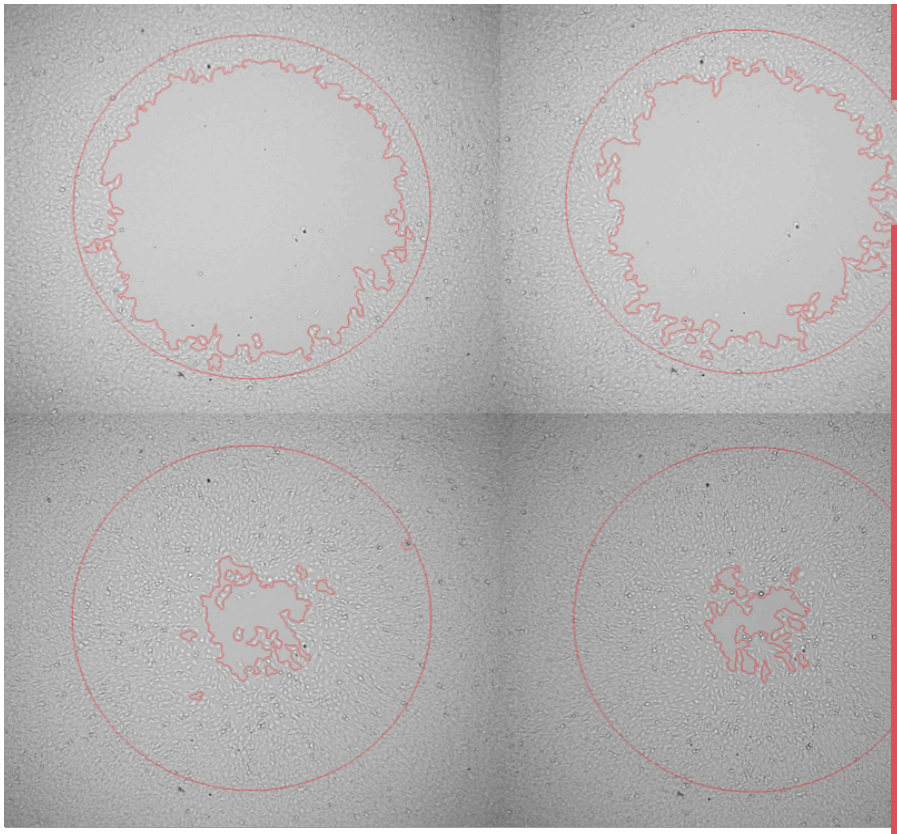

APPLICATION NOTE

Multimode Detection

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A New Approach for Automated Quantification of Cell Migration Using EnSight Multimode Plate Reader

Introduction

Cell migration occurs during diverse physiological processes including embryonic development, wound healing and the immune response, as well as many pathologic conditions such as cancer metastasis[1]. Therefore, cell migration is frequently addressed by scientists to investigate the underlying mechanisms and as a key phenotype for various biological responses. These underlying mechanisms are also attractive targets for therapeutic intervention. Widely used methods to observe the migration process are scratch or exclusion zone assays[2]. These assays are performed by creating a cell free gap in a confluent monolayer either by damaging the cell layer through a scratch or by removing a growth barrier. Subsequently, cells move into the cell-free zone which is detected by image acquisition. The migration process is assessed by comparing images at different time points to reveal information about the migration characteristics such as gap closure velocity or confluency level. Quantification of these characteristics is a time consuming task if data analysis is performed manually and is additionally limited by subjectivity and the lack of quantifiable metrics[3].

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The EnSight™ Multimode Plate Reader with well-imaging technology is an ideal device for fast, reproducible and automated determination of cell migration characteristics. Data acquisition from brightfield images is achieved by using predefined or, in this case, customized image analysis methods provided by the Kaleido™ Software for Data Acquisition and Analysis which is integrated in the EnSight benchtop system. Simultaneously it is possible to obtain other important cell specific data such as cell proliferation rates and cell morphology from the same data set. Due to the use of the EnSight system's brightfield imaging mode and its built-in image evaluation, no cell staining is needed. Maintenance of cell viability during measurements is achieved by integrated temperature control, while sample quality can be verified using additional measurement modes such as the recording of the absorption level of the corresponding culture medium to monitor changes in pH.

