Analyzing Dose-Dependent Effects of Cisplatin Using NYONE® and YT®-Software

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Abstract

In drug screening and development, it is important to analyze more than one concentration of the screened compounds. Here, we analyzed the dose-dependent effect of the chemotherapeutic drug cisplatin on the ovarian cancer cell line HEY using SYNENTEC's cell imager NYONE®-Scientific and its YT®-software. Firstly, we measured the confluence of the cells after 24, 48 and 72 h as a measure for cell growth. In a second step, we analyzed the reduction of alamarBlue as a measure for viability.

Lastly, the actual cell count was determined by the number of Hoechst-stained nuclei. For all three parameters, dose response curves were generated and the inhibitory concentration 50 (IC50) was calculated. All three steps were subsequently performed in the same 96 microwell plate. They generated comparable IC50 values. We show that SYNENTEC's imaging devices are capable of high-throughput and high-content analyses and therefore are well suited for drug discovery and development.
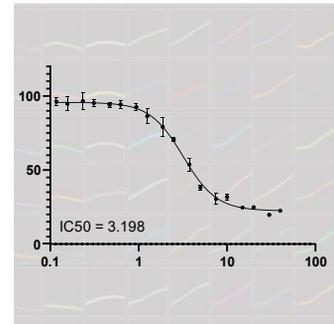
KEYWORDS:
DOSE RESPONSE, IC50, EC50, CURVE FITTING, CYTOSTATIC DRUGS, CANCER RESEARCH, CELL CONFLUENCE, NUCLEI COUNT, NUCLEUS SIZE, NUCLEUS FLUORESCENT INTENSITY, CELL VIABILITY, VIABILITY ASSAY, HOECHST, ALAMAR BLUE, HIGH-CONTENT SCREENING, HIGH-THROUGHPUT SCREENING, HTS, ACTIVE TO HIT, HIT TO LEAD, MTT, XTT, CASPASE, TARGET VALIDATION, DRUG TARGET IDENTIFICATION, COMPOUND SCREENING, APOPTOSIS MONITORING, IN VIVO CELL BASED ASSAYS, NON-INVASIVE, IMMUNOLOGY, DRUG DISCOVERY

Introduction

Drug development is a very long, expensive and time-consuming procedure. The first steps in this process are the screening of new compounds and the identification of hit substances[1][2]. In order to save time and money at this stage, high-throughput screens (HTS) are more and more used[3]. Usually only one parameter is screened at a time e.g. viability. This is often done by a viability test using the reduction of tetrazolium salts like MTT or XTT by living cells. However, these assays can be influenced by changes in the metabolism of the cells and do not show the actual cell number[4]. Therefore, new methods analyzing more than one parameter are desirable. This can be achieved by high-content screening (HCS). In these screens, more than one end-point parameter is analyzed. In this note, we describe the subsequent measurement of cell confluence, viability by an alamarBlue assay and quantification of Hoechst-stained nuclei. Cell confluence is an indirect measure for the growth of adherent cells and can be non-invasively observed over time. However, confluence can also be influenced if the cells change their morphology and e.g. become enlarged after drug treatment. Therefore, other parameters should be analyzed in parallel. One other parameter often used for screening is viability. Common tests are MTT and XTT (see above).

A cheap and easy, one-step alternative is the alamarBlue/resazurin assay. In this assay, the non-toxic, cell-permeable dye resazurin (blue in color and virtually non-fluorescent) is reduced to resorufin upon entering living cells. Resorufin is red in color and highly fluorescent and can thus be measured either by an absorbance- or fluorescence-based plate reader[5]. Here, we show that imaging devices can also be used for the measurement of alamarBlue.

However, also these viability assays are an indirect measure und do not quantify the cell number. In order to count the cells, we used the nucleic acid dye Hoechst33342 in this study. This dye penetrates the cell membrane and stains the nuclei[6]. We also analyzed morphologic changes of the cell nuclei as observed during apoptosis.

Moreover, to better characterize the effects of substances, more than just one concentration should be analyzed. Ideally, many concentrations are used and the concentration/dose-dependent effects are measured. From these results, dose response curves and the half maximal inhibitory or effective concentration (IC50/EC50) of the substance can be generated. We aimed to develop a multi-step process, in which the dose-dependent effect of drugs on confluence, viability and cell count can be observed. For this, we used SYNENTEC's automated cell imagers (NYONE® or CELLAVISTA®).

