APPLICATION NOTE

Volume Contrast, the analysis software of NIS-Elements

Low Phototoxicity Long-Term Live Cell Apoptosis Assay Using Label-Free Live Cell Imaging

Defective apoptosis is implicated in various diseases such as cancer, neurodegenerative diseases and autoimmune diseases. Drug efficacy tests using apoptosis as an index are widely used in drug screening. Pharmacological tests require consideration of various conditions such as drug concentration and treatment time, but the efficiency of experiments can be improved with time-lapse imaging, since it is able to obtain multiple time point data using only one well-plate. In addition, since live cell assays can analyze the same cell population over time, results can be obtained with a minimum of variability.
Apoptosis can be quantitatively evaluated by the ratio of the number of apoptotic cells to the total number of cells. In short-term live cell assays, nuclear stain reagents such as Hoechst are used for measuring the total number of cells, but in long-term assays, cell proliferation is suppressed by dye toxicity and fluorescence excitation phototoxicity, and this causes a decrease in reproducibility in the quantitative analysis of drug efficacy.
This application note introduces an example of a long-term live cell apoptosis assay with low phototoxicity. Low phototoxicity was achieved by generating a phase distribution image (Volume Contrast image) that reflects the cell structure from multiple brightfield images captured in different focal planes, and by measuring the total number of cells over time in the field of view, enabling a long-term apoptosis assay.

Issues of comparison and normalization between wells in long-term live cell assays

In a long-term live cell assay, a drug efficacy comparison, performed by measuring the cell occupancy area in the field of view using brightfield images obtained with transmitted light and performing normalization between wells using the cell occupancy area as an index, is used. However, the area of each cell depends on cell density, and the area per cell generally becomes smaller in regions of high cell density (Fig. 1).

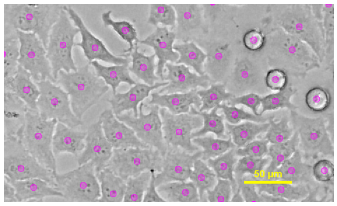


Fig. 1: Cells with different areas
Brightfield image overlaid with the cell count masks (Pink) generated from the VC image.

Objective: 10X
Scale bar: 50 µm

Cell area varies depending on which phase of the cell cycle the cells are in, since cells divide by undergoing repeated extension and contraction (Fig. 2). In addition, because cell area varies greatly depending on the type of drug, it is important to compare wells using the number of cells in the field of view rather than the area as an index.

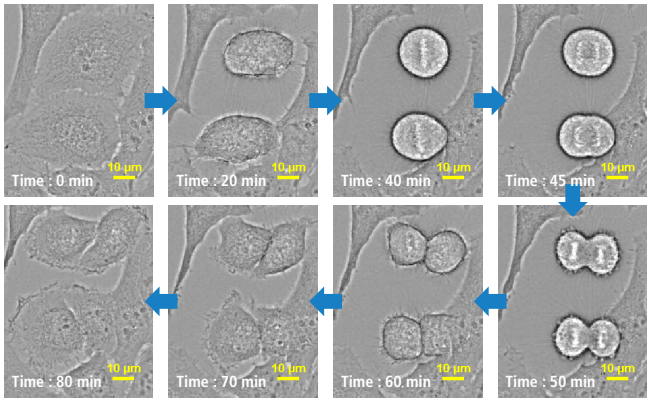


Fig. 2: Brightfield time-lapse images during cell division
Area varies depending on the cell cycle phase.

Objective: 20X
Scale bar: 10 µm

Apoptotic cell morphological characteristics and detection methods

Due to apoptosis (programmed cell death), cells show morphological changes such as cell shrinkage, membrane blebbing, chromatin condensation, nuclear and DNA fragmentation, and formation of apoptotic bodies. Apoptosis can be qualitatively identified by observing these morphological features. In addition, quantitative analysis is possible by using a fluorescent probe (Fig. 3).
When the proteolytic enzyme caspase is activated in the cytoplasm by the induction of apoptosis, the DNA binding dye of the apoptosis detection marker Caspase-3/7 moves to the nucleus, and the nucleus fluoresces as green (Fig. 3 (c)). When apoptotic bodies are formed and the cell membrane is damaged in late apoptosis, the fluorescence of Caspase-3/7 gradually disappears and the cells become dead cells. Also, ethidium homodimer-1 (EthD-1) flows into the cell from damaged cell membranes and emits red fluorescence by binding to the DNA in the nucleus (Fig. 3 (d)). Since the fluorescence intensity and area of the cell differ depending on the timing, it is necessary to use the number of cells as the index for quantitative analysis.

