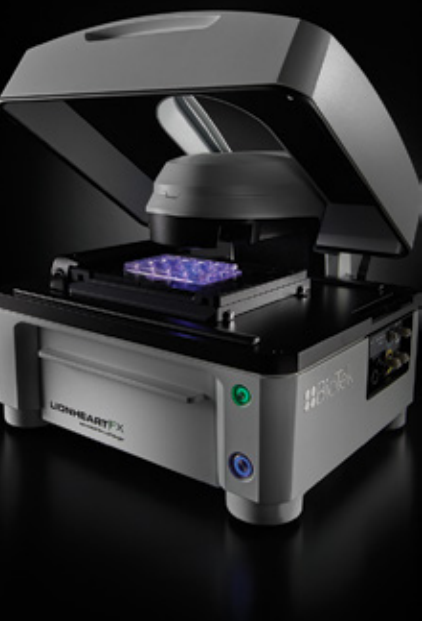
Performance of a Label-Free Image-Based 2D Scratch Wound Healing Assay to monitor Cell Migration and its Inhibition

Leonie Rieger and Brad Larson, Applications Department, BioTek Instruments, Inc., Winooski, VT
Ulf Rädler, ibidi GmbH, Martinsried, Munich, Germany



Key Words:

Wound Healing
Scratch Assay
Oncology
Dermatology
Cell Migration
% Confluency in the Wound
ibidi
35 mm µ-Dish

BioTek Instruments, Inc.
P.O. Box 998, Highland Park,
Winooski, Vermont 05404-0998 USA
Phone: 888-451-5171
Outside the USA: 802-655-4740
Email: **customercare@biotek.com**
www.biotek.com

Introduction

Cell migration- the random movement of cells plays a central role in physiological and pathological processes. Cell migration is an integral part of basic biological processes like tissue formation, wound healing and the immune response, as well as processes like cancer invasion and metastasis[1,2]. The basic principal of cell movement is a cyclical 5-step procedure starting with cell polarization and protrusion of the leading edge. In the second step the leading edge attaches to the substrate followed by the proteolytic degradation of physical barriers e.g. tissue components and actomyosin contraction. Finally the cell rear slides forward. The main modification of collective cell movement is the remaining inter-cellular cell-cell connections of the leading edge. Therefore the principle steps are simultaneously coordinated[2]. An advanced knowledge and understanding of the mechanism of collective cell migration is crucial in order to develop novel therapeutic strategies to control e.g. cellular invasion, the key role in metastasis of cancer.

A powerful tool to investigate cell migration is the wound healing assay. After creating an artificial gap into a confluent cell monolayer, either in the form of a scratch or by removing a growth barrier, the assay follows cell migration from the edges over time until the establishment of new cell-cell contact and the gap is closed again. The extent of wound healing and wound healing rate provide information of the migration characteristics, cell health and ability to interact with other cells; in addition to how test molecules can affect each of these parameters.

In this application we performed experiments to track the ability of various cell types to move into a created gap, or heal the wound. In addition, inhibition of wound healing with Cytochalasin D was also shown. Culture-Inserts and 35 mm µ-Dishes, provided by ibidi, were incorporated to perform the wound healing experiment process. Wound closure was monitored by BioTek's Lionheart™ FX Automated Live Cell Imager and cellular analysis of the kinetic images captured was automatically performed using Gen5 software.

Materials and Methods

Materials

ibidi Culture-Insert

The ibidi Culture-Insert was mainly developed to replace classical scratch assays. Silicon inserts, placed on a cell culture surface, provide two cell culture reservoirs. These two chambers are separated with a 500 µm thick wall. The culture insert sticks to the bottom of a µ-Dish or 24-well plate due to a specially designed surface. Due to the adhesive design the cells are not able to grow under the wall. For the experiments performed here, the Culture-Inserts 2 well in a µ-Dish$^{35mm,\ high}$ (Catalog No. 81176) were used and provided by ibidi (ibidi GmbH, Munich, Germany).

Cells

HT-1080 fibrosarcoma cells (Catalog No. CCL-121) and immortalized keratinocytes (Catalog No. CRL-2309) were purchased from ATCC (Manassas, VA). RFP expressing human neonatal dermal fibroblasts (Catalog No. cAP-0008RFP) were obtained from Angio-Proteomie (Boston, MA).

Wound Healing Inhibitor

The compound Cytochalasin D (Catalog No. 1233, R&D Systems; Minneapolis; MN) was used to inhibit cell migration due to its interfering with actin polymerization and potent disruptor of actin filament function[3].

Lionheart™ FX Automated Live Cell Imager

The Lionheart™ FX Automated Live Cell Imager with Augmented Microscopy™ provides digital microscopy with high magnification images, using up to 100x objectives. In addition the microscopy module provides microscopy in fluorescence, brightfield, color brightfield and phase contrast. Due to environmental control, including temperature, gas control and a humidity chamber the system is optimized for long-term kinetic assays and live cell imaging. The instrument was used to monitor wound closure in phase contrast.

