Automated high-throughput live cell monitoring of endothelial cell migration

Kevin Schmidt¹, Dominik Lerm², Arne Schmidt¹, Nicholas Dickel⁴, Jan Fiedler¹,²,³, Thomas Thum¹, and Meik Kunz²

¹Medizinische Hochschule Hannover
²FAU
³Fraunhofer-Institut für Toxikologie und Experimentelle Medizin ITEM

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Abstract

Background: Cellular migration is important for physiological and pathological processes. As such, angiogenesis and regenerative wound healing are reliant on the promotion of distinct endothelial cell phenotypes exhibiting increased migratory capacity. Functional monitoring of these hallmark events in vitro is invaluable for discovering novel therapeutics. However, while respective methods are usually simple and economic, they often lack a high-throughput character or accurate analysis tools, which are essential for effective screening suitability. Experimental approach: We stained nuclei of confluent human umbilical vein endothelial cells with Hoechst33342 prior to induction of an artificial scratch wound. Treatments with various growth factors and several concentrations of nintedanib were performed to evaluate impacts on wound closure. Images were taken frequently over 24 h to achieve high time-resolution. We developed an ImageJ macro and a Python script for automated analysis of these image sets. Utilizing cell-free area measuring or cellular density evaluation, respectively, cellular migration behavior was assessed well-wise for each time point. Key results: We proved the functionality of our novel tools and identified pro-migratory effects of interleukin 1β as well as inhibitory actions of nintedanib. Hoechst33342 staining allowed for cell counting which could be excluded as a contributing factor to wound closure in our assay. Conclusion: We herein developed a cost-effective, high-throughput pipeline that allows to monitor endothelial cell migration in vitro. We believe that our protocol will significantly accelerate pre-clinical screenings not only for medications targeting angiogenic processes but also drug discovery research in a broad range of diseases with altered vascularization.
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Experimental approach: We stained nuclei of confluent human umbilical vein endothelial cells with Hoechst33342 prior to induction of an artificial scratch wound. Treatments with various growth factors and several concentrations of nintedanib were performed to evaluate impacts on wound closure. Images were taken frequently over 24 h to achieve high time-resolution. We developed an ImageJ macro and a Python script for automated analysis of these image sets. Utilizing cell-free area measuring or cellular density evaluation, respectively, cellular migration behavior was assessed well-wise for each time point.

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Conclusion: We herein developed a cost-effective, high-throughput pipeline that allows to monitor endothelial cell migration in vitro. We believe that our protocol will significantly accelerate pre-clinical screenings not only for medications targeting angiogenic processes but also drug discovery research in a broad range of diseases with altered vascularization.

Keywords: HUVEC, scratch wound assay, high-throughput, image analysis, cell migration, proliferation, angiogenesis

Key Points
- In this study, we provide a protocol for the assessment of cellular migratory capacity. Infliction of an artificial wound in a confluent cell layer instigates collective and single cell migration which is monitored microscopically. Nuclei staining with Hoechst33342 allows for easier wound closure measurement as well as cell counting.
- We provide two novel methods for expeditious analysis of acquired scratch wound images: (1) a Fiji tool that assesses pixel lane intensity profiles across the image for wound border identification and (2) a Python-based application measuring the cell density within the initial wound outline.
- Proposed analysis methods were evaluated and compared to a previously published tool using a set of 213 image sequences containing 13 time points each. We screened for impacts of different cytokines on migration of human umbilical vein endothelial cells (HUVECs) identifying interleukin 1β as a pro-migratory stimulus.
- Pharmacological relevance of our pipeline was proven by evaluation of impacts of several concentrations of nintedanib on HUVEC migration. Nintedanib potently reduced HUVEC migration when applied in concentrations of 0.6 μM or higher while excessive doses (15 μM) lead to cell death.
- Our Fiji tool showed increased robustness versus non-cohesive wounds which available tools lacked. The main advantage of the Python algorithm eliminates biases caused by inconsistency in cell layer confluence. Both tools show high-throughput character superior to existing ones.