Material & Methods

Material:

- HEY (adherent monolayer-forming human ovarian carcinoma cells)
- VLE RPMI 1640 (with 10 % FCS, 2.0 g/l $NaHCO_3$, 300 mg/l L-Glutamine; e.g. Merck cat. # FG1415)
- 1% Trypsin in PBS
- Trypan Blue (according to SYNENTEC's Application Note)
- Cisplatin (in NaCl)
- Physiological NaCl

- Triton X-100
- 96-well cell culture plate, black or clear, flat clear bottom (e.g. Corning Life Science cat. # 734-1609 or Sarstedt cat. # 83.3924)
- AlamarBlue® (e.g. BioRad cat. # BUFO12A)
- Hoechst33342 (e.g. Invitrogen cat. # H1399; 100 mg)
- SYNENTEC's imaging systems (here: NYONE®-Scientific imaging system)
- SYNENTEC's YT®-software

Methods:

Cell culture and cell seeding

The ovarian cancer cells HEY were cultured under standard cell culture conditions (humidified atmosphere, 37 °C, 5 % CO_2) with VLE RPMI including stabile glutamine and 10 % FCS. After trypsinization, the cells were counted with NYONE® Scientific using SYNENTEC's Trypan Blue application (TN-B004-XVII-19). Afterwards, the required amount of cells (here $1.5 * 10^4$ cells per well) was calculated based on the viable cell density (VCD). The cells were seeded into a 96-well plate using a multistep pipette. As the cells in the outer wells of the plates often grow differently because of quicker evaporation of the medium or higher sensitivity to temperature changes, no cells were seeded in these wells. Instead, they were filled with PBS to avoid evaporation of the cell culture medium of the interior wells (cell culture medium can also be used alternatively).

Treatment with the cytostatic drug cisplatin

After seeding the cells into a 96-well plate, the cells were incubated for 24 h to allow adherence of the cells. Afterwards, the first confluence measurement was performed (0 h). This measurement shows the confluence before treatment and reveals any differences in cell seeding. It is therefore a good parameter for quality control. For the cisplatin treatment, one solution was prepared containing 40 µM cisplatin in standard medium and another with 30 µM. Subsequently, both solutions were serially diluted 1:2. This resulted in 18 different cisplatin concentrations (Fig. 1). The cells were treated in triplicate wells. As a solvent control, NaCl was used and Triton X-100 was used as a control for 100 % cell death.

Cell confluence measurement

For the confluence measurements, SYNENTEC´s *Cell Confluence* application of YT®-software was chosen. The cells were imaged before cisplatin treatment (0 h) and 24, 48 and 72 h after treatment. The acquisition and processing settings are shown in table **2**.

AlamarBlue measurement

For the alamarBlue assay, the cell culture medium was removed after 72 h of cisplatin treatment and replaced with 200 µl new cell culture medium containing 10 % alamarBlue solution. We included this medium change to avoid differences in the amount of medium and thus alamarBlue concentration due to evaporation. However, in many cases, this step is not necessary and alamarBlue can directly be added to the medium. The cells were incubated with the alamarBlue solution at 37 °C for 4 h and the average fluorescence intensity was measured with the *Image Statistics* operator of YT®-software. Table **2** shows the excitation and emission settings for the alamarBlue measurements with NYONE®. As a comparison, the assay was also measured by a plate reader (Tecan, Ex 545 / Em 595).

	1	2	3	4	5	6	7	8	9	10
A										
B	NaCL	40 µM	20 µM	10 µM	5 µM	2.5 µM	1.25 µM	0.625 µM	0.3125 µM	0.1563 µM
C	NaCL	40 µM	20 µM	10 µM	5 µM	2.5 µM	1.25 µM	0.625 µM	0.3125 µM	0.1563 µM
D	NaCL	40 µM	20 µM	10 µM	5 µM	2.5 µM	1.25 µM	0.625 µM	0.3125 µM	0.1563 µM
E	Triton	30 µM	15 µM	7.5 µM	3.25 µM	1.625 µM	0.8125 µM	0.4063 µM	0.2032 µM	0.1016 µM
F	Triton	30 µM	15 µM	7.5 µM	3.25 µM	1.625 µM	0.8125 µM	0.4063 µM	0.2032 µM	0.1016 µM
G	Triton	30 µM	15 µM	7.5 µM	3.25 µM	1.625 µM	0.8125 µM	0.4063 µM	0.2032 µM	0.1016 µM
H										

