Phenotypic Characterization of Compound Effects on iPSC-derived Cardiac and Liver Spheroids Using Fast Kinetic Fluorescence and 3D Image Analysis

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INTRODUCTION

Cell models are becoming more complex in order to better mimic the *in vivo* environment and provide greater predictivity for compound efficacy and toxicity. Accordingly, the development of quantitative assays in higher throughput using 3D cultures is an active area of investigation.

In this study, we have developed methods for the formation of 3D spheroids derived from human iPS cells. Using both high-content and fast kinetic fluorescence imaging, we have measured the impact of various compounds on the beating rate and pattern of cardiac spheroids as monitored by changes in intracellular Ca^{2+} levels with calcium-sensitive dyes. The assay was optimized for HTS in 384-well plates and allows for the characterization of beating profiles by using multi-parametric analysis outputs such as beating rate, peak frequency and width, or waveform irregularities. Additionally, the impact of drug treatment on cell viability and mitochondrial integrity was evaluated by high-content imaging.

We have also developed and optimized methods for the formation of 3D liver spheroids derived from human iPS cells and using those for toxicity assessment. We used confocal imaging and 3D image analysis to characterize cellular information from a 3D matrix to enable multi-parametric comparison of different spheroid phenotypes. The characterization of compound toxicities was done by evaluation of spheroid size (volume) and shape, cell number and spatial distribution, nuclear characterization, number and distribution of cells expressing viability, apoptosis, mitochondrial potential, and viability marker intensities. In addition, changes in the content of live, dead, and apoptotic cells as a consequence of compound exposure were characterized. Our results indicate that phenotypic assays using 3D systems formed with human iPSC-derived cells are suitable for high-throughput screening (HTS) and can be used for cardiotoxicity and hepatotoxicity assessment *in vitro*.

OBJECTIVES

The objectives of this study were to: (1) optimize spheroid formation and assay workflows using human iPSC-derived cells, and (2) use these 3D cell models to develop HTS-compatible imaging and analysis methods.

MATERIALS & METHODS

Human iPSC-derived hepatocytes: Cryopreserved iCell® Hepatocytes (Cellular Dynamics International) were thawed and maintained for 1 week in 2D culture format on collagen I-coated plates. To prepare spheroid cultures, the cells were gently dissociated with StemPro® Accutase® (ThermoFisher Scientific), the cell suspension was supplemented with a 10% final volume / volume of Geltrex® hESC-qualified ready-to-use matrix (ThermoFisher Scientific), and then re-plated into low attachment spheroid plates (InSphero or Corning) at 1,000 cells/well. Spheroids were formed within 24-48 hours.

Human iPSC-derived cardiomyocytes: Cryopreserved iCell® Cardiomyocytes[2] (Cellular Dynamics International) were thawed and plated at 20,000 cells/well (96-well format) or 10,000 cells/well (384-well format) directly into ultra-low attachment (ULA) plates (Corning) and incubated for 4 days in maintenance media to form spheroid cultures. The presence of strong synchronous contractions in the 3D cultures was confirmed visually prior to running experiments.

High-Content Imaging: For liver spheroids, images were acquired using the ImageXpress® Micro Confocal High-Content Imaging System (Molecular Devices), with a 10x Plan Fluor and 20x Plan Fluor objective. A stack of 11-17 images separated by 5-10 µm was acquired, starting at the well bottom and covering approximately the lower half of each spheroid (~100-120 µm).

Image acquisition with cardiac spheroids was done using time-lapse image acquisition with 10 reads/second, 5-10 second reads or longer, and 20x or 10x magnification with FITC excitation and emission filters.

Ca^{2+} Flux Assay: FLIPR® Calcium 6 dye (Molecular Devices) was used to monitor changes in Ca^{2+} flux, which occurs synchronously with cell beating. The reagent (2X concentration) was added to the plates and incubated for 2 hours at 37 °C, 5% CO_2. A pre-drug read was acquired at ~8 frames per second using a FLIPR Tetra® High-Throughput Cellular Screening System (485 nm excitation and 530 nm emission settings). Additional reads were acquired during and after compound addition at prescribed times (~ 2 min read times).

