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Multiparametric Live-Cell Cytotoxicity Studies on Primary Human Hepatocytes Using the Operetta/Harmony Imaging Platform

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1 Introduction

The use of cultured primary human hepatocytes in predictive *in vitro* studies on drug metabolism and hepatotoxicity in different stages of the drug discovery process has increased in recent years[1]. Primary liver cells are recognized to be the closest *in vitro* model to human liver function as they express the entire hepatic contingent of drug-metabolizing enzymes and transporter proteins[1].

The commercial availability of good-quality cryopreserved human hepatocytes provided the opportunity to implement routine high-content screening assays. Image-based *in vitro* assays are simultaneously monitoring multiple cytotoxic effects in different pathways following hepatocellular injury, resulting in a more meaningful assessment of the hepatotoxic potential of the compound under investigation[2].

To investigate drug-induced cytotoxicity we used cryopreserved single-donor hepatocytes seeded into microplates in a conventional monolayer and treated them with three model hepatotoxins. After applying a convenient no-wash staining protocol live cells were imaged directly on the Operetta® High Content Screening system. This straightforward approach perfectly met the special sensitivity and finite life span requirements primary cells possess.

2 Experimental Procedure

Single-donor hepatocytes (h NHEPS®, Lonza, CC-2591, lot 7F3066) were seeded at a density of 33 000 cells per well in a 384-well CellCarrier™ microtiter plate freshly coated with 8 µg/cm² collagen I and cultured over night (14 hr) at 37 °C, 5 % CO_2. Next day, growth medium was replaced by FCCP[1], tacrine[2] and acetaminophen[3] (= AAP) serially diluted in growth medium. After 24 hr incubation with each compound and DMSO controls a fluorophore dye cocktail containing Hoechst 33342, BOBO™-3 and MitoTracker® Deep Red in growth medium was added and incubated for 45 min (37 °C, 5 % CO_2). Finally, images of live cells were acquired on the Operetta® non-confocally using a 20X long WD objective.

3 Resulting Images and Analysis Strategy

Based on the application of a fluorophore dye cocktail containing the cell-permeant nuclear dye Hoechst, the cell-impermeant nuclear dye BOBO™-3 and MitoTracker® Deep Red, a cell-permeant organelle dye that accumulates in the matrix of metabolically active mitochondria, significant hepatotoxin-induced cellular changes could be detected (Fig. 1):

