Phenotypic Assessment of Toxicity Using a Human Hepatocyte Co-Culture Model

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INTRODUCTION

Development of predictive *in vitro* assays for early toxicity evaluation is extremely important for improving the drug development process and reducing drug attrition rates during clinical trials. A promising assay for assessing liver toxicity was developed with Hepregen's HepatoPac® *in vitro* liver model. HepatoPac is a 96-well microplate with micro-patterned primary human hepatocytes plus supportive fibroblasts that mimics the physiological microenvironment of the liver. Since primary hepatocytes demonstrate a rapid loss in phenotypic function when cultured, even in extracellular matrix, alternative models such as this one are attractive since the cells remain functional over prolonged treatment periods. We developed phenotypic assays for assessing multi-parameter readouts of general and mechanism-specific hepatotoxicity. The hepatocyte islands (~500 μm diameter) were imaged with an ImageXpress® Micro XLS high-content imaging system. Automated analysis was performed using an optimized Custom Module in MetaXpress® software. The analysis was configured to identify hepatocyte islands using a combination of fluorescent and transmitted light signal and cell health was determined using multiparametric hepatotoxicity algorithms. Output parameters included island size, hepatocyte viability, mitochondria intensity as well as viability of the supporting fibroblasts. The assay performance was tested by measuring concentration responses for selected test compounds.

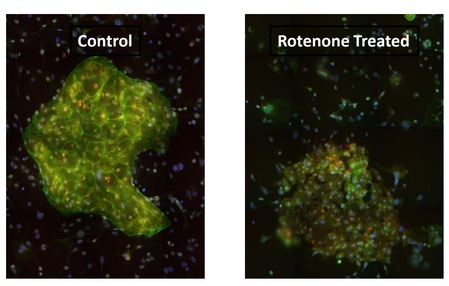

Figure 1. Visible differences in both the hepatocyte island and fibroblasts can be noted with compound treatment. Actin (green), nuclei (blue) and mitochondria (red) are shown in these fixed cells. Images are a stitched montage of 20 images acquired with a 40X PA objective for illustrative purposes only.

MATERIALS & METHODS

- HepatoPac (P/N HPHU-HCI-96S) plates were received fresh. Hepatocytes were incubated for 72 hours with various compounds at 7 different concentrations and then stained with the following dyes: Calcein AM to determine cell viability, MitoTracker Orange CMXRos to determine mitochondria integrity, Cell Explorer™ Fixable Dead Cell Stain, and Hoechst to determine total nuclear count and aid in cell segmentation.
- ImageXpress Micro XLS widefield high content imaging system (Molecular Devices) was used to acquire images of the wells with 4X and 10X objectives using transmitted light as well as standard filter cubes for DAPI, FITC, TRITC, & CY5 wavelengths. The images were analyzed with a Custom Module in MetaXpress software to detect multiple parameters indicating toxicity in the hepatocyte islands and supporting fibroblasts.

USE OF TL & FLUORESCENCE FOR ISLAND SEGMENTATION

To analyze toxic responses of fibroblasts independent of human hepatocytes, different segmentation masks were generated. The combination of transmitted light (TL) and fluorescence (FL) adds flexibility and robustness to the process. Many various features can be identified only from the part of the image that is beneath the white area of each mask. Alternatively, the entire well or field-of-view can be analyzed together.