Gen5™ Microplate Reader and Imager Software

For automated image capturing and analysis the Gen5™ 3.0 Microplate Reader and Imager Software was used. Image acquisition is completely automated from sample translation, focusing and exposure control. The included cell analysis tool calculates precise values in variable parameters, e.g. object area and size, as well as the object intensity. Using the customized transformation tool the calculated values can be converted into the preferred presentation method.

MetaVi Labs Cell Analysis Software

To validate the data provided and calculated by Gen5, a second independent analysis tool was used. Images captured over the total incubation period were uploaded to their website. Pixel resolution values were also required by the software for calculation. After the images were analyzed a detailed report was provided by the software including graphs showing different wound healing determinations.

Methods

Cell Preparation

HT-1080 fibrosarcoma cells, immortalized keratinocytes and the neonatal dermal fibroblasts were cultured in a T75-Flask until they reached 80% confluency. Accutase was used to detach the cells from the flask. Each cell type was resuspended to a concentration of $5.0x10^5$ cells/mL and 70 µL of cell suspension (35,000 cells per reservoir) were added to each chamber of the insert (Figure 1). The cells were allowed to attach to the coated bottom of the dish and to reach confluency over night. The plastic inserts were removed with sterile forceps. After washing the cell patch with 2 mL of fresh culture medium, it was replaced with 2 mL of culture medium containing 10,000 nM, 100 nM or 0 nM Cytochalasin D.

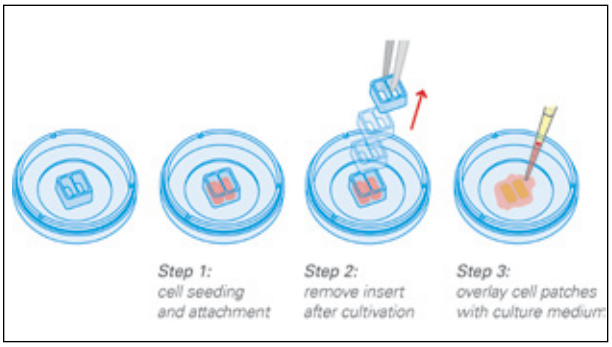


Figure 1. **Performed experimental workflow for the wound healing assay in the ibidi Culture-Insert.** The medium used in step 3 contained either 10,000, 100 or 0 nM Cytochalasin D.

Imaging

The dishes were placed in the µ-Dish Microscopy Rack (ibidi, Catalog No. 80035) and fixed with the magnetic lid for additional stabilization. The rack was placed in the Lionheart™ FX, previously set to 37 °C and 5% CO_2. Imaging of the wound area was performed every 30 min over a 20 hr period using phase contrast and a 4x objective.

Image-based Analysis of Cell Movement

Cellular analysis was performed on all 4x phase contrast images captured over the complete incubation period. Using the Cellular Analysis Parameters in Table 1, Gen5 automatically places masks around the objects meeting the designated criteria.

Cellular Analysis Parameters	
Channel	Phase Contrast
Threshold	500
Background	Dark
Split Touching Objects	Uncheck
Fill holes in masks	Checked
Min. Object Size	100 µm
Max. Object Size	10000 µm
Include Primary Edge Objects	Checked
Analyze the entire image	Uncheck
Plug	Width: 700 µm; Length: 1457 µm
Advanced Options	
Evaluate Background On	1%
Image Smoothing Strength	20
Background Flattening Size	25 µm (Rolling Ball diameter)

Table 1. **Gen5 cellular analysis parameters for object mask placement.** Objects meeting the designated criteria will be marked with a yellow line.

In addition to the Cellular Analysis Parameters, Advanced Detection Options were used. Evaluation background was lowered to 1% allow accurate identification of areas containing cells and empty areas within the image plug. Background Flattening was applied to enhance the contrast between object and background and reduce subtle changes is signal within migrating cell areas. Finally, image smoothing was used to further smooth out variations in phase contrast signal within cellular areas to accurately mask the collective cell monolayer. Instead of analyzing the entire field of view, an image plug with 700 µm width and 1457 µm in length was applied to the entire 4x image (Figure 2). A width of 700 µm was chosen to ensure to include not only the complete wound area but also a small cell area on each side to use and compare phase contrast for cell analysis. The plug length of 1457 µm equaled the 4x field of view in that dimension.

