APPLICATION NOTE

High-content 3D toxicity assay using iPSC-derived hepatocyte spheroids

Background

There is increasing interest in exploring the use of three-dimensional (3D) spheroids for modeling developmental and tissue biology with the goal of accelerating translational research in these areas.[1-3] As a result, the development of higher throughput quantitative assays using 3D cultures is an active area of investigation. In this study, we developed and optimized methods for the formation of 3D liver spheroids derived from human iPS cells as well as confocal imaging and analysis methods for toxicity assessment (Figure 1).

Formation of liver spheroids

Human iPSC-derived hepatocytes[4], iCell Hepatocytes 2.0 (Cellular Dynamics International) and HepG2 (ATCC) were used in the study. Cryopreserved cells were thawed and maintained according to manufacturer protocols. To prepare spheroid cultures of human iPSC-derived hepatocytes, iCell Hepatocytes 2.0 were pre-plated in 2D culture, then detached with Accutase, mixed with a Geltrex solution (ThermoFisher Scientific), and plated into low attachment spheroid plates (InSphero or Corning) at 1000 cells/well. Next, we centrifuged at 300 x g for 2 minutes to settle the cells and remove any bubbles prior to placing the plate into a humidified incubator at 37°C, 5% CO_2. HepG2 cultures did not require the addition of matrix. Spheroids were formed within 24–48 hours.

Assess hepatotoxicity using no-wash staining protocol

Following a 72 hour incubation with hepatotoxic test compounds, spheroids were stained with a mixture of three dyes prepared in sterile phosphate buffered saline (PBS): 2 µM calcein AM, 3 µM of EthD-1, and 10 µM Hoechst 33342 (Life Technologies). In separate experiments, CellEvent Caspase 3/7 reagent, 7.5 µM and MitoTracker Orange, 200 nM (Life Technologies) were used to evaluate the compounds' ability to trigger apoptosis signaling or affect mitochondria.

Benefits

- **Form hepato-spheroids using human iPSC-derived hepatocytes**
- **Assess hepatotoxicity using 3D models for *in vitro* screening**
- **Analyze 3D images to determine appropriate detection and segmentation of objects**

