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APPLICATION NOTE

Multiparametric live-cell cytotoxicity analysis using the Operetta high-content analysis system.

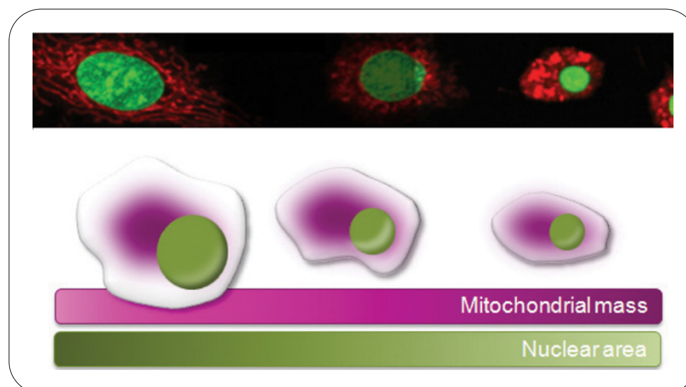
Key features

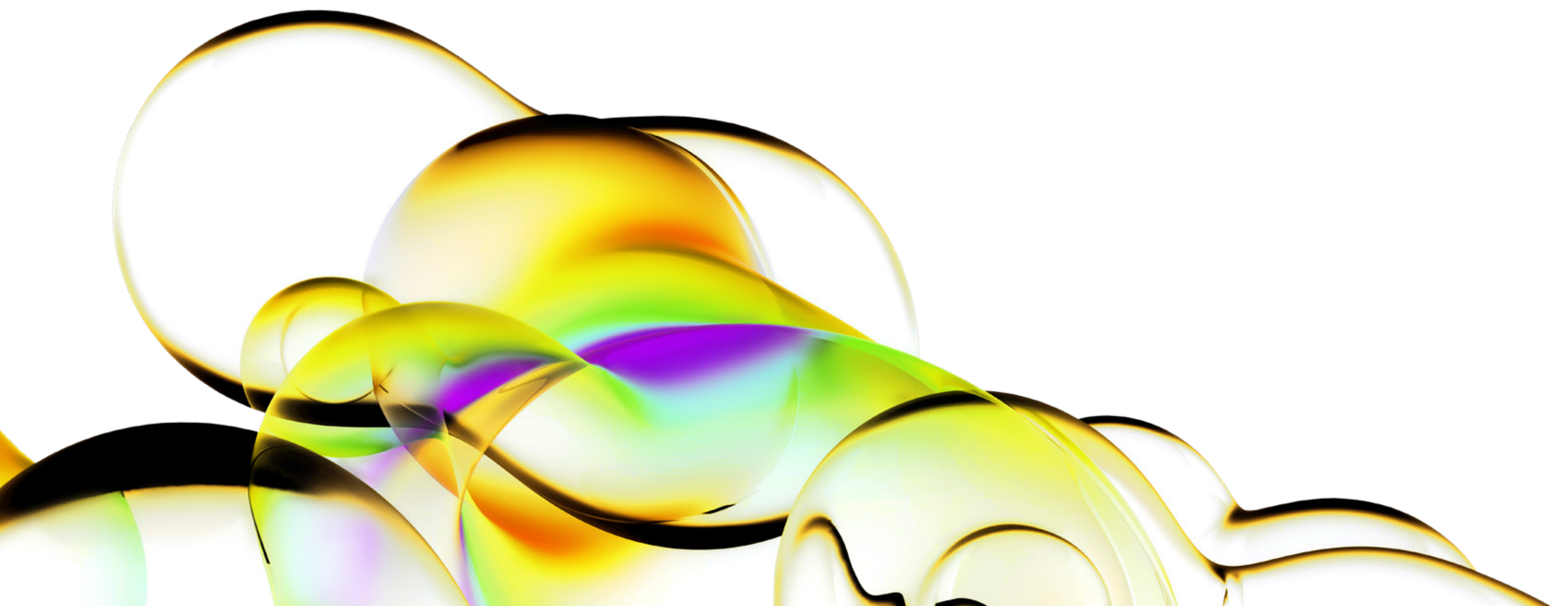
- Automated image acquisition using the Operetta™ high-content analysis system
- Data analysis using the versatile Harmony® software
- Image-based quantification of drug-induced cytotoxic effects on hepatocytes

