Application Note
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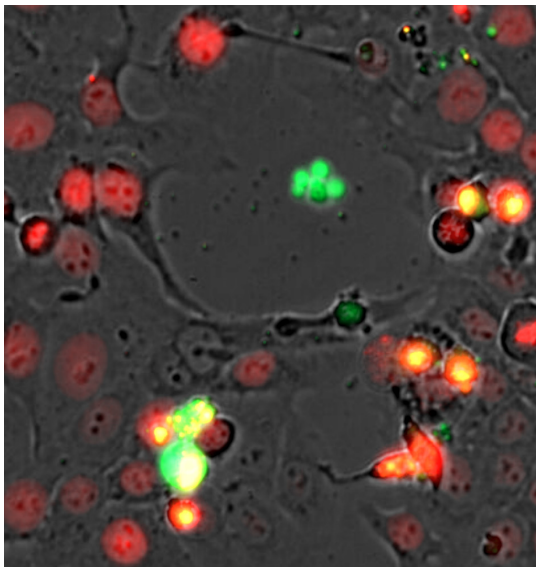
High-content analysis of gemcitabine-induced caspase activity using NYONE® and YT®-Software

Schaefer W[1], Christmann T[1], Sebens S[2], Werdelmann B[1] & Geisen R[1]
[1]SYNENTEC GmbH, Elmshorn, Germany
[2]Institute for Experimental Cancer Research, CAU + UKSH Kiel, Germany

ABSTRACT

Apoptosis is a form of programmed cell death involved in many physiological and pathophysiological processes. Important key players of apoptotic pathways are the effector caspases-3 und 7. Therefore, analysis of their activation is an interesting target for drug screening. Here, we demonstrate the over-time detection of caspase activity in a simple add, no-wash, no-lyse assay suitable for automation and high-throughput screening. To do so, we added the chemotherapeutic drug gemcitabine, CellEvent™ Caspase-3/7 Green Detection Reagent and SPY650-DNA to pancreatic cancer cells. SPY650-DNA stains the nuclei of the cells and was used to normalize for the overall cell number. We imaged the cells daily for 72 h using NYONE® Scientific and YT®-Software. In the end, we performed a viability assay in the same plate and measured it also with NYONE® Scientific. This high-content approach resulted in IC50/EC50 values of different parameters (confluence, growth factor, nuclei count, caspase-positive cells, viability). Moreover, in contrast to simple plate reader assays, cell morphology could also be examined. Treated cells showed clear signs of apoptosis like cell shrinkage, membrane blebbing, DNA condensation/fragmentation and extracellular vesicles. Thus, NYONE® and YT®-Software prove once again to be valuable tools for high-content screening.



KEYWORDS: DRUG SCREENING, APOPTOSIS, CELL DEATH, CASPASE, HIGH-CONTENT SCREENING, HIGH-THROUGHPUT SCREENING

INTRODUCTION

Cell death plays a major role in health and disease. One of the best-known forms of programmed cell death is apoptosis. Apoptosis is activated either by intrinsic signals like cellular stress, DNA damage or mitotic defects or by extrinsic signals like death receptor activation [1]. Both pathways converge in the activation of the so-called executioner caspases-3 and 7. Their activity initiates many of the morphological and biochemical correlates of apoptosis, including DNA condensation and fragmentation, membrane blebbing and the formation of apoptotic bodies [1]–[3].

Thus, assays detecting caspase activity are widely used to analyze apoptotic events in high-throughput screening. However, most assays analyzing caspase activity in a microplate format either require washing steps (e.g. PhiPhiLux® and FAM-FLICA® fluorogenic substrates or ELISA-based assays) or cell lysis (e.g. Caspase-Glo® or Ac-DEVD-AMC assays), making them unsuitable for live-cell imaging. Other caspase activity assays rely on the transfection of cells with a recombinant caspase substrate (e.g. pGloSensor™-30F DEVDG Vector), making them rather time-consuming, and due to the transformation of the original cell line, the physiological process might be altered [4].

To enable live cell imaging in a high-content screening format, we searched for a simple fluorescent no-wash assay. For us, the elimination of washing steps was important, as caspase 3-positive cells might be detached from the cell culture dish [5], and washing possibly removes these cells. For our purpose, the CellEvent™ Caspase-3/7 Green Detection Reagent appeared like a well-suited assay. This cell-permeant reagent consists of a nucleic acid binding dye conjugated to a four-amino acid peptide (DEVD), which inhibits its binding to DNA making it non-fluorescent in healthy cells. However, in apoptotic cells, the DEVD peptide is cleaved by activated caspase-3 or caspase-7, enabling the dye to bind to DNA and produce a bright, fluorogenic response with absorption/emission maxima of 502/530 nm (Fig. 1 A) [6]. A very similar assay principle is used by DEVD-NucView488™ and was successfully described in a high-content assay over time by Antczak et al. [4].