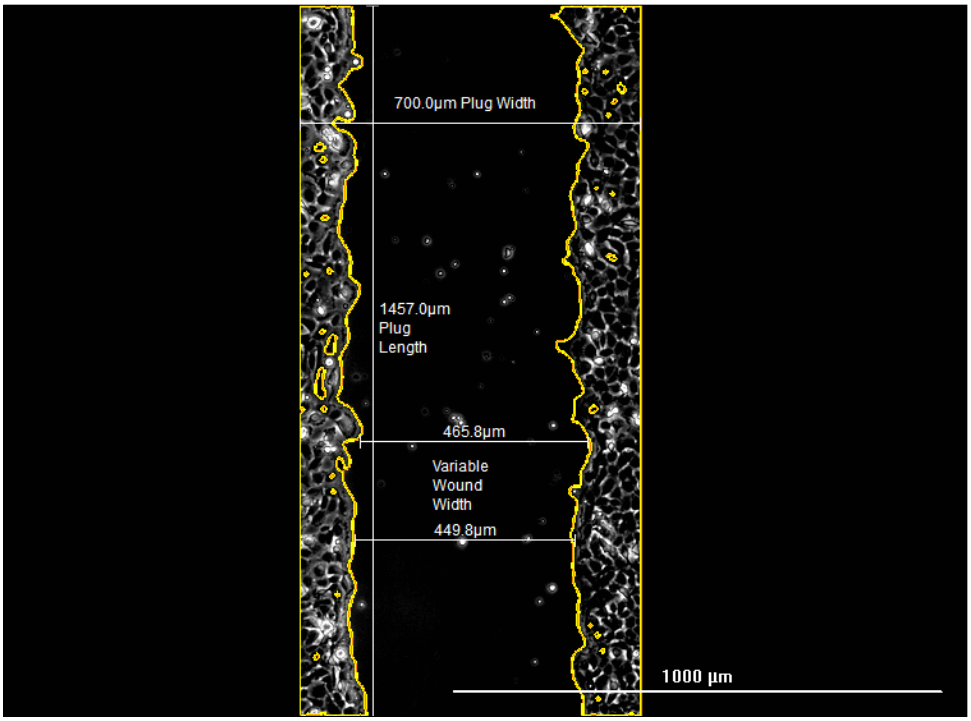


Figure 2. **Used metrics for determination of wound healing results.** Width of the plug is 700 µm, the length is 1457 µm. Two representative lines were drawn to show the variable wound width across the entire wound length. The yellow line shows the objects masked placed by Gen5 using the parameters defined in Table 1.

Calculation of Wound Healing Metrics

The Cellular Analysis Parameters listed in Table 1 were used to define a primary mask that quantified the area in the image plug that contained cells. This mask is depicted in Figure 2. Gen5 Calculated Metric of $(\text{Sum Area})_t$ obtained from the mask can be used to quantify progression of cell migration as a function of time in the kinetic assay using a series of data reduction steps outlined below.

Average Wound Width as Function of Time

While the Culture Inserts provide a simple method to create a cell free gap, an intra- and inter-experiment variability in its width remains. This can easily be accounted for using for Gen5 image analysis by calculating the average wound width per time point.

Formula 1. Average Wound Width as Function of Time

$$W_t = \frac{P_A\text{-}(\text{Sum Area})_t}{P_L}$$

Where W_t is the average wound width (µm) as a function of time; P_A is the image plug area (µm^2); P_L is the image plug length (µm) and the $(\text{Sum Area})_t$ is the Calculated Metric form Gen5 (µm^2).

Percent Confluency within the Wound Area

The average wound width at the first kinetic time point was then also used to calculate the % confluency within the wound area according to the following formula.

Formula 2. % Confluency within the Wound Area

$$\%\ \text{Confluency} = \frac{(\text{Sum Area})_t - P_L(P_w\text{-}W_{t=0})}{P_L W_{t=0}}$$

Where $W_{t=0}$ is the average wound width (µm) at the first time point; P_W is the image plug width (µm); P_L is the image plug length (µm) and the $(Sum\ Area)_t$ is the Calculated Metric form Gen5 (μm^2).

Percent Confluency in a Fixed Wound Width Gap

In addition, % confluency in a fixed wound area (500 µm) and the maximum wound-healing rate were calculated for the Ht10-80 cell line according to the following formula.

Formula 3. % Confluency in a Fixed Wound Gap

$$\%\ Confluency_{Fixed} = \frac{(Sum\ Area)_t - P_L(P_w - W_L W_w)}{W_L W_w}$$

Where W_L is the fixed wound length and W_W is the fixed wound width; P_W is the image plug width (µm); P_L is the image plug length (µm) and the $(Sum\ Area)_t$ is the Calculated Metric form Gen5 (μm^2).

Finally the Kinetic Analysis tool of Gen5 3.0 calculated the highest slope of the actual wound area curve to provide the maximum wound healing rate in μm^2 per minute.

Results and Discussion

Label-free Monitoring of Wound Closure Over Time and its Inhibition with Cytochalasin D

Figures 3-5 illustrate the ability of the Lionheart FX to accurately image the process of gap closure with three different cell types. The cells types were treated with 10,000, 100 or 0 (control) nM Cytochalasin D and imaging was performed over a 20 hr period at 30 minute intervals.

