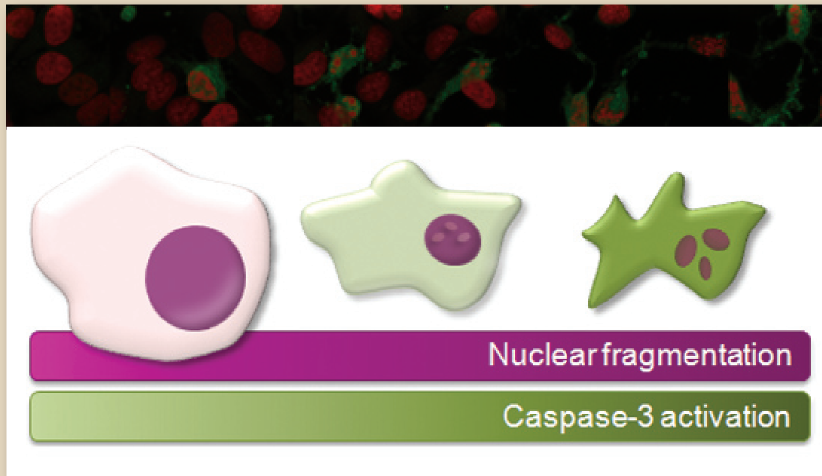
APPLICATION NOTE

# Image-based Quantification of Apoptosis using the Operetta




## Key Features

- Automated image acquisition of fixed cells using the Operetta™ High Content Screening System
- Multiplexing an early (caspase-3) and a late (nuclear fragmentation) apoptotic marker
- Data analysis using the flexible Harmony™ Software

## Nuclear fragmentation, Caspase-3 activation

### Background

Apoptosis, the genetically encoded program that leads to the self destruction of a cell, can be induced via two main pathways; the death receptor-mediated pathway, and the mitochondrial pathway [Hengartner MO, 2000]. Induction of either will finally result in the activation of caspases, a class of intracellular cytokine proteases which are considered to be the central components of the apoptotic response. By breaking down key cellular components that are required for maintaining normal cellular functions, caspases are responsible for executing morphological and biochemical processes directly or indirectly attributed to apoptosis. Caspase-3, which is examined in this Operetta High Content Screening application, belongs to a group of effector caspases[1] which undergo proteolytic activation during apoptosis [Riedl S, Shi Y, 2004]. To experimentally trigger apoptosis in a cell population, we used the cell toxin staurosporine, a naturally occurring alkaloid. By immunofluorescently labeling caspase-3, a strong fluorescence intensity increase became measurable upon induction.

The image analysis application was also complemented by evaluating the major nuclear morphological changes connected to apoptosis, i.e. the size and fragmentation of the nucleus.

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## Application

To initiate apoptosis experimentally, HeLa (human cervix carcinoma) cells were incubated with staurosporine at various concentrations for 4 h. The cells were first seeded in a 384 CellCarrier microtiter plate (6000 cells per well) and after compound incubation were fixed with 3.7 % formaldehyde. The intracellular levels of the activated caspase-3 were detected by applying an Alexa Fluor® 488 conjugated anti-caspase-3 antibody using a standard immunofluorescence protocol [Donaldson JG, 2001]. For counterstaining the nuclei we used DRAQ5™. The resulting images were captured on the Operetta equipped with a LWD 20x objective. The results are shown in Figure 1: while untreated HeLa cells show their "normal" phenotype, i.e. large nuclei (red) and no caspase activity (green), the dramatic impact of a 4 h exposure to staurosporine is apparent. A strong increase in cytoplasmic fluorescence intensity is connected to the activation of caspase-3 and shrunken, fragmented nuclei can be observed. Healthy cells possess the largest nuclei, while early apoptotic nuclei appear condensed (pyknosis) before they finally become fragmented (karyorrhexis).

For the image analysis, the Harmony "Apoptosis-1" module was selected from the Ready-Made Solution collection. As the most significant change in caspase-3 induced fluorescence intensity was located around the nucleus, an enlarged nuclear region was chosen for the image-based evaluation (Figure 2). 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.

By introducing a threshold to the caspase-3 fluorescence (here, a signal intensity of 980), the cells became classified as "apoptosis positive cells", and an $EC_{50}$ for staurosporine was calculated from this numerical data (Figure 3). The numerical output of the changing nuclear morphology is called the "fragmentation index". The assigned indices are directly related to the fluorescence intensity fluctuations of the nuclear stain: in fact, they are the CV values (coefficient of variation; the standard deviation divided by the mean value).