To analyze caspase activity in relation to the cell number, we used the DNA binding dye SPY650-DNA. Like Hoechst 33342, it binds to the minor groove of DNA, but it is linked to a modified silicon rhodamine dye, emitting fluorescent light in the far-red spectrum (Fig. 1 B). It was shown to be non-toxic to viable cells and therefore,

is a good candidate for live-cell imaging [7].
To induce apoptosis, we treated pancreatic cancer cells with the chemotherapeutic agent gemcitabine, which is one of the standard treatments of pancreatic cancer. Gemcitabine is a deoxycytidine analogue preventing the replication of DNA [8] and has been shown to activate caspase-3 [9]. We added the CellEvent™ Caspase-3/7 Green Reagent and SPY650-DNA directly to the cell culture medium and imaged the cells over time using the automated cell imager NYONE® Scientific (Fig. 2).

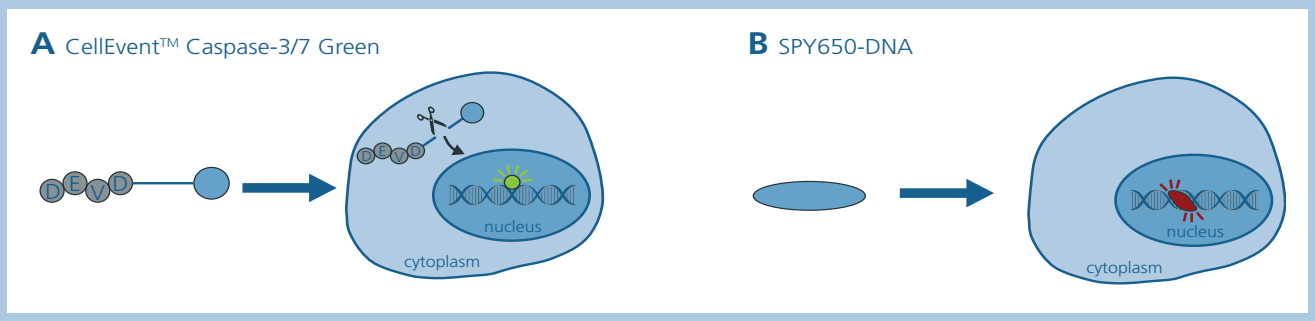


FIG. 1. ASSAY PRINCIPLES

A) In the CellEvent™ Caspase-3/7 Green Detection Reagent kit, a high-affinity DNA binding dye is linked to the caspase-3/7 peptide substrate DEVD. It enters cells, where the dye is only released when caspase-3/7 is activated. It can then enter the nucleus, where it binds to DNA and fluoresces. Picture adapted from biotium.org. B) The non-fluorescent dye SPY650-DNA is membrane-permeable and can thus enter all cells, in which it fluoresces upon DNA binding.

MATERIAL

- Adherent pancreatic cancer cells
- RPMI 1640 (e.g. PAN Biotech Cat. No. P04-17500) supplemented with 10 % (v/v) FCS, 1 % (v/v) Glutamine and 1 % (v/v) Sodium Pyruvate
- 96-well plates e.g. CytoOne
- Gemcitabin Hexal (0.5 mg in 0.5 mL NaCl 0.9 % obtained through pharmacy of the university hospital UKSH)
- CellEvent™ Caspase-3/7 Green Detection Reagent (2 mM in DMSO, Invitrogen™ Cat. No. C10723)
- SPY650-DNA (1000 x in DMSO, Spirochrome Cat. No. SC501)
- alamarBlue® (Biorad, Cat. No. BUF012A)