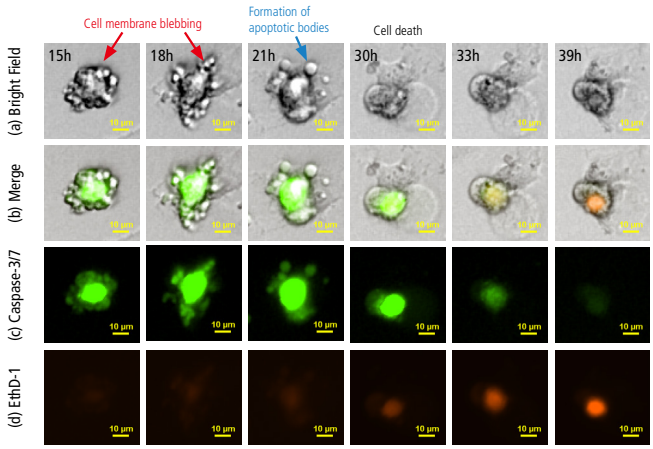


Fig. 3: Time-lapse images of an apoptotic cell

Objective: 10X
Scale bar: 10 µm

Generating a cell mask by binarization of a VC image

In the VC image, large pixel values are output for thick areas of the cell. The lamellipodium region has a flat shape, so its intensity value in the VC image is low, while the region around the nucleus is thick, so its intensity value is high (Fig. 4).

A cell mask that includes the nuclear region can be generated by utilizing the feature quantity of the cell thickness, defining the region with a high intensity value in the VC image as the cell region surrounding the nucleus and binarizing it (Fig. 4).

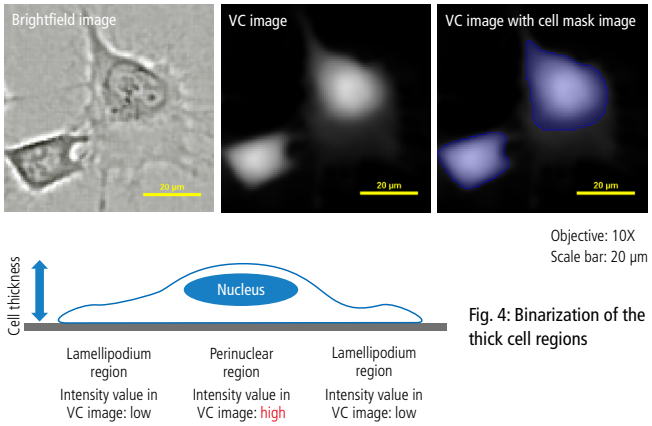


Fig. 4: Binarization of the thick cell regions

Accurate quantitative analysis of apoptotic cell numbers is possible even if they are undergoing chromatin condensation and nuclear fragmentation

In general cell count measurement, cells are defined as having one nucleus each, and a method of estimating cell counts based on the number of nuclei visualized with a fluorescent probe is widely used. However, since apoptosis generates multiple nuclear fragments inside the cell, accurate measurement of the number of apoptotic cells or dead cells from the number of objects (nuclear fragments) is not possible using Caspase-3/7 and EthD-1 (Fig. 5 (a-c)).

In contrast, in VC images, the thick areas of cells are identified as cell regions, so even if the nucleus is fragmented, a cell count can be measured by generating a cell mask (Fig. 5 (d and e)).

