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Label-Free High-Throughput Analysis of Wound Healing Assays Using NYONE® Scientific and YT®-Software

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ABSTRACT

A convenient method to study migration of cells *in vitro* is a wound healing assay. In this assay, closure of an artificial wound is monitored over time by microscopy. As with conventional or time-lapse microscopes only a few samples can be measured at a time, we aimed to develop a wound healing assay in a high-throughput format using our automated microscope NYONE® Scientific and YT®-Software. For this, we seeded cells in microplates and generated wounds either by using sterile pipette tips (scratch assay) or by applying commercially available circular or rectangular silicone inserts (Oris™ and ibidi, respectively). The cells were then treated with different concentrations of the inhibitor cytochalasin D. We developed a new wound healing imaging application consisting of two phases of measurement. In the first phase, the image analysis algorithm robustly detected wounds of different lengths, shapes, widths and rotations. It created a region of interest (ROI) in these wounds which was then automatically scanned in the second phase. Only this second phase was needed to monitor and quantify wound closure at later time points - making the analysis very fast (~37 s per 96 well plate for scratch assays). We quantified a dose-dependent inhibition of wound closure by cytochalasin D and calculated IC50 values from the generated data. In summary, the new wound healing application using NYONE® Scientific and YT®-Software is a very robust and fast method, making it an excellent tool for automation and high-throughput screening.

KEYWORDS: WOUND HEALING ASSAY, SCRATCH ASSAY, MIGRATION ASSAY, WOUND CLOSURE, CANCER RESEARCH, IBIDI, ORIS, AUTOMATION, HIGH-THROUGHPUT, IC50, EC50, DRUG SCREENING
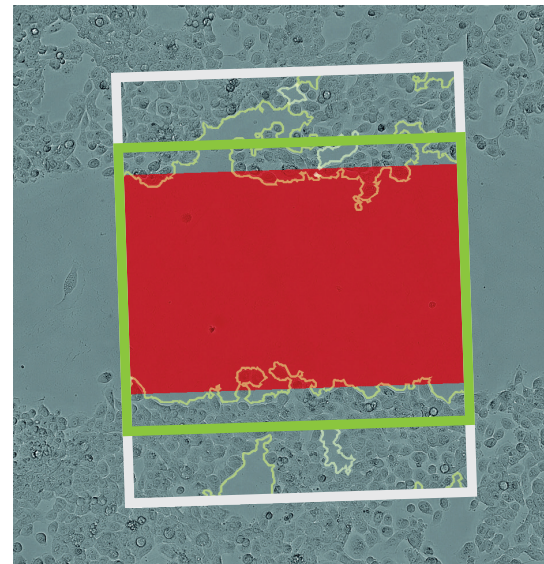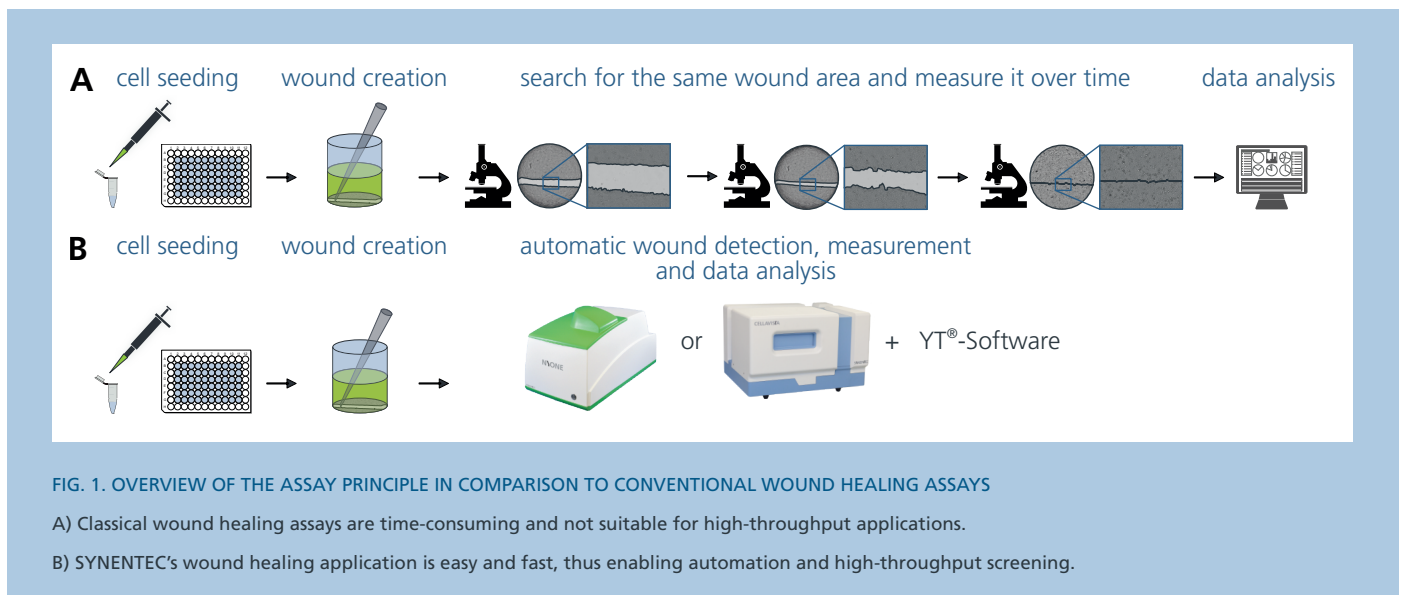
Benefits of the wound healing application at a glance

- The 2-phase measurement design and a robust laser-based autofocus enables very fast imaging and analysis suitable for automation and high-throughput screening.