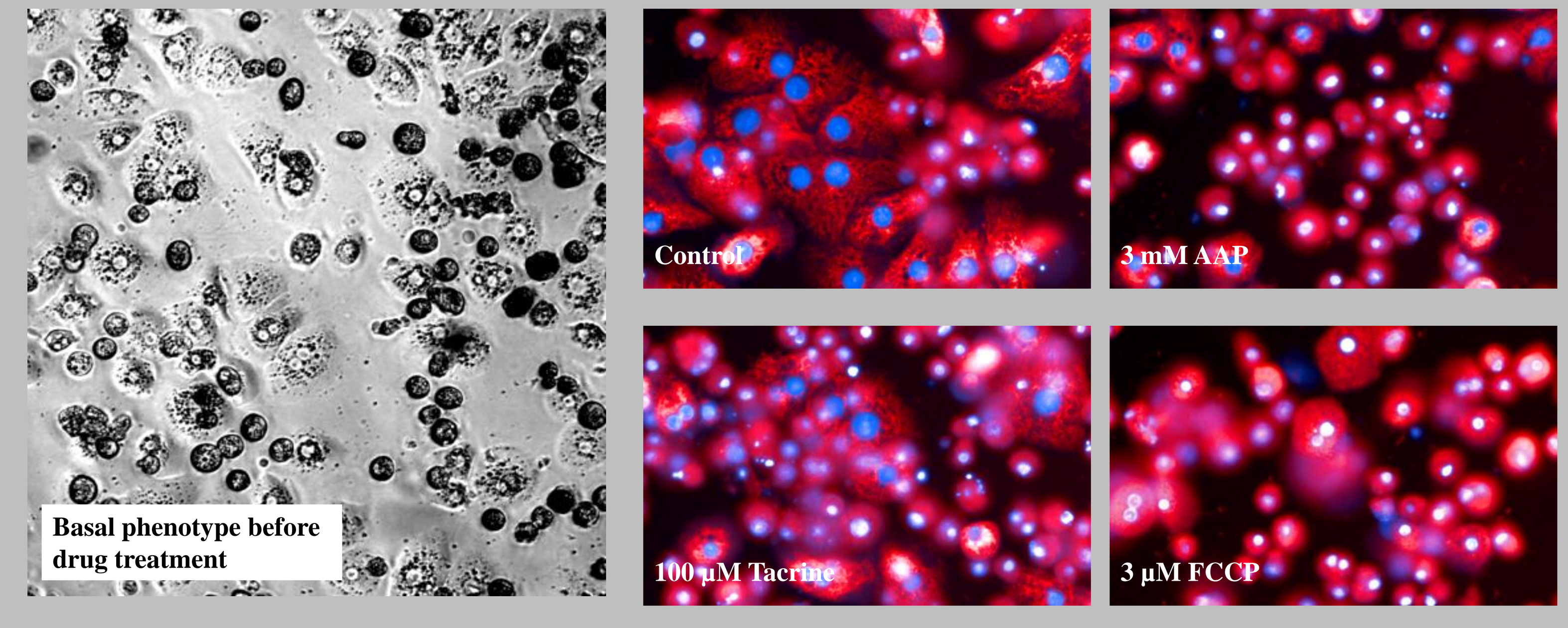

Figure 1. **Left |** 10X phase contrast image of human primary hepatocytes seeded on collagen-coated 384 well microplates in a conventional monolayer, after thawing, over night attachment and one wash step: the majority of the cells adhered and adopted a polygonal shape with a highly granulated cytoplasm; several binuclear cells can be found. Not all primary hepatocytes survived the thawing procedure, leaving some cells detached and rounded up.

Right | Typical phenotypic changes after a 24-hr-treatment with three model hepatotoxins. Cellular toxicity effects change the nuclear (blue, Hoechst) and mitochondrial (red, Mitotracker® Deep Red) status resulting in a general rounding up of the cells, a reduction of the nucleus size and a strong change in the mitochondrial as well as the nuclear staining pattern. Additionally, the number of dead and detached cells is increasing.

The analysis sequence was designed by combining classical segmentation steps with morphological and texture based object characterizations using the image analysis software Harmony® 3.0 (Fig. 2).

Hoechst channel Nuclei segmentation	**MitoTracker®** channel Cytoplasm detection	**BOBO™-3** channel Quantification of intensity
Control	Control	300 µM Tacrine
Readouts		
• Nuclear Size • Nuclear Staining Pattern (Texture) • Viability	• Mitochondrial Staining Pattern (Texture) • Cell Roundness • Viability	BOBO™-3 staining intensity as readout for viability was replaced by another classification system based on machine learning.

Figure 2. Image analysis strategy for quantifying hepatotoxin induced phenotypic changes in primary liver cells. For characterizing changes in the mitochondrial and nuclear texture the SER ("Spots, Edges and Ridges") properties set was used.

[1] FCCP is a very potent uncoupler of oxidative phosphorylation in mitochondria, which functions by degrading the link between the respiratory chain and the phosphorylation system used to generate ATP.

[2] Tacrine: parasympathomimetic and centrally acting cholinesterase inhibitor used in the treatment of Alzheimer's disease. The metabolism of tacrine in the liver results in an active metabolite, which is associated with a high frequency of hepatotoxicity.

[3] AAP: analgesic, well-known to cause potentially fatal liver damage and hepatic necrosis if overdosed. The toxic effect is primarily due to the highly-reactive intermediary metabolite NAPQI.

To improve the robustness of the viability readout the image analysis was extended by the Harmony® 3.0 add-on PhenoLOGIC®. This supervised machine learning algorithm revealed the redundancy of the BOBO™-3 (Fig. 3).