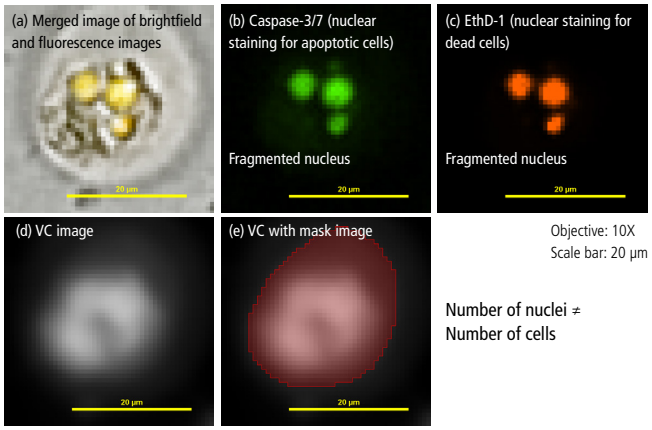


Fig. 5: An apoptotic cell with fragmented nucleus

Cell classification mask for apoptosis assay

Cells can be classified as apoptotic cells or dead cells according to whether the cell mask generated from the VC image overlaps with Caspase-3/7 or EthD-1 fluorescence. At the stage when the integrity of the cell membrane is lost after the formation of apoptotic bodies, there are double positive cells of Caspase-3/7 and EthD-1 (Fig. 3 and Fig. 5). Cells in which both Caspase-3/7 (Green) and EthD-1 (Red) were detected were defined as dead cells where Caspase-3/7 activity remains but the cell membrane is damaged (Table 1).

Cell classification	VC image	Caspase-3/7 (Green)	EthD-1 (Red)	Color of cell classification mask
Non-apoptotic live cells	+	–	–	Blue
Apoptotic cells	+	+	–	Green
Dead cells	+	+/–	+	Red

Table 1: Cell classification mask definition and color

By classifying cells with masks, double counting of cell numbers is prevented and accurate quantitative analysis is possible (Fig. 6 and Fig. 7).

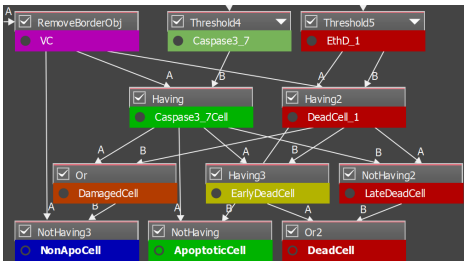


Fig. 6: Analysis block diagram of cell classification mask generated by General Analysis 3 image analysis module of NIS-Elements

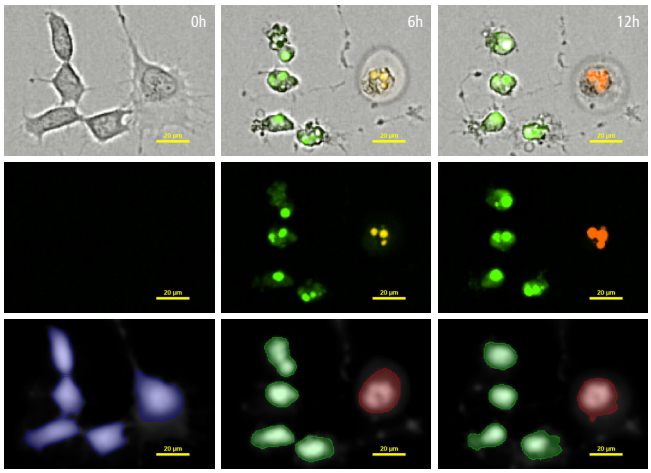


Fig. 7: Time-lapse images after addition of staurosporine

From the left, 0h, 6h, and 12h after addition of staurosporine

Upper row: merged brightfield and fluorescence images, middle row: merged Caspase-3/7 fluorescence and EthD-1 fluorescence images, lower row: VC images with cell classification mask

Blue mask: non-apoptotic live cells, green mask: apoptotic cells, red mask: dead cells

Objective: 10X
Scale bar: 20 µm

Advantages of apoptosis assay with large-FOV imaging

The timing of caspase activation after induction of apoptosis varies from cell to cell. The number of apoptotic and dead cells varies greatly depending on the imaging region in the well. Therefore, reproducible research results can be obtained by capturing and analyzing more cells in a large field of view.