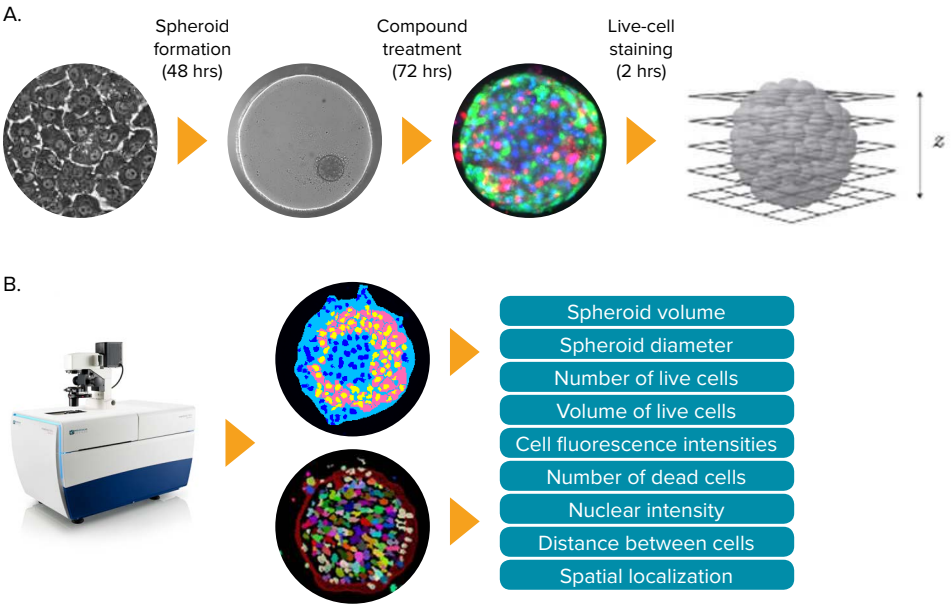


Figure 1. Hepatotoxicity assay using 3D spheroid liver micro tissues derived from iCell Hepatocytes. (A) iCell Hepatocytes were grown in 2D format for 7 days prior to using them to prepare 3D cultures. Following spheroid formation, the liver micro tissues were treated with compounds for 72 hours, then stained for 2 hours and imaged. Z-planes were acquired using the ImageXpress Micro Confocal system. **(B)** The acquired Z-plane images were used to generate sets of 2D and 3D image segmentation, which were analyzed to quantify key phenotypic features of the 3D cultures.

Dye solutions were added onto the spheroid cultures and incubated for 2 hours prior to imaging. The dye solution was not washed out and care was taken during pipetting to avoid spheroid loss, disintegration, or displacement. Representative composite images of spheroids are shown in Figure 2.

Implement high-throughput 3D image acqusition and analysis

Images were acquired using the ImageXpress® Micro Confocal High-Content Imaging System (Molecular Devices), with a 10× Plan Fluor and 20× Plan Fluor objective. A stack of 11-17 images separated by 5-10 µm, a distance of 100 and 120 µm, were acquired using confocal imaging, starting at the well bottom. All individual images including 2D maximum projection images, were saved and used during analysis.

Detect and analyze 3D objects

3D image analysis was performed using MetaXpress® High-Content Image Acquisition and Analysis Software. The most recent Custom Module Editor features a new module (Figure 3) which simplify quantification of 3D structures with volume, fluorescence intensity, and distance measurements in multi-well workflows. The "Find Spherical Objects" option defines roughly spherical objects from organelles to multi-cell, using user-defined settings for the approximate size of the object (minimum and maximum width, minimum and maximum numbers of Z-planes), and object intensity versus background threshold values. For example, nuclei were defined with 5-15 µm widths, 1-2 Z-planes, and threshold values of 200 relative fluorescence units. Hepatic spheroids were defined with 100-300 µm widths, 5-11 Z-planes, and a threshold value of 400 relative fluorescence units. Spherical object analysis may include individual or averaged spheroid volume, spheroid diameter, and average or integrated intensities of spheroids in specific fluorescence channels.