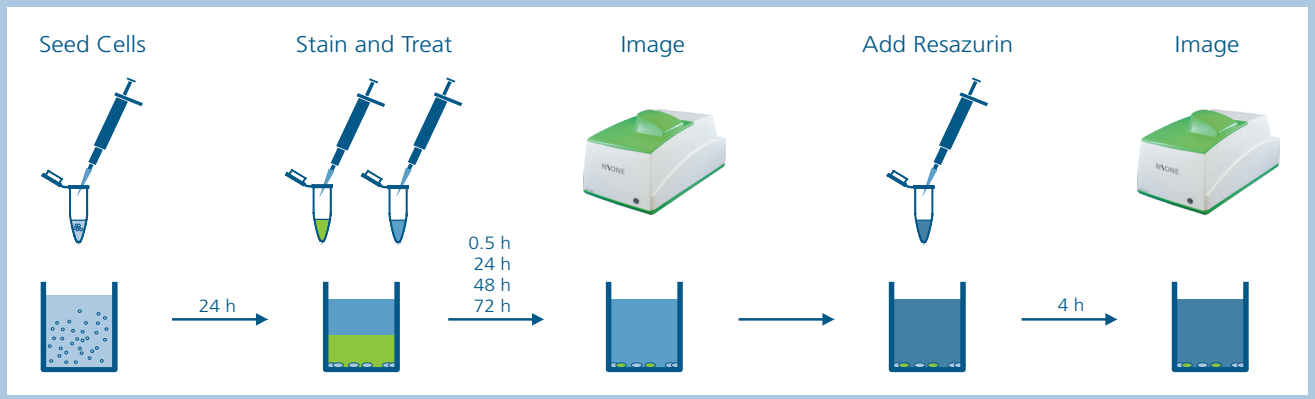


FIG. 2. WORKLOW

We seeded 5 x 10^3 cells per well in a 96 well plate. After 24 h, we added equal amounts of the staining solution containing SPY650-DNA and CellEvent™ Caspase-3/7 Green Detection Reagent (each 2 x concentrated) and treatment solution containing varying concentrations of gemcitabine (also 2 x concentrated). At the indicated time points, the cells were imaged using NYONE® Scientific. After the last imaging 72 h after treament, we added a resazurin solution (alamarBlue®) and incubated the cells for 4 h. Subsequently, the plates were again imaged using NYONE® Scientific or a plate reader as comparison.

METHODS

Cell Culture and Cell Counting

We routinely cultured cells in RPMI 1640 medium containing FCS (see above) using standardized cell culture conditions (37 °C, 5 % CO^2, humidified atmosphere). They were trypsinized and then counted using SYNENTEC's **Trypan Blue** application.

Treatment and Staining of Cells

For the apoptosis assay, we seeded 5 × 10^3 cells/well in a flat bottom 96 well plate. The next day, we washed the cells once with medium and replaced the medium with the staining solution including 2 x SPY650-DNA and 2 x Caspase-3/7 Detection Reagent (end concentration 6 µM). Immediately after pipetting the staining solution, we added an equal amount of treatment solution (gemcitabine diluted to a 2 x concentration in medium) or NaCl (as control, Fig. 2).

Imaging and Image Analysis

Imaging was performed using the 10 x objective of NYONE® Scientific and the settings described in Tab. 1. We imaged the cells directly before staining/treatment and 30 min, 24 h, 48 h and 72 h afterwards. The whole well was imaged. Image analysis was performed using the **Confluence** and **Virtual Cytoplasm (1F)** applications of YT®-Software.

Resazurin/alamarBlue® Assay

After final imaging of the stained cells (72 h after treatment), we added 10 µl of alamarBlue™ reagent to each well. We incubated the cells in the incubator for 4 h and then imaged the plate using the following settings of NYONE® Scientific: Objective: 4 x / 0.13 NA, Ex: Lime, Em: Red-LP, Focus: Each Well, Focus Offset: 0, Application: **Image Statistics**. One image per well was taken. After imaging in NYONE® Scientific, we measured the fluorescence in a Tecan plate reader (Ex: 545 nm, Em: 595 nm). As a last step, we also measured the absorbance in the plate reader (wavelength: 579 nm, reference wavelength: 600 nm).

Statistics

All data were exported and the change of confluence was calculated by the following equation using Microsoft Excel:

$$\Delta confluence(t) = confluence(t) / confluence(t_0)$$

Dose response curves including IC50/EC50 as well as the growth rate k were calculated using statistics software GraphPad Prism.