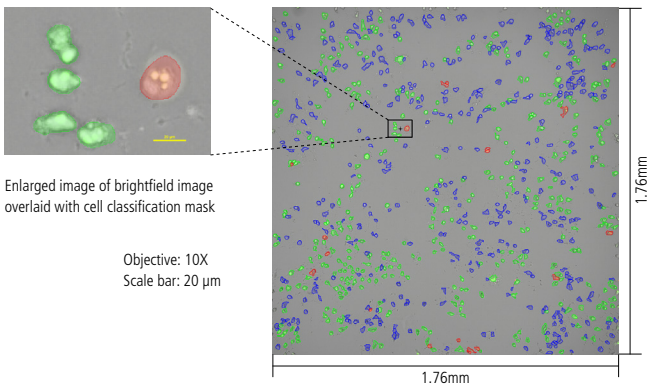
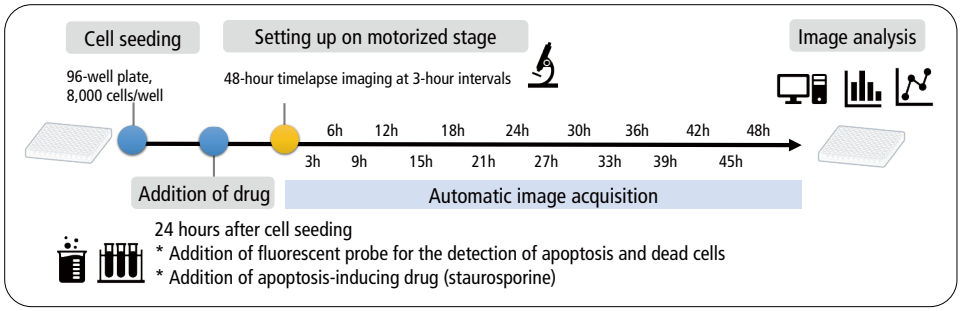
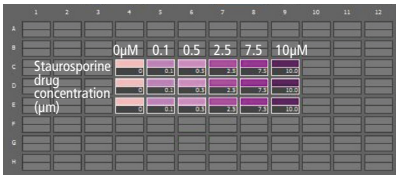


Fig. 8: Imaging range of the DS-Qi2 wide-field camera with 25 mm FOV

A brightfield image of a wide area of 1.76 mm x 1.76 mm, taken in one shot and overlaid with a cell classification mask.

Experiment workflow of apoptosis assay





HCA wizard of NIS-Elements HC
Sample labeling input screen

Cells/Reagents/Materials

- HeLa Human cervical cancer-derived cell line (Riken Cell Bank, RCB0007)
- Minimum Essential Medium Eagle With Earle's salts (Sigma-Aldrich, M4655)
- FluoroBrite™ DMEM (Thermo Fisher Scientific, A1896701)
- Fetal Bovine Serum, qualified, USDA-approved regions (Thermo Fisher Scientific, 10437028)
- L-Glutamine (200mM) (Thermo Fisher Scientific, 25030081)
- Penicillin-Streptomycin (10,000 U/mL) (Thermo Fisher Scientific, 15140122)
- Trypsin-EDTA (0.25%), phenol red (Thermo Fisher Scientific, 25200072)
- DPBS, no calcium, no magnesium (Thermo Fisher Scientific, 14190144)
- EZVIEW® CulturePlate LB (AGCTECHNO GLASS, 5866-096)
- Staurosporine solution (Millipore Sigma, S6942)
- CellEvent™ Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific, C10423)
- Ethidium homodimer-1 (Component B) of LIVE/DEAD™ Viability/ Cytotoxicity Kit (Molecular Probes, L3224)

Observation instruments/Software

- Microscope: Ti2-E (Nikon)
- Objective: CFI Plan Apo Lambda S 10X NA 0.45
- Monochrome camera: DS-Qi2
- Fluorescent LED illumination system: D-LEDI
- Image analysis software: NIS-Elements (Nikon)
- Stage top incubator: STX series (TOKAI HIT)

Methods

After seeding HeLa cells into 18 wells on a 96-well plate with a cell density of 8,000 cells per well, 10% FBS was added to the medium, which was incubated for about 24 hours. The medium was changed about 24 hours after seeding and Caspase-3/7 (7.5 μM), ethidium homodimer-1 (4 μM), and staurosporine were added to each well. Staurosporine at concentrations of 0 μM, 0.1 μM, 0.5 μM, 2.5 μM, 7.5 μM, and 10 μM were added to 3 wells each.