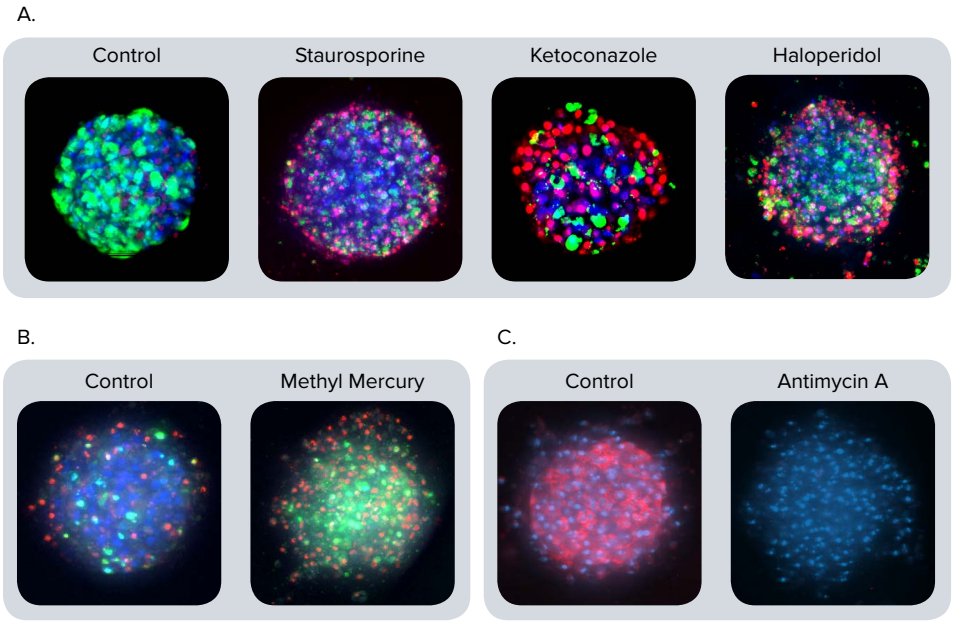


Figure 2. Representative composite images of spheroids stained with different markers. (A) The spheroids were treated with staurosporine 1 µM, ketoconazole 30 µM, or haloperidol 30 µM for 72 hours. Composite images presented after staining with calcein AM (green), Hoechst (blue), EthD-1 (red). **(B)** Apoptosis is apparent in the composite images of spheroids stained with CellEvent Caspase 3/7 (green), EthD-1 (red), and Hoechst (blue). Spheroids treated for 24 hours with methyl mercury (1 µM) exhibit a much higher number of apoptotic cells (green) than the control. **(C)** Composite images for the control and antimycin A (3 µM) treated spheroids stained with MitoTracker Orange (red) and Hoechst (blue). The antimycin A disrupts the mitochondrial membrane integrity. Therefore, no staining of the mitochondria is apparent.