Fig. 1: Pipetting scheme of a 96 well plate for the cisplatin treatment.
The figure shows the controls (NaCl: solvent control, Triton X-100: dead control) and the different cisplatin concentrations used for analyzing and creating dose response curves. The different cisplatin concentrations were tested on an ovarian cancer cell line (HEY). Cells were treated in triplicate wells.
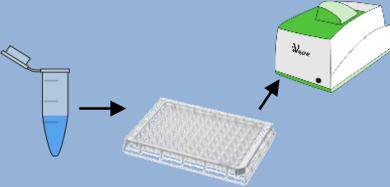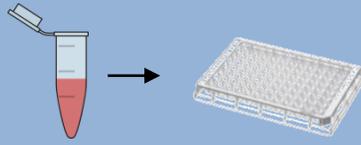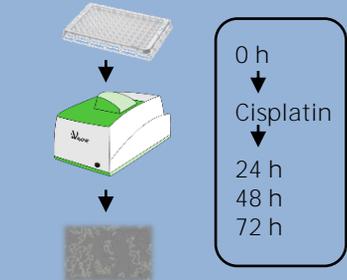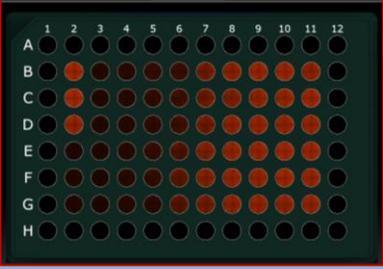
Nuclei count

After the evaluation of cell viability by alamarBlue, the nuclei were stained with 3.25 µM Hoechst33342 for 30 min in order to accurately count the adherent cells. After the incubation, the cells were measured and analyzed by the *Nuclei Count* operator of YT®-software. Table **2** shows the excitation and emission settings for the nuclei count measurements.

Statistical analysis

The results of YT®-software were exported and analyzed using GraphPad Prism. *[Inhibitor] vs. response -- Variable slope (four parameters)* was used to calculate dose response curves and IC50 values.

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Tab. 1: Overview of experimental steps.

Step	Procedure	Information
Trypan Blue viability cell count	Cells were trypsinized and 10 µl of cell suspension were diluted with 90 µl PBS and 100 µl Trypan Blue from the SYNENTEC´s Trypan-Blue-Kit® to a final cell dilution of 1:20. The plate was measured with the NYONE®-Trypan Blue Viability Application.	For detailed information, please refer to SYNENTEC's Technical Note for Trypan Blue measurements (TN-B004-XVII-19).
Cell seeding	After determining the cell number by Trypan Blue viability cell count, the corresponding cell dilution was calculated for the desired number of cells required for the experiment. After calculating the cell number, the cells were seeded in a 96-well plate.	The required cell number depends on the cell line and the experiment. Leave the outer wells of the 96-well plate cell-free but fill with cell culture medium or PBS.
Cell confluence measurements and cisplatin treatment 0 h → Cisplatin → 24 h 48 h 72 h	For the confluence measurements, the *Cell Confluence* operator of YT®-software was used. The first confluence measurement (0 h) was carried out 24 h after cell seeding. Afterwards, the cells were treated with different concentrations of cisplatin and the cell confluence was measured after 24 h, 48 h and 72 h.	For further information about the different *Confluence* operators see the Short Notes and Tech Notes on the SYNENTEC website → downloads.
AlamarBlue cell viability assay	To test the cell viability after 72 h treatment with cisplatin, an alamarBlue assay was performed. The cell culture medium was removed and replaced by 200 µl new cell culture medium containing 10 % alamarBlue. The cells were incubated with the alamarBlue solution at 37 °C for 4 h and the increase in the fluorescent signal was measured by fluorescence microscopy with the *Image Statistics* operator of YT®-software.	The non-toxic, cell-permeable resazurin in this assay (blue in color and virtually non-fluorescent) is reduced to resorufin upon entering living cells. Resorufin is red in color and highly fluorescent.
Nuclei Count	The nuclear dye Hoechst33342 was added to the cells to quantify the number of nuclei after cisplatin treatment. Hoechst was diluted in cell culture medium to a final concentration of 3.25 µM and incubated for about 30 min. Afterwards, the fluorescence of the nuclei was analyzed by the Nuclei Count operator of YT®-software.	AlamarBlue and Hoechst can also be incubated in parallel and measured one after the other. However, it is recommended to measure alamarBlue first, remove the alamarBlue solution and then add Hoechst to the cells to rule out any interference of the dyes.
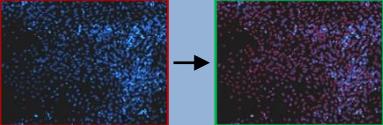
Tab. 2: Settings for cell confluence, alamarBlue and nuclei count measurements.