Background

Cytotoxicity is a very complex process affecting multiple pathways. The ability to measure early indicators of toxicity is an essential part of drug discovery. An important approach to the detection of compound toxicity is a multiparametric analysis at the level of individual cells using high content analysis (HCA) applications [Abraham et al., 2008]. Such cell-based assays, which assess several cytotoxic events in a variety of relevant pathways, are considered valuable predictors of human drug toxicity. The liver, as a primary organ for drug metabolism, is the main target of many toxic effects, therefore many *in vitro* cellular cytotoxicity studies focus on human hepatocytes. [O`Brian et al., 2006]

Here we describe a rapid and flexible image-based live cell approach to study cytotoxicity. Based on the use of a fluorophore dye mixture, we analyzed multiple cellular phenotypic changes following a toxic insult. In order to investigate different drug-induced cellular responses, we treated human hepatocytes (HepG2) with various compounds.





Application

To study *in vitro* cytotoxicity using a high-content analysis approach, we treated HepG2 cells with FCCP[1], Tacrine[2] or AAP[3]. The cells were seeded at a density of 5000 cells / well in 384-well collagen-coated CellCarrier microtiter plates and were cultured overnight. After 24 h incubation with each compound, cells were stained with a fluorophore dye-cocktail (diluted in growth medium), containing Hoechst, BOBO™-3 and MitoTracker® Deep Red. After 40 min incubation with the dye cocktail, measurement of the live-cells was performed on the Operetta system equipped with a LWD 20x objective.

Figure 1 shows images of untreated and FCCP, Tacrine and AAP treated cells. After a 24 h treatment with each compound, significant intracellular and morphological changes of HepG2 cells can be observed.

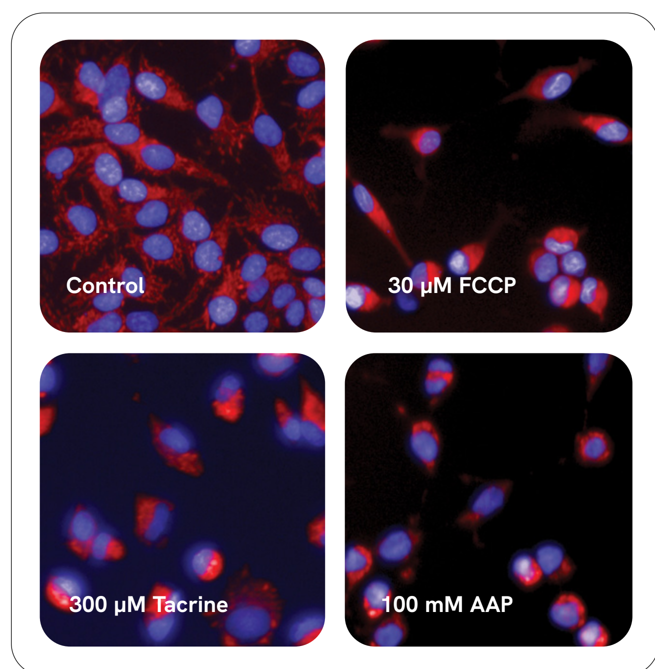


Figure 1: The Hoechst stained nuclei are shown in blue and the MitoTracker® Deep Red stained mitochondria are shown in red. The images show compound induced phenotypic changes, and a loss of both mitochondrial signal and cell number. The treatment of HepG2 cells with each compound results in an enhanced biogenesis of mitochondria and an increase in mitochondrial mass. A reduction of nuclear size due to toxic impact is also observed. The images were acquired on the Operetta system using the LWD 20x objective.

The acquired images were analyzed with the Harmony "Cytotoxicity-1" module. Harmony provides a wide range of building blocks to enable simplified custom image analysis. Each clearly defined building block has primary tuning parameters with optimized values that can be automatically or manually adjusted with real-time visual feedback to facilitate the set-up process. Figure 2 illustrates the image analysis strategy of the "Cytotoxicity-1" module.