- Precise image processing detects wounds of different number, size, width, shape and orientation.

- The analysis is label-free and therefore non-invasive.

- Wound closure is quantified over time and depicted as time charts in YT®-Software using either 'Confluence on Gap' or the 'Average Gap Width'.

- Analysis of confluence on periphery enables calculation of the 'Relative Wound Density' (RWD).

INTRODUCTION

Cell migration plays a crucial role in many physiological processes such as wound healing, tissue formation, and the immune response but it is also pathologically relevant in cancer invasion and metastasis [1,2]. A convenient method to analyze cell migration is a wound-healing assay, in which an artificial cell-free gap (wound) is created on a confluent monolayer of cells. Surrounding cells start to migrate into this cell-free gap and eventually close the wound [1]. The disadvantage of manual scratching (e.g. with a pipette tip) is the diversity in the resulting gaps, making it difficult to compare different conditions and to reproduce results. Moreover, it is not suitable for automation and high-throughput screening. Therefore, commercial scratching tools like IncuCyte™ WoundMaker™, BioTek® AutoScratch™ or robotic pin tools as well as wound healing/migration plates are available. Here, we used circular and rectangular silicone inserts as well as manual scratching with pipette tips.

Closure of the wounds is traditionally tracked with live-cell microscopy [3] or by manual observation of the same wound area at different time points [1]. However, with conventional microscopes, it is difficult to relocate, measure and evaluate the same spot in the wound at every time point making it a very time-consuming method. With many time-lapse microscopes, on the other hand, only very few conditions can be monitored and compared. Therefore, both techniques are not useful for high-throughput applications. In contrast, with automated plate reader microscopes, the imaging process is very easy, fast and reproducible. The aim of this Application Note was to develop a robust screening tool for automated high-throughput analysis of wound healing assays using SYNENTEC's cell imagers

FIG. 1. OVERVIEW OF THE ASSAY PRINCIPLE IN COMPARISON TO CONVENTIONAL WOUND HEALING ASSAYS

A) Classical wound healing assays are time-consuming and not suitable for high-throughput applications.

B) SYNENTEC's wound healing application is easy and fast, thus enabling automation and high-throughput screening.

MATERIAL

- H6c7-Kras (adherent, premalignant pancreatic duct epithelial cell line)
- HPDE-Medium
  - > 50 % (v/v) Keratinocyte-serum free medium supplemented with 50 µg/ml bovine pituitary extract and 5 ng/ml EGF
  - > 50 % (v/v) RPMI 1640 supplemented with 10 % (v/v) FCS, 1 % (v/v) L-Glutamine, 0.5 µg/ml Puromycin
- Trypsin 0.05 %/ EDTA 0.02 % (e.g. PAN Biotech)
- 96 well plates, 24 well plates (e.g. Eppendorf)
- cytochalasin D (from *Zygosporium mansonii*; 5 mg/mL in DMSO, 0.2 µm-filtered; e.g. Sigma Aldrich Cat. No. C2618)
- DMSO (sterile)
- device to scratch (here 100 µl pipette-tip)
- sterile forceps
- Oris™ cell migration assay kit (96 well, Platypus Technologies)
- Culture-Insert 2 Well in µ-Dish 35 mm (Cat.-No.: 801176) or for self-insertion (Cat.-No.: 80209, ibidi®)
- Culture Insert 4 Well µ-Dish 35 mm (Cat.-No. 80466) or for self-insertion (Cat.-No.: 80469, ibidi®)
- SYNENTEC´s imaging device (here NYONE® Scientific)
- SYNENTEC´s YT®-Software

METHODS

Cell culture and cell counting

We routinely cultured the pre-malignant pancreatic ductal epithelial cells H6c7-Kras in HPDE 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.

Cell seeding and migration experiments

For experiments using commercially available silicone inserts (Oris™ and ibidi®), we placed them in microplates using sterile forceps. Then, we seeded the optimal number of cells (previosuly determined, see Tab. 1) into the wells or cavities. After 48 h, we removed the inserts. For scratch assays, we seeded 4×10^4 cells / well into a 96 well plate. We did not use the outermost wells of the plate for cells but filled them with PBS instead to avoid evaporation effects. After 48 h, we manually scratched a wound into the confluent monolayer of cells with a 100 µl pipette tip.

After creating the wounds, we rinsed the cells with PBS and replaced the cell culture medium by FCS-free cell culture medium. Directly after the medium exchange, we measured the plates using the *wound healing* application of YT®-Software (time point 0 h). Afterwards, we aspirated the medium again and replaced it with FCS-free medium containing different concentrations of cytochalasin D.

