# Cytotoxicity studies on live primary human hepatocytes using the Operetta high-content analysis system.

## Key features

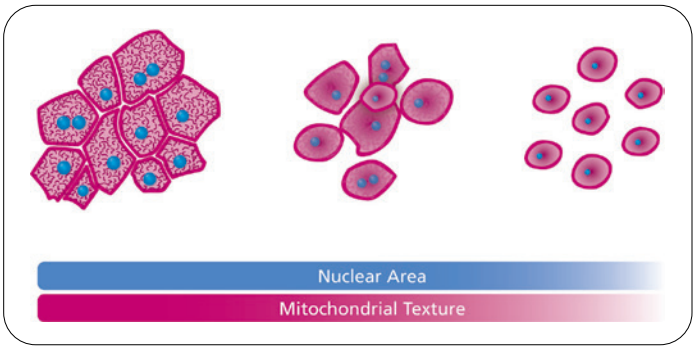
- Multiparametric live-cell cytotoxicity analysis in primary human hepatocytes
- Image analysis combining morphological and texture-based readouts using the flexible Harmony® software
- Improved robustness of the assay by using PhenoLOGIC™ supervised machine learning

## Background

The use of cultured primary human hepatocytes in predictive *in vitro* studies of drug metabolism and hepatotoxicity, in different stages of the drug discovery process, has increased in recent years. Primary liver cells are recognized to be the closest and most relevant *in vitro* model to the human liver as they express the entire hepatic contingent of drug-metabolizing enzymes and transporter proteins [Hewitt et al., 2007].

The commercial availability of high quality cryopreserved human hepatocytes provides the opportunity to implement routine high content screening assays. Image-based *in vitro* assays simultaneously monitor multiple cytotoxic effects in different pathways following hepatocellular injury, resulting in a more meaningful assessment of the hepatotoxic potential of the compound under investigation [Abraham et al., 2008].

To investigate drug-induced cytotoxicity, cryopreserved single-donor hepatocytes were seeded into microplates in a monolayer and were treated with three model hepatotoxins. After applying a convenient no-wash staining protocol, live cells were immediately imaged on the Operetta® high-content analysis system. This straightforward approach is well-suited to the special requirements of primary cells' sensitivity and their finite life span.



## Application

Single-donor hepatocytes (h NHEPS®, Lonza, CC-2591, lot 7F3066) were thawed and cultivated according to the manufacturer's protocol. One cryovial of h NHEPS® hepatocytes was thawed quickly in a 37 °C water bath, 12 hr prior to treatment, and the cell suspension transferred into 20 mL cold hepatocyte medium (HCM™ BulletKit™, CC-3198). Cells were then centrifuged (50 X g, 4 °C, 3 min) and the pellet re-suspended in 10 mL cold hepatocyte medium. The cell number was determined on a CASY® counter (Roche Applied Science). Hepatocytes were seeded at a density of 33 000 cells per well in a 384-well CellCarrier™ microtiter plate freshly coated with 8 µg/cm$^2$ collagen I (BD Biosciences, 354236). After overnight cultivation (14 hr at 37 °C, 5% $CO_2$), the growth medium was replaced with FCCP1 (carbonylcyanide 4-(trifluoromethoxy) phenylhydrazone), tacrine[2] and acetaminophen[3] (AAP) serially diluted in growth medium. A phase contrast image was recorded on a standard Olympus® IX 50 microscope with a 10X objective (10X/0.30 Ph1). After 24 hr incubation with each compound and DMSO controls, a fluorophore dye cocktail containing Hoechst 33342, BOBO™-3 and MitoTracker® Deep Red (Invitrogen, H3570, M22426, B3586) in growth medium was added and incubated for 45 min (37 °C, 5% $CO_2$). Finally, non-confocal images of live cells were acquired on the Operetta system using a 20X long WD objective.

The fluorophore dye cocktail contained 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. The application of the dye cocktail enabled the detection of significant hepatotoxin-induced cellular changes, as indicated by reduced nuclear size, increased cell roundness and changes in the mitochondrial texture (Figure 1).