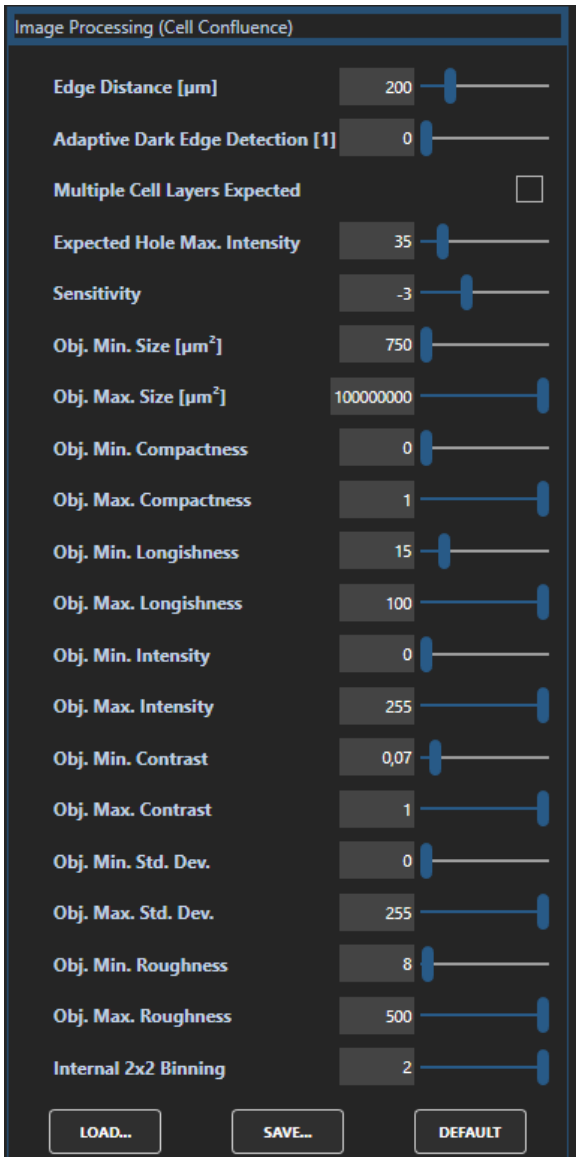


FIG. 3. EXAMPLE IMAGE ROCESSING SETTINGS FOR THE CONFLUENCE APPLICATION

TAB. 1: IMAGING SETTINGS

Dye/Assay	Excitation LED [nm]	Emission Filter [nm]
CellEvent™ Caspase-3/7 Green Reagent	Blue (475/28)	Green (530/43)
SPY650-DNA	Red (632/22)	Far Red (685/40)
Resazurin	Lime (562/40)	Red-LP (593-LP)