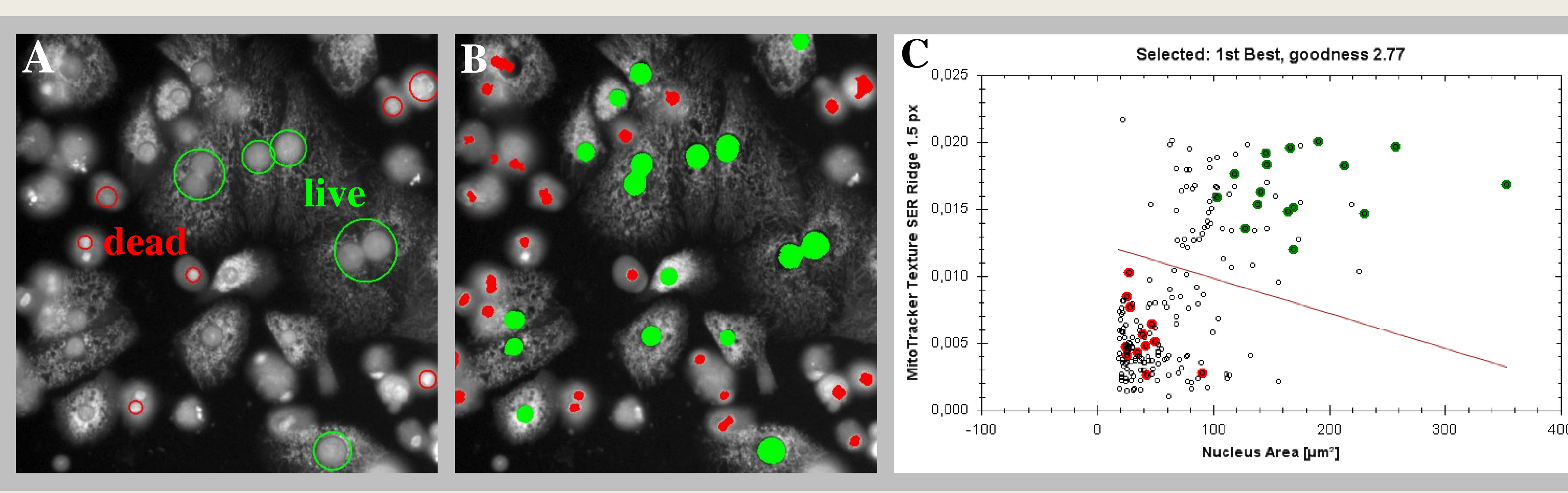

Figure 3. Cell viability: supervised machine learning strategy for classifying the two phenotypes live (green) and dead (red) cells in the population. **A |** The PhenoLOGIC® analysis starts with a training phase in which the user manually selects a few cells belonging to either one or the other class. **B |** Then a classifier is computed and applied to all objects in the image. The algorithm evaluates a user defined set of properties (here 11) to identify the property pair with the highest discriminative power for the two classes. Cells classified dead also displayed high BOBO™-3 intensities as toxicity-induced membrane disruption allowed for the dye to enter the cells to bind DNA. **C |** The best pair – displayed in the scatter plot of the training well - was identified to be the nuclear area correlated to the mitochondrial texture parameter "ridge".

4 Quantification of Cytotoxic Effects

By applying the previously defined image analysis strategy we quantified five cellular parameters describing typical phenotypic changes upon treatment with the model hepatotoxins (Fig. 4). As primary cells are inherently more sensitive than cell lines the population appears more heterogeneous (Fig. 1). Therefore, intensity based readouts were replaced by more robust texture analyses.