The analysis sequence was designed by combining classical segmentation steps with morphological and texture-based (using the SER properties set, "Spots, Edges and Ridges") object measures, using Harmony image analysis software. The Hoechst channel was used for nuclei segmentation and for quantification of the nuclear size and staining pattern (texture). The use of MitoTracker® Deep Red enabled the detection of the cell shape and was also used to evaluate the cell roundness, as well as the distribution and quantity of

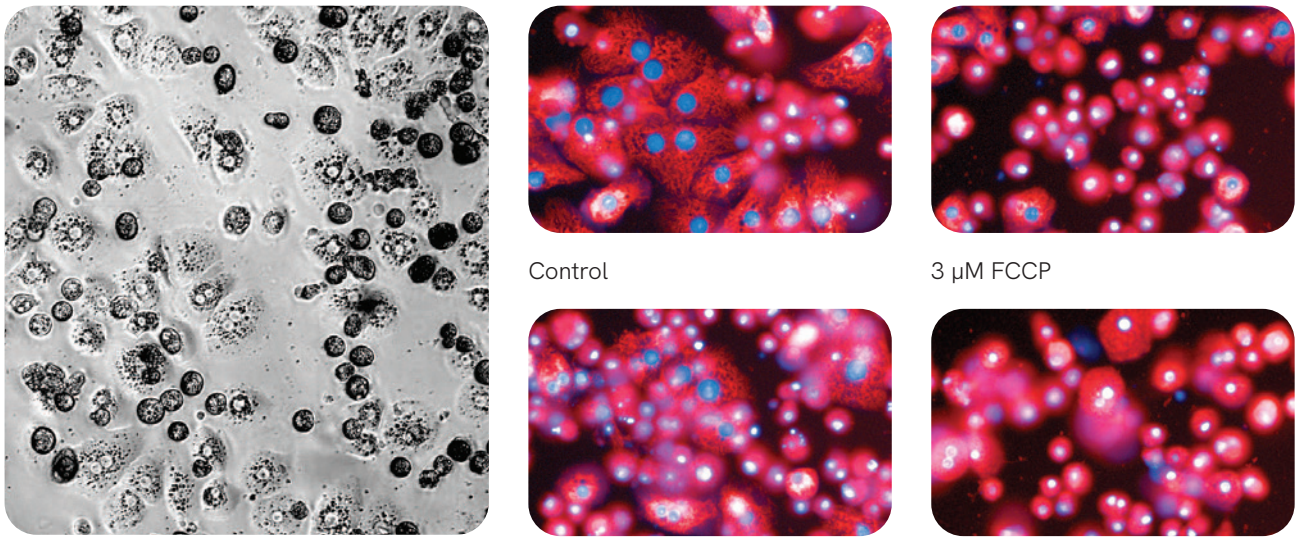



Figure 1: Left. 10X phase contrast image of human primary hepatocytes seeded on collagen-coated 384-well CellCarrier™ microplates in a conventional monolayer, after thawing, overnight attachment and one wash step. The majority of the cells adhered and adopted a polygonal shape with a highly granulated cytoplasm; several binuclear cells could be observed. Most primary hepatocytes survived the thawing procedure, leaving few cells detached and rounded.

Right. Typical phenotypic changes observed after a 24 hr treatment with three model hepatotoxins. Cellular toxicity effects change the nuclei (blue, Hoechst) and mitochondria (red, MitoTracker® Deep Red), resulting in a general rounding of the cells, a reduction in the nuclear size and a strong change in both the mitochondrial and nuclear staining patterns. Additionally, the number of dead and detached cells increased.

mitochondria, reported as mitochondrial texture.
The intensity of the BOBO™-3 stain was initially intended to assess the population´s viability. The supervised PhenoLOGIC algorithm was used to identify the optimal parameter pairing to differentiate between healthy and damaged cells.

Using this image analysis strategy, five cellular parameters were quantified that describe typical phenotypic changes upon treatment with three model hepatotoxins (Figure 3).
As primary cells are inherently more sensitive than cell lines, the population appeared more heterogeneous (Figure 1). Therefore, intensity based readouts were replaced by more robust texture analyses.

The robustness of the viability readout was further improved by using the Harmony plug-in, PhenoLOGIC. This supervised machine learning algorithm revealed the redundancy of BOBO™-3 by identifying a classifier with higher discriminative power for live and dead cells based on the correlation of two parameters strongly connected with the health status of a cell: the nuclear area and the mitochondrial texture (Figure 2).

