

## Multimode Detection

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## Multiparametric Analysis of Cytotoxicity using Cell Imaging in Combination with Luminescence

### Introduction

Cytotoxicity can be caused by different types of agents, including drugs, pathogens, immune cells and external stress factors such as heat. Affected cells die by apoptosis, necrosis or even by necroptosis, a recently discovered

pathway [Vandenabeele *et al.*, 2010]. All of these processes include complex signaling programs that lead to a variety of different phenotypes. Morphological changes include nuclear fragmentation, cell shrinkage, membrane blebbing or loss of membrane integrity, leakage of cell content, and swollen nuclei.

Cytotoxicity is an ideal application to be analyzed using cell imaging, in addition to conventional cytotoxicity assays [O'Brien, 2014]. Particularly during the drug development process, where cytotoxicity remains one of the major causes of drug withdrawal, there is an urgent need for reliable and time-saving assay workflows. The EnSight™ Multimode Plate Reader combines well-imaging technology with label-free and standard labeled technologies, meaning that orthogonal assays can be performed on the same instrument and in the same well, leading to more robust data, more quickly.

This application note shows how multiparametric analysis of cytotoxicity, using different technologies, can be easily performed on just one sample to gain a complete picture of the effects of cytotoxicity. We have investigated cytotoxic effects using pre-defined image analysis algorithms provided by the EnSight system's Kaleido™ Data Acquisition and Analysis Software. Analysis of stainless brightfield images was combined with nucleus and cytoplasmic enzyme activity analysis using fluorescent images. Our imaging results were verified with a standard luminescence ATP detection assay.

## Materials and Methods

### Cell Seeding

Frozen stocks of HeLa cells were thawed in a water bath for one minute at 37 °C, washed once to remove the freezing solution and re-suspended in phenol red-free cell culture medium following regular cell culture protocols. The cell number was determined using an automated cell counter. The cells were then seeded in 30 µl per well of filtered, FCS-containing, phenol red-free cell culture medium at a density of 1.5 K cells per well in a ViewPlate™ -384 microplate (PerkinElmer, #6007460). The plate was incubated under the hood for 20-30 minutes to let cells settle. Cells were then incubated at 37 °C and 5 % CO<sub>2</sub> for 18-24 h before treatment.

### Cell Treatment

Cells were treated with 10 µl per well of a serial dilution of DMSO in the same cell culture medium as used for seeding. To do this, 40 % DMSO in cell culture medium was serially diluted 1:1.5 for 11 steps, resulting in a dilution series with 10 % DMSO as the highest concentration. Z' high and Z' low samples were treated with 10 % DMSO (final concentration) and cell culture medium only, respectively. The cells were incubated for 24 h at 37 °C and 5 % CO<sub>2</sub>.

### Cell Staining

After DMSO treatment and incubation, the cells were stained with Hoechst 33342 (Life Technologies, #H3570) and Calcein AM (Life Technologies, #C3100MP). Hoechst 33342 is a well-known nucleic acid stain. It is cell-permeant (stains live and dead cells) and emits blue fluorescence when bound to dsDNA. Calcein AM is also cell-permeant, but is converted to a fluorescent dye and retained in live cells only. The non-fluorescent Calcein AM is converted to a green-fluorescent Calcein only after acetoxymethyl ester hydrolysis by intracellular esterases. It can, therefore, be used to determine cell viability in most eukaryotic cells. Cell staining was achieved by preparing a 2 x concentrated staining solution, consisting of both dyes and the same cell culture medium as used for seeding. 40 µl of staining solution was then added to each well using the JANUS® MDT Automated Workstation, resulting in a final dye concentration of 5 µg per ml Hoechst 33342 and 0.5 µM Calcein. Cells were incubated for 60 minutes at 37 °C.

### Image Acquisition and Evaluation

Images were acquired using the EnSight Multimode Plate Reader, equipped with well-imaging module, and Kaleido Data Acquisition and Analysis Software. Brightfield images were taken before and 24 h after DMSO treatment to allow for a confluency comparison between both time points. Confluency was determined using the pre-defined *Brightfield Confluency* algorithm provided by the

Kaleido software. Fluorescent images were collected only after 1 h stain incubation. To analyze nuclear and cytoplasmic characteristics, the pre-defined Kaleido *CytoNuc* algorithm was used.