Using the EnSight Multimode Plate Reader, we show in this application note how to obtain reliable and reproducible data from cell migration assays in an automated process.

Materials and Methods

Cell Seeding

Frozen stocks of NIH/3T3 cells (ATCC® CRL-1658™, hereinafter referred to as 3T3s) as well as Clonetics™ Immortalized Human Coronary Artery Smooth Muscle Cells (CASMC XS12C1, hereinafter referred to as SMCs) were thawed and subsequently resuspended and cultured in complete growth medium, following recommended cell culture protocols.

For cell migration experiments, cell number was determined using Scepter™ Cell Counter (Merck Millipore). Cells were seeded in 96-well plates following Oris™ Cell Migration Assay Kit (Platypus Technologies) instructions, at a density of $12.5x10^3$ cells per well in a total volume of 100 µl. Due to the silicone based seeding stoppers included in the kit, cell seeding and adherence is restricted to the periphery of the assay wells. After seeding, cells were allowed to spread and attach for four hours at 37 °C in a humidified atmosphere with 5% CO_2 (Figure 1).

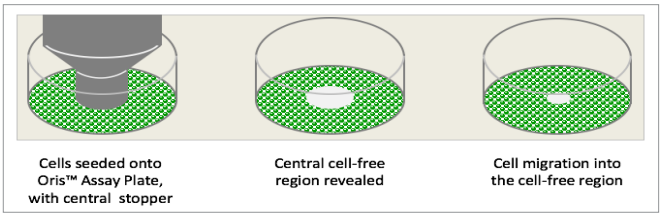

Figure 1. Schematic sequence of Oris™ Cell Migration Assay procedure (adapted from: Oris™ Cell Migration Assay manual).

Cell Treatment

Besides manual addition of 100 µL cell suspension in Oris™ assay wells, all liquid handling steps were executed using the JANUS G3 automated workstation with MDT head (PerkinElmer). After removal of Oris™ Cell Seeding Stoppers from the 96-well plate, a cell-free area in the center of each assay well is revealed. Supernatant medium was aspirated and cells were gently washed with corresponding culture medium. In order to restrain cell proliferation, DNA replication was suppressed by adding 10 µg/ml Mitomycin C (Abcam) to cell culture medium of 3T3 cells and 20 µg/mL in the case of SMCs. After a three hour incubation period, Mitomycin C solution was removed and each well was washed 3 times with culture medium. For investigation of dose response relationships, both cell types were treated with Latrunculin A (Enzo Life Sciences), a known inhibitor of actin dynamics. The dose response measurements were performed in triplicate, while Latrunculin A was serially diluted in cell culture medium in a concentration range of 0.03 – 2.00 µM for 3T3 cells and 0.001 – 1.00 µM for SMCs, respectively. Cell migration was observed over 48 hours.

Data Acquisition and Evaluation

Cell migration within the Oris™ assay system was monitored using the EnSight Multimode Plate Reader which was fully integrated into a plate::handler™ automated workstation (PerkinElmer). During the incubation period, brightfield images were acquired every two hours and between individual measurements, plates were moved into the incubator.

The measurement time per plate, including online analysis of the images and a control measurement for the pH of the medium (data not shown here), was about six min. The EnSight's Kaleido™ Software automatically analyzed the acquired images based on detection masks for different well regions (Figure 2). The custom designed "ORIS Brightfield Migration" analysis method (version 0.9)* provides information about cell confluency level and texture for the initial cell-covered area as well as for the initial cell-free area in which cell migration occurs (Table 1). Secondary data analysis was performed using TIBCO Spotfire® software. For characterization of cell migration, the average slope of the evaluated readout parameter over 48 hours was considered.

Points and error bars in the time dependent figures represent average and standard deviations of the three replicate wells unless stated otherwise.

Table 1. Read-out parameters of the EnSight Kaleido Software used for this analysis.

Parameter	Read Out
Center Confluency	Level of confluency in the region initially blocked by the stopper. Cells migrate into this cell free center region. Characterized as percentage of area covered by cells.
Outside Confluency	Level of confluency in the area where cells have initially been seeded. Characterized as percentage of area covered by cells.
Foreground Roughness	Characterization of the strength of cellular texture. Changes are associated with morphological changes like rounding up of cellular bodies. Unit-free measure.
Diameter of cell free area	Diameter of a circle defined by the boundaries of the cells around the cell free center. This value decreases upon migration of cells into the center. Measured in µm.

