

Image-Based Quantification of Apoptosis by Caspase-3-Activation and Nuclear Fragmentation using the Opera™ High Content Screening Platform

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1 Introduction

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 targeted in this Opera™ application belongs to the group of effector caspases¹ which undergo proteolytic activation during apoptosis. To experimentally trigger apoptosis in a cell population we chose the cell toxin **staurosporine**, a natural occurring alkaloid. By **immunofluorescently** labelling the consequently activated caspase-3 enzyme, a strong fluorescence intensity increase becomes measurable on a single cell basis. In addition, we evaluate **nuclear morphological changes** related to apoptosis: the size as well as the fragmentation of the nucleus. Healthy cells possess the largest nuclei, while early apoptotic nuclei appear condensed (pyknosis) and later on highly fragmented (karyorrhexis)². This Opera™ / Acapella™ application clearly demonstrates high-content imaging to be a powerful tool in drug discovery: approaching apoptosis on a **multiparameter** level (combination of morphological with biomarker-based read-outs) and on a single cell basis is leading to a reliable evaluation of apoptosis in any cell-based experimental system.

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.

²: The term nuclear fragmentation in this context describes the segmentation of the nucleus into smaller, compact compartments and shall not be mixed with the also typically occurring DNA fragmentation during apoptosis.

2 Material and Methods

HeLa (human cervix carcinoma) cells were cultured in RPMI 1640 containing GlutaMAX and 25 mM HEPES supplemented with 10% FCS. For sub-cultivation, cells were detached with trypsin/EDTA after washing them once with PBS. 6000 cells were seeded in early passages in 50 µl growth medium per well into 384-well CellCarrier microplates and incubated for 16 h at 37 °C, 5% CO₂ before the apoptosis inducer staurosporine was added. **Staurosporine** stock solution (5mM) was prepared in DMSO and diluted in growth medium. Cells were incubated with 25 µl of inducer for either 4 h or the time intervals given in the results section when performing kinetic studies. The **caspase-3-immunofluorescence** (IF) labeling was performed using the following steps: first cells were fixed with formaldehyde (4% (v/v), 15 min, room temperature = RT), followed by permeabilization of their membranes with Triton-X-100 (0.3% (v/v), 5 min, RT) and a 30 min blocking step. The Alexa® fluor 488 conjugated anti-caspase-3 antibody (diluted to 0.75 µg/ml in 3% (w/v) BSA) was incubated for 60 min at RT with the cells. After washing the cells (0.05% Tween® 20), their nuclei were stained with 10µM **DRAQ5** for 15 min at RT before the measurement took place.

3 Image Acquisition on the Opera™

Images were recorded confocally on the high-content screening platform **Opera™ QEHS** which – equipped with up to 5 excitation sources (4 lasers: 405, 488, 561, 640nm, UV lamp) and 4 parallel recording detection channels (3 CCD cameras, one CCD camera is non-confocal for UV) – allows to combine high speed with high resolution and high content. Further features of this high-end imaging device are a fast working laser-based autofocus system, an optional climate-control setup for live-cell imaging applications and an optional automated dispensing unit. For image recording and simultaneous analysis a high resolution 20x (NA 0.7) water immersion objective was used. **DRAQ5™** was excited with the 640nm laser and the emission was captured on camera 3 using a 690/50 bandpass. Cleaved caspase-3-Alexa 488 conjugated antibody was excited with the 488nm laser, emitted fluorescence was collected on camera 1 using the 540/75 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.



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 ready made analysis algorithms - known as “**scripts**” - are available for a broad range of standard application like counting, translocation, spot analysis, etc. **CellRegionAnalysis** and **Nuclear Fragmentation** were used to create the data presented here. Due to its high speed Acapella™ is ideally suited for on the fly analysis as well as analysis of large data sets.