Secondary data analysis was performed using TIBCO Spotfire® software. All error bars shown in the dose-response curves indicate standard deviation for five replicates. Z' values were determined using 12 replicates each of cells treated with medium only or 10 % DMSO.

### Luminescence Measurement

After well-imaging, the JANUS MDT Automated Workstation was used to remove 40 µl of staining solution from each well and to add 40 µl of ATPlite one step Luminescence assay solution (PerkinElmer, #6016731). All subsequent steps were performed according to the kit instructions. To reduce cross talk, the plate was covered with BackSeal, a white adhesive bottom seal (PerkinElmer, #6005199), prior to luminescence measurements. Luminescence was measured using the default settings on the EnSight system.

## Results

### One Sample – Three Assays

For the multiparametric cytotoxicity analysis, we used HeLa cells as a model system. The cells were treated with a serial dilution of our test compound DMSO to determine the effect of increasing concentrations on different aspects of cytotoxicity, such as cell attachment, nuclear characteristics, intracellular esterase activity and ATP quantity. These analyses require different technologies to be employed. The EnSight system enabled us to combine stainless brightfield and fluorescent imaging approaches with luminescence measurements within the same well (Figure 1). This easy and time-saving workflow permits investigation of the same cells from different angles leading to a reduction in artifacts caused by inter-well variability and allowing additional insights into biological effects.

### Brightfield Confluency Analysis Reveals DMSO-Dependent Cell Shrinking and Detachment

Confluency analysis, which determines the percentage of the image area covered by cells, is a well-known, indirect readout for the health status of cells. Toxic compounds often bring about detachment and condensation of adherent cells. As a result of this the cells appear as round, high-contrast objects in the image compared to healthy cells which show a normal adherent morphology (Figure 2, A2 and B2).

We determined the confluency of cells before and after DMSO treatment. By considering only the confluency determined after DMSO incubation, we showed that the confluency changes in a dose-dependent fashion, with higher DMSO concentrations leading to lower confluency values (Figure 3, left). However, this analysis does not allow distinction between cytostatic (cell growth inhibition) or cytotoxic (cell death) effects. In contrast, subtraction of the confluency determined before DMSO addition clearly reveals that cells treated with more than 4 % DMSO are not only inhibited in cell growth (change in confluency = 0 %), but also suffer from cell death (negative values for cell confluency change, Figure 3, right). Additionally, this normalization leads to a more robust assay with Z' values of 0.77 compared to a non-reference assay.