**The most recent version of the analysis method can be obtained from PerkinElmer.*

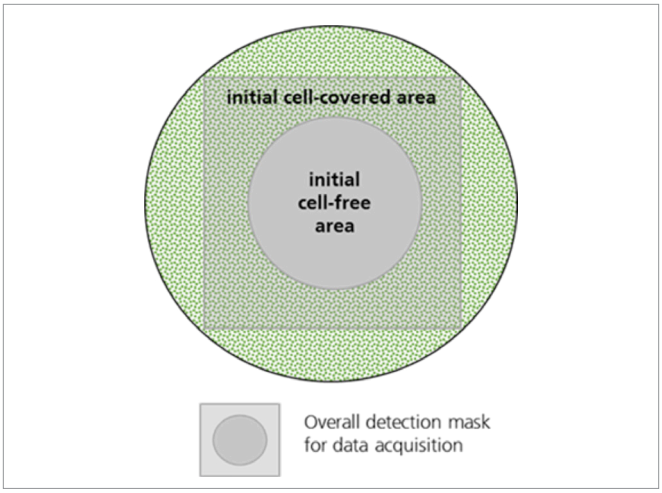

Figure 2. The EnSight's Kaleido™ Software automatically analyzed the acquired images based on detection masks for different well regions. The well area with cells seeded initially is marked in green.

Results

Discrimination Between Proliferation and Migration

In order to distinguish between proliferation and migration in the migration assay system, DNA replication was suppressed by treatment with Mitomycin C. Mitomycin C induces cell-cycle arrest due to its ability to cross-link complementary strands of the DNA double helix thereby suppressing the proliferation of examined cells.

Migration is characterized by changes in the density of cells in the initial cell-free area (stopper region). In contrast, the initial cell-covered area ("outside of the stopper" region) provides confluency data defined by the Kaleido™ image analysis parameter "outside confluency". A constant level of this parameter thereby provides information about the effectiveness of the Mitomycin C treatment and suppressed cell proliferation rates. This is shown in Figure 3, where both cell lines treated with Mitomycin C exhibit a constant outside confluency level, while untreated cells still proliferate and show increasing confluency levels. The Mitomycin C treatment used here is therefore sufficient to prevent cell proliferation which would otherwise interfere with monitored cell migration, leading to false results.

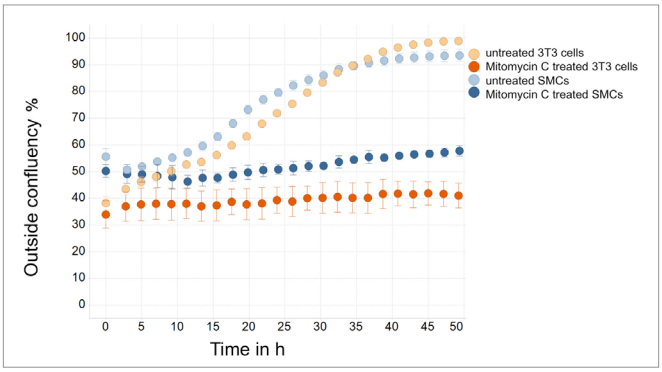

Figure 3. **Growth of cells with and without proliferation suppressor (Mitomycin C).** Time dependency of cell confluency level in the initial cell-covered area (outside of the stopper region) for Mitomycin-treated (suppressed proliferation) and untreated 3T3 cells and SMCs, respectively. The Mitomycin treated cells do not show proliferation, while untreated cells proliferate to confluent cell layers.

