## Caspase-3 Activation: An Indicator of Apoptosis in Image-Based Assays

# Application Note

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## Introduction

The understanding of the cellular signaling processes leading to programmed cell death ("apoptosis") is of the utmost importance in the study of autoimmune diseases such as rheumatoid arthritis, Parkinson's, and Alzheimer's disease. In these types of diseases, errors in the signaling cascade are believed to lead to premature apoptosis in the affected tissue. At the same time, damaged cells that do not enter the apoptotic pathway may proliferate unchecked and become a cancerous cell mass. Thus, the study of apoptosis benefits many different fields of medicine by offering multiple targets for specific compounds investigated as a part of drug discovery research.

Caspases (cysteinyl aspartate-specific proteases) are a family of important signaling molecules with various tasks depending on the subtype and organ involved. The activation of caspases also is a marker for cellular damage in diseases such as stroke and myocardial infarction.[1] Although the precise role in the initiation and progression of apoptosis is not known for all caspases, their involvement as an indicator alone and as a potential leverage point for drug research makes them widely researched molecules.

We explored the use of the active form of caspase-3 for the detection of apoptotic events. This protease has been implicated as an "effector" caspase associated with the initiation of the "death cascade" and is therefore an important marker of the cell's entry point into the apoptotic signaling pathway.[2] Caspase-3 is activated by the upstream caspase-8 and caspase-9, and since it serves as a convergence point for different signaling pathways, it is well suited as a read-out in an apoptosis assay. Detection of active caspase-3 can be used in different cell lines or primary cells, does not require the use of transfection techniques, and can be multiplexed with other probes to get an in-depth understanding of signaling events with cell-by-cell resolution. Since there have been reports that cells progressively undergo apoptosis when exposed to staurosporine over time,[3] we investigated the time dependence of apoptosis as measured by caspase-3 activation.

## Methods

HeLa cells (ATCC, CCL-2, a human cervical cancer cell line) were maintained and grown in DMEM supplemented with 2 mM of L-glutamine, 1 mM of sodium pyruvate, and 10% fetal bovine serum.

Cells were seeded in BD Falcon™ 96-well black/clear bottom tissue culture plates optimized for imaging applications at 10,000 cells per well. After the cells had been incubated for 16 hours, staurosporine (Sigma-Aldrich, S4400) was added to the cells for 4 hours. Staurosporine is a broad-spectrum kinase inhibitor known to activate the apoptosis pathway. To generate data on cellular response, staurosporine was serially diluted in growth medium, and equal volumes of a 2X final concentration were added to the wells. The final vehicle (DMSO) concentration did not exceed 0.5%.

After incubation, cells were fixed with 3.7% formaldehyde (in phosphate buffered saline [PBS]) for 1 hour at room temperature and permeabilized with 100% methanol for 10 minutes at –20°C. Non-specific binding was blocked for 1 hour with 5% goat serum in PBS buffer containing 0.3% Triton™ X-100. Cells were then incubated with cleaved caspase-3 (Asp 175) rabbit polyclonal antibody in 5% goat serum and 0.3% Triton X-100 overnight at 4°C. Additional wash steps removed unbound antibody prior to the addition of the secondary Alexa Fluor® 488 goat anti-rabbit antibody and Hoechst 33342 dye (5 µg/mL) in 5% goat serum and 0.3% Triton X-100 for 1 hour at room temperature. The cells were then washed and imaged. For time-course experiments, the exposure to staurosporine was staggered in hourly intervals, and cells were fixed at the end of the experiment.