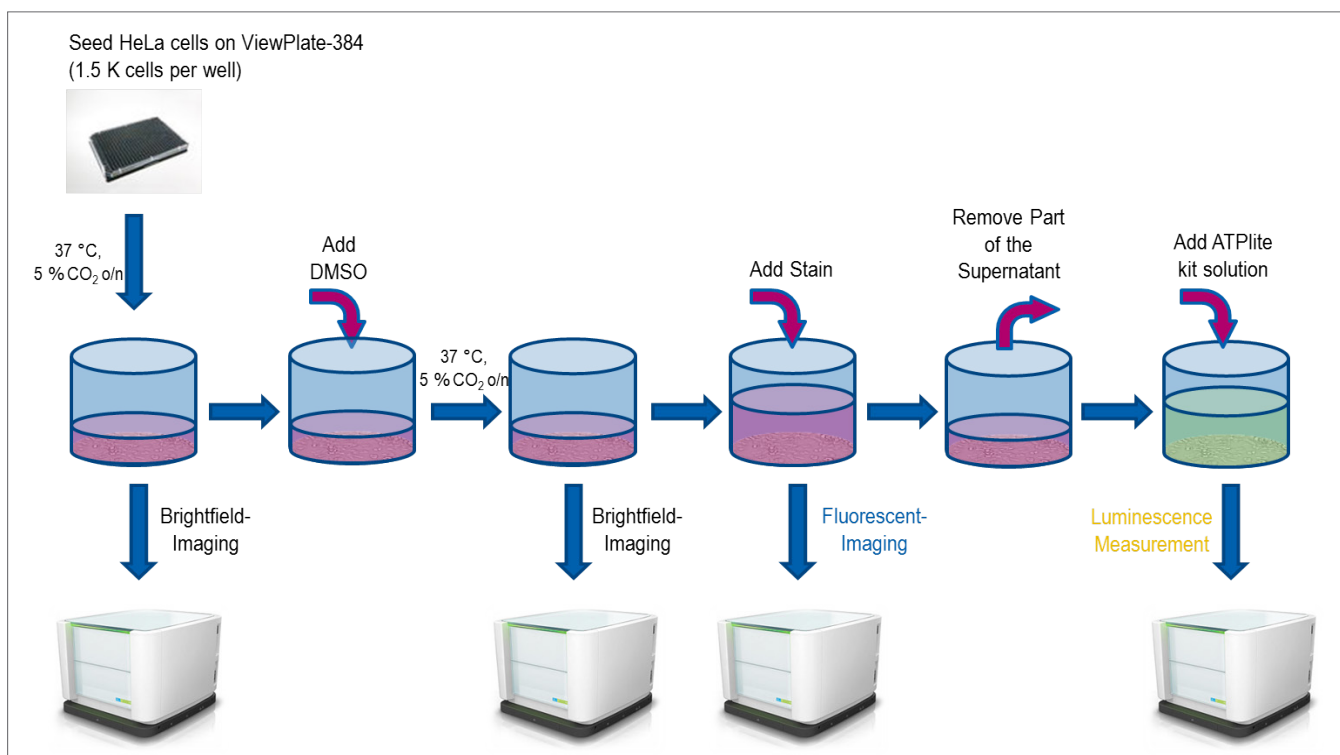


Figure 1. The workflow for multiparametric cytotoxicity analysis combines brightfield and fluorescent imaging with a luminescence readout to investigate different aspects of cytotoxicity in the same well.