The HT-1080 cells showed a collective phenotypic movement, causing the gap to close after 8 hr (Figure 3A). While wound healing was mildly inhibited with 100 nM Cytochalasin D (Figure 3B) the higher concentration of 10,000 nM Cytochalasin D completely prevented cell migration compared to the control (Figure 3C)

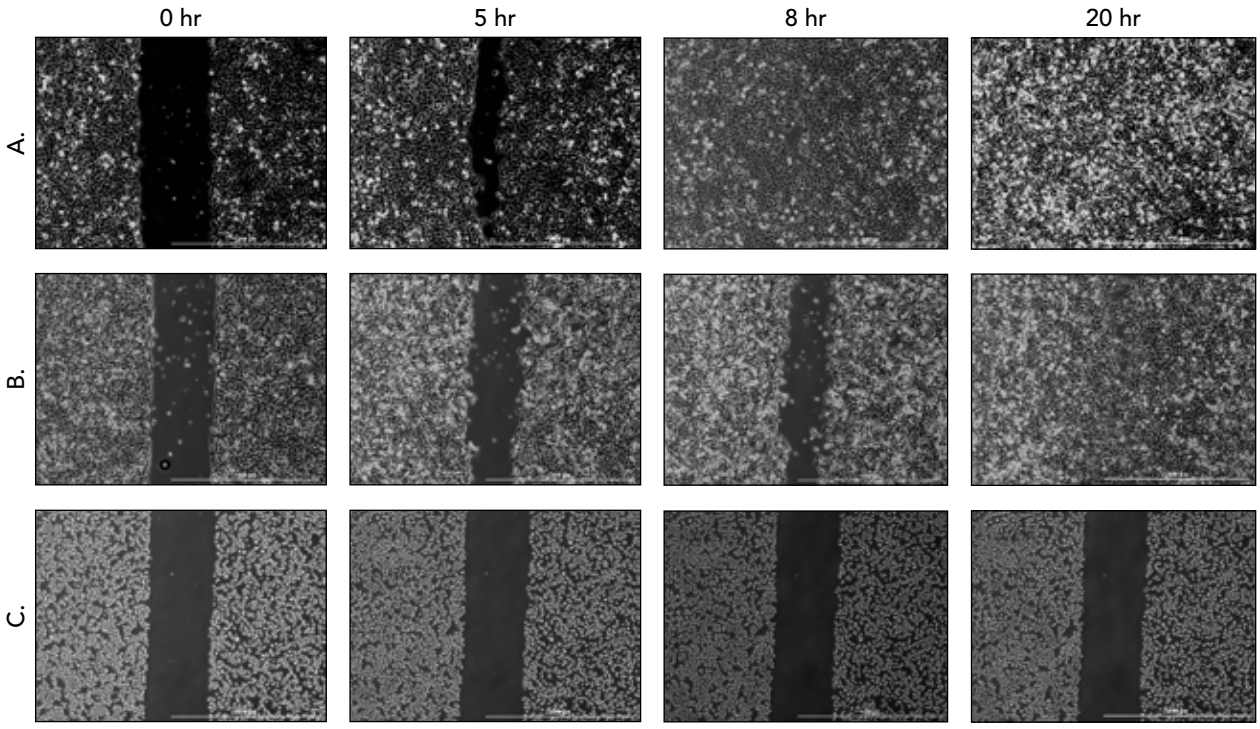


Figure 3. **Image-based monitoring of HT-1080 migration. Imaging was performed over 20 hr using phase contrast and 4x magnification.** Kinetic images shown for cells treated with (**A**) 0; (**B**) 100; or (**C**) 10,000 nM Cytochalasin D.

Keratinocytes, in comparison, started migrating as single cells and ended up closing the wound after 9.5 hr (Figure 4A). Compared to the control the lower concentration of Cytochalasin D had an impact on total gap closure and even after 20 hr the artificially created wound was not closed (Figure 4B). Keratinocytes treated with 10,000 nM compound showed no cell movement compared to the control and therefore no migration (Figure 4C).

