

Image-Based Quantification of Cytotoxicity by Vital Dyes using the Opera™ High Content Screening Platform

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1 Introduction

Cytotoxicity is a very complex process affecting multiple pathways and is not manifested by determining one morphological or intracellular parameter. However, the ability to measure early indicators of toxicity is an essential part of drug discovery. Cell-based High Content Applications are a powerful tool for determining several events in cytotoxicity simultaneously. Here we describe a rapid and flexible **dye-based** high content cytotoxicity assay performed with the widely-used HepG2 (human hepatocellular carcinoma) cells. In order to show different effects of cellular cytotoxic responses to compound treatment, the impact of Tacrine¹, FCCP² and Acetaminophen³ (AAP) on the **nuclear size, cell membrane integrity, mitochondria** and on **cell proliferation** is investigated. Our **live cell** High Content Assay presented here is based on the application of a fluorophore cocktail consisting of three different organelle dyes to analyze multiple read-outs simultaneously. Furthermore **no washing** steps are included in the assay procedure after compound treatment thus no false negative read-outs because of cell-loss are recorded.

We demonstrate that our High Content Cytotoxicity approach using the Opera™ platform in combination with the Acapella™ image processing software is well suited for providing reliable results of compound toxicity on HepG2 cells.

¹: Parasympathomimetic and centrally acting cholinesterase inhibitor used in the treatment of Alzheimer's disease. The metabolism of tacrine results in an active metabolite, which is associated with a high frequency of hepatotoxicity.

²: A very potent uncoupler of oxidative phosphorylation in mitochondria which acts through degrading the linkage between respiratory chain and the phosphorylation system used to generate ATP.

³: Well-known as Paracetamol and to cause potentially fatal liver damage and hepatic necrosis if overdosed. Primarily responsible for the toxic effect is the highly-reactive intermediary metabolite NAPQI.

2 Material & Methods

HepG2 cells were cultured in growth medium, i.e. DMEM-F12 supplemented with 10% FBS and 2 mM L-Glutamine. For subcultivation cells were detached with trypsin/EDTA after washing them once with PBS. Low passage (5-20) cells were plated in Collagen I coated 384-well CellCarrier microplates (PerkinElmer Inc) at a density of 4000 cells in 50 µl growth medium per well and incubated for 16 h to 24 h at 37°C, 5% CO₂ before compounds were added. After removing the growth medium, cells were exposed to various concentrations of **compounds** diluted in growth medium for **24 h** treatment at 37°C, 5% CO₂. Afterwards cells were stained with a **fluorophore cocktail** containing *Hoechst* (1 µM) *BOBO-3* (0.75 µM) and *MitoTracker® Deep Red* (0.3 µM) (Invitrogen). 25 µl of a 3-fold concentrated dye-cocktail was added to the wells and incubated for further 45 min at 37°C and 5% CO₂. The measurement of the plate was subsequently performed on the Opera™ High Content Screening System.

3 Image Acquisition on the Opera™

Images were acquired on the high content screening platform Opera™ QEHS, which is equipped with up to five excitation sources (405, 488, 561, 640 nm lasers and UV lamp). Simultaneous detection of up to four channels (three CCD cameras on confocal light paths, one CCD for non-confocal UV), together with water immersion lenses, allows high speed acquisition with high resolution and high content. The Opera includes a fast working laser-based autofocus system, with options to add climate control for live cell imaging applications and an automated dispensing.

For this experiment a high resolution 20x water immersion objective was used. Hoechst was excited with the 405 nm laser and the emission was captured on camera 1 using a 450/50 bandpass. BOBO-3 was excited with the 561 nm laser, emitted fluorescence was collected on camera 2 using the 600/40 bandpass filter. MitoTracker Deep Red was excited with the 635 nm laser and the emitted fluorescence was captured on camera 3 using a 690/50 bandpass filter. Images together with **Acapella™** analysis results can be stored in the **Columbus** Gallery, a database system that allows complex HCS multi-channel images to be stored and accessed by multiple users, providing a convenient and easy to use solution to high volume image management.