RESULTS

Hepatotoxicity assay using 3D spheroid liver micro tissues derived from iCell® Hepatocytes

Assay Workflow

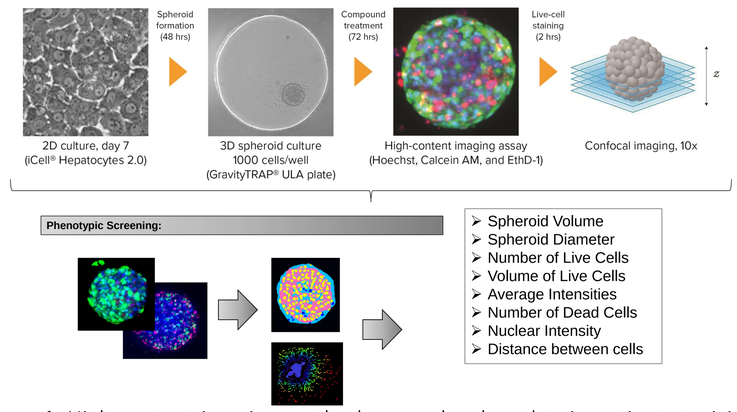

Figure 1. High-content imaging methods were developed to investigate toxicity effects on the morphology of 3D liver spheroids using confocal imaging and 3D image analysis. Spheroids were treated with hepatotoxic compounds for 72 hours. Following incubation with test compounds, spheroids were stained with a mixture of three dyes: 2 µM calcein AM (live cell stain), 3 µM EthD-1 (dead cell stain), and 10 µM Hoechst 33342 (nuclei stain, ThermoFisher Scientific). Dye solutions were added directly to the media (no wash) and incubated for 2 hours prior to imaging.

Confocal Imaging

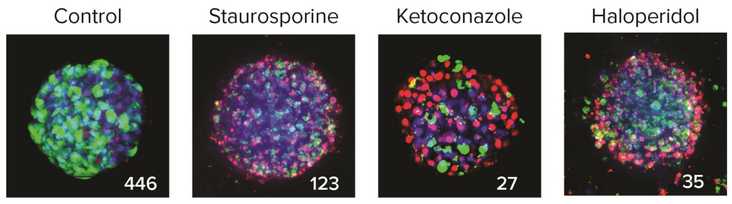

Figure 2. Composite confocal images of liver spheroids treated with known hepatotoxic compounds. Hoechst-blue, Calcein AM-green, EtHD-1-red; the number of Calcein AM positive (live) cells counted is indicated. Analysis was performed using new 3D features in MetaXpress Software. This module converts a stack of 2D images into a 3D space with appropriate detection and object segmentation. The Find Spherical Objects option defines roughly spherical objects from organelles to multi-cell spheroids, using user-defined settings for the approximate size of spheroid by selecting minimum and maximum width, minimum and maximum numbers of Z-planes, and object intensity versus background threshold values.

Phenotypic Analysis

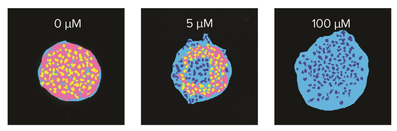

Figure 3. Quantitative analysis of the images included derivation of parameters to assess morphological features of spheroids, cell content, volumes, and complexity. Analysis masks display nuclei of live cells in yellow, live cell cytoplasm staining in pink, and nuclei of dead cells in dark blue

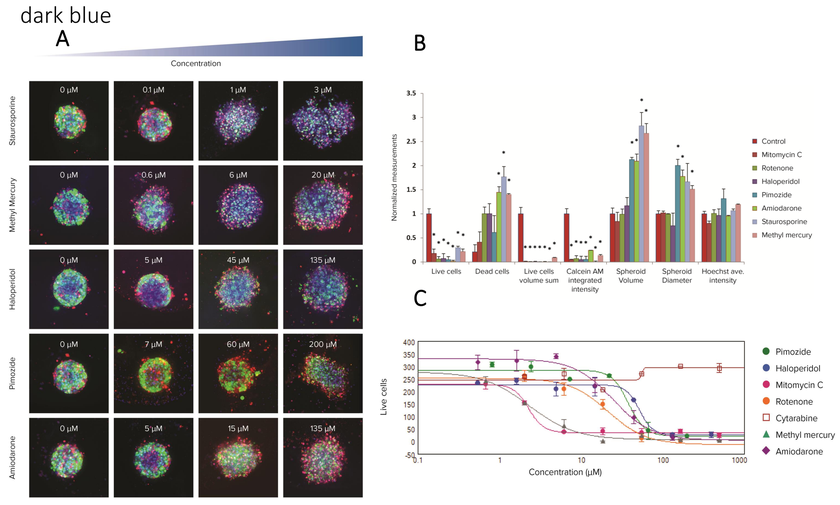

Figure 4. **(A)** Image examples of concentration-dependent hepatotoxicity effects on liver spheroid morphology. **(B)** Quantitative analysis of the images enabled derivation of several parameters to assess morphological features of spheroids, cell content, intensities, and volumes. Normalized measurements that differed significantly from the vehicle control are indicated with an asterick. **(C)** Examples of dose-response curves that were generated from the data set.