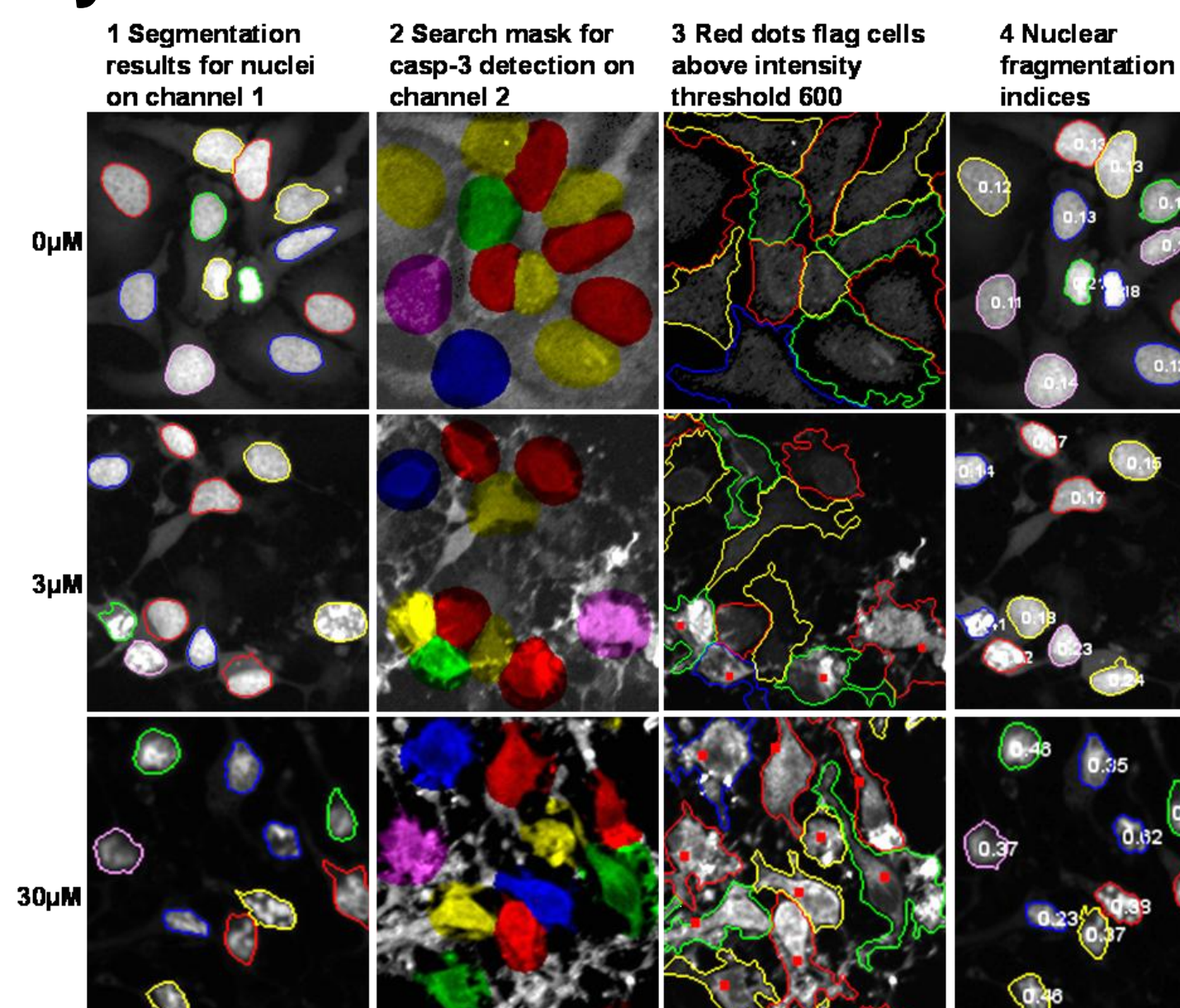


Figure 2 | Image analysis strategy for quantifying activated caspase-3 fluorescence and nuclear fragmentation in HeLa cells after a 4 hour treatment with 0, 3 and 30µM staurosporine with the Acapella™ scripts **CellRegionAnalysis** (CRA) and **NuclearFragmentation** (NF). **Panel 1:** segmentation results after applying the nuclei detection algorithm, DRAQ5-stained nuclei are detected on channel 1. **Panel 2:** search region created by enlarging the nucleus mask by 10 to 15 pixels. This region is applied to quantify fluorescence intensities on channel 2 capturing emissions belonging to activated caspase-3 (CRA). As the most significant change in caspase-caused fluorescence intensity was located around the nucleus this region was chosen for the image-based evaluation. **Panel 3:** red dots flag cells which belong to the subpopulation having caspase-3 intensities larger than 600 (as determined within the enlarged nucleus mask) (CRA script). Cells exceeding this threshold were identified as apoptotic cells. **Panel 4:** numerical values of indices increase with increasing degree of nuclear fragmentation (visual feedback generated by NF script).