Introduction

Many pathological and physiological processes largely depend on cellular migration (Wu and Lin, 2011; Charraus and Sahai, 2014). Besides the pivotal roles migrating cells play in tumor invasion and metastasis (Friedl and Gilmour, 2009; Mayor and Etienne-Manneville, 2016; van Helvert et al., 2018) as well as immune reactions (Ridley et al., 2003; Friedl and Gilmour, 2009; van Helvert et al., 2018), tissue turnover (Ridley et al., 2003; Mayor and Etienne-Manneville, 2016; van Helvert et al., 2018) and developmental biology (Ridley et al., 2003; Mayor and Etienne-Manneville, 2016), migratory capacity of endothelial cells (ECs) is essential within these biological systems. For instance, the formation of new blood vessels from existing ones, called
angiogenesis, is indispensable for successful development of functional organs, reperfusion of infarcted areas, but also cancer growth (Carmeliet, 2005; Lamalice et al., 2007; Ellis and Hicklin, 2008). Angiogenesis is initiated by differentiation of a selected EC to a tip cell and orchestrated in a programmed manner. Subsequently, such angiogenic ECs start to increasingly secrete proteases for digestion of subjacent tissue thereby enabling invasion. Migration of tip cells is chemotactically guided by various pro-angiogenic factors such as vascular endothelial growth factor (VEGF) (Gerhardt et al., 2003; Tammela et al., 2008). Moving tip cells are followed by proliferating and migrating stalk cells allowing for facilitation and growth of the new vessel (Carmeliet et al., 2009). Apart from angiogenesis, migratory behavior of ECs has been observed in wound healing processes, e.g. after injury of the endothelial wall of blood vessels (Haudenschild and Schwartz, 1979; Liang et al., 2007).

On experimental side, the scratch wound assay is a cost-effective method to assess migratory behavior of cells in vitro. It has been established for many cell types over the past decades and is still frequently used in biomedical research (Bian et al., 2022; Dasgupta et al., 2022; Ortega-Paz et al., 2022). Commonly, cells are seeded in multi-well plates, which have been coated with extracellular matrix components beforehand to enhance migration (Liang et al., 2007), and cultured until a confluent monolayer is achieved. Subsequently, an artificial scratch wound is introduced to the monolayer, for instance by scratching with a pipette tip. Alternatively, culture-insert systems, e.g. from ibidi®, can be utilized in which removal of the insert simulates wound induction. While these systems may be gentler to the cells and enhance reproducibility of the wounding process, they are more expensive and scalability is limited. Regardless of the wound induction strategy, wound closure is generally monitored under a microscope over time (Liang et al., 2007).

Assessment of only a few selected conditions or time points in such scratch wound assays may result in manageable datasets suitable for manual analysis of acquired images for quantification of the wound area over time. However, drug screening approaches usually comprise a multitude of candidates that need to be evaluated, thus requiring expeditious automated analysis methods. Over the recent years, different automated tools have been developed for commercially available softwares or freewares such as ImageJ/Fiji (Schindelin et al., 2012). While some of these algorithms allow for analysis of migratory behavior of single cells, e.g. the Chemotaxis tool for ImageJ (Pijuan et al., 2019), others rather focus on quantification of the collective migration by tracing the leading edge of the monolayer. For example, Suarez-Arnedo et al. recently published an easy-to-use ImageJ plugin that can be used for high-throughput assessment of wound healing assay images (Suarez-Arnedo et al., 2020). Briefly, they utilized pixel-to-pixel variance analysis coupled with hole filling to segment the image into occupied and cell-free areas. Output measurements allow for quantification of migration via two methods: (1) based on area difference to starting time point and (2) development of average wound width over time normalized to starting time point (Suarez-Arnedo et al., 2020). In contrast, Bobadilla et al. applied GrowCut algorithm (Canny, 1986; Vezhnevets and Konouchine, 2005; Shrivakshnan and Chandrasekar, 2012) to delineate leading edges in images and propose a different analysis method called “monolayer edge velocimetry” (Bobadilla et al., 2019). Here, the leading edge was further segmented into smaller windows in which the movement speed of the edge is quantified individually (Bobadilla et al., 2019). In general though, one major drawback of current in vitro scratch assays is the incapability of distinguishing the influence of proliferative and migratory behavior on wound closure (Ascione et al., 2016; De Ieso and Pei, 2018). Therefore, mostly combined effects are considered or proliferation has to be assessed in a separate assay such as measuring incorporation of labeled thymidine or 5-bromodeoxyuridine (BrdU) (Rothaeusler and Baumgarth, 2007).

