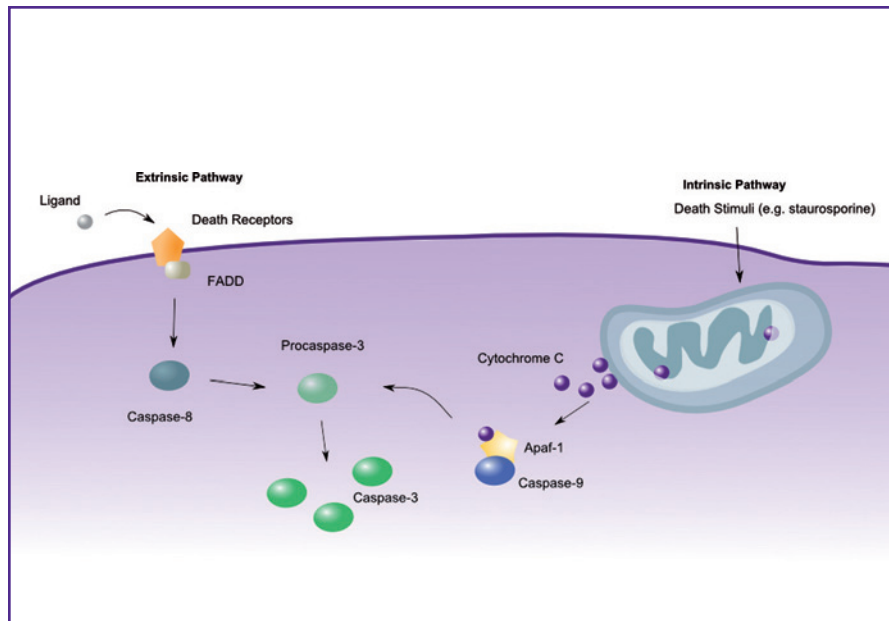


Image-based Quantification of Apoptosis
by Caspase-3 Activation using the Opera

Key Features

- Confocal image acquisition of fixed cells using the Opera™ High Content Screening system
- Image-based quantification of two key markers in the apoptotic process: level of activated caspase-3 and nuclear size
- Acapella™ analysis using the versatile CellRegionAnalysis Script

Caspase-3 activation, nuclear size

Background

Apoptosis – the genetically coded program leading to the self-destruction of a cell – can be induced via two main pathways, the death receptor-mediated pathway, and the mitochondrial pathway. Induction of either finally results 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 consequences directly or indirectly attributed to apoptosis.

Caspase-3 which is biochemically targeted in this application belongs to the group of effector caspases¹ which undergo proteolytic activation during apoptosis. To trigger apoptosis in a cell population, we chose the cell toxin staurosporine, a natural occurring alkaloid. By immunofluorescently labeling the activated enzyme, a strong fluorescence intensity increase became measurable.

The image analysis application was complemented by evaluating one nuclear morphological change connected to apoptosis: the size of the nucleus. Healthy cells possess the largest nuclei, while early apoptotic nuclei appear condensed (pyknosis) before they finally become fragmented.

Application

To initiate apoptosis, HeLa (human cervix carcinoma) cells were incubated with staurosporine in variable concentrations for 4 h. The intracellular levels of activated caspase-3 were detected by applying an Alexa Fluor® 488 conjugated anticaspase-3 antibody. For counterstaining, the nuclei DRAQ5™ was used. Resulting images were recorded confocally on the Opera using a high NA 20X water immersion objective.

As the most significant change in caspase-caused fluorescence intensity was located around the nucleus, an enlarged nucleus region was chosen for the image-based evaluation. For this task, the algorithm CellRegionAnalysis was selected from the script collection running with the built-in image analysis software Acapella. The evaluation was further refined by introducing a threshold to the caspase-3-related fluorescence intensity in order to quantify cells according to their apoptotic state. This way the subpopulation of apoptotic cells became classified, and the resulting numerical readout was called “percentage of apoptosis positive cells”. This 3-step evaluation scheme is shown in Figure 1.

The resulting caspase-3 related phenotype is displayed in Figure 2A: while untreated HeLa cells show their typical appearance with well formed, large nuclei (red) and no caspase activity (green), the dramatic impact of a 4 h exposure of HeLa cells to staurosporine becomes apparent. A strong increase in cytoplasmic fluorescence intensity connected to the activation of caspase-3 can be observed and translated into numerical data resulting in the dose-response curve shown in Figure 2B. Taking the read-out mean intensity caspase-3 as “apoptosis positive cells”, an EC_{50} of 2.2 μM for staurosporine was calculated. The Z' was calculated to be 0.96 confirming this assay design to be very robust.