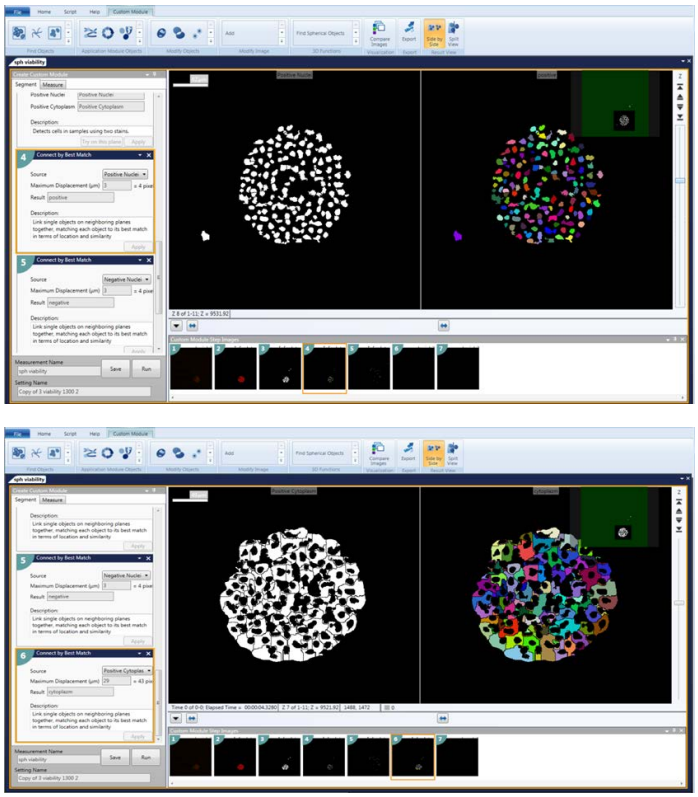


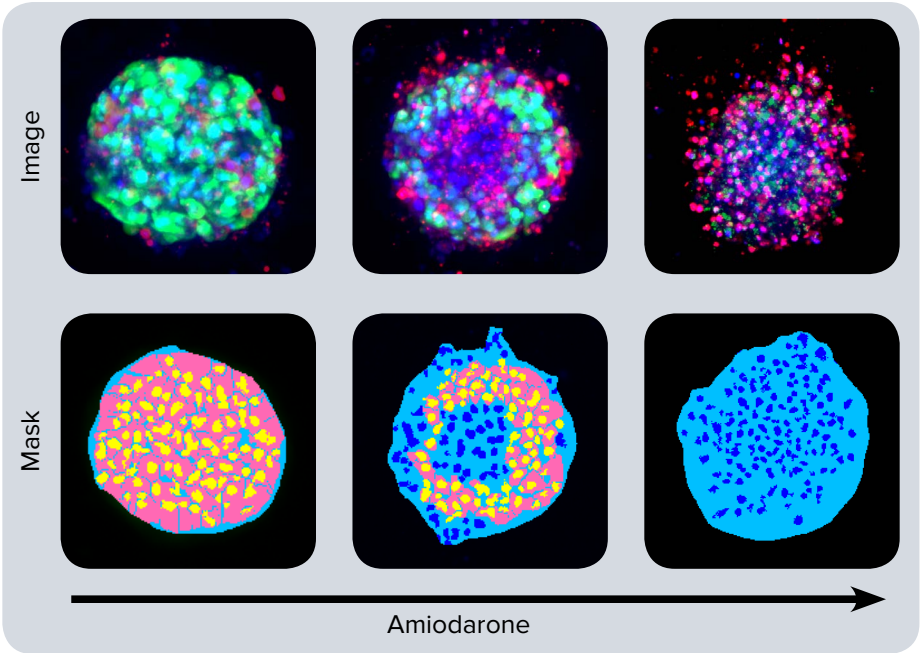
Figure 3. The Custom Module Editor can be used for defining spheroids. Once defined, spheroids can be analyzed with counting and characterizing individual cells, nuclei, or cytoplasm positive or negative for the marker of interest.

Find Spherical Objects analysis can also be used to define individual nuclei and cells as well as appropriate characteristics of individual cells as separate objects. Additional capabilities of the 3D structure analysis module include the ability to connect objects from different planes according to the user defined options “Connect by Best Match”, “Connect by Touching”, and “Do Not Connect Objects”.

Individual Z-planes are first segmented and analyzed as 2D images, for measurements such as nuclei count, live/dead analysis, and cell scoring. Then objects are “Connected by Best Match” with user defined maximum displacement of each object, e.g. 3-6 µm for maximum displacement of nuclei and 20-30 µm for cytoplasm. As a result, nuclei or individual cells are segmented and scored in the 3D volume, without missing objects or counting any object twice. These steps allow all cells such as calcein AM positive or negative cells, and Ethidium homodimer positive and negative cells to be defined and enumerated.

Different colors can be used on individual cells to segment integrated and average intensities, volumes, diameters, distance between objects, and location of objects in three-dimensional space. Then, spheroid masking can be used to enumerate smaller objects that should be included or excluded from the mask. For example, in a hepatocyte spheroid assay which contains one spheroid per well, single-cell masking allows all cells to be enumerated and is able to distinguish cells included or excluded from the spheroid. This step is important in cases where spheroid integrity has been compromised. In the case of several or multiple spheroids per well (e.g. spheroids in a matrigel assay) using a similar analysis can also define the cellular content of each spheroid and report averaged measurements. Figure 4A illustrates the result of cell scoring analysis for calcein AM positive cells in different 2D planes. Finally, objects are connected in 3D space using the “Connect by Best Match” option. A similar approach was used to define ethidium homodimer positive and negative cells (live/dead analysis) and can be applied to the analysis of mitochondrial, intensity, apoptosis, or other desired stains and markers.

A.



B.

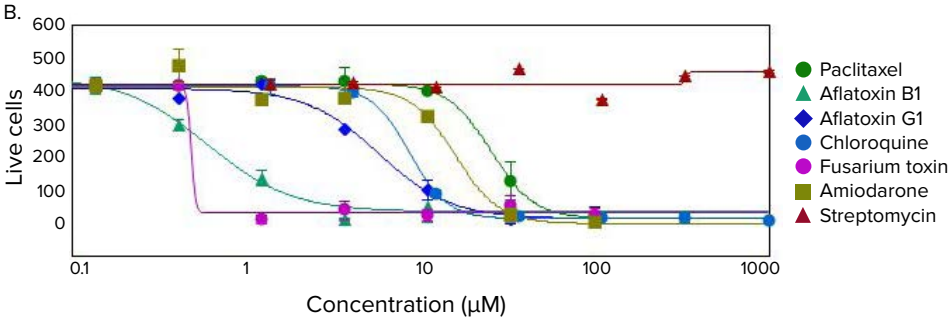


Figure 4. Phenotypic changes in the spheroids after compound treatment. (A) iPSC-derived hepatocyte spheroids treated with increasing concentrations of hepatotoxic compound (amiodarone) and stained with viability markers calcein AM (green), Hoechst (blue), EthD-1 (red). Image analysis masks are shown below the composite images. **(B)** Dose response curves for selected compounds.