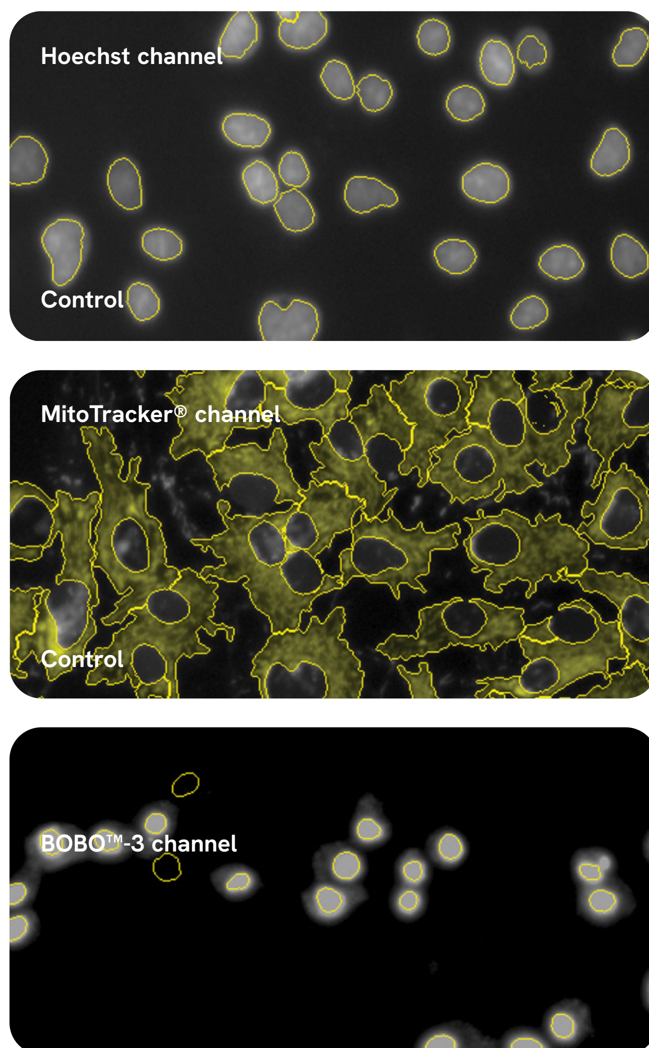


Figure 2: Image analysis strategy for quantification of compound cytotoxicity.
Top) The determination of the cell count, nuclear intensity and area is based on the detection of Hoechst stained nuclei.
Middle) A mask generated by finding the cytoplasmic outlines and excluding the nuclei is applied to determine the mitochondrial mass, calculated by measurement of the MitoTracker® intensity.
Bottom) The region used to calculate the intensity of BOBO™-3 staining is defined through nuclei detection. By introduction of a threshold (here: 1200), cells with disrupted membranes were classified as "dead".

The early effects of cytotoxicity can damage both cell structure and function. Almost all cellular structures and pathways are involved in cell proliferation and therefore, determination of the cell count is a very sensitive indicator of cell stress [O`Brian et al., 2006]. All three compounds examined here cause significant decrease in cell count. The sensitivity of this readout is demonstrated by the very low EC_{50} values (Figure 3 A).

Mitochondria are essential for the life of eukaryotic cells and their responses to toxic impact depend on the type of compound, on its concentration and on the specific mitochondrial function that is affected. Here, higher mitochondrial respiration leads to enhanced biogenesis of mitochondria, which results in increasing mitochondrial mass after 24 h treatment with each compound [O`Brian et al., 2006] (Figure 3 B).

Nuclear shrinkage and condensation as a result of toxic impact on cells typically occurs with cell injury. When cells were treated with FCCP and Tacrine, we observed a dose-dependent reduction in nuclear size to half of the initial value (Figure 3 C). This morphological change was accompanied by nuclear condensation, as determined by increasing nuclear intensity (Figure 3 D). AAP treatment initially results in a swelling of the nuclei, caused most probably by compound induced necrosis, prior to nuclear shrinkage (as described by Mirochnitchenko et al., 1999). The intensity of the nuclei correlates to the nuclear size; large nuclei show low intensities and small nuclei show high intensities (Figure 3 C and 3 D).