The cells were maintained at 37°C and 5% CO_2 in a stage-top incubator. Three Z-stack images each taken with brightfield, red fluorescence wavelength, and green fluorescence wavelength illumination, of an area (approx. 1.76 mm x 1.76 mm) at the center of each well were captured with a 10X objective and a wide (25 mm) field-of-view camera every 3 hours for 2 days after drug addition.

	Brightfield	Caspase-3/7 (fluorescence: green)	EthD-1 (fluorescence: red)
Light source	Diascopic LED	D-LEDI (Power: 10%)	D-LEDI (Power: 10%)
Ex/Em (nm)	—	502/530 nm	528/617 nm
Exposure time	35 ms	200 ms	200 ms

The obtained brightfield images were converted to VC images using the General Analysis 3 image analysis module of NIS-Elements, and binarized to generate a cell mask. The cells overlapping with the fluorescent image were classified with a mask to create an apoptosis assay analysis recipe. The apoptosis assay analysis recipe was executed on all images using Batch GA3 of NIS-Elements, and the measured values were output in CSV format. The measurements were then imported into the Microsoft Power BI® data visualization tool to create a graph and analyze the measurement results.

A staurosporine dose-response curve was created with an online tool, "Quest Graph™ EC50 Calculator," (AAT Bioquest, Inc., 31 Jul. 2021, https://www.aatbio.com/tools/ec50-calculator) and an EC_{50} value was obtained.

Results

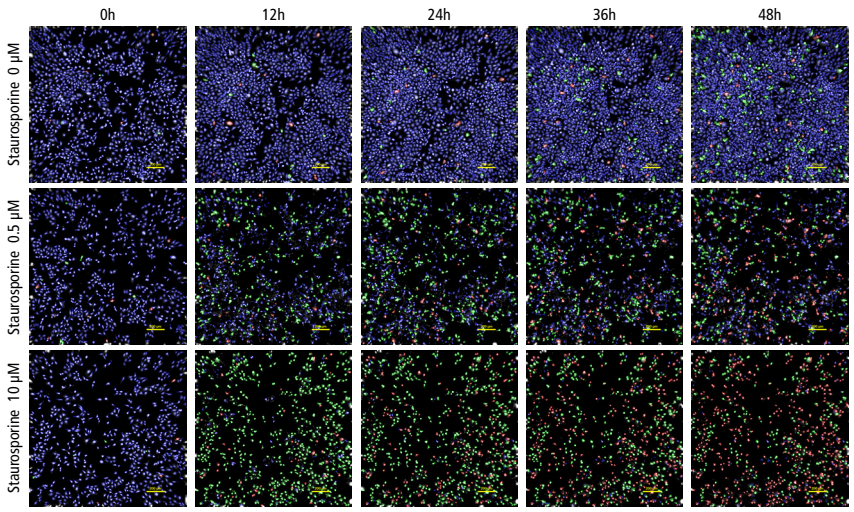


Fig. 9: Analysis results showing that apoptosis was induced in a concentration-dependent manner for staurosporine (STS)

VC images were overlaid with a cell classification mask
Blue: Non-apoptotic live cells
Green: Apoptotic cells
Red: Dead cells
Upper row: STS 0μM
Middle row: STS 0.5 μM
Lower row: STS 10 μM
From the left: 0h, 12h, 24h, 36h, 48h after drug was added