5 Results

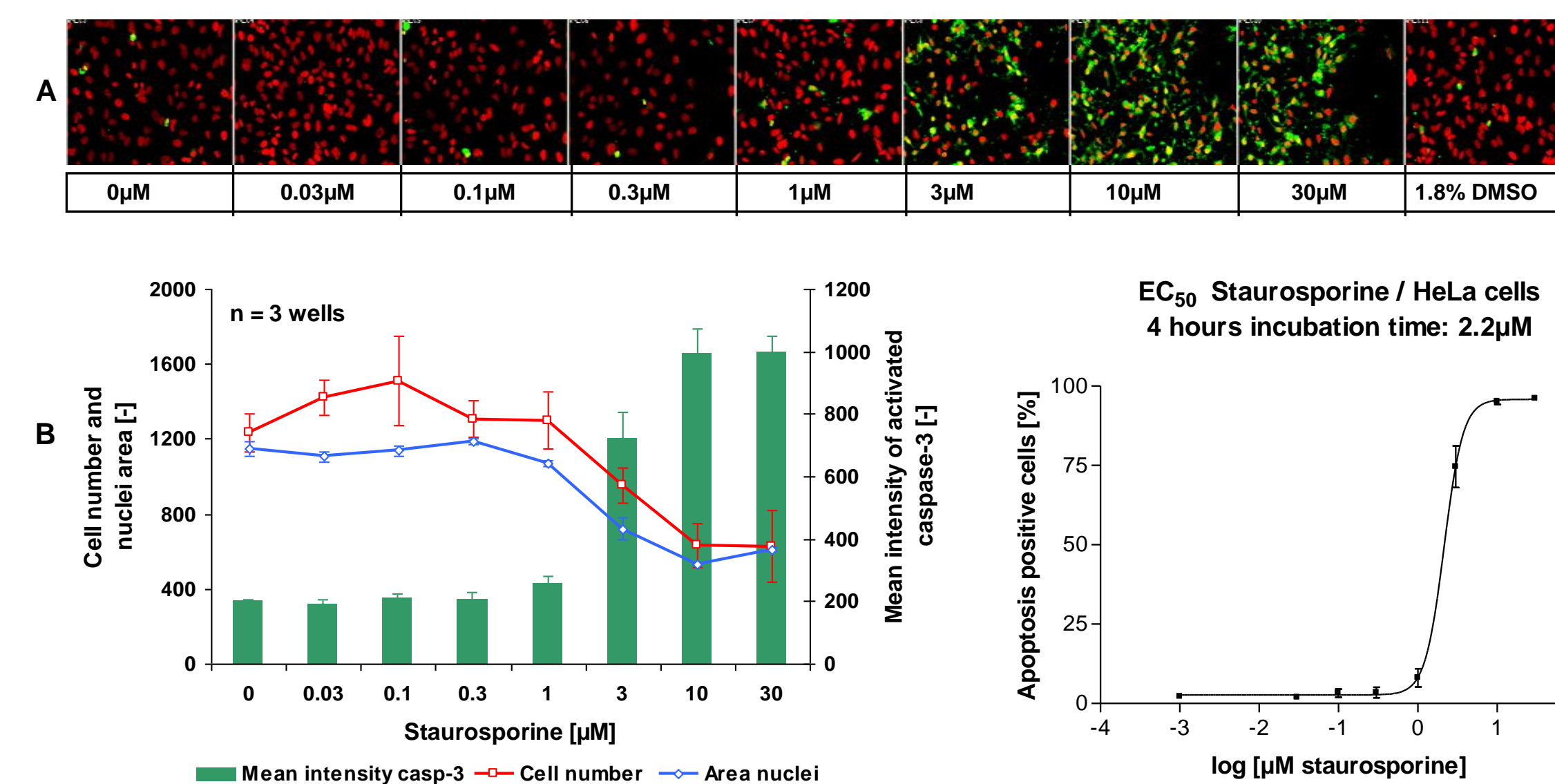


Figure 3
A | Assemble of images originating from one field per well by applying the **PlateMontage**. The resulting thumbnail composition of images reflects the dose-dependent increase of active caspase-3 accompanied by a decrease in cell number in the highest concentrated staurosporine wells. The monochromatic emissions captured on channels 1 and 2 were merged and a Color Look Up Table (CLUT) was applied - red (nuclei) and green (caspase-3).
B | shows numerical data assembled to a **dose-response curve** after applying the **CellRegionAnalysis** script (HeLa cells after a staurosporine-treatment). Apoptosis manifests itself by an increasing fluorescence intensity of labelled activated caspase-3 as well as the increasing appearance of pyknotic, smaller nuclei. The intensity of casp-3 was converted into “percentage of apoptotic cells” by applying a threshold on these values, thereby classifying the subpopulation of apoptotic cells. The resulting values were the basis for determining the EC₅₀ being 2.2 µM for staurosporine. At an inducer concentration of about 1 µM the **nuclear size** (area) also starts decreasing indicating the starting point of the typical morphological changes of the nucleus connected to apoptosis - pyknosis and karyorrhexis. Additionally plotting the decrease in **cell number** indicates the cytotoxic character of the inducer.