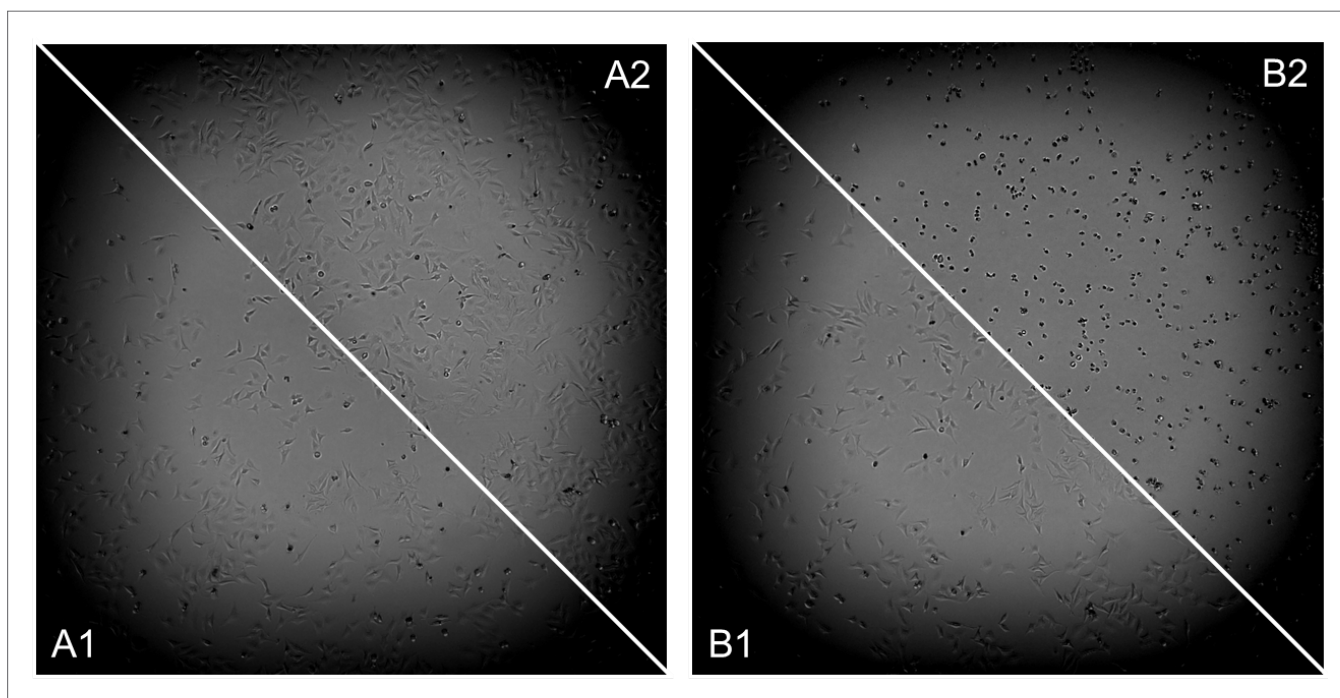


Figure 2. Brightfield images of two wells before (A1 + B1) and after 24 h incubation (A2 + B2). Cells were incubated in either cell culture medium only (A2) or in 10% DMSO (B2). A difference in cell morphology between non-treated (A2) and treated (B2) cells is clearly visible.

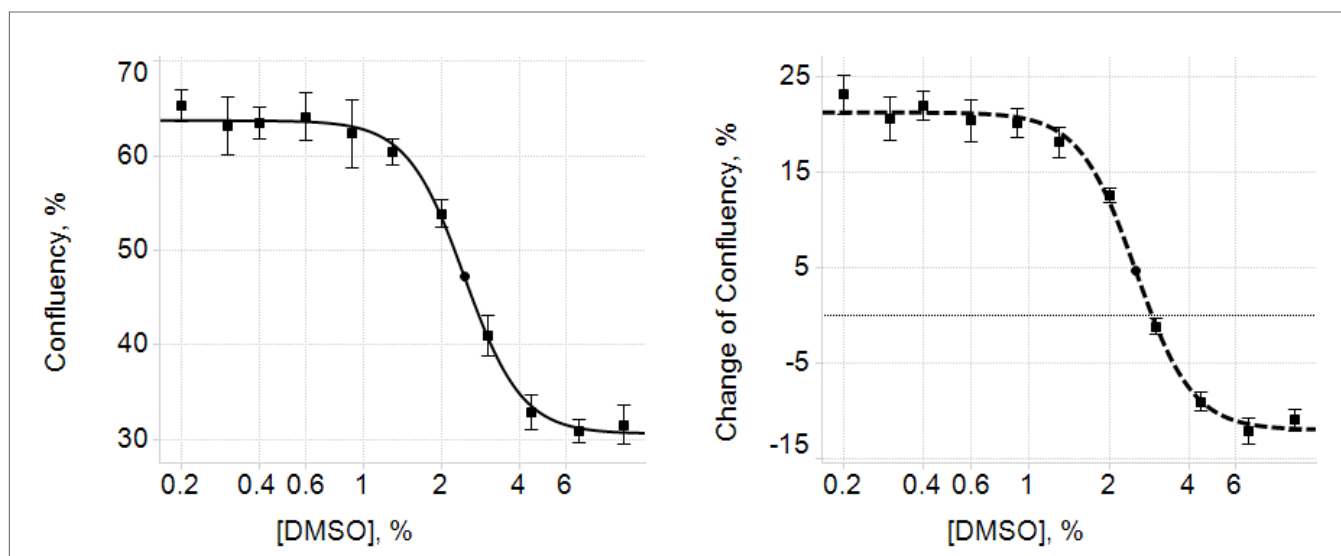


Figure 3. Cell confluency was analyzed based on brightfield images using a pre-defined Kaleido algorithm. After treatment, the confluency decreases in a dose-dependent manner with increasing DMSO concentrations.  $EC_{50}$  was determined as 2.44 % DMSO with a  $Z'$  of 0.73 (left). Subtraction of the confluency determined before the treatment leads to a comparable  $EC_{50}$  of 2.52 % DMSO, but a more robust assay as represented by an enhanced  $Z'$  value of 0.77, and allows for analysis of cytostatic and cytotoxic effects (right).

Another parameter that is provided by the pre-defined *Brightfield Confluency* algorithm is the foreground roughness parameter. This describes the texture and pattern of the area covered by cells and can also be used to reliably characterize cytotoxicity. As shown in Figure 4, the foreground roughness increases with elevated DMSO concentrations due to rounding up and detachment of dying cells that appear with higher contrast, also visible in Figure 2, B2.