Settings	Confluence	AlamarBlue	Nuclei Count
Image Processing Operator	Confluence	Image Statistics	Nuclei Count
Excitation	Brightfield (BF)	562/40 (Lime)	377/50 (UV)
Emission	Brightfield (Green)	593 (Red-LP)	452/45 (Blue)
Intensity [%]	100	100	100
Exposure Time [ms]	~ 10	~ 20	~ 40-60

Results & Discussion

High-content screening with SYNENTEC's imaging devices
With SYNENTEC's imaging devices (here NYONE® Scientific), it is possible to execute high-content and high-throughput screenings quickly and easily. In this note, the cell confluence of an ovarian cancer cell line as well as the cell viability and the analysis of the cell nuclei under treatment conditions were investigated with only one 96-well plate.

Confluence measurements reflect the dose-dependent effects of cisplatin on cells over time
To analyze dose response effects of cytostatic drugs on cancer cells, the ovarian cancer cell line HEY was treated with different concentrations of the cytostatic drug cisplatin. The cells were imaged after 24 h, 48 h and 72 h using NYONE® and analyzed with the *Cell Confluence* operator of YT®-software. Fig. 2A shows representative pictures of cells treated with 40 µM of cisplatin. YT®-software detects the confluent cell areas and marks these areas with a yellow overlay. The confluence of the control treatment with NaCl increased over time (Fig. 2A). This increase was inhibited by cisplatin treatment. This effect can easily be observed at a glance using either the time charts (Fig. 2B) or heat map (Fig. 2C) of the analyst tab of YT®-software. From the exported confluence data, dose-response curves were generated and IC50 values calculated (Fig. 2D). In the example shown, the standard deviation of triplicate wells was high. An explanation might be, that the cells were quite confluent at 72 h, especially the control cells and cells treated with low cisplatin concentrations. They started to grow on top of each other, which was difficult to distinguish by image processing. This can be mitigated by seeding cells at a lower density.
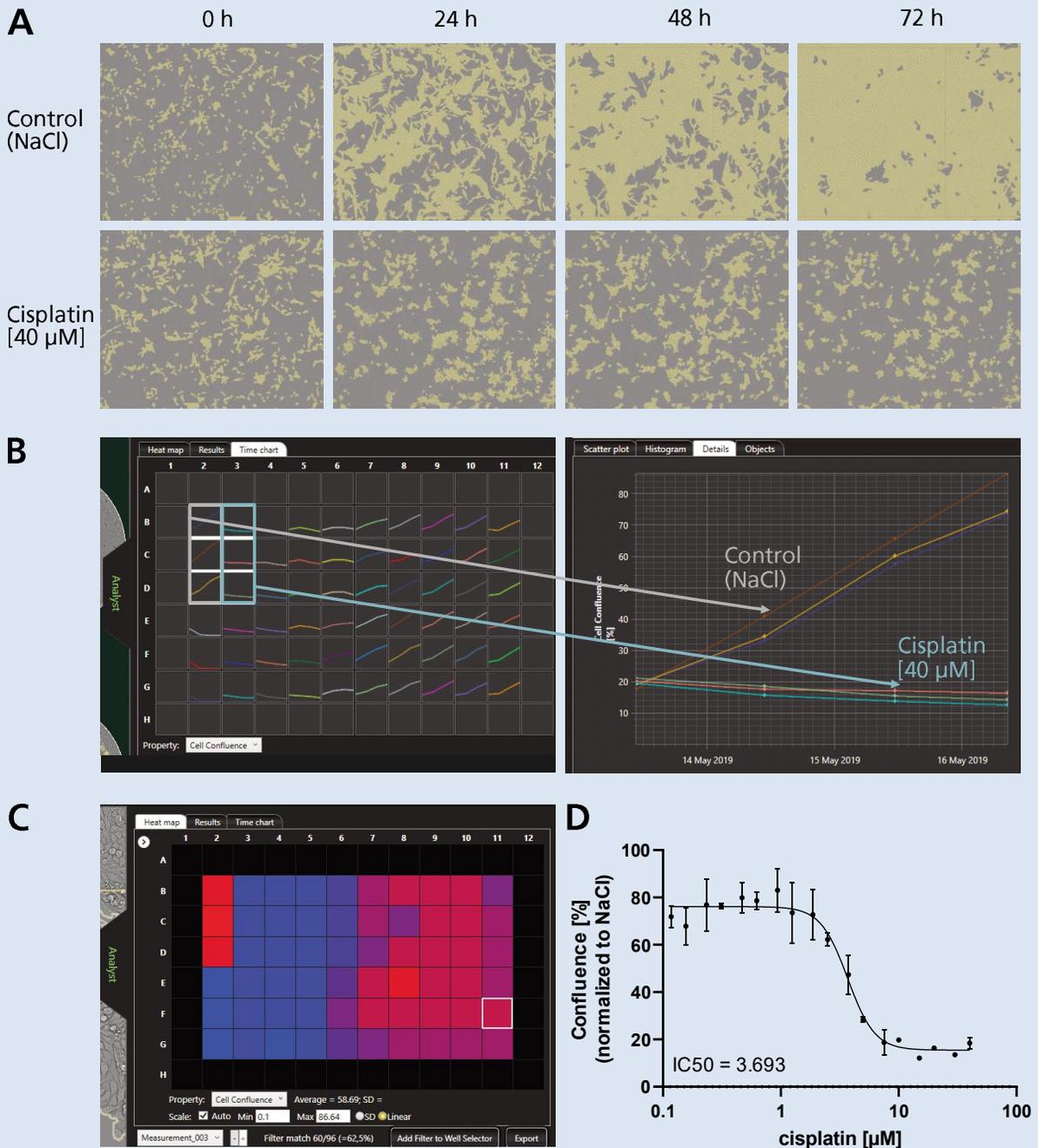
Fig. 2: Confluence measurements reflect the dose-dependent effects of cisplatin on cells over time. The ovarian cancer cell line HEY was seeded into 96-well plates and treated with different concentrations of cisplatin (see Fig. 1). Before (0 h) and 24, 48 and 72 h after treatment, the cells were imaged using the *Cell Confluence* operator of NYONE®. A) YT®-software detects the confluent area and overlays it yellow. Representative pictures of cells treated with NaCL (as control) or 40 µM cisplatin are shown. B) YT®-software detects the confluent area and generates time charts. Single wells can be selected and observed in the details of the Analyst tab. Here, representative growth curves of NaCL-treated and cisplatin-treated cells are shown. C) For each time point, a heat map of the confluence can be observed in the Analyst tab. D) The results were exported and analyzed with GraphPad Prism to generate dose response curves and calculate IC50 values (n=3, error bars = standard deviation).