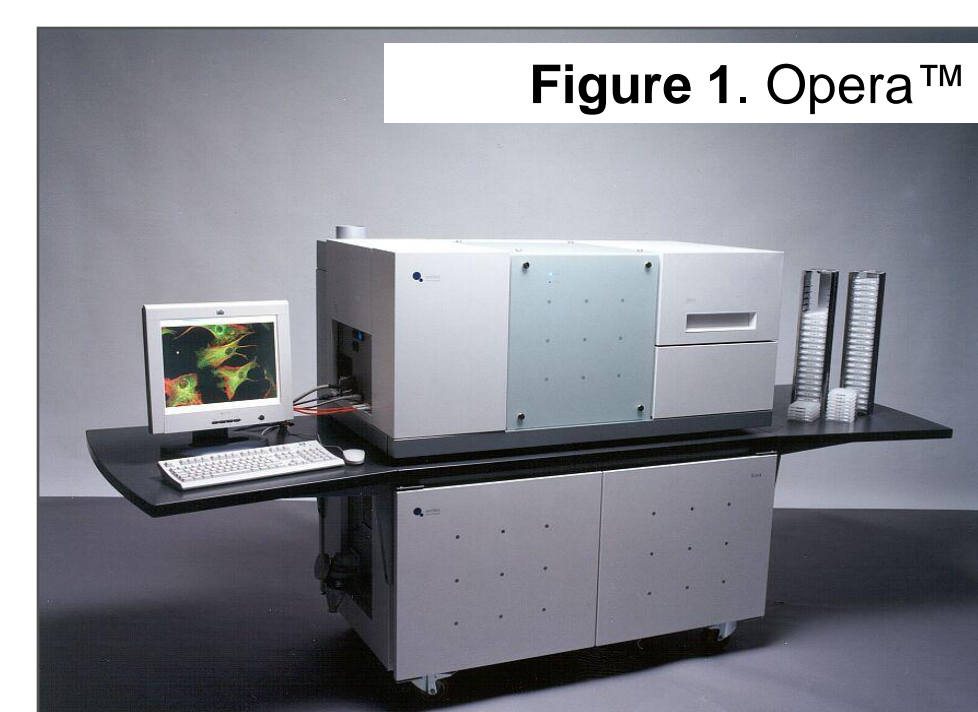


Figure 1. Opera™

4 Acapella™ Image Analysis

The Acapella™ image analysis platform allows precise quantification of the location, shape, structure and amount of fluorescence on an individual cell level. A set of readymade analysis algorithms - known as **Scripts** - are available for a broad range of standard situations like counting, translocation, spot analysis, etc. These Scripts have been used to create the data presented here. Due to its high speed Acapella™ is ideally suited for on the fly analysis and analysis of large data sets. We analyzed the acquired images using the **LiveDead** script to determine the cell permeability. Cells with intact membranes were counted on the Hoechst channel and cells with disrupted membranes on the BOBO-3 channel. Based on the Hoechst stained images the **CellRegionAnalysis** script calculated the nuclear area and the cell count, based on the MitoTracker® stained images the mitochondrial mass was calculated (Fig.2).