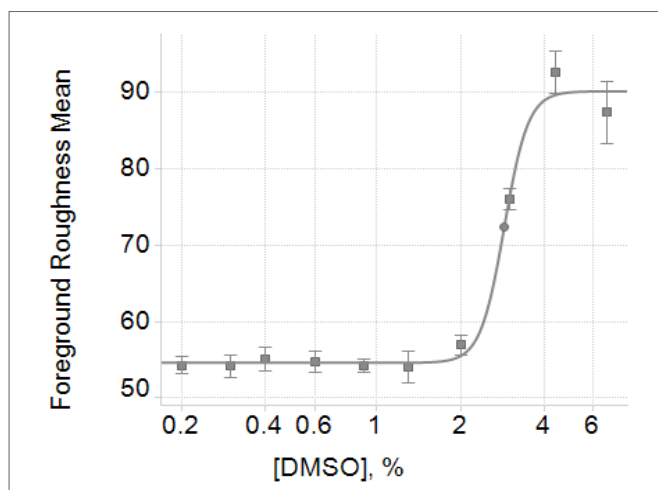


Figure 4. The mean foreground roughness (texture of the area covered by cells) is enhanced at higher DMSO concentrations and reflects an increase in roundness and detachment of cells.  $EC_{50}$  was determined as 2.87 % DMSO.

## Fluorescent Imaging of the Nucleus and Cytoplasm Uncovers Cell Death

In contrast to brightfield imaging, which mainly determines morphological properties of cells, fluorescent imaging allows for more detailed analysis by distinguishing between the nucleus and the cytoplasm. Furthermore, it can be used for suspension cells whose health status is difficult to determine in brightfield images based on their round morphology.

There are many different dyes available with diverse biochemical modes of action that can be used to study toxicity. Here, we have focused on Hoechst 33342 as a cell-permeant nuclear stain which stains all cells (live/dead) in the assay. Furthermore, we used the cell-permeant Calcein AM stain that shows fluorescence only if it is cleaved by intracellular esterases and which is only retained in cells with intact cell membranes. Together, both dyes allow for detailed analysis of the effects of toxicity on the nucleus and enzyme activity in the cytoplasm. Figure 5 shows representative detailed images for both dyes, acquired using the EnSight system.

Image analysis of the nucleus and cytoplasm was performed using the pre-defined Kaleido *CytoNuc* algorithm. This was set to detect the nuclei of cells based on Hoechst 33342 staining, and to determine the Calcein AM intensity of the cytoplasm in a ring structure around the nucleus. This analysis provides several output parameters that describe the properties of the nucleus and cytoplasm in treated versus untreated cells, and therefore reflects cytotoxic effects.

As shown in Figure 6, the number and median area of the nuclei decrease with increasing DMSO concentrations, indicating that the cells die and the nuclei shrink. The latter is often associated with chromatin condensation in apoptotic cells and leads to elevated median nucleus intensities in the images.