3D calcium flux assay

Assay Workflow

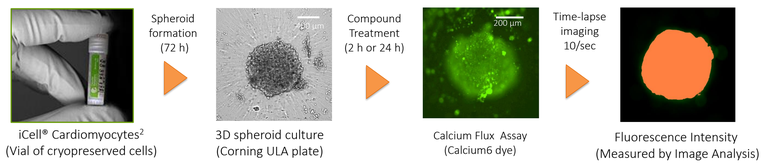

Figure 5. Cardiac spheroids were efficiently formed and started contracting spontaneously after 3-4 days in culture. These 3D cell models were used for cardiotoxicity assessment by staining them (2 hour loading time) with a Ca^{2+} sensitive dye (Calcium 6) and exposing them to compounds for 1 or 24 hours. Changes in the fluorescent intensities in response to Ca^{2+} flux were used as a surrogate marker for spheroid contraction. Significant alterations to the base-line beating pattern were observed in response to cardioactive and cardiotoxic compounds using either an ImageXpress time-lapse protocol or the FLIPR® Tetra System. Automatic data analysis was performed using the ScreenWorks ® Peak Pro® Software.

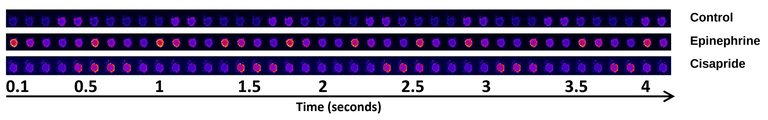

Figure 6. Time-lapse image series of contracting iPSC-derived cardiomyocyte spheroids loaded with a Ca^{2+} sensitive dye. Each image was taken at 100 ms intervals; yellow/red (false color scale) indicates a high Ca^{2+} concentration.

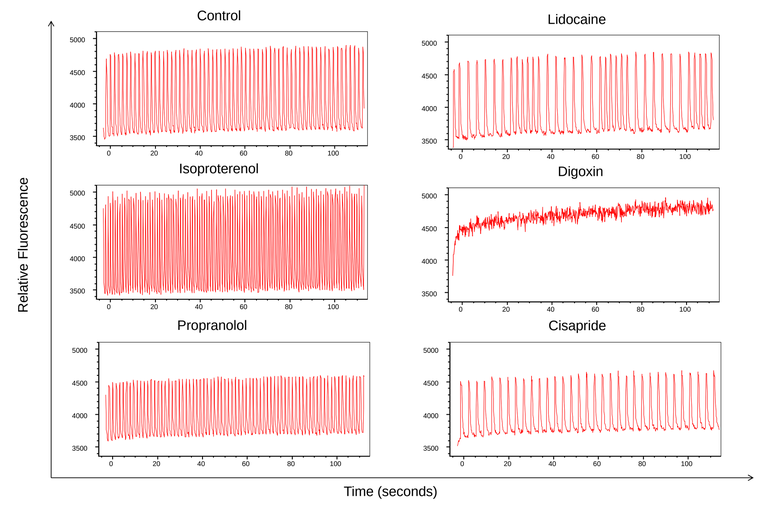

Figure 7. Calcium Flux assay using 3D cardiac spheroids treated with select compounds. Measurements done using FLIPR instrument. Phenotypic changes observed included: positive or negative chronotropic and inotropic effects with isoproterenol and propranolol, blocking of beating and Ca^{2+} flux with digoxin, and beat irregularity with lidocaine. The vehicle control (0.3% DMSO) did not result in alterations of the beating pattern.

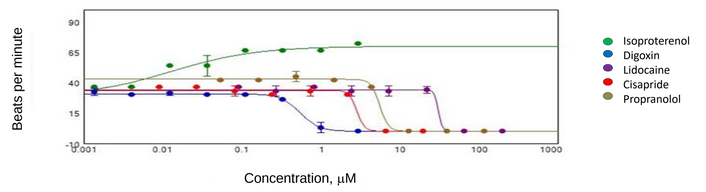

Figure 8. Graph of the dose-dependent changes in beating frequency caused by selected compounds.

SUMMARY

We have developed quantitative high-throughput assays which enable assessment of the viability, morphological changes, and physiologic activity of iPSC-derived liver and cardiac 3D spheroids.

Confocal imaging and 3D analysis allows higher resolution and multi-parametric analysis that includes single cell counting and classification to statistically characterize spheroid phenotypes and compound effects.

Calcium flux assay using fast kinetic fluorescence allows evaluation of physiological changes caused by cardiotoxic compounds in 3D spheroids.

Phenotypic assays using 3D systems formed with human iPSC-derived cells are suitable for high-throughput screening (HTS) and can be used for cardiotoxicity and hepatotoxicity assessment *in vitro*.