Images were acquired using 20×/NA 0.75 objective on an automatised inverted imaging system equipped with fluorescent lamps. Hoechst 33342 and Alexa Fluor® images were acquired using standard filter sets for dyes in this range, with careful consideration of bleed-through artifacts. Caspase-3 is located primarily in the cytoplasm, and the algorithm was set to sample an area around the nucleus as representative of the cytoplasm of the imaged cells.
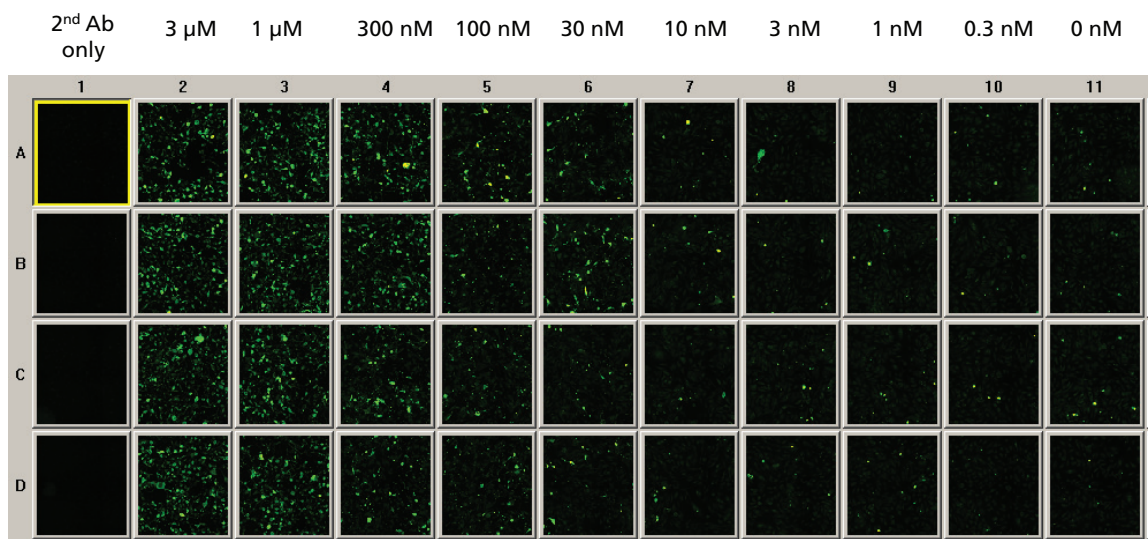


**Figure 1.** Screenshot of thumbnail images after caspase-3 activation.

*Notice the dose-response relationship (from 3 µM to 0.3 nM, left to right). For clarity, only the Alexa Fluor® 488 signal is shown.*
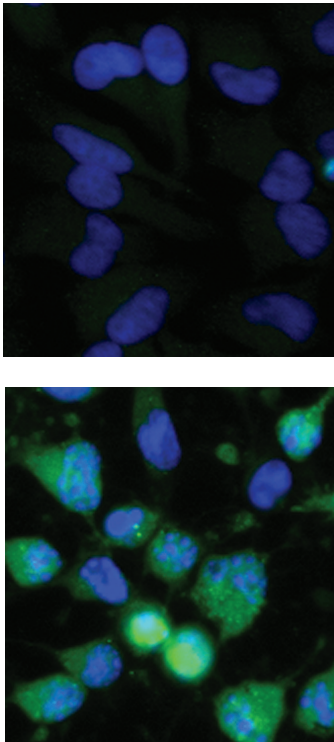
## Results

Images acquired showed a distinct labeling pattern of the activated caspase-3 in response to the apoptosis-inducing staurosporine (Figure 1). The relationship is highlighted in Figure 1, which shows an overview of the acquired images.