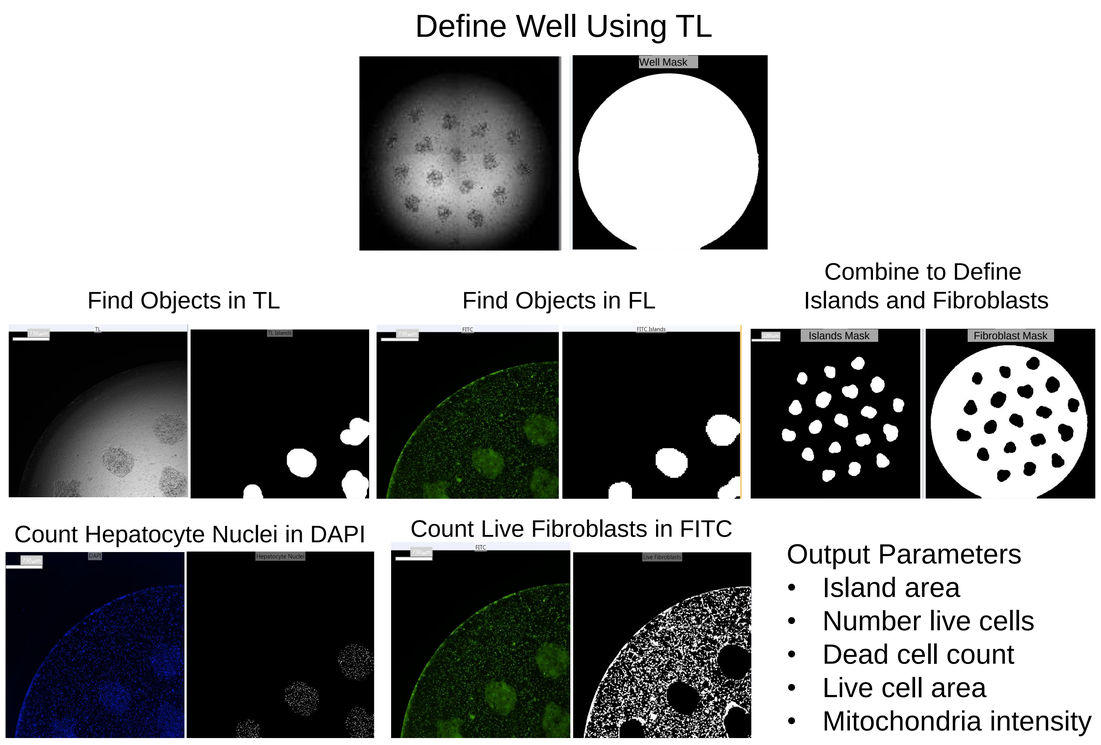

Figure 2. TL and FL images are used to define the well and location of islands in a Custom Module image analysis process. The TL contribution to the mask is necessary if the cells in the island are dead or dying so remain unstained by the fluorescent dye. In addition, a fibroblast mask is defined to allow interrogation of the supportive cells between the hepatocyte islands. Cells in the image are segmented using nuclear stain (DAPI) and then classified as Live or Dead using Calcein AM (FITC) and a dead cell marker (Cy5 example not shown).

AUTOMATED CUSTOM IMAGE ANALYSIS

Once Island masks are defined then standard high-content analysis algorithms can be employed to interrogate the health of hepatocytes as well as surrounding fibroblasts. Here we show a combination of Cell Count, Live/Dead Hepatocyte, Live/Dead Fibroblast, and Mitochondria Health analyses. Outputs are provided on a well-by-well or island-by-island basis.

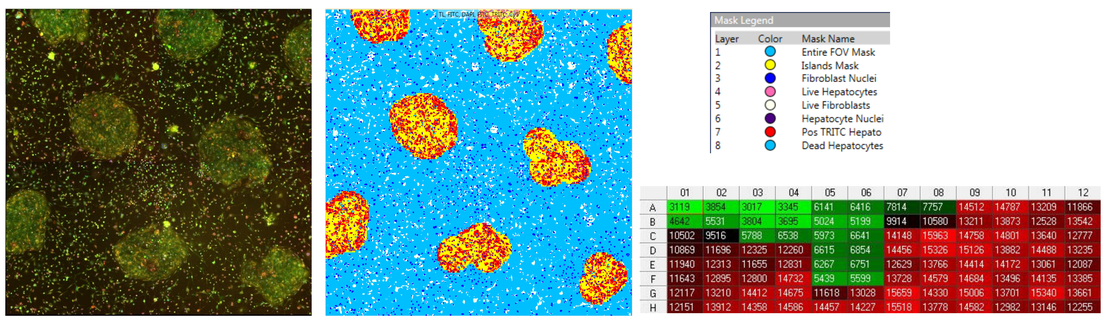

Figure 3. (Left to right) A representative overlay of Calcein AM (green), Hoechst nuclei (blue), MitoTracker Orange (yellow) and Dead Cell Stain (red) taken with a 10X objective. An example of the segmentation of 8 specific objects, each of which may yield multiple measurements. The legend of the color-coded segmentation overlay. A heat map showing trending across the wells of the 96 well plate.

TOXIC EFFECTS OF COMPOUNDS

The assay performance was evaluated using select test compounds. HepatoPac plates were incubated with dilution series of the compounds for 72 hours. Images were acquired using ImageXpress Micro XLS under environmental control (37° C, 5% CO2). The entire area of each well was imaged by taking 4 sites using a 4X objective. The resulting 4 fields-of-view were stitched together to create a single whole-well image and analyzed using the Custom Module described above. An average of 17 islands per well were identified using this method (see Table 1).