In this report, we attempted to generate a user-friendly protocol for assessment of migratory behavior of endothelial cells cultured in vitro. A high-throughput character was achieved by combining a 96-well cell culture plate format with automated microscopy. To account for the proliferative factor, we labeled cell nuclei with Hoechst33342 based on which a simple cell count with commonly known methods in ImageJ/Fiji or other softwares such as CellProfiler (Carpenter et al., 2006) is possible. We furthermore provide two alternative approaches, including an ImageJ macro and a python-based application, for expeditious analysis of the acquired images and compare those to the earlier described ImageJ/Fiji plugin (Suarez-Arnedo et al., 2020).
Material and Methods

General cell culture

Human umbilical vein endothelial cells (HUVECs, Lonza, Basel, Switzerland) were cultivated at 37 °C and 5% CO$_2$ in EBM-2 Basal Medium (CC-3156, Lonza) supplemented with Hydrocortisone, hFGF-B, VEGF, R3-IGF-1, Ascorbic Acid, hEGF and GA-1000, all from EGM-2 SingleQuots Supplement Pack (CC-4176, Lonza) according to manufacturer’s instructions. Additionally, 10% (volume/volume) fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) was added. Medium on cultured cells was regularly exchanged to fresh culture medium and cells were passaged weekly according to best cell culture practice. In brief, medium was aspirated and cells were washed with Dulbecco’s phosphate buffered saline (PBS, Invitrogen, Waltham, MA, USA). Subsequently, 0.05% Trypsin-EDTA solution (Invitrogen) was used to detach adherent cells for 5 min. Cells were spun down for 5 min at 4 degC and 300degxg and resuspended in fresh culture medium. Counting of cell concentration was performed with Countess II (Thermo Fisher Scientific) and 300000 cells per flask were seeded for maintenance culture in T75 flasks (Sarstedt, Numbrecht, Germany).

Migration assay

HUVECs were pelleted and seeded in culture medium at a density of 30000 cells per well in a 96-well plate (TPP, Trasadingen, Switzerland) that had been coated a priori with gelatin (#G9382, Sigma-Aldrich, St. Louis, MO, USA) solution (0.1% weight/volume in water) for 30 min at 37 degC. Afterwards, cells were allowed to adhere for 24 h before start of treatments. For the assay, HUVECs were stained with 5 μM Hoechst33342 (Thermo Fisher Scientific) in culture medium light-protected at 37 °C for 20 min. Afterwards, scratches were introduced to the cell layer using a 20 μL pipette tip (Sarstedt). Scraped-off cells were washed away with PBS and 100 μL culture medium or starvation medium with treatments or respective controls were added. Cellular migration was imaged for 24 h at 37 °C and 5% CO$_2$ using a Cytation1 combined with a BioSpa8 device and respective Gen5 (version 3.11) and BioSpa OnDemand (version 1.03) softwares (all BioTek, Winooski, VT, USA). To ease automatization of analysis, image files were named after following scheme where the different items were separated by an underscore: (1) well name, (2) Gen5 operating step, (3) imaging channel number, (4) image number in well at time point, (5) channel name, (6) time point. Background in images acquired with the Cytation1 device was subtracted with the image processing module of the Gen5 software selecting an adequate rolling ball radius of approximately twice the average nuclei size.

Treatment of cells

HUVECs seeded for migration assays were either kept in culture medium as control or treated with EBM-2 Basal Medium supplemented with 0.1% FBS and 1% PenStrep (Gibco). Media were supplemented with (combinations of) following concentrations of (growth) factors or respective controls: 10 ng/mL transforming growth factor beta (TGF-β, 240-B, R&D Systems, Minneapolis, MN, USA) dissolved in 0.1% (weight/volume) bovine serum albumin (BSA, 810683, Sigma-Aldrich) in 4 mM HCl, 10 ng/mL interleukin 1 beta (IL-1β, 200-01B, PeproTech, Cranbury, NJ, USA), 25 ng/mL VEGF (293-VE, R&D Systems) both dissolved in 0.1% BSA in PBS, 3 μg/mL polyinosinic:polycytidylic acid (polyI:C, P9582, Sigma-Aldrich) dissolved in PBS. Conditioned media were applied 24 h before scratch wound infliction and during image acquisition. Nintedanib (S1010, Selleckchem, Planegg, Germany) treatment in the described concentrations was applied either in culture medium alone or in combination with aforementioned growth factors (TGF-β, IL-1β, VEGF) following the wound infliction. Here, 1x CellTox Green Dye (G8741, Promega, Madison, WI, USA) was added to lable necrotic cells.

Image analysis using Wound Healing Size Tool

Images were analyzed with the “Wound Healing Size Tool” by Suarez-Arnedo et al. (Suarez-Arnedo et al., 2020). To expedite the analysis, images were combined with the “Images to Stack” function in Fiji and the stack analysis mode of the plugin was utilized with following parameters: variance window radius, 20; threshold value, 50; percentage of saturated pixels, 0.3; set scale global, “yes”; scratch is diagonal, “yes”.
Migration Assay Analysis Macro

For automated quantification of wound closure in acquired images sets with Fiji (v1.52p or higher), we developed a macro combining several image modification steps with intensity plot assessment for wound identification. For adjustment of settings for image analysis, a user interface was created (Figure 1A).