Evaluation of Compound Concentration Range for Cell Migration Experiments

Commonly known signs of decreased cell viability are morphological changes, such as cell detachment or rounding up of cellular bodies. However, these morphological changes can also occur at non-cytotoxic concentrations if inhibitors of actin dynamics distort the actin cytoskeleton. Occurrence of these morphological changes is associated with a change of the cellular texture, characterized by an increase of the parameter "foreground roughness". The dimensionless foreground roughness parameter refers to fluctuation of image intensities caused by the cell layer. Changes in 3-dimensional cell shape as well as detachment and rounding up cells affect this value and increase the foreground roughness values. Low concentrations of actin inhibitors do not induce distinct changes in the texture, while increasing concentrations of Latrunculin A lead to an increase of the foreground roughness values indicating morphological changes. Figure 4 shows the foreground roughness for the initial cell-covered area after 48 hours corresponding to different concentrations of Latrunculin A.

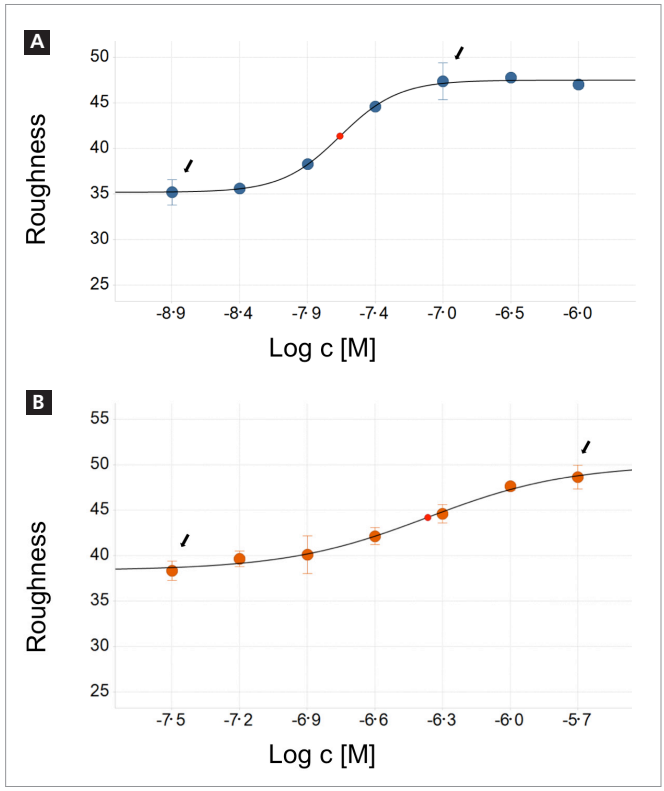

Figure 4. **Cellular texture in response to Latrunculin A.** Average of foreground roughness of the initial cell-covered area after 48 hours corresponding to Latrunculin A. (A): Dose response relation of SMCs treated with Latrunculin A; (B): Dose response relation of 3T3 cells treated with Latrunculin A. Note the differences in the concentration scales of both panes. Minimum and maximum effect is marked by black arrows.

The obtained IC_{50} based on the foreground roughness parameter for Latrunculin A treatment amounts to 0.020 µM for SMCs and 0.437 µM for 3T3 cells, respectively. Although the foreground roughness parameter does not provide direct information about cell migration, it is an ideal measure to estimate an appropriate concentration range for further investigation of cellular motility. Evaluation of foreground roughness data revealed that approximately a five fold concentration of foreground roughness derived IC_{50} values marks the maximum effect on cell morphology, while one-fifth of the IC_{50} concentration marks the minimum effect on cell morphology.

Figure 5 shows a morphological assessment of corresponding EnSight images 48 hours after compound addition. SMCs exposed to 0.111 µM Latrunculin A, corresponding to the maximum effect on SMCs, exhibit a rounded cell shape and a foreground roughness value of about 47.3 (Figure 5, right). In contrast to that, cells treated with 0.004 µM Latrunculin A, corresponding to the minimum effect on SMCs, show no morphological changes compared to untreated cells and exhibit a foreground roughness value of about 35.2 (Figure 5, left) similar to untreated cells (data not shown). Maximum and minimum effect concentrations determined by the roughness value can therefore be used to apply optimal and migration-relevant compound concentrations. The risk of killing cells and thus leading to non-relevant migration data is reduced. When using unknown compounds in a screening application, the roughness parameter also indicates cytotoxic effects.