Imaging and processing

We measured the microplates directly after wound generation (before treatment) and at different time points after the treatment using the *wound healing* application of YT®-Software.
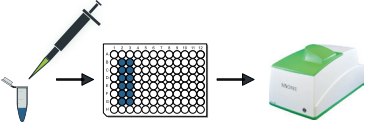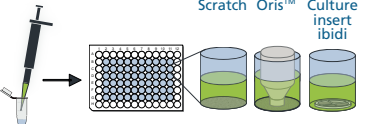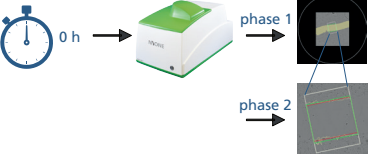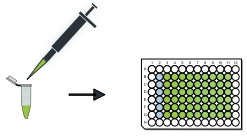
Data evaluation

We exported the data of the processed experiments from YT®-Software and subsequently analyzed them with the statistics software GraphPad Prism. IC50 (Inhibitory concentration 50) and EC50 (effective concentration 50) are the concentrations that give a response half way between bottom and top plateau of the sigmoidal curve. To calculate these values, we used the '[Inhibitor/Agonist] vs. response - Variable slope' equations of GraphPad Prism.

TAB. 1: OVERVIEW OF DIFFERENT WOUND ASSAY FORMATS

Name	Manufacturer	Wound Generated by	Plate Format	Cells per Well / Cavity
Scratch	any microplate	sterile pipette tip	96 well	4×10^4 cells
Oris™	Platypus	circular silicone inserts	96 well	4×10^4 cells
Culture-Insert 2 well	ibidi®	rectangular silicone inserts (two cavities)	24 well	5.33×10^4 cells / 4×10^4 cells
Culture-Insert 4 well	ibidi®	rectangular silicone inserts (four cavities)	12 well	8×10^4 cells / 4×10^4 cells

EXPERIMENTAL WORKFLOW

TAB. 2: SHORT DESCRIPTION OF THE EXPERIMENTAL SETUP

Step	Procedure	Information
Count cells with Trypan Blue Application	Trypsinize the cells and determine the viable cell density using SYNENTEC's Trypan Blue Viability Application.	For detailed information please refer to SYNENTEC's Technical Note for Trypan Blue measurements (TN-B004-XV-17).
Seed cells	Prepare a cell suspension containing the optimal number of cells (refer to Tab. 1). Seed 100 µl of the cell suspension into 96 well plates and Culture-Insert 4 wells or 70 µl into Culture-Inserts 2 well.	The required cell number may vary from cell line to cell line. The cells should form a confluent monolayer at the time of wound generation without being over-confluent. When using 96-well plates, do not use the outer wells of the plate but fill them with culture medium or PBS to avoid unintended side effects due to evaporation.
Create wounds	After 48 h, create a cell free gap by scratching the cell layer with a sterile pipette tip or by removing the culture insert. Wash the cells with preheated (37 °C) FCS-free cell culture medium and replace it with the same medium afterwards.	The time depends on the used cell lines. Premalignant cells do not grow as fast as e.g. malignant cell lines or fibroblasts. Monitor growth using SYNENTEC's Confluence Application and wait until a confluent monolayer is formed.
Measure in NYONE®	Image the plates directly after wound generation (time point 0 h) using NYONE® and the *wound healing* application of YT®-Software.	Only at this very first measurement, both phases of the *wound healing* application are imaged. Make sure, that YT®-Software correctly detects the wounds in phase 1 and places green ROIs inside them. Otherwise, optimize the image processing settings before proceeding to phase 2.
Treat with cytochalasin D	Treat the cells with cytochalasin D (different concentrations) and repeat the phase 2 measurements after 2, 4, 6, and 24 h.	Cytochalasin D is an inhibitor of actin polymerisation and thus inhibits migration of cells.
Measure in NYONE®	Measure the plates at different time points (e.g. 2, 4, 6, 24 h) using NYONE® and the *wound healing* application of YT®-Software.	In these measurements, only the second phase of the *wound healing* application is used and only small parts of the wells are imaged based on the wound healing detection in phase 1.

RESULTS & DISCUSSION

1. The 2-phase wound healing application enabled very fast imaging suitable for high-throughput screening

In order to develop a wound healing assay suitable for automation and high-throughput screening, we developed a new application consisting of a 2-phase measurement procedure.

In the first phase, which is only measured once at the beginning of the experiment, most of the well including the whole wound is imaged using the 4 x objective. Compared to the 10 x objective, this generates less data and makes the imaging very fast. The imaging times for the first phase are between 50 s for scratches in 96 well plates and 84 s for 12 well plates (summarized in Tab. 1). In phase 1, the software automatically detects the wounds and places wound healing ROIs (region of interests, green areas) inside the gaps (Fig. 2). The amount and size of this wound healing ROI can be defined by the user. Then, a white periphery ROI area is automatically placed around the green wound healing ROI. The size of this box depends on the size of the wound healing ROI (% of the green one) and can be defined by the user (see Technical Note for details).