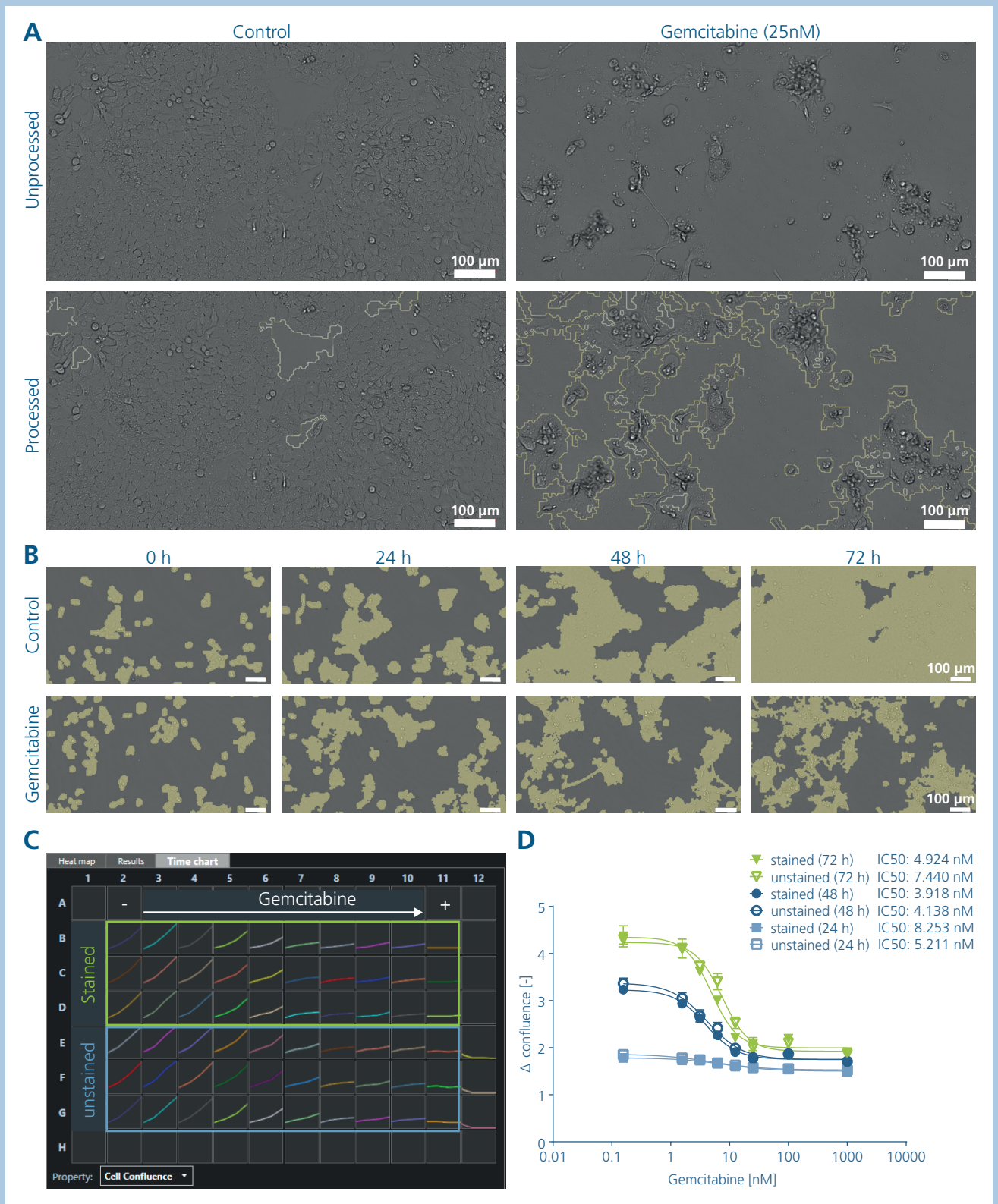


FIG. 4. THE CONFLUENCE APPLICATION OF YT®-SOFTWARE DETECTED DOSE-DEPENDENT EFFECTS OF GEMCITABINE

5 × 10^3 cancer cells were seeded into a 96 well plate, treated with gemcitabine, NaCl (negative control, -) or staurosporin (positive control, +) and imaged daily using NYONE® Scientific. A) Representative pictures after 72 h. The area covered by cells was analyzed (processed) with the Confluence application (yellow lines). B) Representative processed images of control- or gemcitabine-treated cells (yellow area). C) The Time chart function of YT®-Software depicts the cell confluence over time. D) Data were exported and dose response curves/IC50 values calculated with GraphPad Prism.

RESULTS AND DISCUSSION

The Confluence application of YT®-Software detected dose-dependent effects of gemcitabine

At first, we analyzed the effect of gemcitabine on the confluence of the cells as an indirect measure for cell death using the **Confluence** application of YT®-Software. This analysis is label-free, cheap and easy to perform. While control-treated cells proliferate and their confluence increases, dying cells do not divide anymore. Therefore, we expected their confluence to be lower than that of control cells.

For this analysis, it might be necessary to adjust the image processing settings as a lot of debris is usually visible in wells containing apoptotic/dying cells. This might falsely be recognized as cell confluence using the default settings. Parameters that help to exclude debris are "sensitivity", "Obj. Min. Size", "Obj. Min. Longishness", "Obj. Min. Contrast" or "Obj. Min. Roughness". Example settings are shown in Fig. 3.

As expected, the cell confluence of untreated cells increased over time, while gemcitabine-treated cells were less confluent after 72 h of treatment (Fig. 4 A+B). We could easily observe these effects using the Time chart function of the Analyst tab in YT®-Software (Fig. 4 C). After exporting the data and analyzing them with the statistics software GraphPad Prism, we clearly saw a dose-dependent effect of gemcitabine treatment on the cell confluence (Fig. 4 D) and the confluence-derived growth factor (data not shown).

As we wanted to add the CellEvent™ Caspase-3/7 Reagent and SPY650-DNA directly to the cell culture medium, we also analyzed, whether addition of these dyes had any effect on cell growth or their sensitivity towards gemcitabine. The cell growth of untreated cells did not show any difference between stained and unstained cells (Fig. 4 C). However, stained cells were slightly more sensitive to gemcitabine treatment (Fig. 4 C+D). If such subtle changes are important for respective research questions, running the kinetic assay only in preliminary experiments to determine the optimal time point for caspase activation can be considered. Subsequently, the cells can be treated without staining and the dyes can be added and used as an endpoint measurement at this optimal time point to determine the exact IC/EC50.

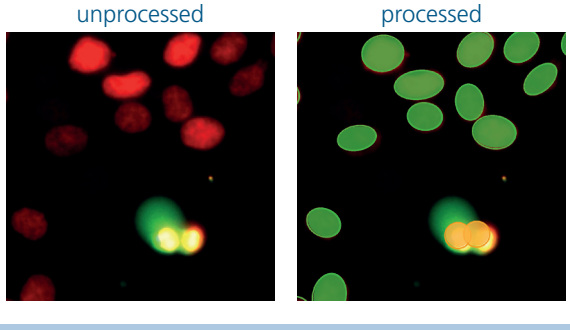


FIG. 5. THE VIRTUAL CYTOPLASM (1F) APPLICATION DETECTED THE NUCLEI AND CELLS WITH CASPASE-3/7 ACTIVITY

SPY650-DNA-positive nuclei are marked with a green ellipse and cells with caspase-3/7 activity with an orange circle (right image).