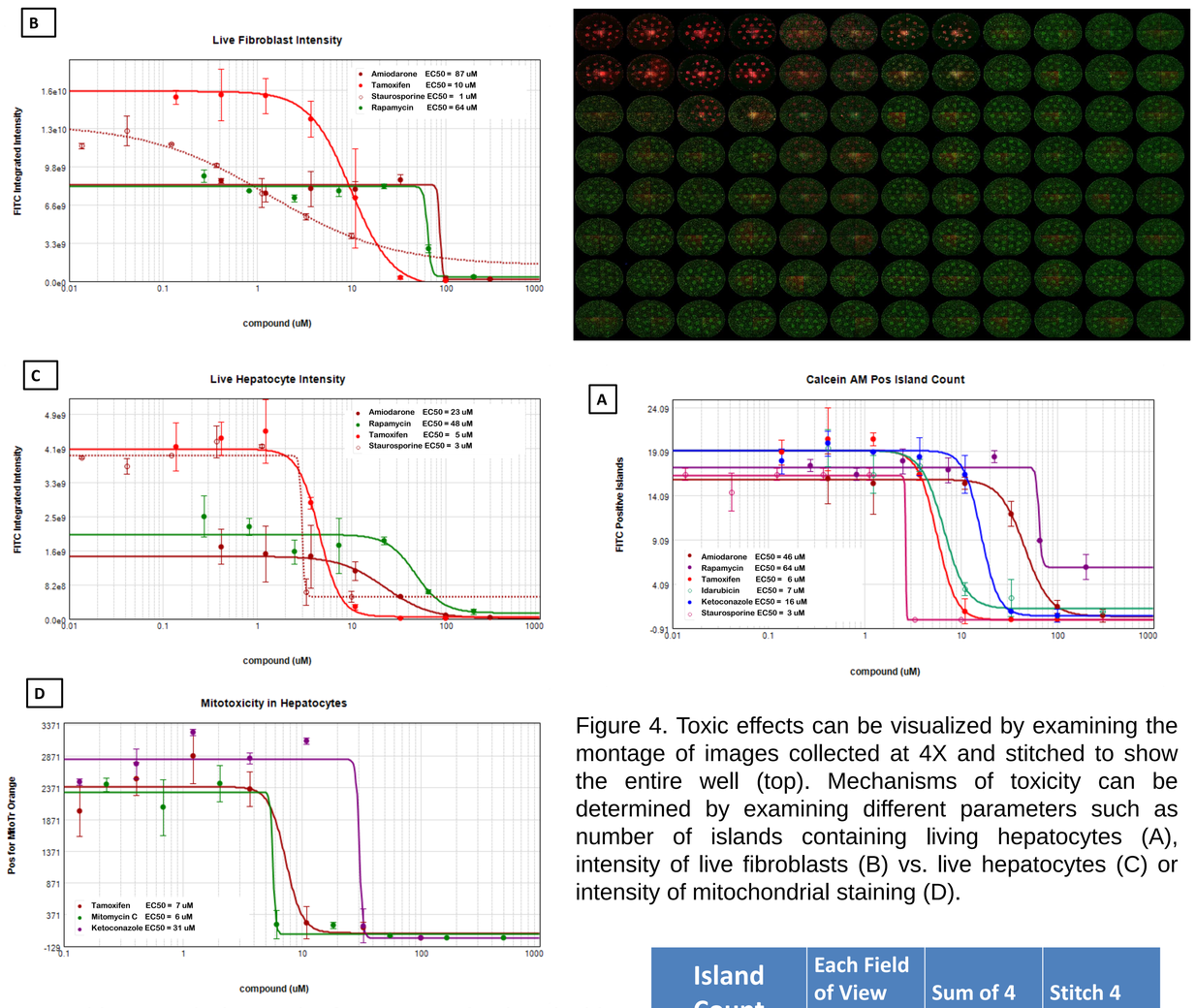

Figure 4. Toxic effects can be visualized by examining the montage of images collected at 4X and stitched to show the entire well (top). Mechanisms of toxicity can be determined by examining different parameters such as number of islands containing living hepatocytes (A), intensity of live fibroblasts (B) vs. live hepatocytes (C) or intensity of mitochondrial staining (D).

Table 1. Analyzing each of 4 FOV can lead to double-counting of islands that cross over the image borders so numbers or area of hepatocyte islands is not accurate but other intensity parameters for live/dead staining or mitochondrial toxicity are still relevant.

Island Count	Each Field of View (FOV)	Sum of 4 FOV	Stitch 4 FOV
4X objective	5.5	21.9	16.9
10X objective	1.6	7.0	8.4

CONCLUSIONS

- High-content automated imaging assays using the HepatoPac liver model are feasible and have potential to improve safety and efficacy assessment of drugs and chemicals.
- The ImageXpress Micro XLS is well suited for this assay as it allows capture of a whole well with 4 images and can combine TL & FL images for enhanced analysis capability.
- The Custom Module Editor in MetaXpress software enables identification of both islands and fibroblast areas and provides multi-parametric assessment of hepatocyte health.

MOLECULAR DEVICES