In the second phase, solely the white ROI is imaged using the 10 x objective (Fig. 2). Only this phase is used for all subsequent measurements. This procedure saves a lot of time because merely a few pictures are taken in the second phase instead of scanning the whole well. This
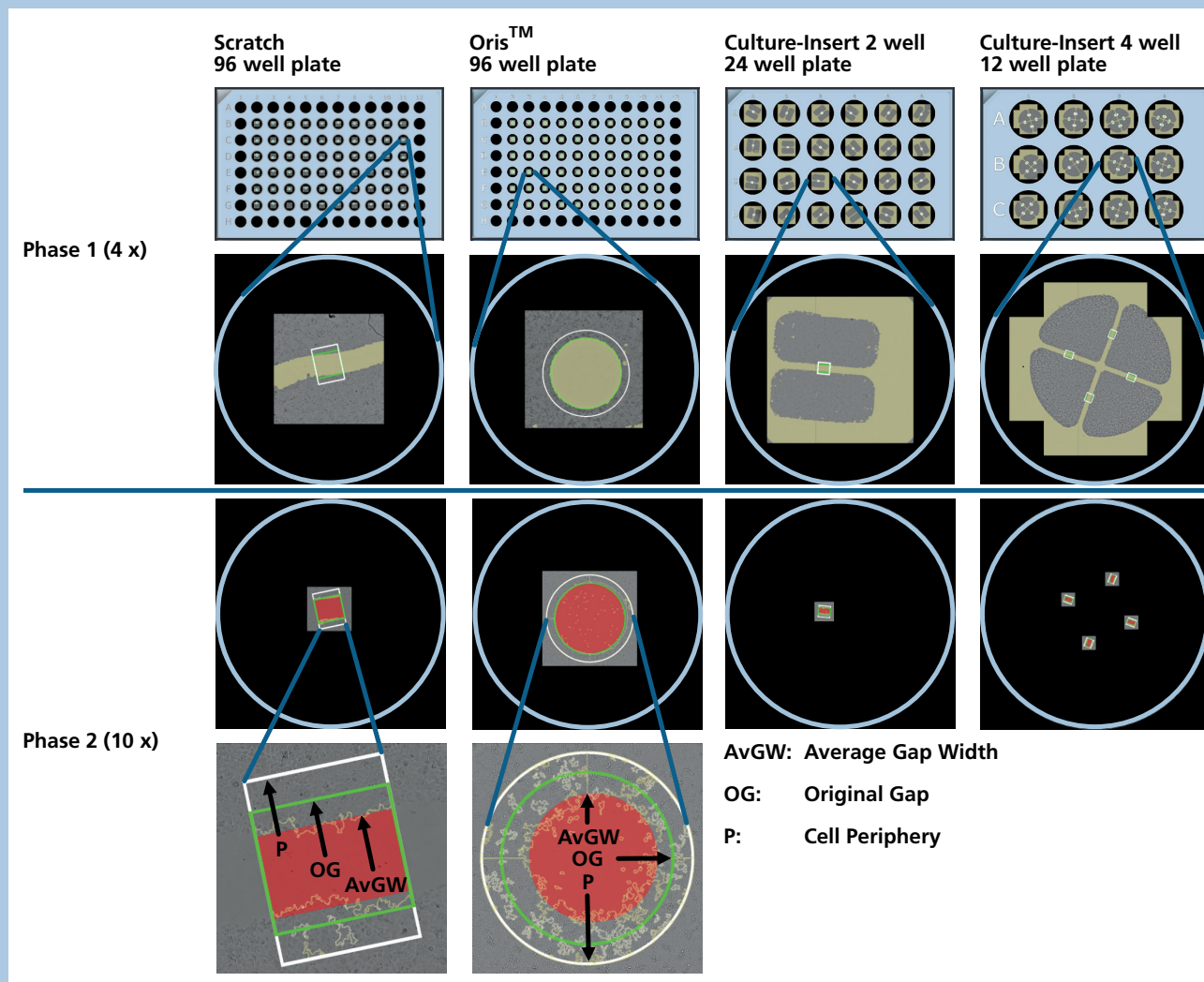

FIG. 2. THE 2-PHASE WOUND HEALING APPLICATION ROBUSTLY DETECTED WOUNDS OF DIFFERENT SIZE AND SHAPE

We seeded H6c7-Kras cells in 96 well plates with or without Oris™ inserts or in Culture-Inserts placed in 24 or 12 well plates. We generated wounds by manual scratching or by removing the inserts. Then, we imaged the plates using NYONE® and the *wound healing* application of YT®Software. It consists of two phases. In phase 1, the wound is recognized and marked by a wound healing region of interest (ROI, green box). A white ROI is added to analyze the confluence in the wound periphery. In phase 2, only this small white ROI is imaged. Both ROIs from phase 1 are used to quantify the confluence (yellow overlay) in the original gap (OG, green box) as well as in the periphery (P, white box) in phase 2. Additionally, the algorithm determines the average gap width (AvGW, red box) by recognizing and smoothing the cell fronts.

effect was minimal for Oris™ inserts as the wounds are quite big and always located at the same place in the middle of the well. Therefore, the region imaged in the second phase is not much smaller than in the first phase. However, especially for Culture-Inserts 2 and 4 well, the region imaged in the second phase is significantly smaller than in the first phase leading to a large reduction of the imaging time even though a higher magnification was used than in the first phase (see Fig. 2 and Tab. 3). In general, after wound detection in phase 1, all assays could be imaged within a minute (Tab. 3) enabling a high-troughput of microplates per day.