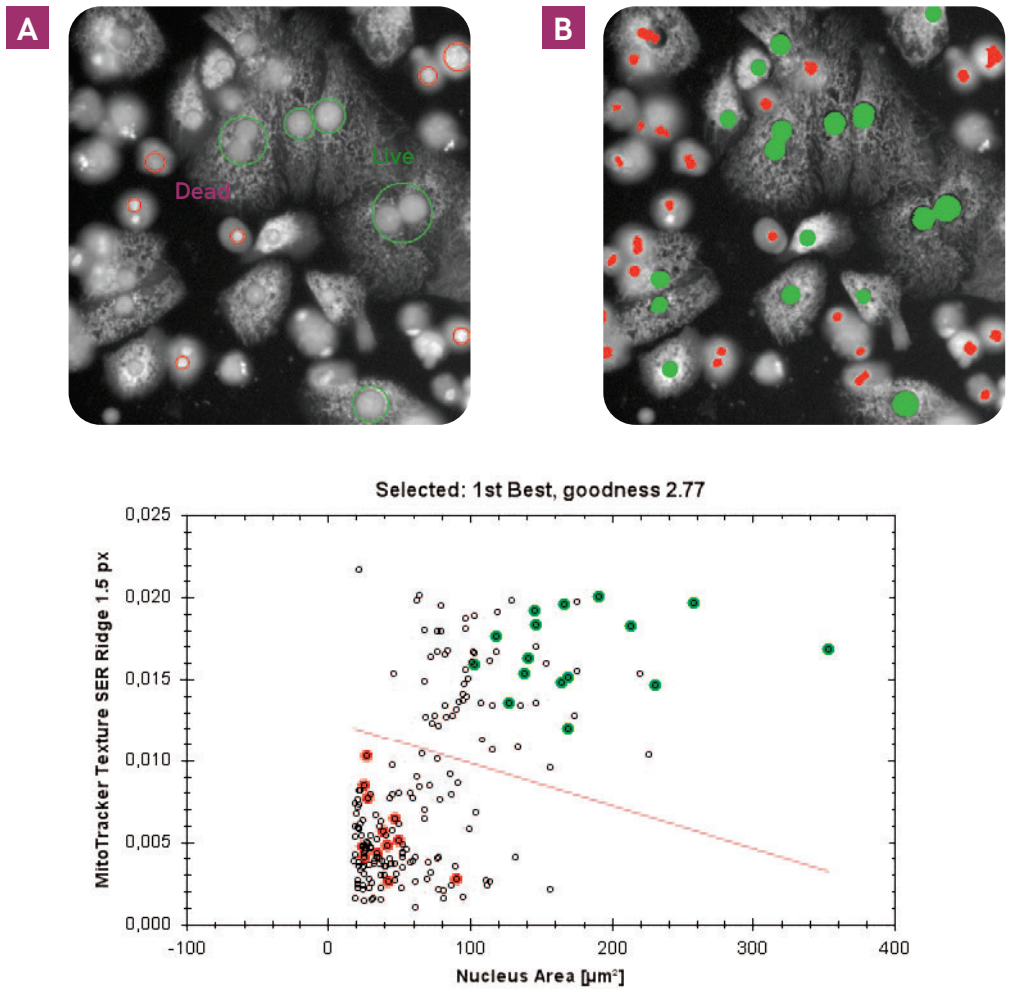



Figure 2: Cell viability: supervised machine learning strategy for classifying two phenotypes; live (green) and dead (red) cells in the population.
A) The PhenoLOGIC analysis starts with a training phase in which the user manually selects a few cells belonging to either one class or the other.
B) A classifier 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 as dead also displayed high BOBO™-3 intensities as toxicity-induced membrane disruption allowed for the dye to enter the cells and bind the 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".