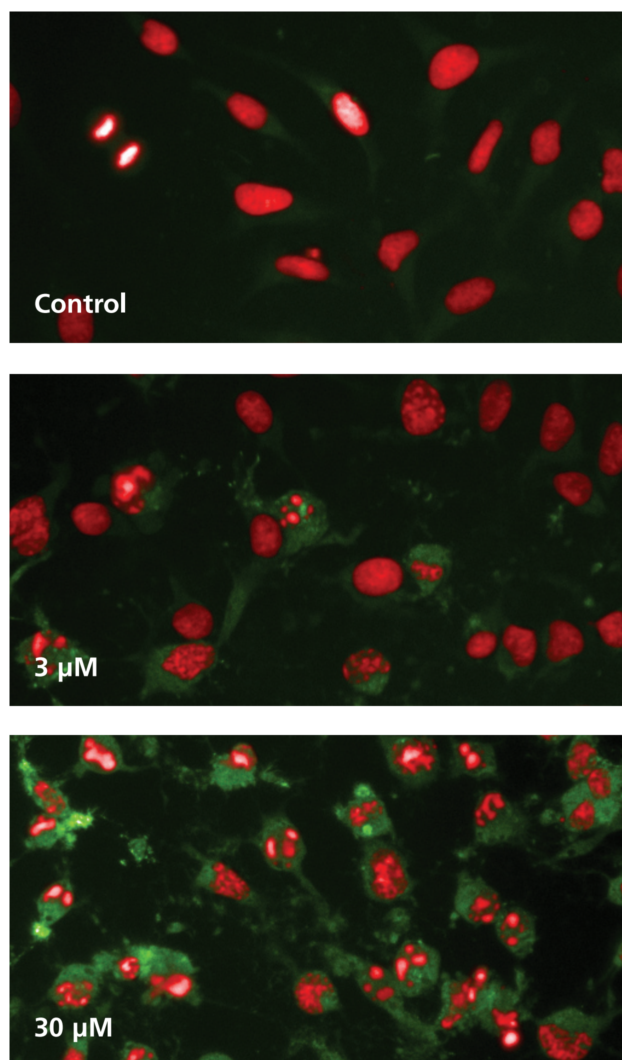
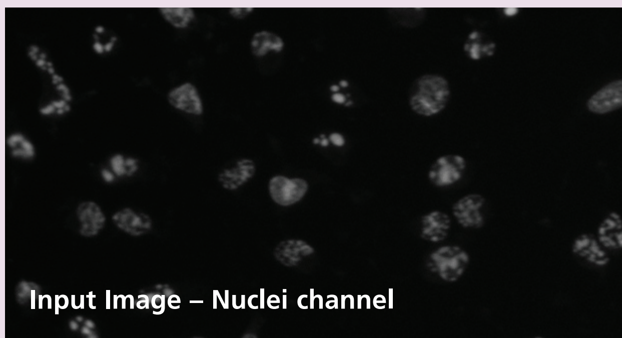
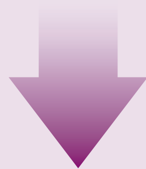
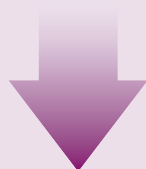



*Figure 1.* Images were captured using a 20x objective. A false color overlay of nuclear (red) and caspase-3 (green) fluorescence is shown. The increasing fluorescence of labeled activated caspase-3 becomes apparent as the compound concentration of staurosporine is increased. The nuclei display the typical nuclear phenotypes associated with apoptosis. After shrinking (which condenses the nuclear stain onto smaller area) the nuclei appear brighter, and in a later stage of the apopototic process the nuclei appear more fragmented.