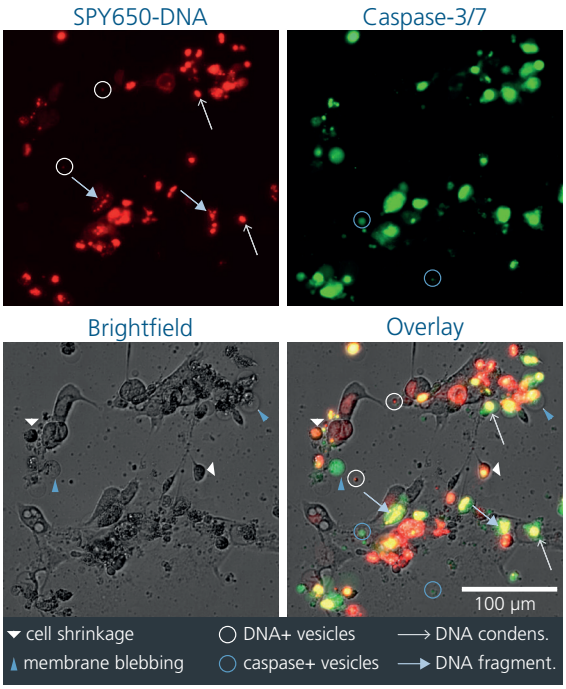


FIG. 6. GEMCITABINE-TREATED CELLS SHOWED MORPHOLOGICAL SIGNS OF APOPTOSIS

Cells were treated with 25 nM gemcitabine. After 48 h - 72 h, they showed classical signs of apoptosis like cell shrinkage, membrane blebbing, DNA condensation or fragmentation and extracellular vesicles containing DNA or active caspase-3/7.

The Virtual Cytoplasm (1F) application of YT®-Software quantified the cell nuclei and the percentage of cells with caspase-3/7 activity

Confluence measurements are fast, cheap and label-free and give a first impression. However, they do not show whether the cells die or just stop proliferating. To prove that the cells actually die after gemcitabine treatment, we stained them with CellEvent™ Caspase-3/7 Reagent and SPY650-DNA. Then, we quantified the number of cells and the percentage of cells with caspase activity using the **Virtual Cytoplasm (1F)** application of YT®-Software. This application recognized the SPY650-DNA-stained nuclei in the red channel and determined whether a CellEvent™-positive signal in the green channel was visible in a virtual circle surrounding these nuclei (Fig. 5). We determined the optimal thresholds for the analysis using the control-treated cells and morphological observation so that healthy cells with no or very low caspase activity were regarded as negative while cells that exceeded a