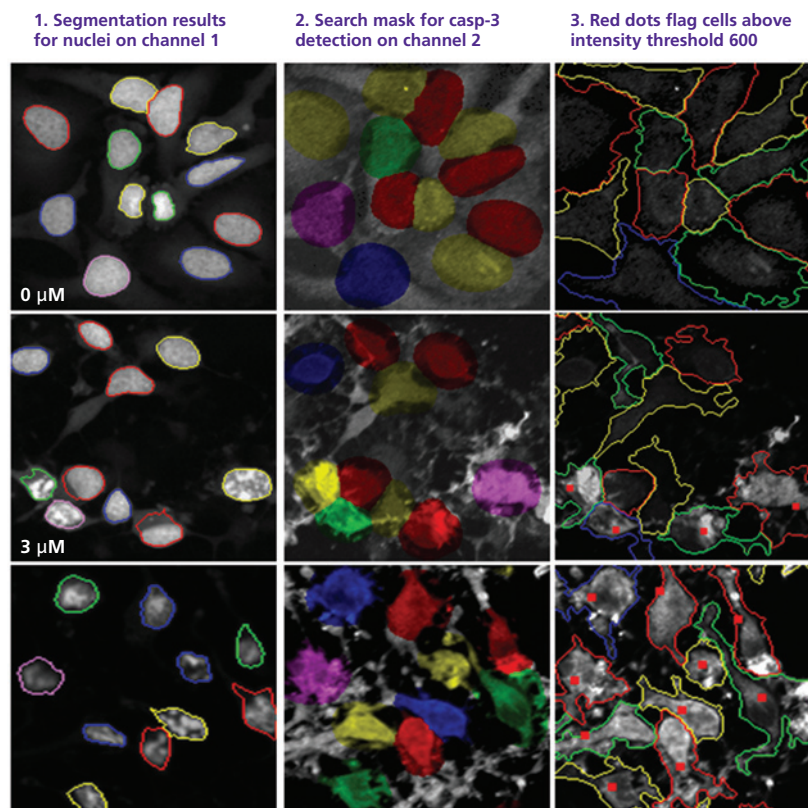


Figure 1: Image analysis strategy for quantifying activated caspase-3 fluorescence in HeLa cells after a 4 h treatment with 0, 3 and 30 μM staurosporine with the Acapella script CellRegionAnalysis.

Panel 1: segmentation results after applying the nuclei detection algorithm on channel 1 (note that the nuclei become increasingly smaller in size and more fragmented).

Panel 2: search region created by enlarging the nucleus mask from channel 1. This region is applied to quantify fluorescence intensities on channel 2 which captures the emission belonging to activated caspase-3.

Panel 3: red dots flag cells which belong to the subpopulation have caspase-3 intensities larger than intensity threshold 600.

Conclusions

Figure 2B highlights how this high content assay described here gives the researcher a valid and robust tool to approach apoptosis on a multiparameter level: by plotting the decrease in nuclear size, the apoptotic switch intensity of caspase-3 and cell number. At a staurosporine concentration of about 1 μM the nuclear size starts decreasing indicating the starting point of the typical morphological changes of the nucleus connected to apoptosis. Furthermore, we found the cell number to be a useful “by-product” of this imaging evaluation. This way, a first assessment of the cytotoxic impact of any compound on the viability of the cells is possible.

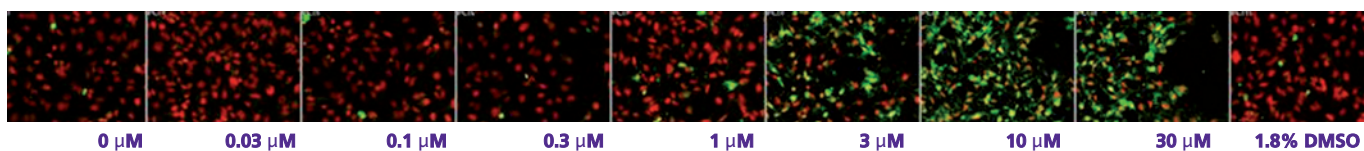
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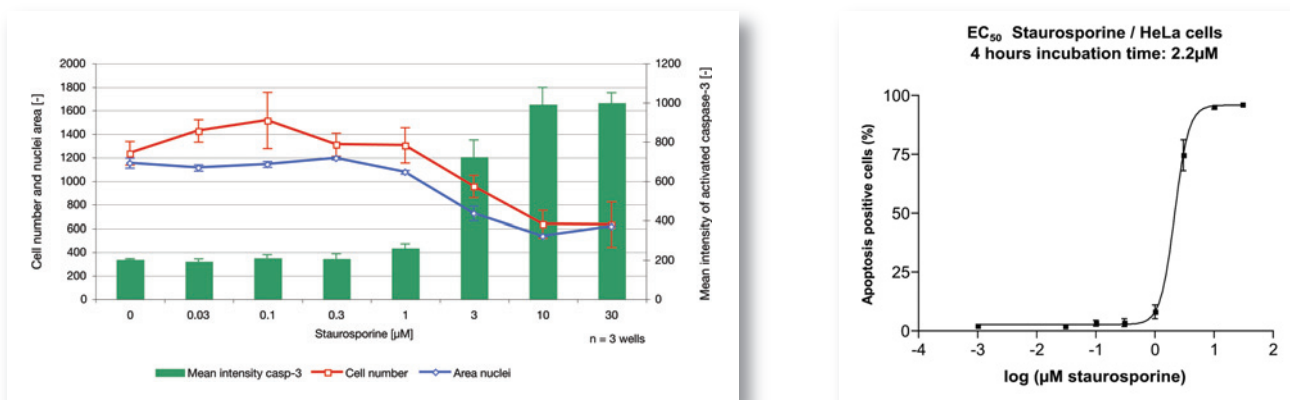


Figure 2: A. Montage of images originating from one exemplarily chosen subfield per well (20X magnification), distributed on a 384 well CellCarrier microplate by applying the PlateMontage Script. The two detection channels were merged and a Color Look-up Table applied to each channel: red (nuclei) and green (caspase-3 activation). HeLa cells were exposed to different concentrations of staurosporine for 4 h and show a dose-dependent reaction to this inducer. DMSO does not show any influence in the highest vehicle concentration used in this experiment.

B. Apoptosis manifests itself by an increasing fluorescence intensity of labelled activated caspase-3 as well as the increasing appearance of pyknotic, smaller nuclei. The fluorescence intensity of caspase-3 was converted into “percentage of apoptotic cells” by applying a certain threshold (here 600) on the intensity values. These threshold-corrected values were the basis for determining the EC₅₀ being 2.2 μM for staurosporine.

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

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