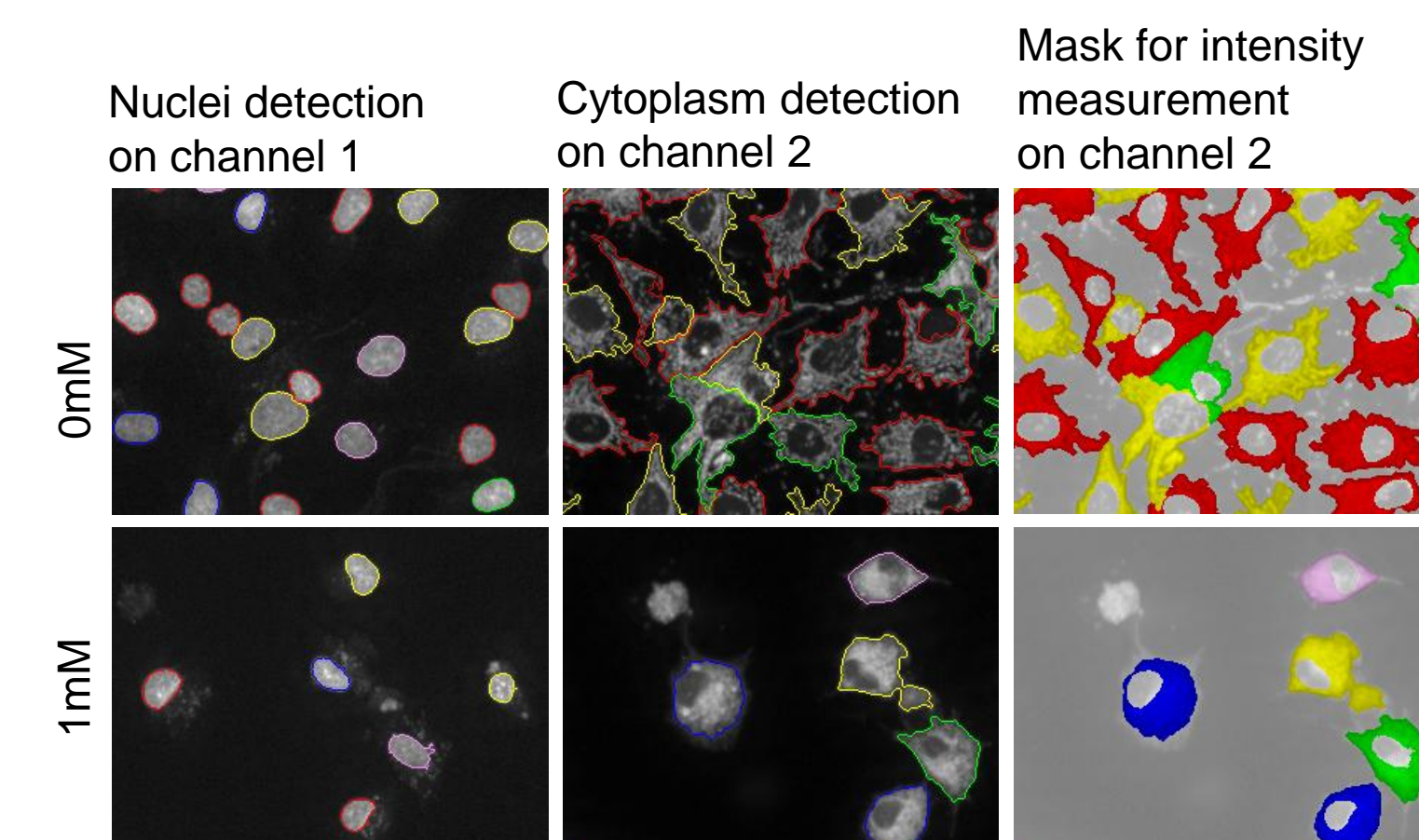


Figure 2. Image analysis strategy for quantifying cell count, nuclei size and mitochondrial mass in untreated and 1mM (after 24 h) Tacrine treated HepG2 cells with the Acapella™ script **CellRegionAnalysis**.

Left: Channel 1, which represents the Hoechst stained nuclei, was used for the nuclei detection and for the calculation of the read-out parameters nuclear area as well as cell number.

Middle: Channel 2, the MitoTracker® Deep Red stained Mitochondria was used for cytoplasm detection and thereafter for determining the intensity of the mitochondria.

Right: Based on the results of the cytoplasm detection a mask, excluding the nuclei, for the intensity calculation of stained mitochondria was generated. By defining the cytoplasm region based on the stained mitochondria we created a variable mask for the intensity measurement adapting to mitochondria relocation due to compound treatment.