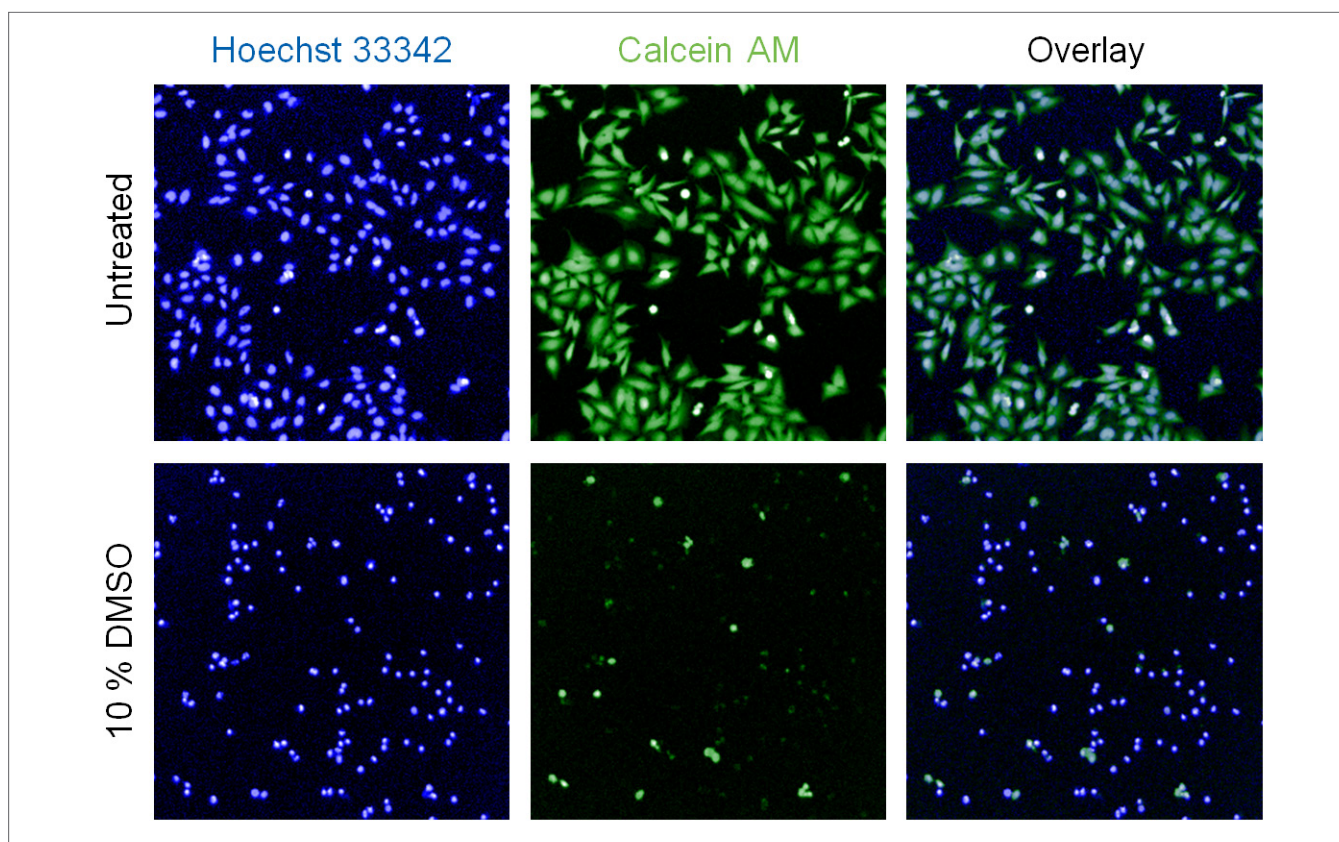


Figure 5. Untreated HeLa cells were incubated in cell culture medium for 24 h before staining (top). They show both homogeneous Hoechst 33342 staining in the nucleus and Calcein AM staining in the cytoplasm. In contrast, cells treated with 10 % DMSO (bottom) result in small Hoechst 33342-stained nuclei and only a small number of cells that are positive for Calcein AM.