2. The wound healing application robustly detected wounds of different size, width, orientation and shape

We show that the image processing operator robustly detected wounds of different size and shape. Round wounds as in the Oris™ migration assay were detected as well as rectangular wounds generated by manual scratching or ibidi inserts (Fig. 2). Manual scratches

TAB. 3: EXAMPLE SCANNING TIMES

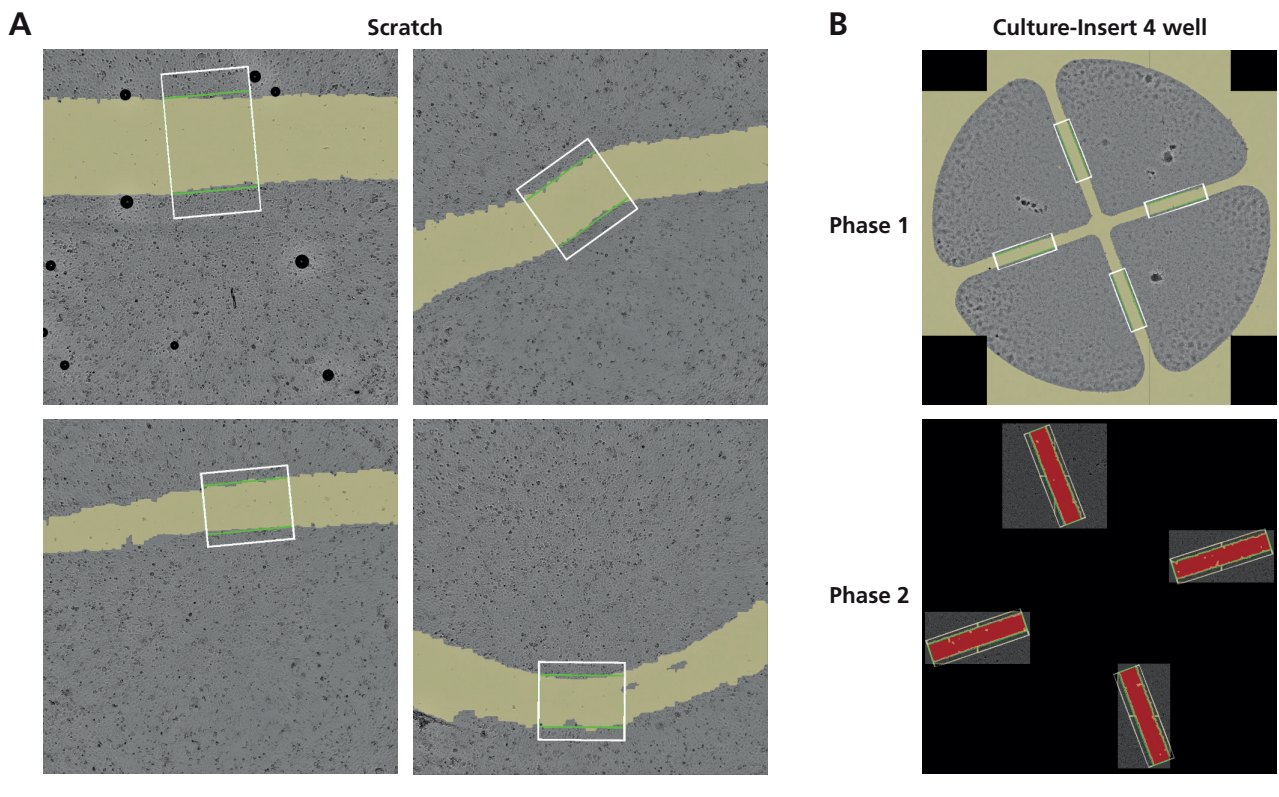| Name	Plate format	Imaging Time Phase 1 [s]	Imaging Time Phase 2 [s]
Scratch	96 well	50	37
Oris™	96 well	55	60
Culture-Insert 2 well	24 well	60	20
Culture-Insert 4 well	12 well	84	44

FIG. 3. THE IMAGE PROCESSING DETECTED WOUNDS OF DIFFERENT WIDTH, ORIENTATION AND ROTATION

A) We manually scratched a wound into a monolayer of H6c7-Kras cells using a sterile pipette tip. The *Wound Healing* application of YT®-Software automatically recognized the cell-free area (yellow area) and placed a region of interest (ROI, green box) in it. The algorithm is very robust and even detected scratches that were crooked or of uneven width. B) We seeded H6c7-Kras into culture insert 4 wells from ibidi and generated wounds by removing the insert. The image processing of the *Wound Healing* application detected the wounds even if the insert was rotated. YT®-Software automatically determined the minimal number of pictures needed to image the ROI from the first phase in the second phase and merged these subimages to one image, which was then analyzed. In this example, four subimages were needed for the upper ROI while two were enough for the other three ROIs.

were robustly recognized even though they often vary in width and orientation and are sometimes crooked (Fig. 3 A). This is a huge benefit because manual scratching is very cheap and easy to perform. Morever, wounds of ibidi inserts were recognized even if they were rotated, which often happens when they are manually inserted.

Moreover, the software algorithm always determines the minimal number of pictures needed to capture the ROI in the second phase. It automatically merges these subimages to one image which is then quantified (Fig. 3 B). Again, this procedure makes the application very fast.

3. The wound healing application enabled a label-free quantification of wound closure over time and the generation of dose-response curves

As described in Fig. 2, the images of the second phase were automatically quantified by YT®-Software. Using the green wound healing ROI, the algorithm determines the cell confluence on the gap. Moreover, it analyzes the confluence on the periphery (white box). With these two parameters the user could calculate the 'relative wound density'. This metric is often used to normalize for changes in cell density which may occur outside the wound due to cell proliferation and/or pharmacological effects [1]. Additionally, the application recognizes the cell fronts and determines the average gap width (red box). The parameters are summarized in Tab. 4 (a more detailed description of all parameters can be found in the Technical Note).