5 Results

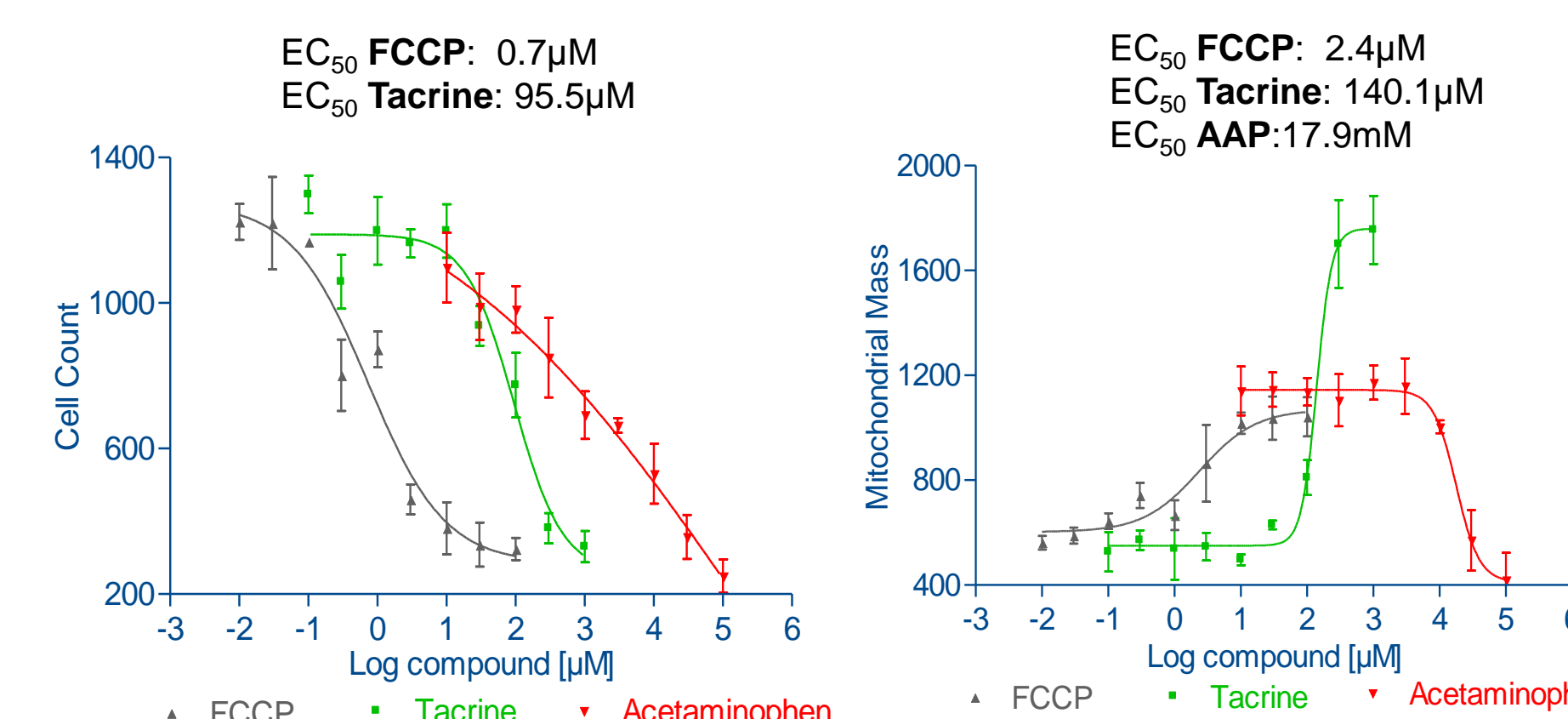
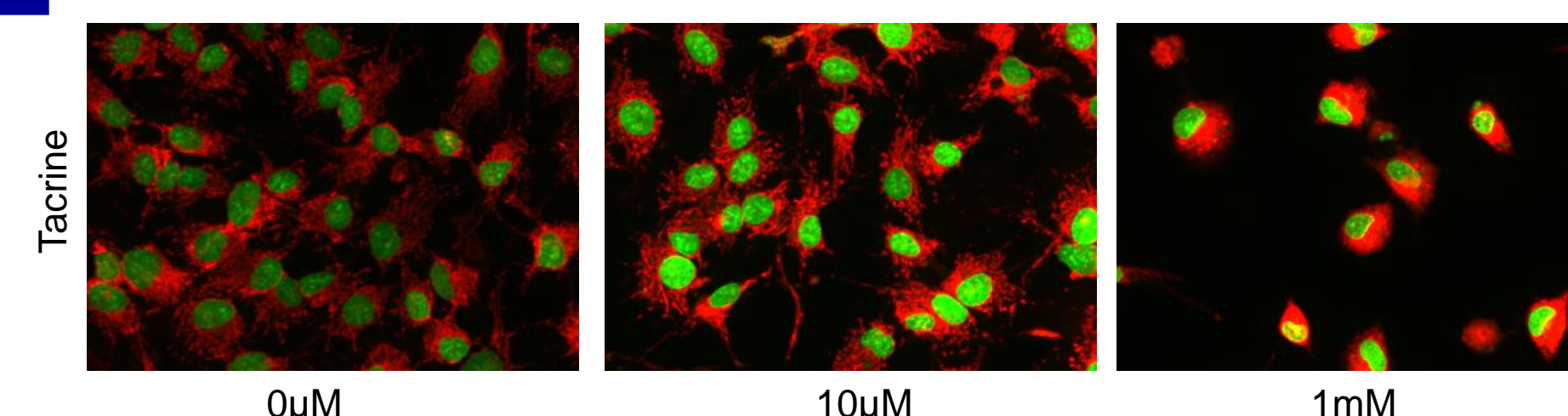


Figure 3.
A | Tacrine treated cells at various compound concentrations. The images show a significant dose-dependent increase of mitochondrial signal caused by enhanced mitochondrial biogenesis as well as loss of cells accompanied by phenotypic changes due to damages in cell structure and function (green – nuclei & red – mitochondria).
B | Numerical data of compound-generated dose response curves deduced from **cell count** (left) and **mitochondrial mass** (right).