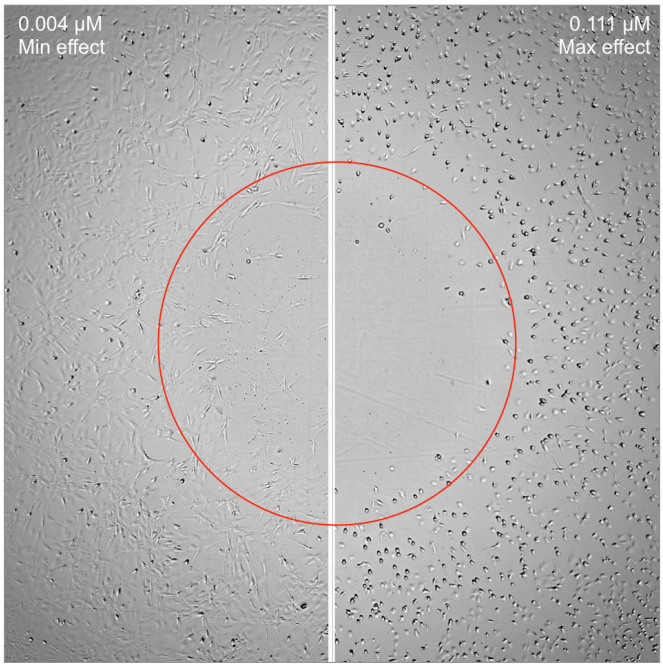

Figure 5. **Morphological Assessment of SMCs in Response to Latrunculin A.** SMCs in Oris™ Cell Migration Assay well 48 hours after addition of Latrunculin A. The red circle marks the area of the initial cell-free gap and detection mask used for confluency analysis, which determines the cell texture. Left: Foreground roughness = 35.2 Right: Foreground roughness = 47.3.

Quantification of Cell Migration

The following analysis shows two different approaches for characterization of cell migration:

a) The diameter of the cell-free center is similar to the standard used in scratch assays and results in a 'gap closure velocity' measured in µm / hour.

b) The degree of confluency found in a fixed mask yields a derived gap closure velocity measured in % per hour.

Both approaches compare the change of the derived parameter over time, which is calculated using the slope of the respective parameters (Figure 6).

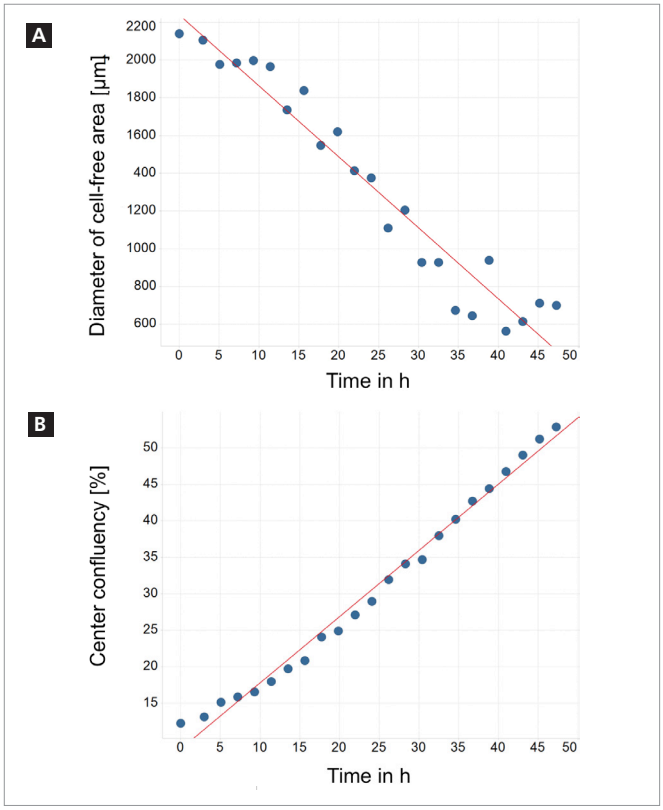

Figure 6. **Changes in the parameter "Diameter of cell-free area" (A) and "Center Confluency" (B) over time for SMCs in an untreated assay well (negative control).** Slope of the respective parameters is displayed by the red line, the respective value of the slope provides the "gap closure velocities".

a) Diameter Based Analysis

A widely used parameter for quantification of cellular migration is the distance moved by cells over time. Determination of this distance generally requires a reference which is usually the boundary between cell-covered and cell-free area before migration starts. The version of the Kaleido migration analysis method used in this application note* provides a parameter which determines the "diameter of the cell-free area" at every time point measured. If migration occurs, the diameter decreases over time. The slope of the diameter of the cell-free gap over time offers thereby a direct correlation of gap closure velocity of examined cells.