The user is allowed to insert a username as well as an experiment title which both are added to the names of the analyzed images that are saved for a posteriori manual scrutiny of wound detection, as well as the text file containing comma-separated calculated wound areas. The following six parameters are needed for the actual image analysis. Selection of the wound orientation determines whether images are rotated at the beginning. Background intensity and minimal cell size are used to selectively exclude signals in the image that are below these thresholds. Wound detection accuracy determines the height of the rectangles drawn to generate the intensity plots over the image width, the higher the accuracy, the lower the rectangle height. This parameter should be adjusted proportionally to the cell density. Initially detected wound borders are subjected to a correction for putative outliers, the stringency of which can be adjusted with the maximal deviation angle from the wound orientation. Optionally, a Gaussian blur step can be added to the image processing by inputting a number that is used to set the radius. This function is opted out by default but should be considered if images show high background noise. The last two checkboxes encircled in red (Figure 1A) determine whether images are either analyzed individually or well-wise. If image names contain well and time point identifiers which are clearly distinguishable by a separator, image names can be disassembled accordingly and identifiers can be assigned. Additionally, if the dataset contains more than one image channel, the user is also asked to specify the channel based on which wounds should be detected (Figure 1B). In case of more than one imaged channel, it is also possible to subtract a second channel from the analyzed one to avoid false positive signal detection. This is a particularly useful feature in combination with additional labeling of necrotic cells, e.g. with a cytotoxicity dye. It is also possible to choose the starting time point of a sequence; the rest is analyzed according to default array sorting by Fiji.

Per well, scratch wounds are initially determined in the image of the selected reference time point. A schematic illustration of the algorithm for image adjustment explained below can be found in Figure 2A. In our Fiji pipeline, the image is first rotated to orient the scratch wound vertically. Approx. 95% of the image height is analyzed further to avoid errors caused by artefacts at image borders. Background signals are subtracted using a rolling ball radius of twice the “minimal cell size”-input and brightness and contrast of the image are adjusted assuming a portion of saturated pixels of 0.35. To reduce data size, the image is converted into an 8-bit format. If defined in the user interface, Gaussian blur with the given sigma radius is applied to further reduce background noise thereby enhancing the effectiveness of the following pixel-to-pixel variance assessment step. Here, comparable to the Wound Healing Size Tool (Suarez-Arnedo et al., 2020), Fiji’s variance filter function is applied using a radius based on the user-defined “minimal cell size”-input to achieve an image contrasting high pixel intensities in the cell layer versus low pixel intensities in the cell-free wound. In the processed image, a rectangle with a height specified by the “accuracy”-input spanning from the top left to right image border is drawn (Figure 2B). The profile plot of this rectangle is then screened for edges indicating potential wound borders where intensity drops over the input background threshold are tagged as left and subsequent intensity rises as appurtenant right borders. If the pixel distance from the right border of one pair to the left border of the following pair is smaller than the defined “minimal cell size”, the values are excluded creating a new pair consisting of the left border of pair one and the right border of the following pair. The border pair with the maximal left to right distance is then selected for wound outline. This process is repeated with the next image spanning rectangle starting at the bottom of the previous one until the whole height of the image is covered. All preliminarily determined left and right border coordinates are screened for outliers. To set a threshold, X-coordinates of left and right borders are sorted in arrays and the respective median values are returned, from or to which a tolerance value T is subtracted or added, respectively. T for image k is calculated according to equation (1) with h being the height of image k in pixels and α being the angle in degree of the maximal deviation specified in the user interface.

\[ T_k = \frac{h_k}{2} \times \tan\left(\alpha \times \frac{\pi}{180}\right) \]
In case outliers have been identified at a certain Y-coordinate, new borders are searched at this spot as previously described but with a rectangle spanning only between tolerance thresholds for left and right borders. Eventually, the scratch wound is outlined and the area is measured using the corrected coordinates. Along the timeline, wound borders identified in the previous image are used as boundaries for respective rectangles for edge identification based on the assumption that wounds will not enlarge over time. However, to cope with smaller local expansions of the cell-free area which might occur as a result of single cell motion, a deviation factor based on “minimal cell size” and “accuracy” parameters is added. In case of individual assessment, images are handled as described above for the reference time point.