Left: The toxic effects on cell proliferation are similar for tested compounds; loss of cells with increasing concentrations. Determining the cell count in a live cell assay is accepted as the most sensitive indicator of cell stress as almost all cellular structures and pathways are involved in cell proliferation.

Right: Mitochondrial responses to cytotoxins depend on the type of compound, on its concentration and on the specific mitochondrial function that is affected. The effect of Tacrine and FCCP on the mitochondria of HepG2 cells leads to an increase in mitochondrial respiration by decreasing the mitochondrial transmembrane potential. Loss of mitochondrial ion balance and inhibition of mitochondrial respiration by the reactive metabolite NAPQI is involved in the toxic mechanism of AAP on liver cells.

N = 4 wells

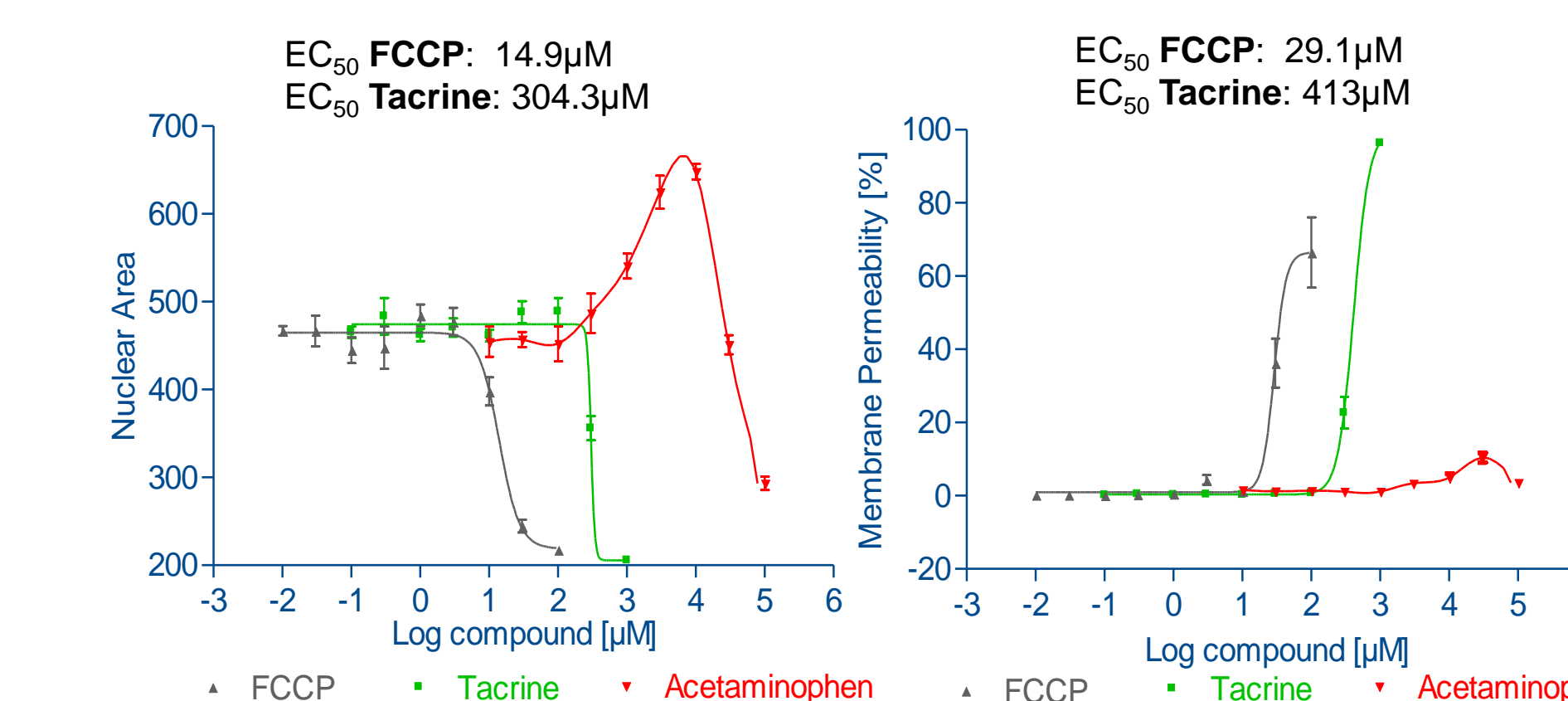
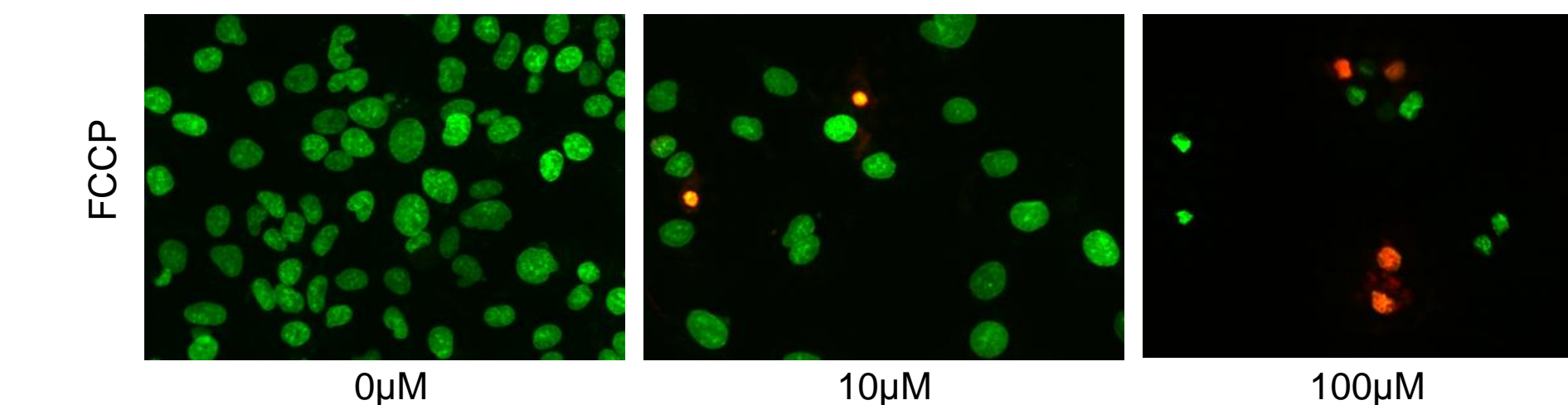