Scale bar: 200 μm

Click to view the video

Sample video

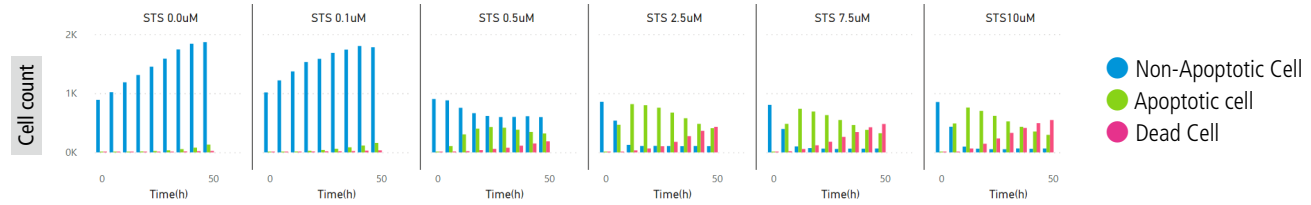
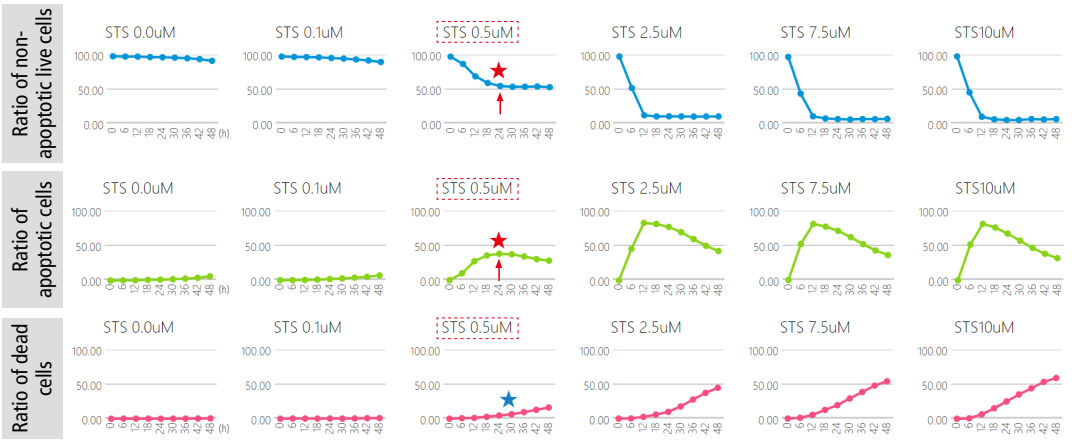


Fig. 10: Changes in cell count every 6 hours after addition of staurosporine (STS) (from 0h to 48h)
STS concentration: From left: 0 µM, 0.1 µM, 0.5 µM, 2.5 µM, 7.5 µM, and 10 µM
X-axis: time, Y-axis: cell count, Blue: non-apoptotic live cells, Green: apoptotic cells, Red: dead cells



★Under the condition of staurosporine 0.5 µM, the rate of non-apoptotic live cells (blue) and apoptotic cells (green) reached a plateau 24 hours after drug treatment.

★Under the condition of staurosporine 0.5 µM, the rate of dead cells (red) gradually increased from 30 hours after drug treatment.

Fig. 11: Changes in percentages of non-apoptotic live cells, apoptotic cells, and dead cells every 6 hours after addition of staurosporine (STS) (from 0h to 48h)
STS concentration: from left, 0 µM, 0.1 µM, 0.5 µM, 2.5 µM, 7.5 µM, and 10 µM
X-axis: time, Y-axis: ratio of cell quantities, Upper row: non-apoptotic live cells, middle row: apoptotic cells, lower row: dead cells

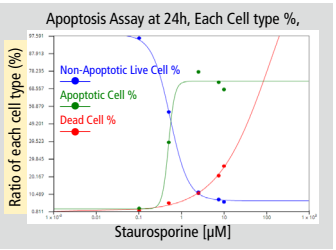


Fig. 12: Staurosporine dose-response curve over 24 hours after drug treatment
Blue: ratio of non-apoptotic live cells
Green: ratio of apoptotic cells
Red: ratio of dead cells