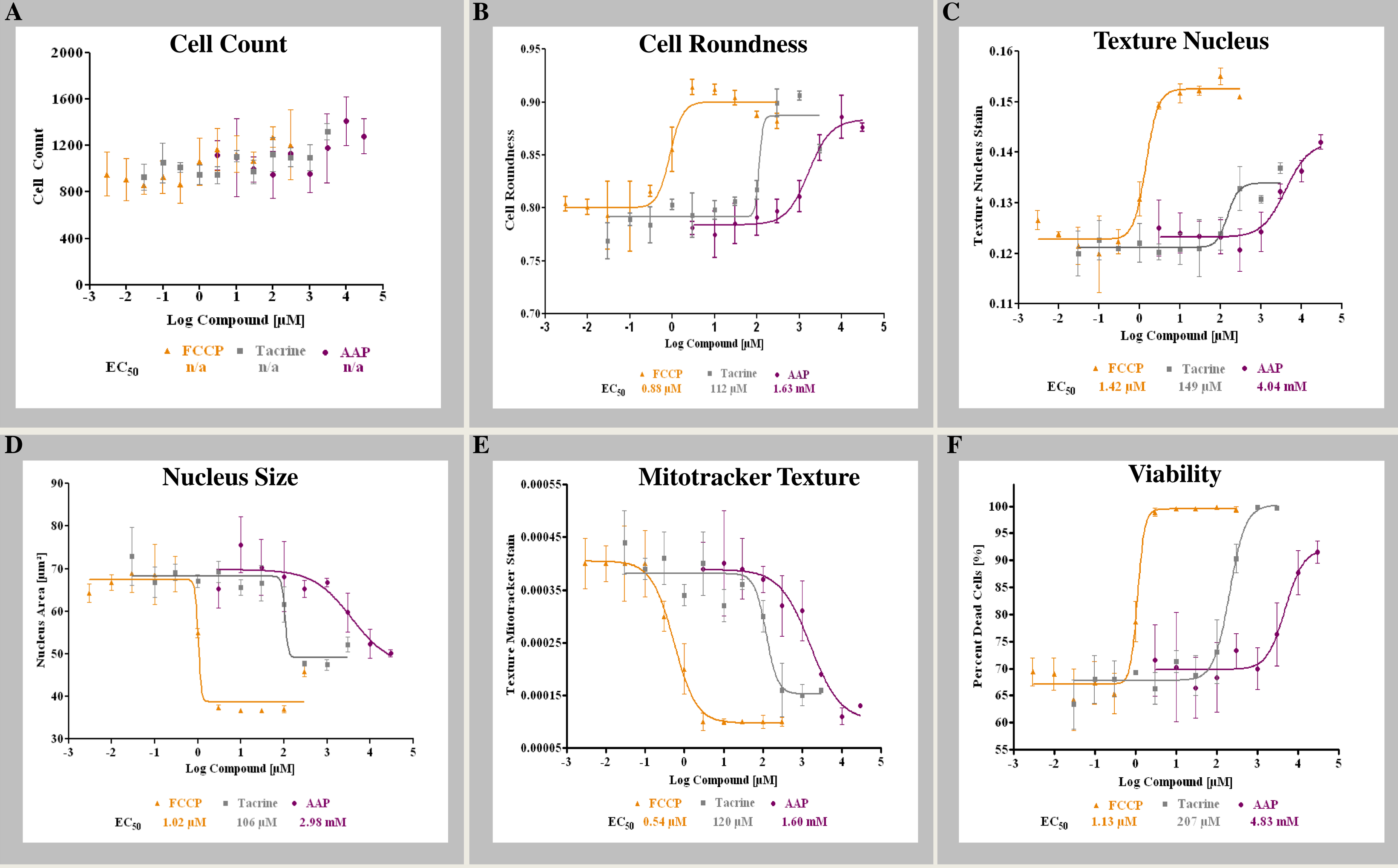

Figure 4. Quantification of hepatotoxicity in human liver cells: FCCP, tacrine and acetaminophen-generated dose-response curves (fitted with GraphPad Prism® software) deduced from 5 readouts reflecting major phenotypic changes (n = 3 wells).

A | As primary hepatocytes do not proliferate in culture the cell count is only used as a control readout.

B | All three compounds are gradually damaging the cell structure leading to a successive increase in cell roundness and a disruption of the cell adhesion.

C | and D | The nuclear integrity is a simple and powerful indicator for cell toxicity: characteristic morphologic changes include nuclear shrinkage, condensation and fragmentation which can be quantified by evaluating the area (D) as well as the texture of the nuclear stain (edge, 1 px, C). FCCP showed the most dramatic dose-dependent reduction in nuclear size.

E | As mitochondria are central and essential for the life of eukaryotic cells any toxic influence affecting the metabolic function of mitochondria need to be monitored closely. Here, the change in mitochondrial texture (hole, 3 px) serves as an indirect yet sensitive indication for their impaired functionality.

F | The loss of cell viability is typically a late-stage effect and was quantified here by correlating the nuclear size with the mitochondrial texture (machine learning algorithm). Cells classified as dead on this basis also showed a high BOBO-3 intensity indicating an increasing loss of the membrane barrier function. Characteristic for this lot of primary hepatocytes is a relatively low viability rate in the control.

5 Conclusions and Summary

Predicting and understanding drug-induced hepatotoxicity is still considered a challenge. Several studies have shown that the primary hepatocyte model has a higher predictive power for the *in vivo* metabolic profile of a drug[1].

The high content approach presented here is a rapid and straightforward live cell assay which perfectly meets the special requirements of a primary cell model (sensitivity, survival rate, limited life span, and more heterogeneous appearance).

Three model hepatotoxins were profiled for multiple effects on typical cytotoxicity markers in single-donor cells. We could show that the combination of texture analysis and supervised machine learning on the Operetta/Harmony® imaging platform is especially suited for a robust and sensitive detection of these effects.

The commercial availability of single-donor primary cells enables users to readily compare cytotoxicity profiles in hepatocytes from different donors. This provides the opportunity to assess inter-individual differences in a controlled environment.

[1] Hewitt N et al.: Primary Hepatocytes: Current Understanding of the Regulation of Metabolic Enzymes and Transporter Proteins, and Pharmaceutical Practice for the Use of Hepatocytes in Metabolism, Enzyme Induction, Transporter, Clearance, and Hepatotoxicity Studies, Drug Metabolism Reviews, 39, 159-234 (2007)

[2] Abraham V et al.: Application of a High-Content Multiparameter Cytotoxicity Assay to Prioritize Compounds Based on Toxicity Potential in Humans, J Biomol Screen, 13, 527-537 (2008)

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