A

B

C

IC50 = 3.198
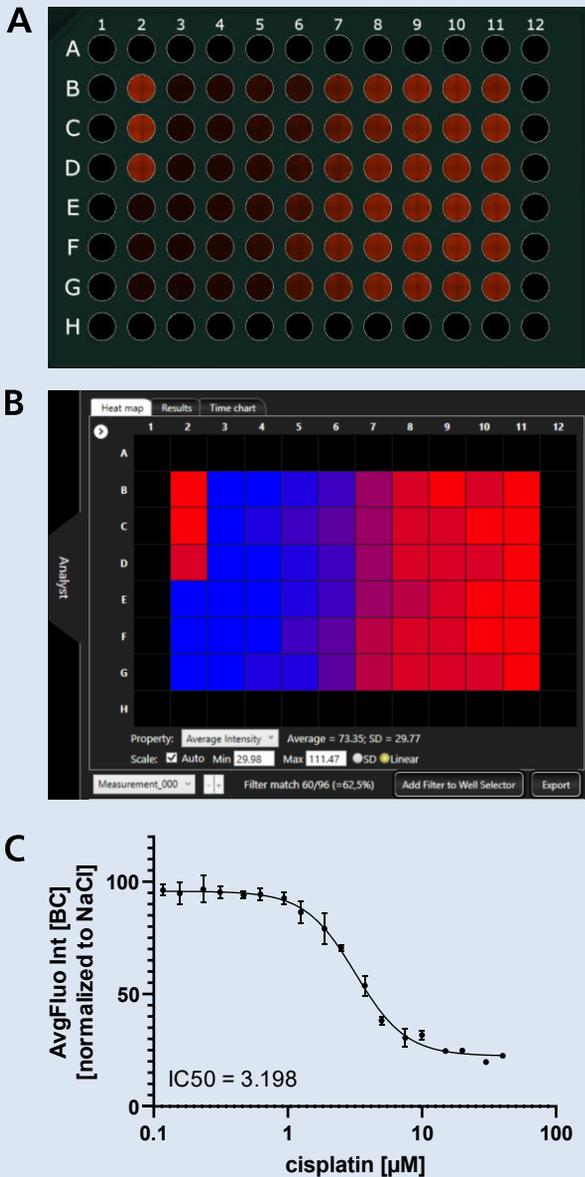
Fig. 3: The viability of the cells after cisplatin treatment decreased as observed by alamarBlue staining and evaluation with the *Image Statistics* operator