To verify that the quantification parameters result in meaningful data, we treated H6c7-Kras cells with different concentrations of cytochalasin D, which inhibits actin polymerization and thus migration [4]. Fig. 4 shows that the DMSO-treated cells (solvent control) almost closed the wound after 6 h and completely closed it after 24 h. Thus, the red box visualizing the average gap width diminished to a thin line. In contrast, the highest concentration of cytochalasin D [5000 ng/ml] completely inhibited wound closure and thus, the average gap width did not change much within 24 h. A slow-down of wound closure was observed after treatment with 40 ng/ml cytochalasin D. YT®-Software autmatically depicts these results as time charts in the Analyst tab (Fig. 5). As expected, the confluence on gap increased over time in control-treated cells while the average gap width decreased.

To generate dose response curves and calculate IC50/EC50 values, we exported the results and analyzed them using GraphPad Prism (Fig. 6). The confluence on the gap was dose-dependently decreased by cytochalasin D while the average gap width was dose-dependently increased. The IC50 values calculated from the dose-dependent decrease of the confluence were very similar to the EC50 values calculated
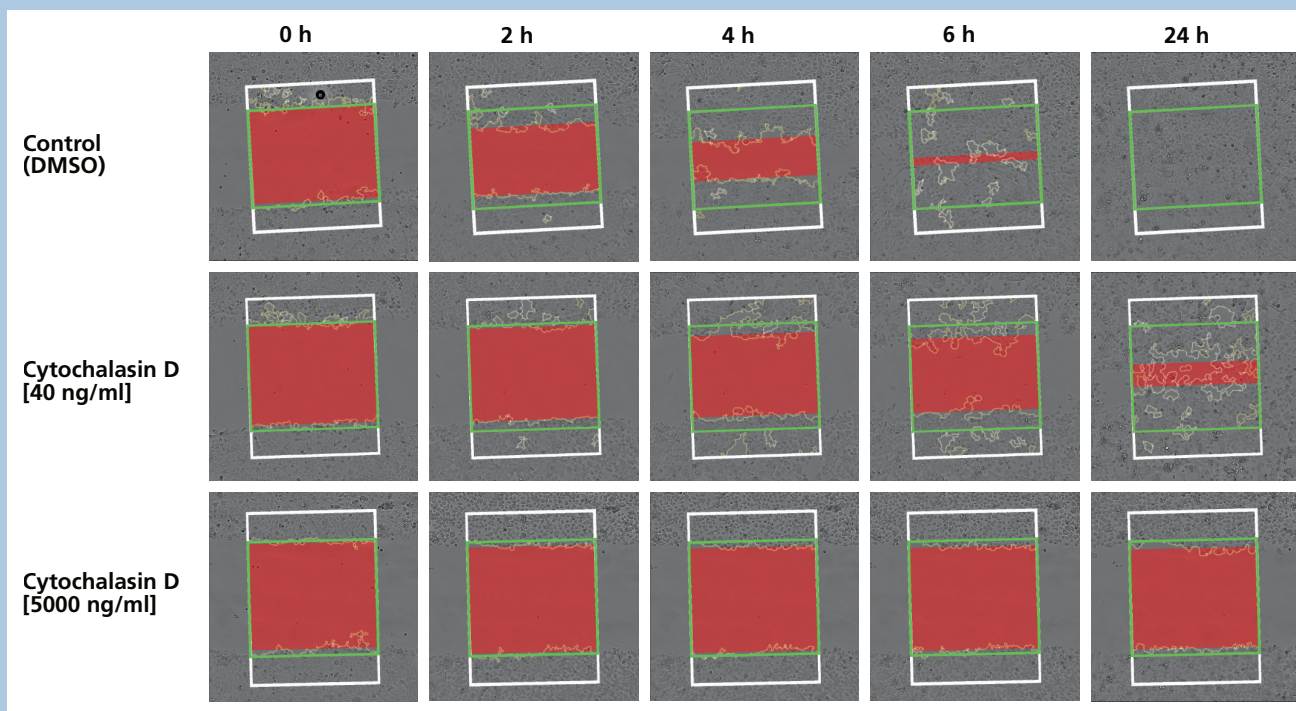

FIG. 4. THE WOUND HEALING APPLICATION ENABLED A LABEL-FREE VISUALIZATION OF WOUND CLOSURE OVER TIME

We seeded H6c7-Kras in 96 well plates, and generated wounds by manual scratching using a sterile pipette tip. After scratching, we treated the cells with different concentrations of cytochalasin D or DMSO as solvent control. We observed wound closure at multiple time points using NYONE® and the *wound healing* application of YT®-Software. The green box visualizes the original gap, the white box the cell periphery and the red box the average gap width. Cell confluence was analyzed in the original gap as well as in the periphery (yellow overlay).

from the effect on the average gap width. The values are comparable in the first six hours, when the cell migration followed a linear curve. But after 24 h, when the gap was closed, the IC50/EC50 values were higher, demonstrating that the choice of the optimal time point is important. With our application, cells do not need to be fixed and can therefore be imaged multiple times to find the optimal time for each cell line.