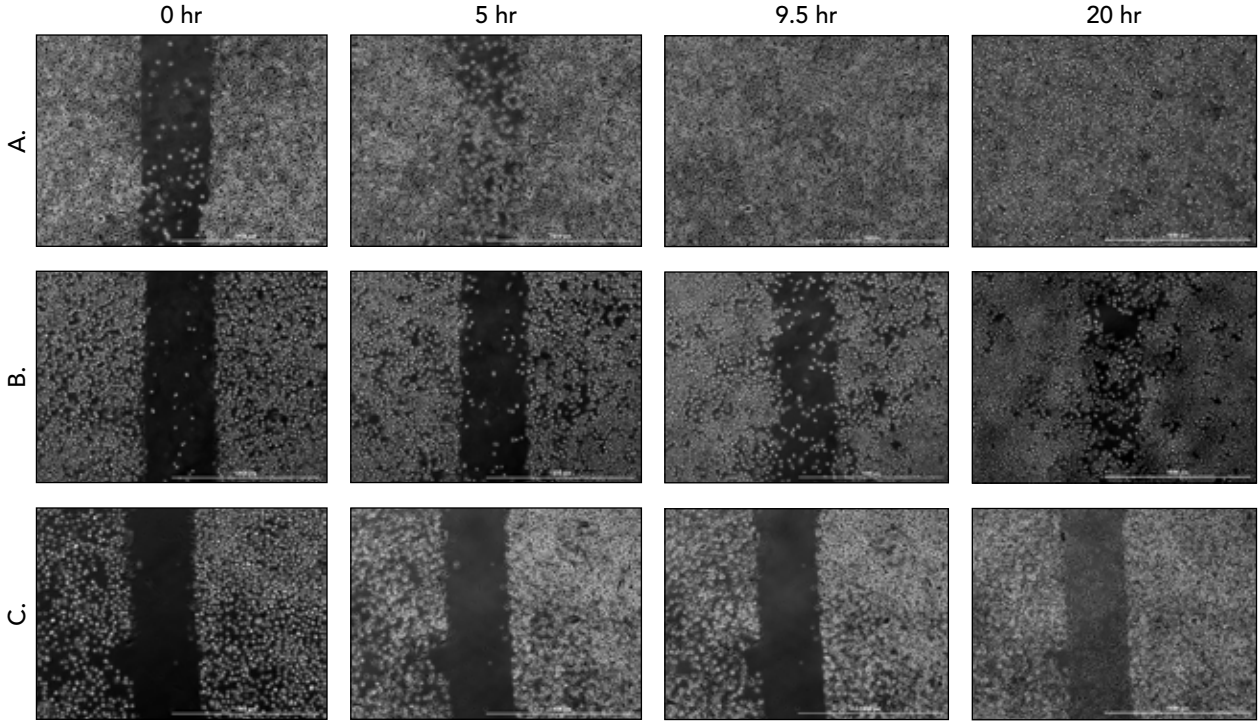


Figure 4. **Visual confirmation of keratinocyte wound healing. Imaging was performed over 20 hr using phase contrast and 4x magnification.** Kinetic images shown for cells treated with (**A**) 0; (**B**) 100; or (**C**) 10,000 nM Cytochalasin D.

Finally, it was also possible to monitor the larger, filamentous fibroblasts moving across the gap (Figure 5A). Within the 20 hr incubation period, the fibroblasts were not able to reach confluency within the wound area. Adding 100 nM Cytochalasin D to the medium slowed down wound healing compared to the control (Figure 5B). The highest concentration of Cytochalasin D had the greatest observed impact of all tested cell models. As illustrated in Figure 5C, not only cell migration but also cell adherence is affected, indicating a potential necrotic effect on the fibroblasts.