Cell membrane integrity is a well-known and common indicator of cell viability. Here, we measured membrane disruption by quantifying the influx of an extracellular dye, BOBO™-3 (Figure 4). As loss of membrane integrity is a late-stage effect of cytotoxicity, the EC_{50} values calculated by this readout (Figure 4) are considerably higher than the EC_{50} values of all other readouts (Figure 3 A-D).

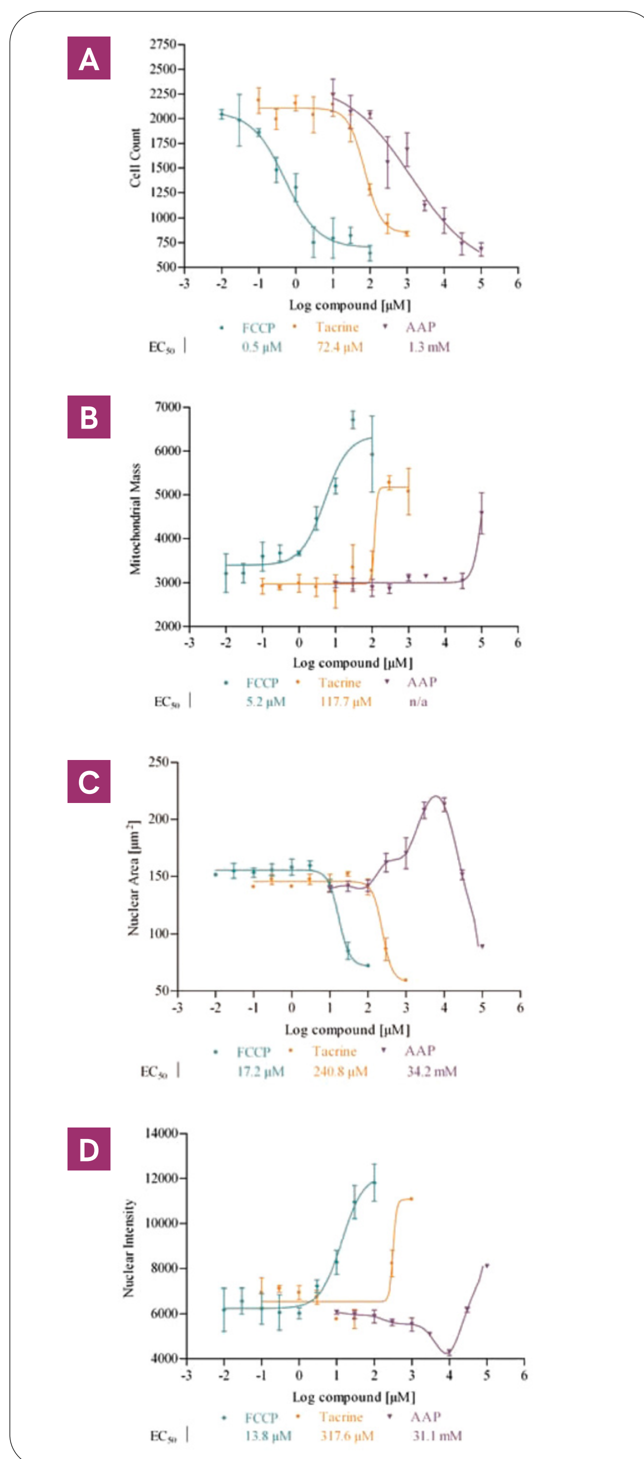


Figure 3: Compound-generated dose-response curves deduced from cell count (A), mitochondrial mass (B), nuclear area (C) and nuclear intensity (D). A) The effects of cytotoxicity on cell proliferation are similar for all three tested compounds; a significant loss of cells was observed with increasing concentrations of each compound. B) Compound treatment of HepG2 cells leads to an increase in mitochondrial mass caused by enhanced biogenesis of mitochondria. C/D) The phenotypic changes induced by FCCP and Tacrine manifests in nuclear shrinkage accompanied by nuclear condensation. AAP treatment initially causes nuclear swelling prior to nuclear shrinkage and condensation. N = 3 wells.