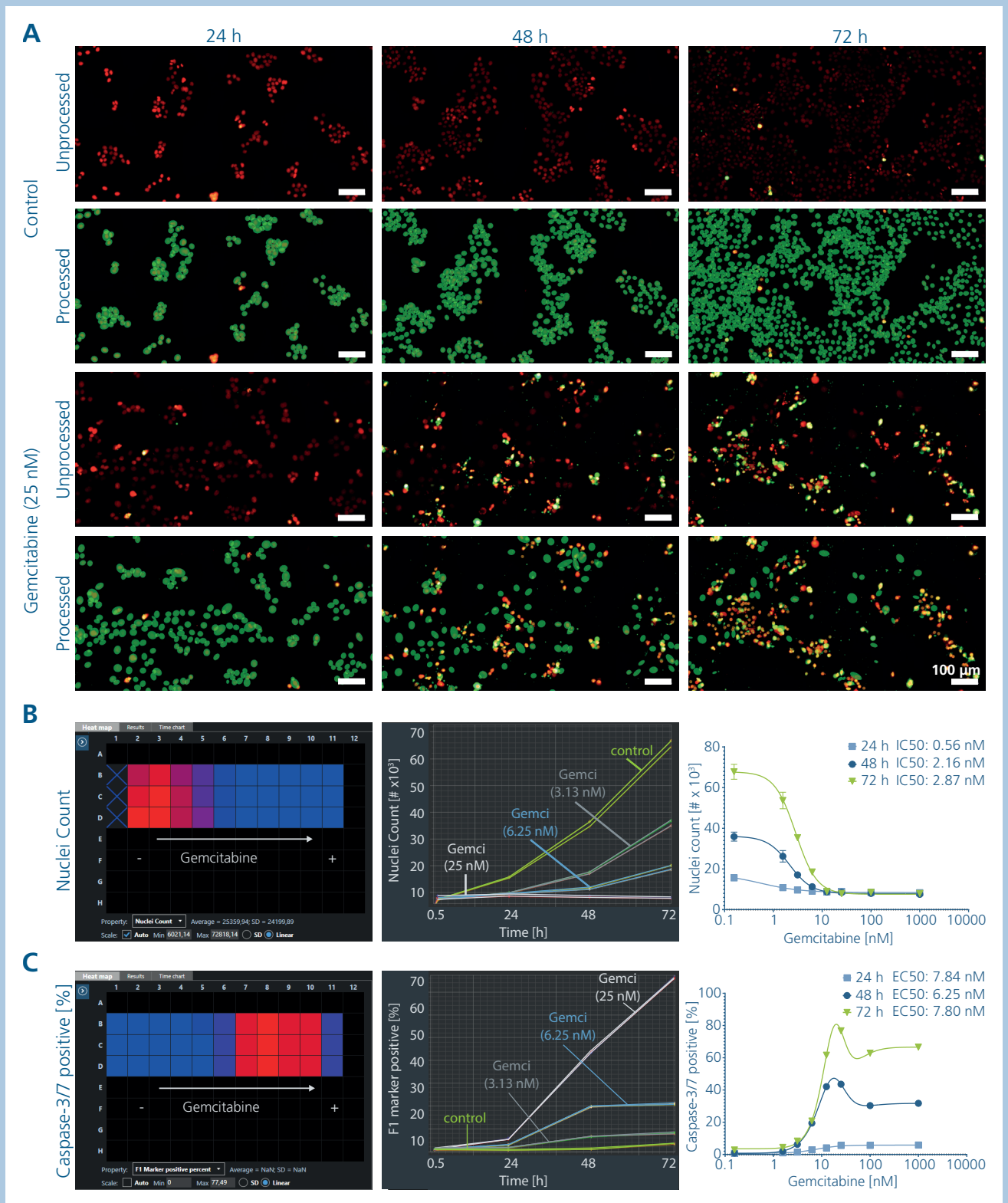


FIG. 7. GEMCITABINE TREATMENT DECREASED THE NUCLEI COUNT AND INCREASED THE PERCENTAGE OF CELLS WITH CASPASE-3/7 ACTIVITY

We seeded cells in 96 well plates, stained them with SPY650-DNA (red) and CellEvent™ Caspase-3/7 Reagent (green) and treated them with gemcitabine. The plates were analyzed with NYONE® Scientific and the Virtual Cytoplasm (1F) application of YT®-Software. A) SPY650-DNA-positive cells are marked with a green ellipse and SPY650-DNA-/CellEvent™-positive cells with an orange circle. B) The Heat map function gives a fast overview of the results at each time point (here 72 h, blue = low numbers, red= high numbers) and the Time chart function shows the time course of each parameter (duplicate wells of selected treatments are shown). Dose responce curves were generated using GraphPad Prism.

certain caspase threshold were regarded as positive.
Many of the cells with caspase activity showed characteristic signs of apoptosis like cell shrinkage, DNA condensation and fragmentation or apoptotic blebbing (Fig. 6). Moreover, many extracellular vesicles (EVs) were visible in the treated wells, which were most likely derived from apoptotic cells (also called Apo-EVs) [10]. Some of these particles obviously contained DNA as they were SPY650-DNA-positive. This is well-known for Apo-EVs [10]. Interestingly, some also showed Caspase-3/7 activity, a phenomen that has also previously been described [5]. This morphological characterisation is a huge benefit of high-content screening over simple plate reader assays.

Gemcitabine treatment dose-dependently decreased the nuclei count and increased the percentage of cells with caspase-3/7 activity

Using the **Virtual Cytoplasm (1F)** application, we analyzed the effects of gemcitabine over time. In control-treated cells, the number of nuclei increased (Fig. 7 A+B). Some cells with caspase-3/7 activity were also visible, which is normal for most cell culture cells (Fig. 7 A). However, the percentage did not increase over time but remained constant (Fig. 7 C). In contrast, after gemcitabine treatment, the number of nuclei was diminished compared to control cells (Fig. 7 A+B) while the percentage of cells with caspase-3/7 activity increased (Fig. 7 C). Again, these effects were dose-dependent as could easily be observed using the heatmap function of YT®-Software or by dose response curves of the exported data (Fig. 7 B+C).

The IC50 values of the different read-outs varied slightly but were comparable to a conventional viability assay

To compare the results to a standard viability assay, we used the resazurin-based alamarBlue™ assay. We added the reagent to the unstained or previously stained cells as its fluorescence does not interfere with the other wavelengths. After incubation, we measured the fluorescence in NYONE® Scientific or a Tecan plate reader for comparison. The dose response curves and IC50 values of NYONE® and Tecan looked remarkably similar (Fig. 8). Again, we saw that staining slightly sensitized the cells towards gemcitabine treatment.
The IC50 values of the fluorescent read-out of the viability assay were very similar to the EC50 for the caspase assay (Tab. 2). The values for nuclei count, confluence and growth factor were slightly lower. However, the values were in a similar range (~2-8 nM for stained and 5-11 nM for unstained cells).