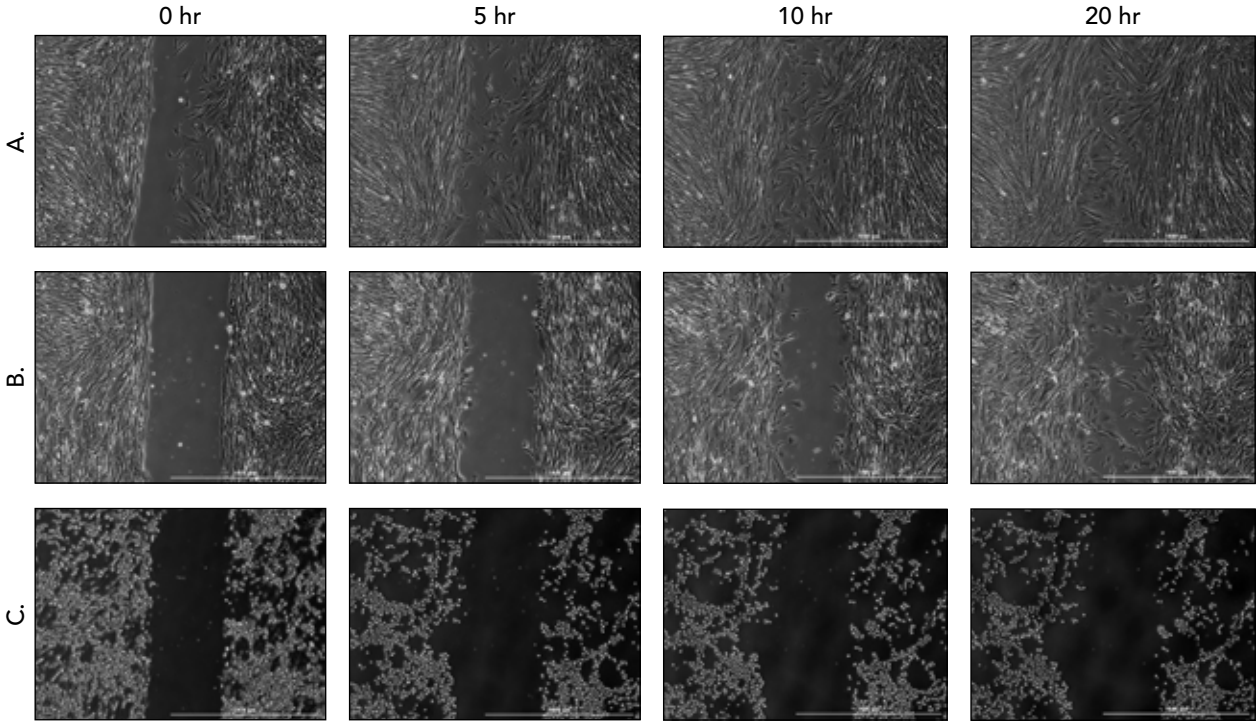


Figure 5. **Image-based monitoring of fibroblasts migration. Imaging was performed over 20 hr using phase contrast and 4x magnification.** Kinetic images shown for cells treated with (**A**) 0; (**B**) 100; or (**C**) 10,000 nM Cytochalasin D.

Quantification of Wound Healing and its Inhibition using Image-based Cellular Analysis

As previously described, the Cellular Analysis parameters used (Table 1) were able to accurately quantify the area occupied by cells in the plug image using a primary mask $(\text{Sum Area})_t$. This is depicted in Figure 6 over three time points for the HT-1080 cell model. Then using Formulas 1 and 2, the average wound width, Wt and % Confluency can be determined in the data reduction tool in Gen5.

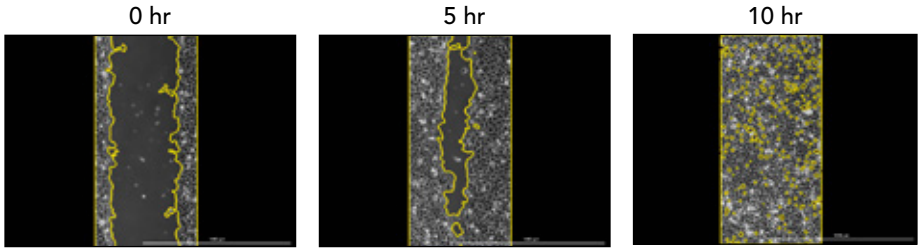


Figure 6. Representative images at different time points showing the applied object mask of the cellular analysis tool provided by Gen5 3.0.

Figure 7 shows kinetic plots spanning a 21 hr period for the three cell models using the three separate Cytochalasin D concentrations. Cell migration is somewhat different between the three cell lines, although in each case, Cytochalasin D demonstrates the ability to inhibit cell migration in a dose dependent manner, albeit to different extents. The cell free gap using the HT-1080 cells was closed after 7-10 hr, while for the keratinocytes it was delayed somewhat to and 8-10 hr. The fibroblasts by comparison only reach 80% confluency after approximately 10 hr of incubation and at the highest inhibitor concentration demonstrated a loss of adherence noted by negative % confluency.

