulation



DMSO Control 30 µM Cyclosporin A

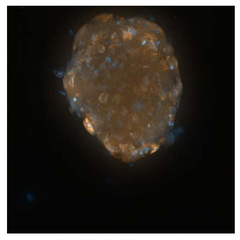


HepG2 spheroids displayed an increase in 1X HCS LipidTOX Green Neutral Lipid stain after 48-hour exposure to Cyclosporin A. Representative composite images were captured using a CellInsight CX7 HCS platform.

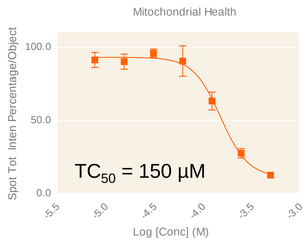
High Content Imaging of Corning HepatoCells Spheroids Tacrine Study

To test this assay model, Corning HepatoCells spheroids were incubated with a dose series of Tacrine for 48 hours. Tacrine is a cholinesterase inhibitor used in the treatment of Alzheimer's disease but it was found to induce liver damage and was withdrawn from the U.S. market in 2012[4]. There has been a lot of research into the mechanisms of toxicity of Tacrine, which include the induction of mitochondrial uncoupling and phospholipidosis without causing steatosis[5].

Mitochondrial Health

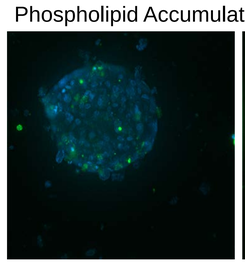


DMSO Control 500 µM

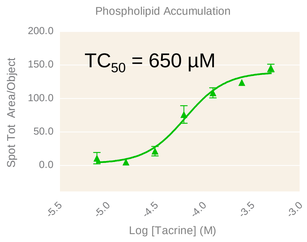


Corning HepatoCells spheroids demonstrated a dose-dependent decrease in mitochondrial health in response to Tacrine exposure for 48 hours. HepatoCells spheroids exposed to Tacrine for 48 hours were stained using 200 nM MitoTracker Orange. The amount of stain per cell was quantified as total spot intensity per cell using a CellInsight CX7 HCS platform. Each dose was tested in duplicate 3 independent times. Representative composite images were taken with a 20X objective.

Phospholipid Accumulation

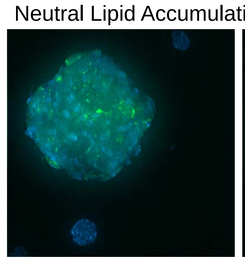


DMSO Control 250 µM

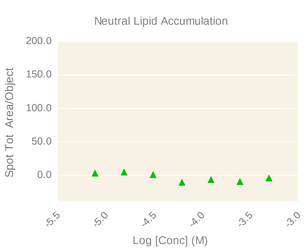


Coring HepatoCells spheroids demonstrated a dose-dependent increase in phospholipids in response to Tacrine exposure for 48 hours. HepatoCells spheroids exposed to Tacrine for 48 hours were stained using 1X HCS LipidTOX Green Phospholipid stain. The amount of stain per cell was quantified as total spot area per cell using a CellInsight CX7 HCS platform. Each dose was tested in duplicate 3 independent times. Representative composite images were taken with a 20X objective.

Neutral Lipid Accumulation



DMSO Control 125 µM



Corning HepatoCells spheroids did not display a dose-dependent increase in neutral lipids in response to Tacrine exposure for 48 hours. HepatoCells spheroids exposed to Tacrine for 48 hours were stained using 1X HCS LipidTOX Green Neutral Lipid stain. The amount of stain per cell was quantified as total spot area per cell using a CellInsight CX7 HCS platform. Each dose was tested in duplicate 3 independent times. Representative composite images were taken with a 20X objective.

Conclusions

- Corning spheroid microplates can be used for the formation of a single spheroid per well.
- Spheroids formed and stained in the spheroid microplate can be imaged and analyzed using a high content screening platform directly in the spheroid microplate.
- Corning HepatoCells can be used to perform 3D liver toxicity assays.

References

1. Atienzar, F.A., et al. (2016). Key Challenges and Opportunities Associated with the Use of In Vitro Models to Detect Human DILI: Integrated Risk Assessment and Mitigation Plans. BioMed Res Int, 2016. doi:10.1155/2016/9737920.
2. Bell, C.C, et al. (2015). Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease. Sci Rep. May 4 (6).doi:10/1038/srep25187.
3. Takahashi, Y., Hori, Y., Yamamoto, T., Urashima, T., Ohara, Y., & Tanaka, H. (2015). 3D spheroid cultures improve the metabolic gene expression profiles of HepaRG cells. Biosci. Rep. 2015 (35), e00208, doi:10.1042/BSR20150034.
4. Halter, M.J. (2017). Psychological Disorders. In *Varcarolis' Foundations of Psychiatric-Mental Health Nursing: A Clinical Approach* (pp.444). Retrieved from http://books.google.com
5. Dykens, J.A. and Will, Y. (2008). Production of Reactive Oxygen Species. In *Drug-Induce Mitochondrial Dysfunction* (pp.83). Retrieved from http://books.google.com.