Evaluate toxicity using multi-parametric phenotypic screening

Significant changes in spheroid phenotypes and cell content were observed after treatment with various hepatotoxins. Many spheroids lost their spherical shape, appeared disintegrated, “loose”, “flattened”, or “irregular”, had cells detach from the main body, or exhibited condensed nuclei due to cell death. These phenotypic changes in the spheroids after compound treatment occurred over a range of concentrations (Figure 4B). Quantitative analysis of the images included derivation of parameters to assess morphological features of spheroids, cell content, and complexity. The volume, diameter, and fluorescence intensities were measured, along with the number of calcein AM-positive cells (live) and the number of EthD-1- positive cells (dead). Total cell number did not decrease in response to compound effects. Instead, an increase in the number of dead cells and a concentration-dependent decrease in the number of viable cells were observed as a result of compound treatment. Average fluorescent intensity for calcein AM was dramatically reduced for the entire spheroid and individual segmented cells (cell cytoplasm).

For further investigation into the cytotoxicity mechanisms, we evaluated apoptotic phenotype and mitochondria integrity. Activation of apoptosis using caspase 3/7 dye was measured 24 hours after compound treatment. Typical activated caspase 3/7 staining patterns for control cells and those treated with methyl mercury are shown in Figure 2B. Treatment with compounds causing apoptosis resulted in an increase of caspase 3/7 stain intensity and the number of caspase 3/7 positive (apoptotic) cells. Mitochondrial potential was evaluated with MitoTracker Orange dye. Treatment of spheroids with compounds affecting mitochondrial integrity resulted in a dose-dependent decrease in MitoTracker Orange staining intensity (Figure 2C).

iCell Hepatocytes		
Compound	IC_{50} 3D Assay (µM)	Description
Methyl mercury	2.82 +/- 0.49	Toxin
Fusarium toxin	0.481	Toxin
Aflatoxin B1	0.595 +/- 0.145	Toxin
Aflatoxin G1	7.78 +/- 2.13	Toxin
Doxorubicin·HCl	5.69 +/- 4.04	DNA-intercalator, anti-cancer
Staurosporine	0.91 +/- 0.12	Kinase inhibitor, apoptosis inducer
Mitomycin C	2.28 +/- 0.246	DNA-intercalator, anti-cancer
Chloroquine	18.60 +/- 0.247	Anti-malaria
Ketoconazole	20.4 +/- 10.7	Anti-fungal
Amiodarone·HCl	22.6 +/- 9.68	Anti-arrhythmia, autophagy inducer
Paclitaxel	26.2 +/- 6.39	Microtubule inhibitor
Fluoxetine	28.9	Anti-depressant
Pimozide	35.1 +/- 1.39	Anti-psychotic
Haloperidol·HCl	52.2 +/- 21.6	Anti-psychotic

Table 2. IC_{50} values measured for selected compounds using number of live cells per spheroid as the readout.

Measured features such as the number of live cells, calcein AM fluorescence intensities, and volumes of calcein AM positive cells resulted in significant assay windows and successfully fit into 4-parametric dose-response curve model (Figure 4B). IC_{50} values (Table 2) were determined using SoftMax® Pro 6 Software (Molecular Devices).

Summary

The 3D liver spheroid model combined with high-content 3D assays shows promise as a sensitive and reproducible screening tool for assessing hepatotoxicity. While assay predictivity compared to animal and clinical data still needs to be established, further development of these methods and models will increase their utility for *in vitro* screening.

References

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