**Enlarged nuclear area**

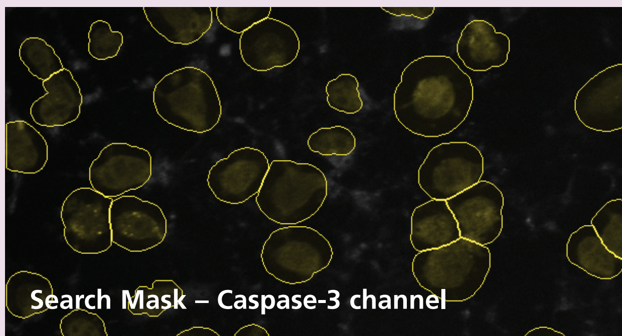
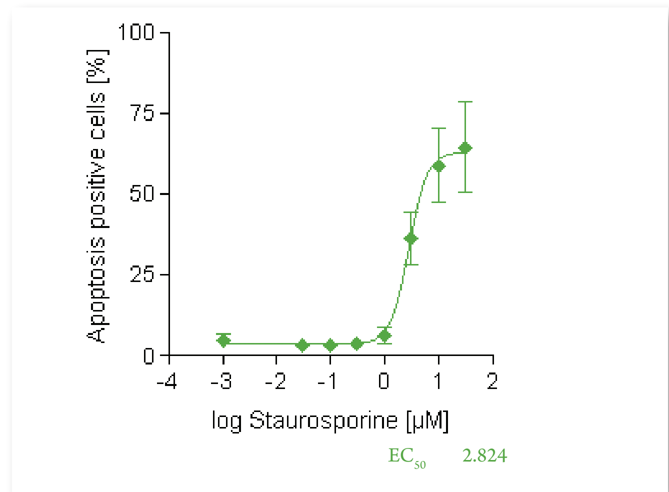



*Figure 2.* Image analysis strategy for caspase-3 detection. The search region for quantifying the caspase-3 fluorescence was created by enlarging the nucleus mask. The region is applied on the caspase-3 detection channel which captures the emission belonging to the activated caspase-3 signal.



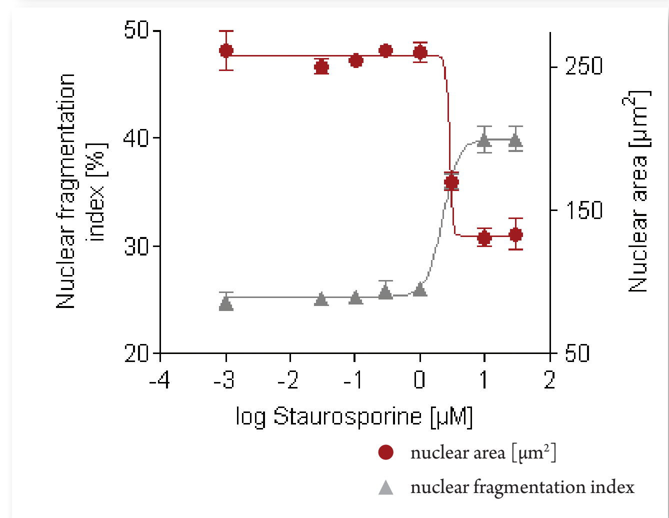




*Figure 3.* The graphs display the staurosporine-generated dose-response curves after 4 h.
*Top* | In order to quantify cells according their apoptotic state a threshold with respect to caspase-3-related fluorescence intensity was introduced to the data (the threshold was variable and was adapted to individual experimental intensity results, here 980). In this way the subpopulation of apoptotic cells became classified, and the resulting numerical readout was called "percentage of apoptosis positive cells". Taking these percentages as a basis, an $EC_{50}$ of 2.8 μM for staurosporine was calculated using the Prism-software to fit the curve.
*Bottom* | The graph shows the fluctuation of the nuclear intensity on one axis (fragmentation index) and the nuclear area on an other axis. It clearly shows the rapid decrease in nuclear area and the increase in fragmentation, upon treatment with 3 μM staurosporine.

## Conclusions

In this study we combined a nuclear morphological read-out (fragmentation, a late apoptotic event) with the measurement of a specific apoptotic marker (caspase-3 activation, an early apoptotic event) to determine programmed cell death.

The high content assay described here provides the researcher with a valid and robust tool to examine apoptosis on a multiparameter level: by measuring the decrease in nuclear size, the nuclear fragmentation index and caspase-3 activation. At a staurosporine concentration of around 3 μM the nuclear size starts to decrease, indicating the start point of the typical nuclear morphological changes associated with apoptosis. In this way, a first assessment of the apoptotic impact of any compound on the viability of the cells is possible.

*1 Two distinct classes of caspases play major roles in apoptosis: the initiators and the effectors. They share structural similarities, but their activation and inhibition are differentially regulated. The regulation of initiator caspases is particularly tight, since they can trigger the activation of downstream caspases which are responsible for proteolytically cleaving a broad spectrum of cellular targets.*

## References


Hengartner MO (2000), The biochemistry of apoptosis, Nature, 407, 770-776.

Riedl S, Shi Y (2004), Molecular Mechanisms of Caspase Regulation During Apoptosis, Nature Cell Biology, 5, 897-907.

Donaldson JG (2001), Unit 4.3 Immunofluorescence Staining in Current Protocols in Cell Biology John Wiley and Sons, Inc.; Published online.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