The entry into the apoptotic pathway is terminal, and once a cell commits, the caspase-3 pathway is completely activated. Therefore, the best measure of the relative apoptotic activity in a well is the percentage of cells above a certain threshold. The data analysis tool allows setting of multiple thresholds and plotting of curves for calculating $EC_{50}$ (Figure 3) and Z' using these percentages.
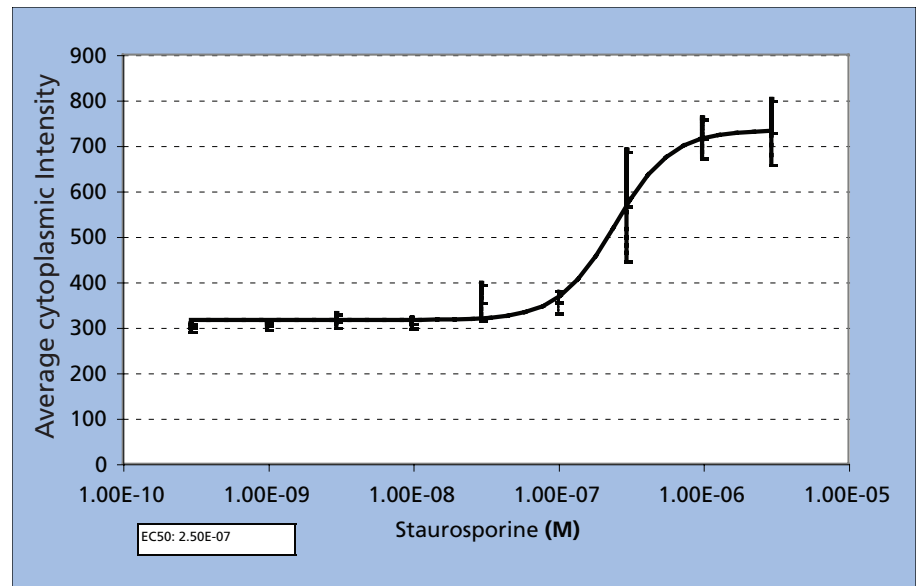


**Figure 2.** Representative image showing untreated (left panel) and staurosporine (1 µM, right panel) treated cells.

*Composite false-colored image of Hoechst 33342-labeled nuclei (blue) and activated caspase-3 (green).*



EC50: 2.50E-07

**Figure 3.** Caspase-3 activation in cells plotted against increasing staurosporine concentrations (n = 4 wells).

*The data is expressed as the percentage of cells that show a cytoplasmic fluorescence intensity greater than 450 (Z'= 0.51).*
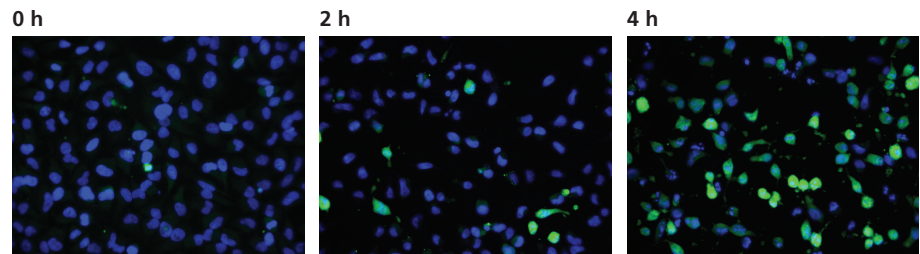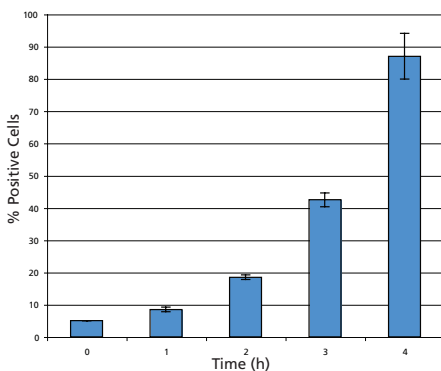


**Figure 4.** Time course of caspase-3 activation.

*Under continuous exposure to 1 µM of staurosporine, increasing numbers of cells stain positive for active caspase-3, indicating the progression of apoptosis across the cell population. Images on the right show nuclei in blue and activated caspase-3 in green for selected time points.*

## Discussion

Activation of the caspase-3 pathway is a hallmark of apoptosis and can be used in cellular assays to quantify activators and inhibitors of the "death cascade." The response is both time and concentration dependent, suggesting that multiple pathways play a role in triggering the caspase-3 activation. One could hypothesize that cells are most susceptible to staurosporine in a specific phase of the cell cycle and therefore, over time, most cells will die, similar to previous findings.[4] We show that this representative apoptosis assay can be used in an automated way and is amenable to development of high-throughput applications. Our data supports findings[5] that report an $EC_{50}$ for staurosporine of 200 nM. We specifically see the usefulness of this assay as a building block in a multiplexed high-content assay. This caspase-3 assay can easily be accommodated in a different fluorescence channel, and many dye combinations are possible.

## References

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