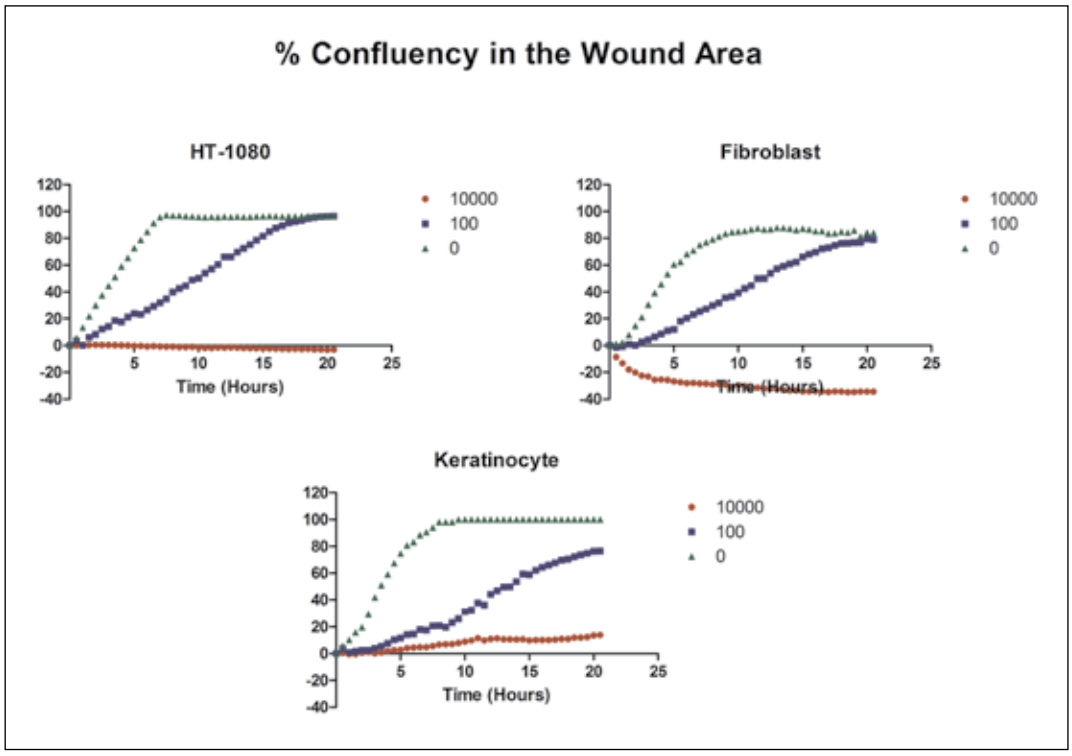


Figure 7. **Quantification of % confluency in the wound area to show cell migration.** Formula 2 was used to calculate the values.

In order to better explain observed phenotypic results, Gen5™ also provides user- friendly methods to monitor wound healing using alternative metrics. These are demonstrated using results from the HT-1080 cell model in Figure 8. Calculations with a fixed wound area, as shown in the upper right graph (Figure 8), can for example help explain variances seen amongst replicates. This is important especially when cells start to migrate before initial imaging takes place (Figure 4A and 5A). To show actual wound closure, plotting the average wound width over time (lower left graph in Figure 8), provides information about cell movement itself. In the absence of Cytochalasin D, HT-1080 cells move together, narrowing the gap to a value approaching 0. In comparison, HT-1080 cells treated with the highest concentration of inhibitor showed a constant average wound width. Finally, the kinetic analysis tool included in Gen5™ evaluates each curve to determine the maximum wound healing rate. As seen in the lower right graph of Figure 8, Cytochalasin D slows wound healing, until the value reaches 0 with the highest concentration tested. Together these metrics, which are automatically determined by Gen5, provide high information content and give a more complete understanding of wound healing and how the process can be altered by adding an inhibiting compound.

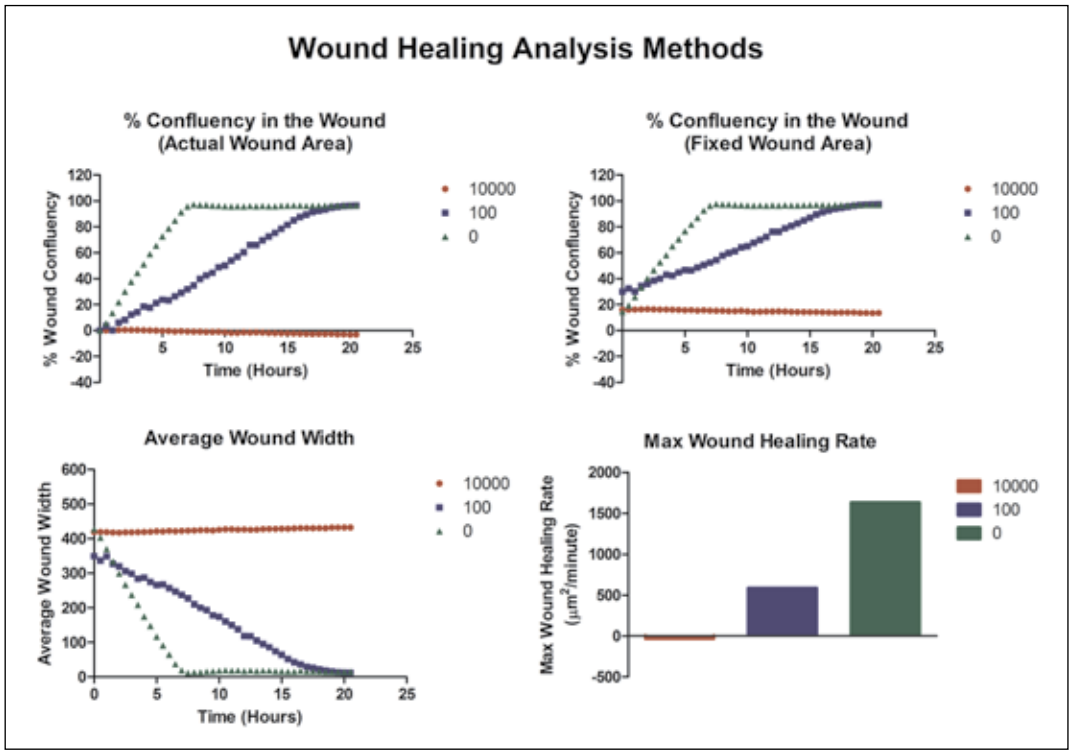


Figure 8. Variable determinations of wound healing and cell migration. Formula 1, 2, and 3 were used to calculate the values.