The ovarian cancer cell line HEY was seeded into 96-well plates and treated with different concentrations of cisplatin (see Fig. 1). 72 h after treatment, alamarBlue was added. A) Four hours later, the plate was imaged using the *Image statistics* operator of NYONE®. B) The *Average (Fluorescent) Intensity* can be visualized in a heat map. C) The results were exported and analyzed with GraphPad Prism to generate dose response curves and calculate IC50 values (n=3, error bars = standard deviation).

Viability of the cells can be displayed by alamarBlue staining and evaluation with the *Image Statistics* operator

To examine the cell viability after cisplatin treatment, an alamarBlue assay was performed. This assay is usually measured using plate readers. However, as it is fluorescence-based and the excitation and emission matches the filter sets of NYONE®, we measured it with NYONE® and quantified the signal using the *Image Statistics* operator of YT®-software. Fig. 3 shows that this was possible and generated an accurate dose response curve with low standard deviation. The low standard deviation for this method in comparison to the others can be explained by the fact, that this assay shows the metabolic activity of the cells regardless if they are in a monolayer or not. The curve and respective IC50 was almost equal to the curve obtained by a plate reader (data not shown).

The actual cell number and the fragmentation of nuclei as observed in apoptosis was observed by Hoechst staining

To quantify cells after cisplatin treatment, the nuclei were stained with the fluorescent dye Hoechst33342 after performing the alamarBlue assay. The nuclei were counted using the *Nuclei Count* operator of YT®-software. Fig. 4A shows example pictures of cells treated with different cisplatin concentrations. The corresponding dose response curve of the exported data is displayed in Fig. 4B. After cisplatin treatment, the morphology of the nuclei changed drastically. With higher cisplatin concentrations, the nuclei became smaller and showed a more intense fluorescence (Fig. 4C+D). This most likely reflects the DNA fragmentation typically observed during apoptosis induction. A fact that could be further confirmed with other apoptotic assays like e.g. JC1 or AnnexinV / PI (Propidium iodide).
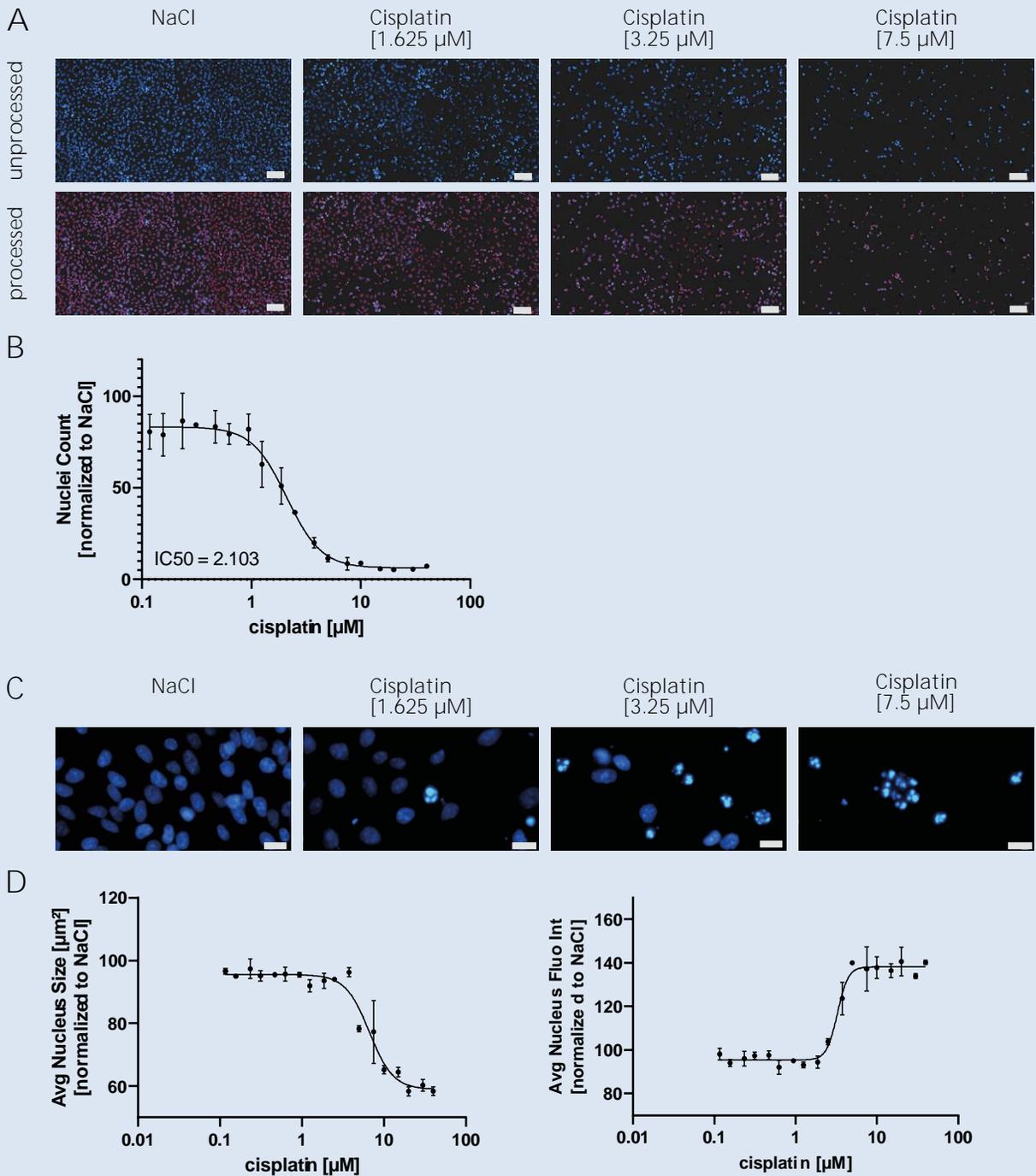