Moreover, we calculated the cell migration rate using equations (1) and (2). First, we determined the change in the average gap width ($\Delta AvGW_{tx}$) by subtracting 'Avg Gap Width' ($AvGW_{tx}$) at each time point from the original 'Avg Gap Width' ($AvGW_{t0}$). Then, we plotted $\Delta AvGW_{tx}$ of each concentration against the time. We plotted only against the first six hours because the curves flattened at later time points and were not linear any more. To calculate the cell migration rate, we calculated the slope of these straight lines using linear regression and divided it by two because cells migrated into the wound from both sides. We used this cell migration rate to plot another concentration response curve and calculate its IC50 value, which was comparable to the one obtained using individual time points in the linear phase of wound closure.
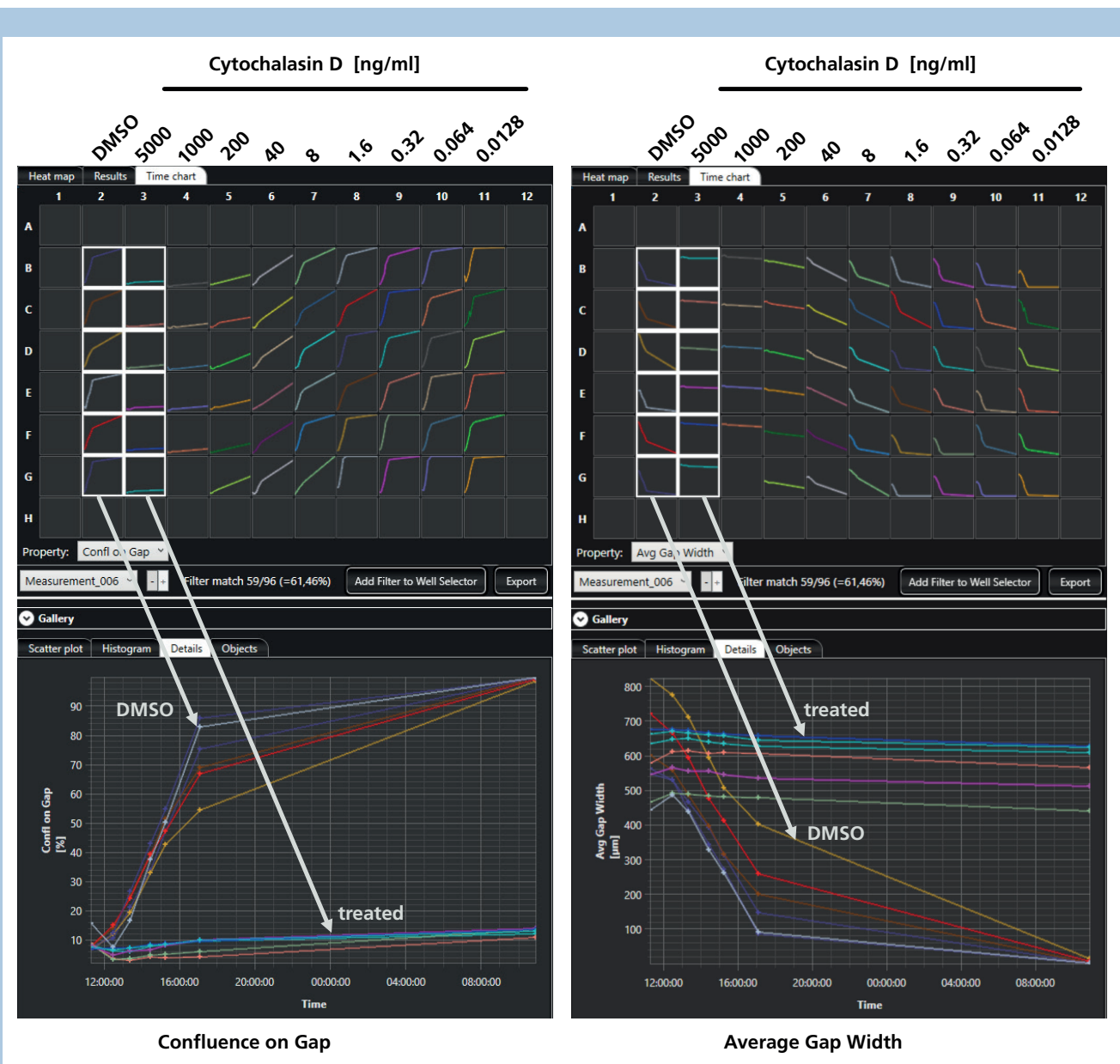

Confluence on Gap   Average Gap Width

FIG. 5. WOUND CLOSURE WAS QUANTIFIED OVER TIME AND DEPICTED AS TIME CHARTS IN YT®-SOFTWARE

We seeded H6c7-Kras in 96 well plates, and generated wounds by manual scratching using a sterile pipette tip. After scratching, we treated the cells with different concentrations of cytochalasin D or DMSO as solvent control. We observed wound closure at multiple time points using NYONE® and the *wound healing* application of YT®-Software. YT®-Software automatically depicts time charts in the Analyst Tab and thus enables a fast overview of the results for the user.

Equations to calculate the cell migration rate:

(1) $\Delta AvGW_{tx}\,[\mu m] = AvGW_{tx}\,[\mu m] - AvGW_{t0}\,[\mu m]$
(2) Cell migration rate $[\mu m/h] = $ Slope $/ 2 = \Delta AvGW_{tx}\,[\mu m]\,/\,2t_x\,[h]$