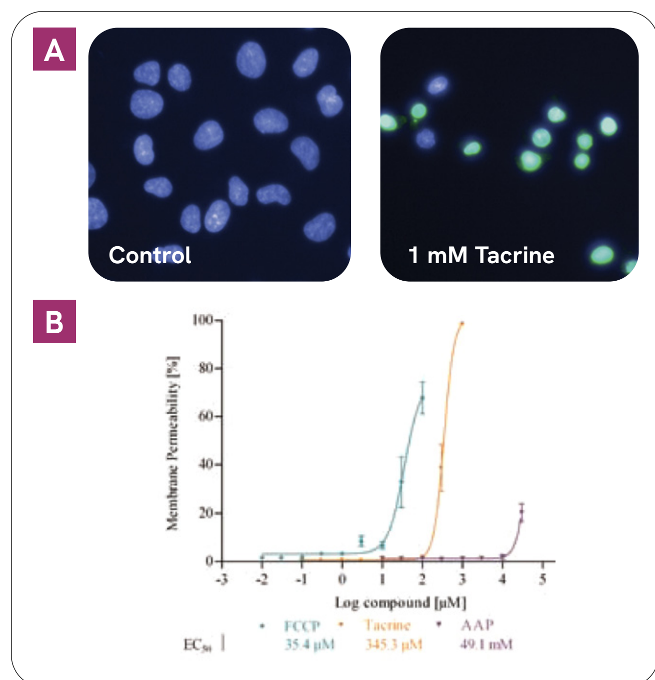


Figure 4: A) False color overlay of untreated and 1 mM Tacrine treated cells, showing Hoechst (blue) and BOBO™-3 (green) stained nuclei. Cells with intact membranes show Hoechst staining only (left). Loss of membrane integrity allows the cell-impermeant dye BOBO™-3 to enter the cells and binding to the DNA (right). B) Dose-response curves for FCCP and Tacrine deduced from membrane permeability, show a significant increase. The cell membrane disruption caused by high AAP concentrations is marginal. N = 3 wells.

Conclusions

The detection of compound cytotoxicity is an essential part of drug discovery. Here, we present an *in vitro* cytotoxicity approach using HepG2 cells, which are used as an effective indicator of the human hepatotoxicity potential of test compounds.

The high content analysis approach presented here is a rapid and robust live cell assay to determine multiple cytotoxic effects simultaneously. Furthermore, this assay detects early as well as late-stage occurrences of cytotoxicity, as the calculated EC_{50} values clearly demonstrated. Moreover, this HCA approach enables flexibility of assay design for adding or replacing an organelle dye.

References

Abraham VC, Towne DL, Waring JF, Warrior U, Burns DJ (2008): Application of a high content Multiparameter Cytotoxicity Assay to Prioritize Compounds Based on Toxicity Potential in Humans. Journal of Biomolecular Screening 13 (6), 527-537.

Mirochnitchenko O, Weisbrot-Lefkowitz M, Reuhl K, Chen L,Yang C, Inouye M (1999): Acetaminophen Toxicity. The Journal of Biological Chemistry 274 (15), 10349-10355

O`Brian P, Haskins R (2006): *In Vitro* Cytotoxicity Assessment. Book Title: High content screening: A Powerful Approach to Systems Cell Biology and Drug Discovery. Methods in Molecular Biology 356, 415- 425.

Authors

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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 is a parasympathomimetic and centrally acting cholinesterase inhibitor used in the treatment of Alzheimer`s disease. The isozyme cytochrome P450 is involved with the metabolism of Tacrine in the liver, resulting in an active metabolite, which is associated with a high frequency of hepatotoxicity.*

3. *Acetaminophen (AAP) is a widely-used analgesic and antipyretic. It is well-known that it causes potentially fatal liver damage and hepatic necrosis if overdosed. The toxic effect of Acetaminophen is primarily due to a highly-reactive intermediary metabolite (NAPQI). NAPQI is metabolized via the hepatic cytochrome P450 enzyme system and at usual doses quickly detoxified by conjugation with glutathione.*

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