Low concentrations of Latrunculin A correlate with high gap closure velocity (Figure 7). Slope values are negative in this case as the diameter shrinks. Increasing concentrations of actin inhibitor lead to impaired cell motility and the "diameter of the cell-free area" parameter approaches zero. The turn of the parameter into positive slope values (i.e. increasing diameter of the initially cell-free center region) at high compound concentrations refers to morphological changes of cells located outside of the center region. High concentrations of Latrunculin A distort the actin cytoskeleton resulting in rounding up and separation of the cells into isolated cells or small patches. Such effects break the clear definition of a boundary of a cell layer and the determination of the diameter of the area not covered by cells can become ambiguous. This effect limits this kind of quantification in general. Depending on the examined compounds and concentrations, differences in cell shape and cellular arrangement may have a considerable effect on data acquisition that is therefore susceptible to imprecise or even misleading regressive migration data. This effect is also visible when very low cell densities are investigated, where a homogenous boundary is absent (data not shown).

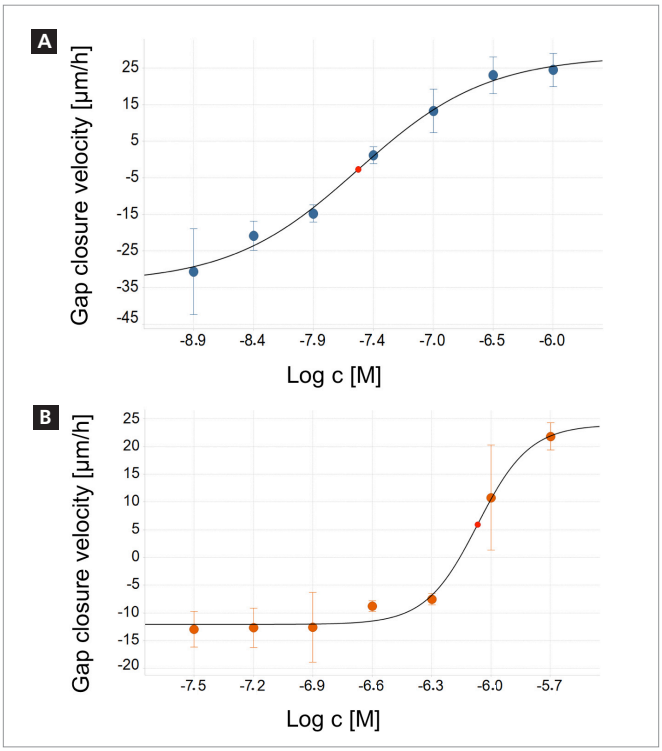

Figure 7. **Gap closure velocity indicated by the slope of "diameter of cell-free area" over time (48 hours) [µm/h].** (A): Dose response relation of SMC treated with Latrunculin A; (B): Dose response relation of 3T3 cells treated with Latrunculin A. Note the different scales of the concentration ranges used. The turn of the parameter into positive slope values at elevated compound concentrations is due to separation of the cell layer into individual cells, which limits this type of quantification.

b) Confluency Based Analysis

In contrast to the diameter based analysis, the "center confluency" parameter overcomes the potential issues in defining the cell layer boundary. The Kaleido image analysis method detects the initial cell-free area automatically and determines the degree of confluency in a mask corresponding to the size of the seeding stopper at this area. This approach makes the read-out independent of the possibly ambiguously defined border of the cell-covered area. The obtained data of increasing confluency level in the initial cell-free area correlates directly and exclusively with cells migrating into the center region. Therefore, the slope of increasing center confluency over time provides robust data for quantification of migrated cells in the initially cell-free area (Figure 8).