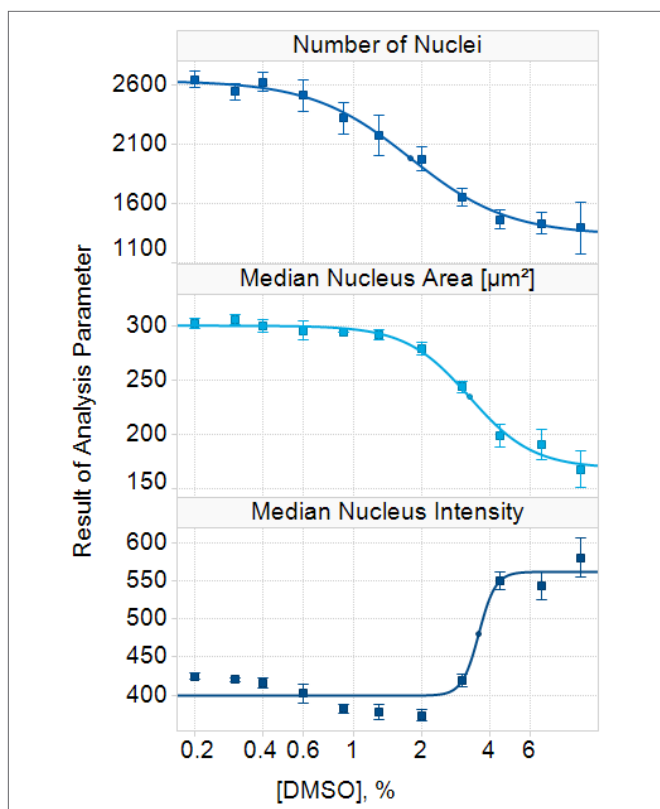


Figure 6. Nuclei characteristics of DMSO-treated cells were determined using pre-defined Kaleido image evaluation algorithms. Cytotoxic effects are indicated by a decreasing nuclei number and median area of nuclei, and an increase in the median nucleus intensity.  $EC_{50}$  value determination was 1.77, 3.24 and 3.56 % DMSO for the nuclei number, area and intensity, respectively.  $Z'$  evaluation yielded values of at least 0.5.

Calcein AM-converting enzyme activity in the cytoplasm was evaluated by determining the median intensity of the Calcein AM signal. To detect the cytoplasm, the *CytoNuc* image evaluation algorithm was used. Firstly, cells were identified using the nuclear stain. Based on this, an adjustable ring structure around the nuclei was defined in which the median Calcein AM signal intensity was determined.

The Kaleido software also enables the user to detect cells based on stainless digital-phase images instead of a nuclear stain. Both image evaluations result in very similar dose-response curves (Figure 7) and show a decrease in enzymatic activity indicating an impaired cell membrane and cell death.

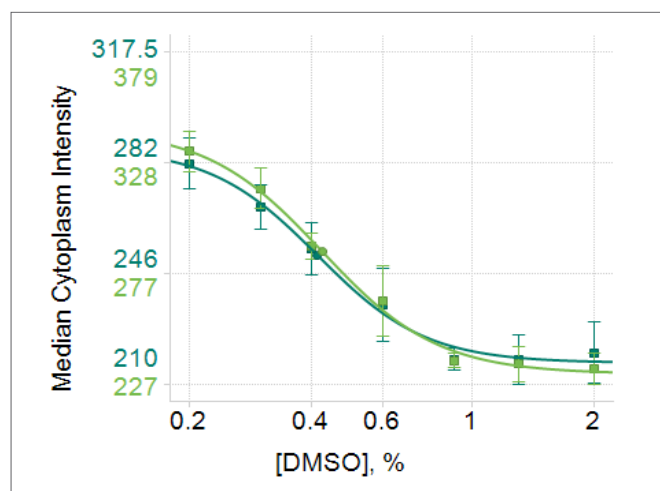


Figure 7. The cytoplasm is characterized by the median intensity of the Calcein AM stain which is fluorescent only in live cells and which diminishes with concentrations up to 2 % DMSO. The intensity was either determined in the ring structure defined around the detected nuclear stain (light green), or in a ring structure defined inside the detected cells using digital-phase images (dark green). EC<sub>50</sub> analysis resulted in 0.42 % DMSO for nuclear based cell detection and 0.41 % DMSO for cell detection using digital-phase imaging.

### Luminescent ATP Detection Assay Confirms Cytotoxicity

A frequently used approach for detecting cell death is determination of the intracellular levels of ATP. This is often measured using assay kits that are based on the luminescent light which is produced if luciferase converts Luciferin. To measure the ATP levels in DMSO treated cells, part of the supernatant was removed after 2 h stain incubation and the ATPlite 1 step assay kit solution was added instead. To keep pipetting variability at a minimum both steps were performed utilizing the JANUS MDT Automated Workstation. Figure 8 shows the DMSO dose-dependent luminescence signal of cells with residual staining solution still present in the supernatant. Unstained cells were tested in parallel and gave similar results (data not shown), indicating that the staining solution neither interferes with the luminescence measurement nor leads to increased cytotoxicity. Therefore, Figure 8 verifies the cytotoxic effect of DMSO, as shown by the loss of ATP, and confirms the well-imaging based results shown above.