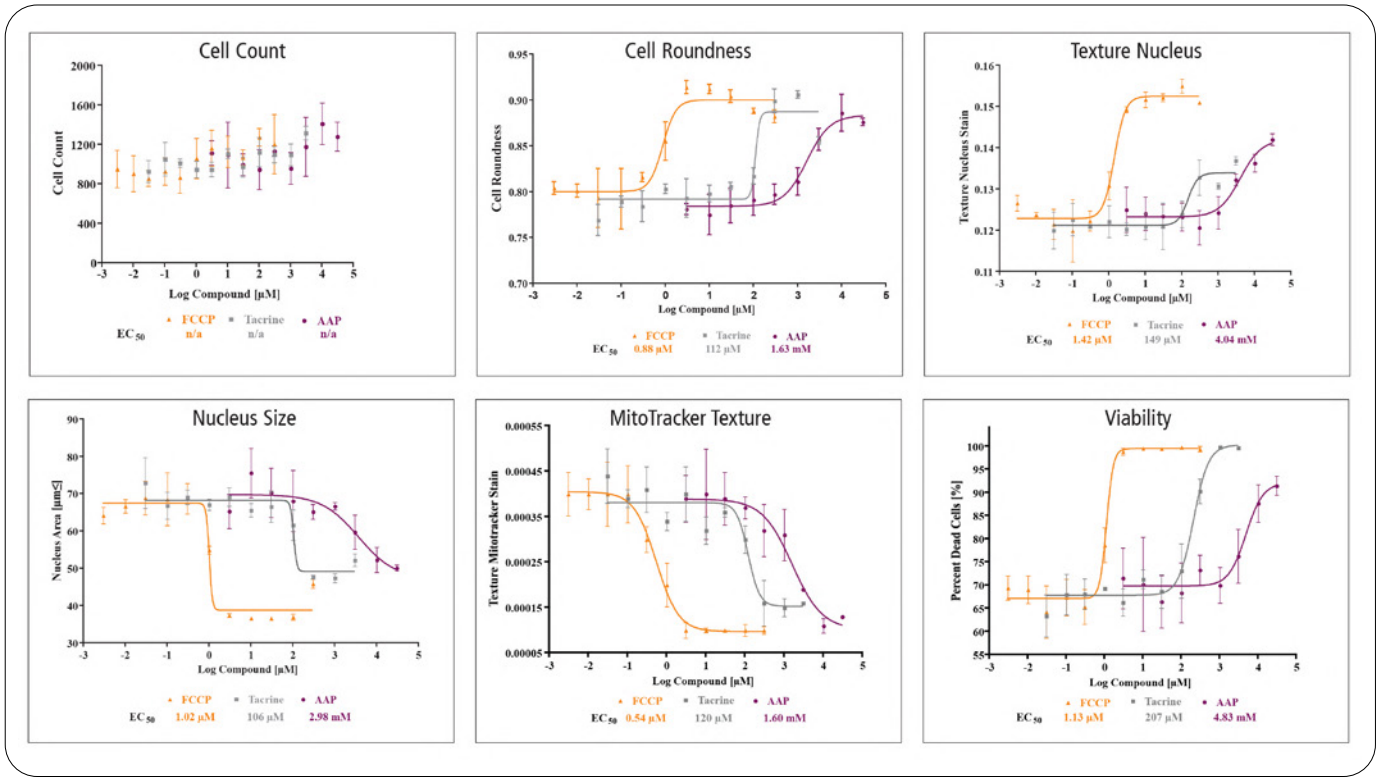



Figure 3: Quantification of hepatotoxicity in human liver cells: FCCP, tacrine and acetaminophen-generated dose-response curves (fitted with GraphPad Prism® software) deduced from 5 readouts, reflecting major phenotypic changes (n = 3 wells).
A) As primary hepatocytes do not proliferate in culture, the cell count is only used as a control readout.
B) All three compounds gradually damage the cell structure leading to a successive increase in cell roundness and disruption of cell adhesion.
C) and D) The nuclear integrity is a simple and powerful indicator of 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 essential for the life of eukaryotic cells, any toxic influence which affects the metabolic function of mitochondria needs to be monitored closely. Here, the change in mitochondrial texture (hole, 3 px) serves as an indirect, yet sensitive, indicator of their impaired functionality.
F) The loss of cell viability is typically a late-stage effect of cell toxicity, and was quantified here by correlating the nuclear size with the mitochondrial texture (using a machine learning algorithm, Figure 2). Cells classified as dead on this basis also showed a high BOBO™-3 intensity, which was indicative of an increasing loss of membrane barrier function. A relatively low viability rate in the control is characteristic of this batch of primary hepatocytes.

## Conclusions

Predicting and understanding drug-induced hepatotoxicity is 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 [Hewitt et al., 2007].

The high content approach presented here is a rapid and straightforward live cell assay which is ideally suited to 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 showed that the combination of texture analysis and supervised machine learning using the Operetta system and Harmony software is especially well suited to providing robust and sensitive detection of these effects.

The commercial availability of single-donor primary cells and robust assay methods enables users to readily assess cytotoxicity profiles of compounds in a model system that is close to the situation in humans. Furthermore, hepatocytes obtained from different donors provide the opportunity to assess inter-individual, pharmacogenomic differences in a controlled environment.

## References

Hewitt N et al. (2007): 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

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

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[1] FCCP: a 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: a 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: an 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.

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