TAB. 4: DESCRIPTION OF THE MAIN RESULT PARAMETERS

| Result	Unit	Description
Confl on Gap	%	cell confluence in the wound healing ROI (area covered by cells/area of wound healing ROI *100) as determined in the first phase (green box)
Confl on Periphery	%	cell confluence in the periphery (area covered by cells/area of cell periphery *100) (white box)
Avg Gap Width	µm	average distance between the migrating/invading edges of the wound (width of red box)
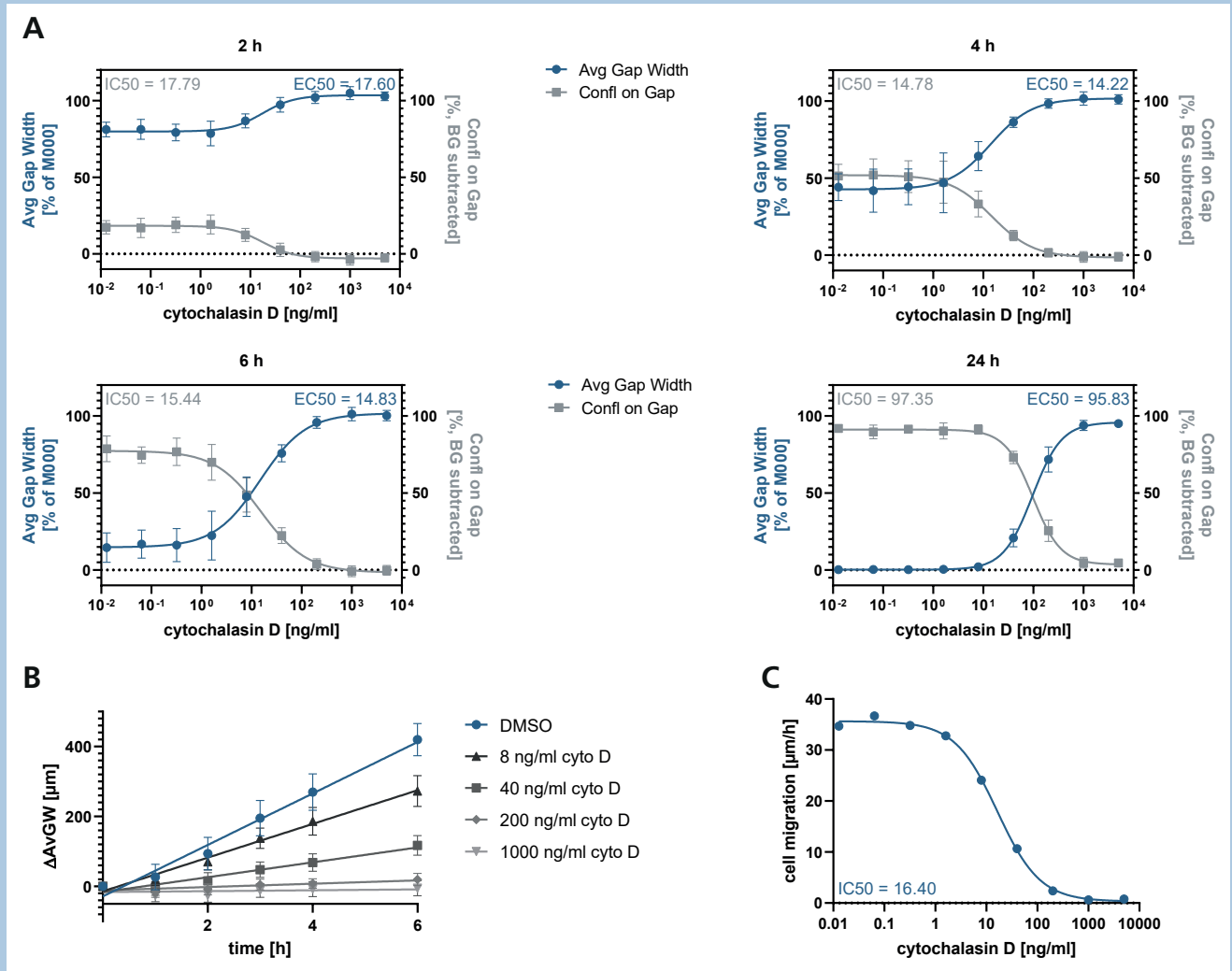
FIG. 6. DOSE-RESPONSE CURVES WERE GENERATED FROM THE EXPORTED DATA

We seeded H6c7-Kras in 96 well plates, and generated wounds by manual scratching using a sterile pipette tip. After scratching, we treated the cells with different concentrations of cytochalasin D or DMSO as solvent control. We observed wound closure at multiple time points using NYONE® and the *wound healing* application of YT®-Software. We exported the results and analyzed them using GraphPad Prism. A) We plotted the parameters 'Avg Gap Width' and 'Confl on Gap' against the concentration and calculated the IC50/EC50. B) We calculated the change in the 'Avg Gap Width' and plottet it over time. C) We calculated the cell migration rate using the slope of the curves in B), plotted the results against the concentration and calculated the IC50 value. n=6 technical replicates, error bard = standard deviation

CONCLUSION

SYNENTEC's cell imagers and YT®-Software enable a very fast, easy and robust analysis of wound healing and migration assays, making it an excellent tool for automation and high-throughput screening. The rapid imaging as well as precise high speed focusing can easily be used to generate accurate and reproducible results in a flexible manner, regardless of the magnification needed due to the automated lens changer. The presented approach to wound healing and migration assays copes with a high variety of experimental parameters due to the robust detection of scratches in various sizes, numbers and shapes by the image processing and the vast compatibility of SYNENTEC's imagers to different cell culture systems. The fast measurement of individual timepoints supersedes an incubation of the plates in NYONE® and CELLAVISTA® and thus enables a considerably higher throughput of plates per imager and day, tailored to the demands in high throughput screening facilities and scientists who require great results in a short amount of time.

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