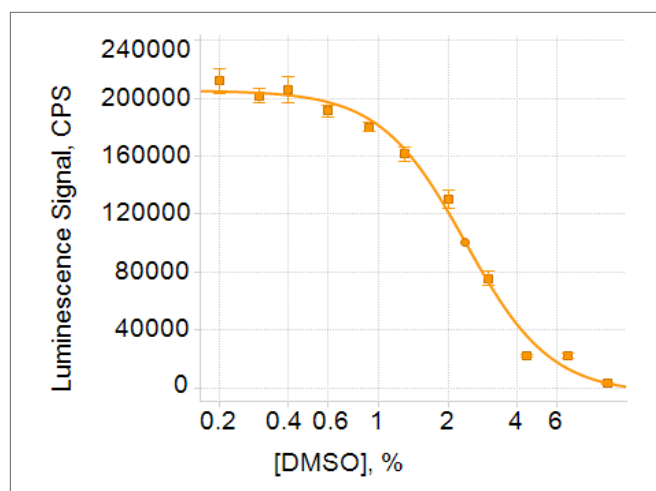


Figure 8. The intracellular ATP levels, represented by the Luminescence readout, decreases in response to increasing DMSO concentrations. The EC<sub>50</sub> was determined as 2.37 % with a high robustness of Z' = 0.8.

### Discussion

In this application note we have demonstrated an easy-to-apply workflow for cytotoxicity analysis. Using the EnSight system and the Kaleido software's pre-defined image analysis algorithms, we examined a significant number of analysis parameters and their dose-dependencies.

Table 1 demonstrates that our test compound DMSO has a potency of around 2-3 % to induce toxicity for all parameters focusing on cell or nuclear morphology. In contrast, the cytoplasmic enzyme activity measured by Calcein AM fluorescence is impaired at much lower DMSO concentrations. Impaired Calcein AM conversion is known to be an early event in apoptotic cells [Gatti *et al.*, 1998], which shows that our multiparametric approach is able to analyze and distinguish between different levels of cytotoxicity using fluorescent dyes.

Stainless brightfield confluency analysis serves as a robust indicator for toxicity in adherent cells. It is non-hazardous to cells, can be easily applied in every labeled or label-free cell-based assay, and also enables discrimination between cytostatic and cytotoxic effects.

Table 1. Summary of EC<sub>50</sub> values, which were determined for each analysis parameter based on the dose-response curves shown above. The colors mark parameters that were collected for the same analysis target.

Analysis Target	Analysis Parameter	Description	DMSO Evaluation Range, (%)	EC <sub>50</sub> , (%)
Cell Morphology	Confluency [%]	Degree of Area Covered by Cells	0-10	2.44
	Change of Confluency [%]	Confluency After Treatment Minus Confluency Before Treatment	0-10	2.52
	Foreground Roughness Mean	Texture of Area Covered by Cells	0-6.7	2.87
Nuclei Characteristics	Number of Nuclei	Number of Hoechst 33342 Stained Nuclei	0-10	1.77
	Median Nucleus Area [µm <sup>2</sup> ]	Median Area of Hoechst 33342 Stained Nuclei	0-10	3.24
	Median Nucleus Intensity	Median of Hoechst 33342 Intensity	0-10	3.56
Enzyme Dependent Cytoplasm Characteristics	Median Cytoplasm Intensity (Nucleus-Based Cell Detection)	Median of Calcein AM Intensity in Ring Around the Nucleus	0-2	0.42
	Median Cytoplasm Intensity (DPI-Based Cell Detection)	Median of Calcein AM Intensity in Ring Within Cells	0-2	0.41
Intracellular ATP Levels	Luminescence signal	ATP-Dependent Luciferin Conversion	0-10	2.38

For comparison between standard multimode detection assays and image-based assays we determined the effect of DMSO on the intracellular ATP levels. The luminescence assay kit used here is representative of other orthogonal assay approaches, such as AlphaLISA® kits to detect Caspase-3 or other apoptotic markers. Any of the analyses described in this application note can serve as a cytotoxicity assay on their own; however, multimodal approaches increase the assay reliability. This is because, in contrast to our test compound DMSO, not all toxic substances can be identified using only one parameter and may even be masked by one parameter or the other. Multimode plate readers with well-imaging technology, such as EnSight system, are therefore ideal systems to save time and money during the drug discovery processes and in basic research.

## References

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