To validate the analysis performed with Gen5, a second independent software provided by MetaVi Labs was also used. Similar analysis results were shown in both analysis software.

The graph showing the 'Percent Gap Closed All Positions' in Figure 9 is comparable with the graph showing the '% Confluency in the fixed Wound Area' presented in Figure 8. The 'Scratch Open Area All Positions' can be compared to the 'Average Wound Width' in Figure 8. While the curve shapes agree the values represent different metrics. Instead of calculating the wound width in µm, the wound area in µm² was calculated. Both metrics show the same progress over time. Finally the software also calculates the 'Gap Closure Speed' (Figure 9), which agrees with Gen5 software kinetic analysis of the 'Max Wound Healing Rate' (Figure 8).

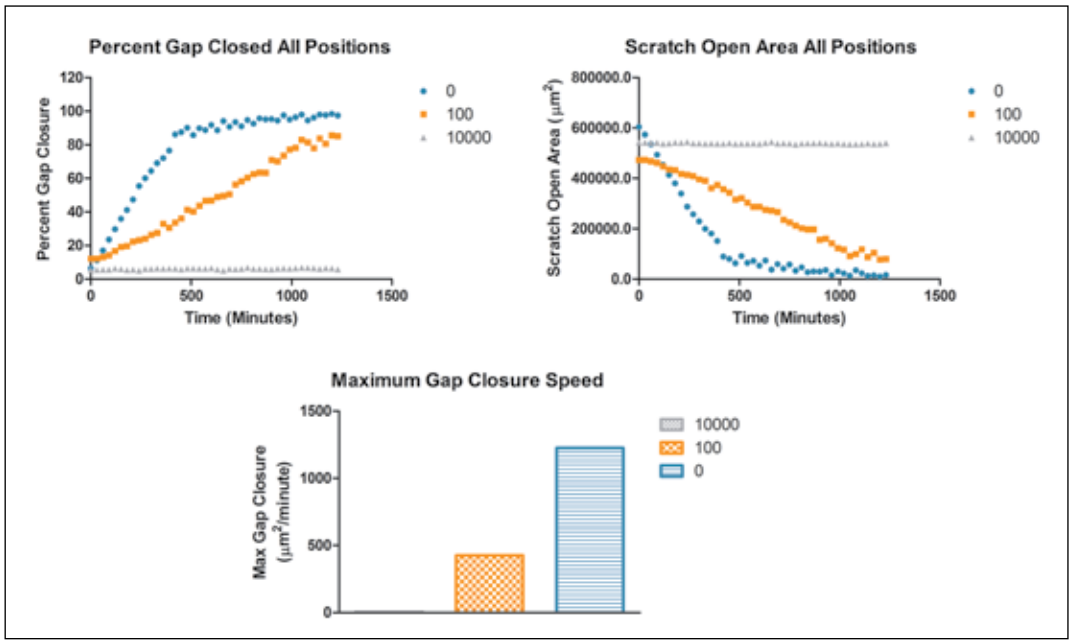


Figure 9. Determination of wound healing using an independent cell analysis software.

Conclusions

The wound healing assay is a powerful method to investigate cell migration. The culture insert and the 35 mm µ-Dish provided by ibidi simplifies the performance and allows easy compound addition. The results seen with the combined experimental procedure delivered results that agree with what has been shown previously in the literature. Biotek's new Lionheat FX automated cell imaging system is perfectly suitable to perform long-term kinetic imaging of the wound healing process. The Cytation 5 is also capable of performing the imaging needed for the experimental set-up. Proper cell health can be ensured through the incorporation of appropriate environmental settings over the incubation time. Integrated Augmented Microscopy™ combines accurate time lapse imaging with precise data analysis. Percent confluency was calculated for each cell type, while various determinations of gap closure can also be automatically calculated using the Gen5 software. The results were verified by using an independent second analyzing software provided by MetaVi Labs. The combination of an easy experimental setup, imaging and precise data analysis creates a powerful tool to investigate cell migration.

References

1. http://www.nature.com/subjects/cell-migration

2. Ilina Olga and Friedl Peter; Mechanisms of Cell migration at a glance; **2009**; *Journal of Cell Science*: 122, 3203-3208.

3. John H. Henson, Ronniel Nazarian, Katrina L. Schulberg, Valerie A. Trabosh, Sarah E. Kolnik, Andrew R. Burns, and Kenneth J. McPartland; Wound Closure in the Lamellipodia of Single Cells: Meditation by Actin Polzmerization in the Absence of an Actomyosin Purse String; **2002**; *Molecular Biology of the Cell*: Vol. 13, 1001-1014.

Acknowledgements

The authors would like to thank Michael Wyler, Joel Mailliet, and Gary Prescott of BioTek Instruments for their contributions to the final analysis methods included in the application note.