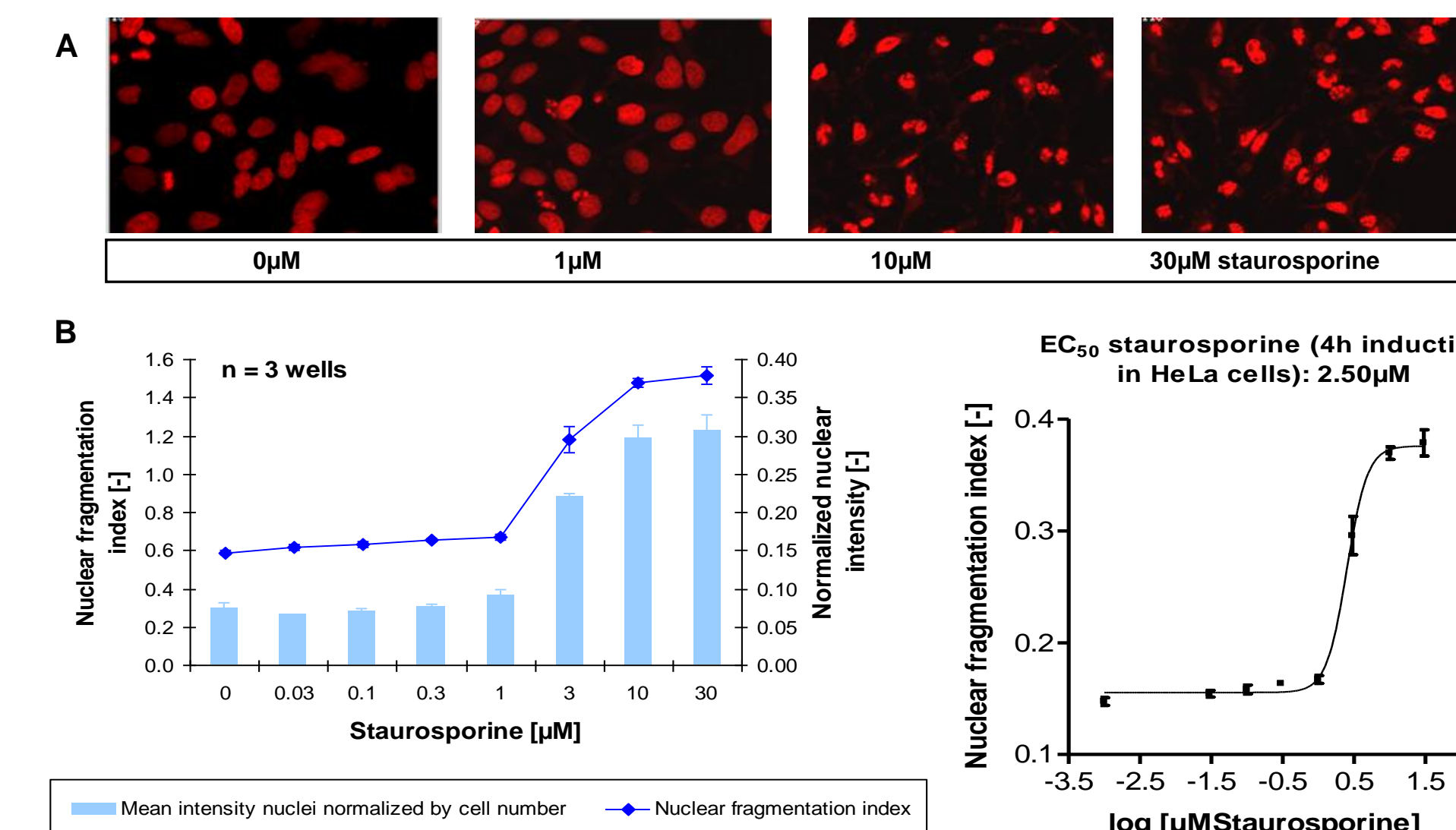


Figure 4
A | The cell nuclei display the nuclear phenotype related to apoptosis: after shrinking and thereby condensing the nuclear stain on a smaller area the nuclei appear brighter and in a later stage of the apoptotic process more fragmented (after treatment with 1, 10 and 30 µM staurosporine for 4 h). Images were acquired using a 40x (NA 0.9) water immersion objective for visualization purposes and a red CLUT was applied.
B | Observing the increasing level of nuclear fragmentation and the normalized nuclear intensity confirmed the course of the resulting dose-response curve. Fitting the nuclear fragmentation indices (with the Prism® software) resulted in an EC₅₀ of 2.5 µM for staurosporine. The image data for this analysis was acquired using a 20x (NA 0.7) water immersion objective.

6 Conclusions

Imaging cell-apoptosis has been the subject of great interest in recent years as the activation or deregulation of the programmed cell death plays a role not only during development but also in neoplasia and other medical disorders. Thus, the discovery of novel compounds that modulate apoptosis pathways could lead to the development of new therapeutic agents.

Using the described HCA approach we have defined the apoptotic switch in a cell population at which major nuclear fragmentation and caspase-3 activation are taking place after staurosporine induction.

This high content assay gives the researcher a flexible and robust tool to approach apoptosis in a primary or secondary screening setup using the Opera™ platform in combination with the Acapella™ image processing software. The quantification of activated caspase-3 and the determination of the level of nuclear fragmentation are well recognized parameters to describe effects of compounds on the apoptotic pathway.

Only two fluorescence emission channels are used in this assay and it could be easily multiplexed with additional biomarkers.