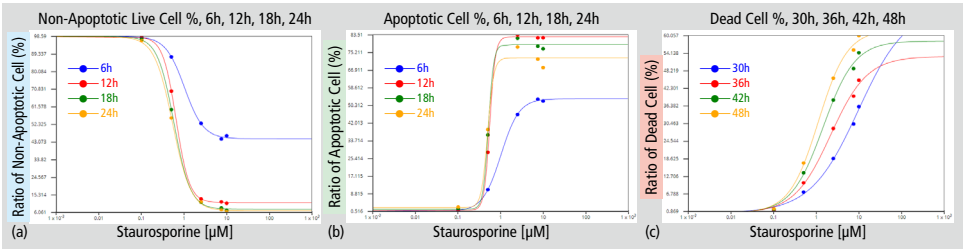


Fig. 13: Staurosporine dose-response curve and EC_{50} value at each drug treatment time
(a) Rate of non-apoptotic live cells: EC_{50} for 24 hours after drug treatment is 0.537 µM.
(b) Rate of apoptotic cells: EC_{50} for 24 hours after drug treatment is 0.493 µM.
(c) Rate of dead cells: EC_{50} for 48 hours after drug treatment is 1.116 µM.
Drug reaction time: (a, b) 6h, 12h, 18h, 24h, (c) 30h, 36h, 42h, and 48h.

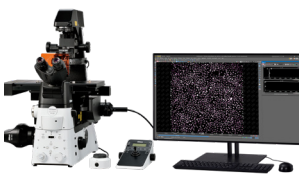
By binarizing VC images to generate a cell mask containing the perinuclear region, even a cell with fragmented nuclei can be recognized as one cell, and the number of cells can be measured (Fig. 5 and Fig. 7).
A decrease in the number of non-apoptotic live cells that are staurosporine dose-dependent was confirmed (Fig. 9).
While the number of cells doubled 48 hours after the start of imaging in the control well (STS 0.0 µM), cells did not proliferate in wells to which staurosporine was added at a concentration of 0.5 µM or higher, and apoptosis was induced (Fig. 10).
Under the condition of staurosporine 0.5 µM, the percentage of apoptotic cells reached its maximum about 24 hours after drug treatment, and the percentage of dead cells gradually increased after 30 hours. The percentage of dead cells reached 50% 48 hours after drug treatment under the condition of 2.5 µM or more (Fig. 11).
A dose-response curve was created using the online tool Quest Graph™ EC50 Calculator (AAT Bioquest, Inc.) to calculate EC_{50} value. This was able to quantitatively evaluate that apoptosis is induced in a staurosporine dose-dependent and time-dependent manner (Fig. 12 and Fig. 13).
The EC_{50} value 24 hours after drug treatment was 0.537 µM for non-apoptotic live cells (%) and 0.493 µM for apoptotic cells (%) (Fig. 12 and Fig. 13 (a, b)). On the other hand, dead cells (%) did not reach a plateau 24 hours after drug treatment (Fig. 12). The percentage of dead cells showed a sigmoid-type dose-response curve 36 hours after drug treatment (Fig. 13 (c)).

Summary

- A long-term live cell apoptosis assay with reduced phototoxicity to live cells was possible by generating cell masks from label-free VC images and using them in combination with images of fluorescent probes for apoptotic/dead cell detection.
- Drug efficacy at multiple time-points during drug treatment could be evaluated in one experiment using one plate, improving efficiency.
- A long-term live cell apoptosis assay utilizing time-lapse imaging and VC images is an effective tool for developing new drugs.
- The timing of apoptosis varies from cell to cell, but highly reproducible results that minimize the bias of cell numbers depending on imaging fields can be obtained, by acquiring a wide field of view in one shot with a DS-Qi2 camera having a FOV of 25 mm to perform cell counting.

Product Information

High content analysis (HCA) microscope system



The High Content Analysis (HCA) option of NIS-Elements imaging software is installed in the Ti2-E microscope and camera combination. Quick and easy execution, from image acquisition to analysis, is possible with one system.