Developing a high content analysis method for identifying mitotoxic effects in the HepG2 liver spheroid model

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Introduction

Quantifying a cell model's biological response to a compound involves many challenges. The purpose of this project was to develop a validated high content imaging assay to assess mitochondrial dysfunction in the HepG2 cell line. It was hypothesized that if the assay was designed properly and it was run against a control compound then it would yield a dose response for mitochondrial depolarization.

Identifying or measuring mitotoxicity was suggested to be completed in *in vitro* cell models through measuring several features of cellular phenotypes that are directly related to key mechanisms of hepatoxicity (Xu et al., 2008), which can be completed through High Content Analysis. This system begins with a High Content Scanner, employing automatic microscopes to capture high quality images of cell models within each well in a 96-well microplate. The scanner utilizes Florescence Confocal Microscopy via laser scanning to acquire high resolution images of three-dimensional cell models. These models contain fluorescent stains for distinct separations between the biological features in the analysis software, HCS Studio™. Prior to analysis, protocol must be designed to designate how the software will pursue measurement acquisition. The protocol determines how the software will identify and isolate the cell model from and the individual biological features, such as nuclei or mitochondria. This protocol is then run against an image set to generate data for the assigned measurements completed in the analysis, such as a biological feature count, average and total area, or average and total intensity within each image.

Methods

Images were captured using a 10X narrow aperture objective with an extended depth of field of 200-300 microns. The spheroids were imaged with 10-15 progressive images at 20-micron intervals, and were projected based on maximum pixel intensity. This image capture methodology produced high resolution images for analysis of HepG2 spheroid shape, live cell analysis, and mitochondrial distribution. The criteria designated a wide-field microscope capturing spheroid shape with a nuclear stain in one channel, and confocal microscopy (Figure 1) to capture individual nuclei, mitochondria, and dead cells in three more separate channels. A Thermo Fisher Array Scan VTI executed the protocol to image spheroids exposed to a known mitotoxic compound, such as CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone).

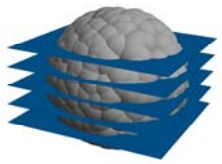


Figure 1. A diagram of confocal microscopy completed on a spheroid. The sphere represents the spheroid composed of individual HepG2 liver cells. The slices through the spheroid are the images captured at varying focal lengths. This figure is based off a diagram by Sirenko et al. (2016).

Methods (continued)

In HCS Studio™, background correction was assigned through 3D Surface Fitting to remove image background until only the spheroid in the image was retained. Thresholding in the wide-field nuclear channel to identify spheroids based upon pixel intensity isolated the spheroid within each image (Figure 2). Isodata, a dynamic thresholding methodology, automatically calculated threshold bounds for each individual image based on its histogram of pixel intensities of the specific fluorescent stain. Objects were rejected if their area was outside the threshold bounds calculated from a known average spheroid diameter of 250 microns. A Circ mask was selected to isolate the region of interest for nuclear identification. Isodata thresholding and intensity segmentation identified and separated individual nuclei from distinct peaks of fluorescent intensity. The mitochondrial analysis mask contracted the spheroid border by 25 pixels since the high intensity of mitochondria around the border induced measurement difficulties. Spot identification (Figures 3 and 4) detected individual mitochondria with a morphological method capability to set a radius of eight pixels for each subgroup of mitochondrial measurements. The dead cell mask was expanded by seven pixels to capture those detached from the spheroid. A radial intensity method compared fluorescent intensity peaks to distinguish between individual dead cells (Figure 5). Protocol altercation occurred when assay output data disagreed with observable morphology or the known response of the compound.

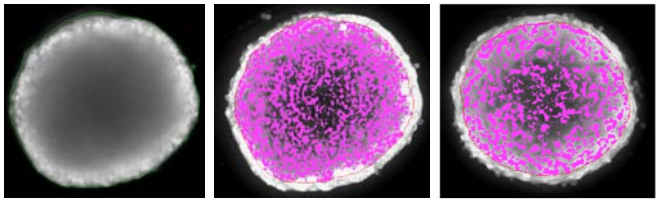


Figure 2 (left), 3 (middle), and 4 (right). All images captured with a 10X objective. The wide-field nuclear image on the left portrays the border (green) created to initially select and isolate the spheroid in each image. Figures 3 and 4 illustrate mitochondrial analysis for a low (middle) and high (right) concentration of CCCP. These two images were compared during parameter selection to standardize the parameters.

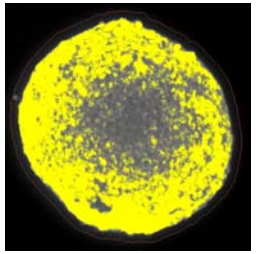
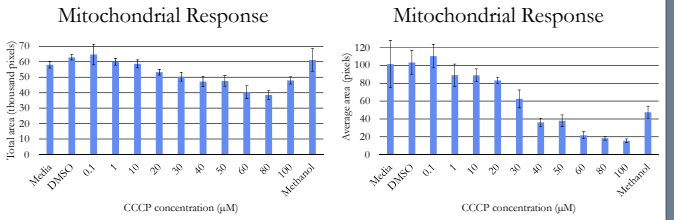


Figure 5. Image acquired with a 10X objective. Dead cell analysis of a spheroid exposed to the dead cell control, methanol. The yellow is the analysis selecting dead cells for measurement. The dead cells in the center of the spheroid may not have been recognized by the analysis because these cells were not in focus.

Results



Graph 1 (left) and 2 (right). Averaged total (left) and average (right) mitochondrial area in the spheroid images with increasing concentrations of CCCP. Error bars are standard deviations.

The data generated from the assay displays the correct dose response to CCCP. The total (Graph 1) and average (Graph 2) mitochondrial area decreases as the CCCP concentration increases, which agrees with the known mitotoxic response, increasing mitochondrial failure. A 2-Sample *t*-test was completed for each graph, with $n = 4$ for each concentration, comparing the lowest (0.1 µM) and highest (100 µM) concentrations where $\alpha = 0.05$. The total area and average area tests yielded *p*-values of 0.09 and 0.0009, respectively, with a null hypothesis of $\mu_1 \neq \mu_2$.

Conclusions

This project pursued the utilization of automated High Content Imaging and Analysis, capable of measuring several cellular health parameters simultaneously. These measurements produce comprehensive high dimensional data that can be mined for cellular mechanistic effects, to provide a reliable and accurate method to identify, analyze, and measure mitochondrial dysfunction. While the results of the 2-Sample *t*-tests suggest that the developed parameters of the assay correctly capture the average area dose response for a known mitotoxic compound exposed to a HepG2 spheroid model, the total area measurements are not conclusive.

For future work, several other known mitotoxic compounds can be tested against the assay to further refine the mitochondrial analysis parameters. Known cytotoxic compounds may also be run against the assay to refine the nuclear and dead cell analysis criteria.

References

Sirenko, O., Hancock, M. K., Hesley, J., Hong, D., Cohen, A., Gentry, J., & Mann, D. A. (2016). Phenotypic characterization of toxic compound effects on liver spheroids derived from iPSC using confocal imaging and three-dimensional image analysis. *ASSAY and Drug Development Technologies*, *14*(7), 381-394.

Xu, J. J., Henstock, P. V., Dunn, M. C., Smith, A. R., Chabot, J. R., & de Graaf, D. (2008). Cellular imaging predictions of clinical drug-induced liver injury. *Toxicological sciences*, *105*(1), 97-105.