Figure 4.
A | Two channel overlay of Hoechst stained nuclei (green) and BOBO-3 stained nuclei (red) of untreated and 100 µM **FCCP** treated HepG2 cells. As expected BOBO-3 stained cells only occur at high compound concentrations. Also clearly visible is the nuclear shrinking, a typical cytotoxic effect on nuclei.

B | Numerical data of compound-generated dose response curves deduced from **nuclear area** (left) and **cell membrane permeability** (right).

Left: Nuclear shrinkage as a result of toxic impact on cells typically appears with cell injury. We showed for FCCP as well as for Tacrine a dose-dependent reduction of nuclear size to half of the initial control value. For AAP treated HepG2 cells we initially observed swelling nuclei caused by compound induced necrosis prior to nuclear shrinkage.

Right: Cell membrane disruption is a common indicator of cell death and usually occurs in late stages of cytotoxicity as the EC₅₀ values calculated demonstrate. FCCP and Tacrine treated HepG2 cells show a significant increase in cell membrane permeability, whereas the AAP treated cells show marginal cell membrane disruption with increasing concentrations.

N = 4 wells

6 Conclusions

Nowadays *in vitro* cytotoxicity cell-based studies are used as an effective indicator of human toxicity of potential drugs.

We demonstrated a rapid, time saving and easy to use live cell HCS cytotoxicity application using the Opera™ platform in combination with the Acapella™ image processing software. This application is well suited for providing reliable results of compound toxicity on HepG2 cells. By applying a multi-labeling dye-cocktail combined with multi-parametric read-outs this application can detect early as well as late stage occurrences of cytotoxicity.

Furthermore, this HCA approach can be easily modified by replacing one organelle dye with another or it can be easily multiplexed with an additional one.