Fig. 4: Hoechst staining was used to analyze the cell count and observe morphological changes of the nuclei.

The ovarian cancer cell line HEY was seeded into 96-well plates and treated with different concentrations of cisplatin (see Fig. 1). 72 h after treatment (and after confluence and alamarBlue measurements), the medium was replaced with Hoechst33342 in cell culture medium. The plate was imaged using the *Nuclei Count* operator of NYONE®. A) Representative images of cells treated with different concentrations of cisplatin before and after image processing (nuclei encircled red, scale bar = 100 µm). B+D) Results were exported and analyzed with GraphPad Prism (n=3, error bars = standard deviation). C) Representative images of stained nuclei treated with different concentrations of cisplatin (scale bar = 20 µm).

Tab. 3: Comparison of the IC50 values of the described methods

Method	IC50
Cell Confluence	3.693 µM
alamarBlue	3.198 µM
Nuclei Count	2.103 µM

IC50 values of all three methods were comparable

The evaluation of the performed experiments and methods (Cell Confluence, alamarBlue and Nuclei Count) resulted in comparable IC50 values (Tab. 3). The comparability of the data underlines the accuracy of the experiments carried out with NYONE® and the experimental setup.

Conclusion & Outlook

The experimental design used for this Application Note points out how easy it is to perform dose response analyses in a high-content mode with SYNENTEC's imaging systems (here NYONE® Scientific). Within a very short time, several results can be obtained from a single 96-well plate (other plates formats like 384-well plates are also possible) with little effort. In addition, it would even be possible to combine a live/dead assay (see Application Note on the website of SYNENTEC), for example with Calcein and Propidium iodide, with Hoechst staining in order to precisely analyze whether the cells with intact cell nuclei are still alive and whether the cells with fragmented cell nuclei are accordingly apoptotic. Additionally, other assays like JC1 or pHH3 could be performed. With the large number of different and easy to use operators of YT®-software, both high-content and high-throughput screenings are virtually unlimited.

All applications are compatible with all models of NYONE® and CELLAVISTA® and are readily available in YT®-software.

CELLAVISTA® 4.0

NYONE®

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