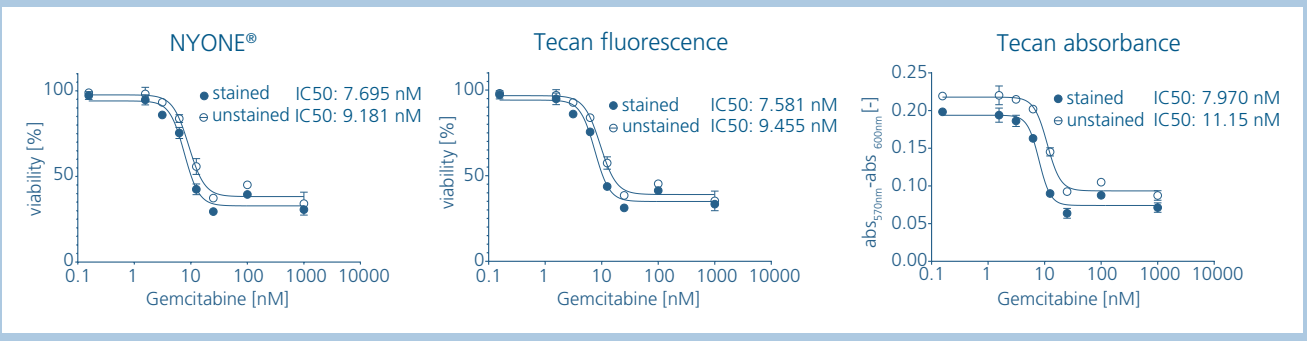


FIG. 8. THE IMAGE STATISTICS APPLICATION OF YT®-SOFTWARE RELIABLY ANALYZED A RESAZURIN-BASED VIABILITY ASSAY

We seeded 5 × 10^3 pancreatic cancer cells per well in a 96 well plate. After 24 24 h, we stained them with SPY650-DNA and CellEvent™ Caspase-3/7 Green Reagent (stained) or left them unstained. Immediately afterwards, we treated them with various concentrations of gemcitabine for 72 h. After the last measurement, we added a resazurin solution (alamarBlue™) and incubated the cells for 4 h. Subsequently, the plates were again imaged using the Image Statistics application of NYONE® Scientific or the fluorescence or absorbance function of a Tecan plate reader as comparison.

TAB. 2: IC/EC50 VALUES OF GEMCITABINE-TREATED CELLS AFTER 72 H

Cells were stained with SPY650-DNA and CellEvent™ Caspase-3/7 Green Reagent or left unstained. Then, they were treated with gemcitabine for 72 h and analyzed using the Confluence or Virtual Cytoplasm (1F) application of YT®-Software. Subsequently, the resazurin-based viability reagent alamarBlue™ was added and analyzed after 4 h using the Image Statistics application of YT®-Software or a Tecan plate reader (Tec.) as a comparison. Data were exported and dose response curves as well as IC50 values generated using GraphPad Prism.

	Confluence	Growth Factor k	Nuclei Count	Caspase+	Resazurin NYONE	Resazurin Tec. fluo	Resazurin Tec abs.
Stained	4.92 nM	3.94 nM	2.87 nM	7.80 nM	7.70 nM	7.58 nM	7.97 nM
Unstained	7.44 nM	5.23 nM	-	-	9.18 nM	9.46 nM	11.15 nM

Conclusion

SYNENTEC's cell imagers and YT®-Software enable detection and analysis of fluorescent caspase assays in combination with nuclei dyes over time. The simple no-wash, no-lyse assay introduced here is suitable for automation and high-content screening. Different parameters like confluence, growth factor, nuclei count, percentage of cells with caspase-activity and viability could be analyzed using just one plate and resulted in comparable IC50 values. Moreover, in contrast to simple plate reader assays, the cells could be morphologically examined for signs of apoptosis like cell shrinkage, membrane blebbing, DNA condensation/fragmentation and extracellular vesicles. Thus, NYONE® and YT®-Software prove once again to be versatile tools for high-content screening.

Applications used in this note

- The **Confluence** application non-invasively detected dose-dependent effects over time.
- The **Virtual Cytoplasm (1F)** application quantified the cell nuclei and the percentage of caspase-positive cells.
- The **Image Statistics** application reliably analyzed a resazurin-based viability assay.

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SYNENTEC GmbH
Otto-Hahn-Str. 9A
25337 Elmshorn/Germany
Phone. +49 (0) 4121 46311-0
Email. appsupport@synentec.com

www.synentec.com