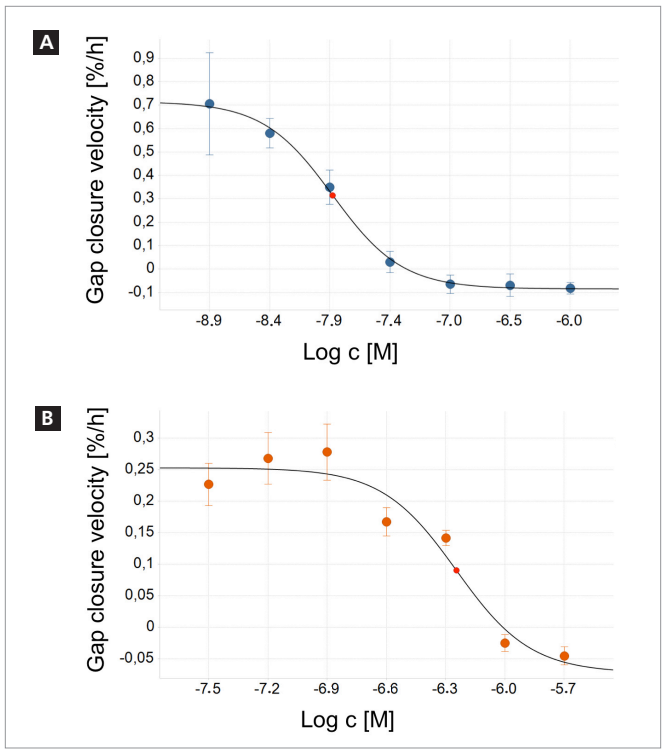

Figure 8. **Gap closure velocity indicated by the slope of "center confluency" over time (48 hours) [%/h].** (A): Dose response relation of SMC treated with Latrunculin A; (B): Dose response relation of 3T3 cells treated with Latrunculin A. This readout measure is independent of the detection of the boundary of the cell-free area and provides robust data for quantification of migrated cells.

Table 2 shows a comparison of IC_{50} values derived from both methods for quantification of cell migration.

Table 2. Comparison of IC_{50} values obtained from different quantification methods of cell migration generated using brightfield images.

	SMCs	3T3 cells
IC_{50} [µM] Center Confluency	0.012	0.562
IC_{50} [µM] Diameter of Cell-free area	0.028	0.851

The comparison of both stainless quantification methods shows that the "center confluency" parameter of the EnSight™ system exhibits distinct lower IC_{50} values for cell migration. Since the measured migration characteristics evolve exclusively from migrating cells, obtained data of the "center confluency" parameter is more reliable and not influenced by an imprecise detection of the ambiguous boundary of the cell free gap.

Discussion

In this application note we demonstrate that the EnSight Multimode Plate Reader enables the monitoring of cell proliferation rates and the differentiation of proliferating and migrating cells without the need for cell staining, whilst toxic effects can also be identified. Therefore, the obtained migration data is not affected by proliferating cells nor impaired by staining-related cellular effects, which leads to more precise results.

The cellular texture characterized by the roughness parameter of the EnSight Cell Migration analysis method provides information about changes in cell morphology. High concentrations of inhibitors of cytoskeletal dynamics, lead to distinct distortions of the cytoskeleton at non-cytotoxic concentrations. Using the foreground roughness parameter of the EnSight benchtop system, we show how optimal concentration ranges for dose-response measurements can be assessed and thus enable the investigation of cellular migration.

The center confluency parameter overcomes the issue of finding a potentially ambiguous cell layer boundary for the cell-free gap that is significantly affected by increasing compound concentrations, leading to imprecise results. The readout of the center confluency parameter provides robust and accurate data to characterize cell migration. Moreover, the data acquisition of the EnSight Multimode Plate Reader, such as the detection and characterization of the cell-free area, is performed in an automatic process that does not require subsequent and time consuming image editing. In contrast to other systems, the EnSight system provides the advantage of a stain-free method for quantifying cell migration using brightfield imaging which avoids the effort and potential cytotoxic effect of cell staining. Therefore a precise, uncomplicated, stainless and fast characterization of dose response relationships for cell migration and determination of derived data such as the IC_{50} value of a certain compound is enabled.

For quality verification and orthogonal measurement approaches, the (stain-free) brightfield imaging method for ORIS™ Cell Migration can be combined with fluorescence imaging as well as with classical detection technologies such as absorbance, fluorescence intensity, luminescence, TRF or Alpha.

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