Finally, a text file containing the input parameters from the UI as well as the calculated wound areas organized well-wise in a comma separated matrix is created and saved to the same directory as chosen for the analyzed images.

For the analysis of our image dataset, we used following parameters: background, 50; minimal cell size, 20; accuracy, 8; maximum deviation angle, 20; additional blur, 0. As previously described, image names contained clearly separable identifiers for well, channel and time point, therefore we enabled timeline-based correction of wound areas. If included, CellTox Green Dye signals were subtracted from analyzed images.

Relative areas covered (RAC) of well $i$ at time point $j$ were calculated from the acquired wound area measurements ($A_i$) according to following equation (2) with $A_{i0}$ being the wound area of well $i$ at the initial time point.

$$RAC_{ij} = 1 - \frac{A_{ij}}{A_{i0}}$$

### Image analysis using Python application

We developed a python-based application for automated cell migration analysis. The program utilizes the open-source frameworks open-cv and NumPy for image analysis and data processing. The workflow of the application encompasses three primary modules: data import, scratch-detection, and migration calculation.

**Figure 3** shows the image analysis steps of the wound detection module.

The initial step of the data preprocessing involves the definition of the working folder, which contains the appropriately named images. Subsequently, the program automatically discerns the number of timelines and the corresponding number of images within each timeline. To ensure consistency, the images are then cropped to match the dimensions of the smallest image in each timeline. Notably, we observed that the microscope images may exhibit uneven illumination due to technical factors. To enhance the quality of subsequent analysis steps, a top hat transformation is applied, mathematically defined as $T_w(G) = G - G \circ S$ with $G$ being the greyscale image, $S$ being the structure element and $\circ$ being the opening operation.

In contrast to initially introduced alternative methodologies, the underlying approach for quantifying the progress of migration does not rely on the measurement of the distance between wound borders. Instead, it calculates migration progress based on the cellular density within the gap. As such, the second module of the analysis pipeline is responsible for determining the size and position of the initial scratch, utilizing the image captured at time point zero for each respective timeline. The module starts by converting the greyscale image into a binary image through the application of a threshold by Otsu’s method. This effectively separates the cells from the background and enables the subsequent dilation operation. The outcome is an enlargement of the cells, consequently leading to reduced intercellular gaps. To ensure complete closure of these cell spaces, a smoothing operation is implemented, utilizing an appropriate kernel size and averaging technique. Following a final thresholding step, the Suzuki contour finding algorithm is employed to identify the largest contour. In order to counteract the artificial shrinkage of the initial wound caused by previous image processing steps, we have added a linear scaling function. The resulting contour is subsequently used to generate a binary mask, where the initial scratch is white (ones) while the background (i.e., the non-wounded area), remains black (zeros).

In the final module of the pipeline, unlike in the second module which utilizes only the first image of each timeline to determine the initial wound area, all images within the series are employed to calculate the
migration progress. Initially, the starting wound area $w$ and background area $b$ are computed according to equation (3) with $M(i,j)$ being the binary mask of the contour and $n$ and $m$ being the dimension of the image.

\[(3)w = \sum_{i=1}^{n} \sum_{j=1}^{m} M(i,j) \quad \text{and} \quad b = nm - w\]

Subsequently, on each image in the time series, the threshold algorithm is once again applied specifically within the region of the wound. The resulting segmented areas correspond to cells that have already migrated into the wound. With the defined binary masks of the migrated cells $C_t(i,j)$ we can calculate the total area of migrated cells $c_t$ and background area of the scratch $s_t$ for a given timepoint $t$ with equation (4).

\[(4)C_t = \sum_{i=1}^{n} \sum_{j=1}^{m} C_t(i,j) \quad \text{and} \quad s_t = w - c_t\]

Finally, the absolute migration progress $mig$ is defined as the ratio of the area occupied by migrated cells in the scratch to the initial scratch area, calculated as a percentage using equation (5).

\[(5)mig_t = 100 \cdot \frac{c_t}{w}\]

The resulting value can be used to compare different samples or conditions. However, it is difficult to estimate the true rate of migration progress based on this ratio, since 100 percent would mean that the whole scratch is populated with cells, without any intercellular space. To address this limitation, we have implemented a linear correction step based on the background cell density. The corrected value is determined using equation (6), where $bd$ represents the background cell density at timepoint zero.

\[(6)mig_{scaled} = \frac{mig_t}{bd}\]

The computed migration value table is saved as a comma-separated text file. Additionally, for visualization and as a control, the first images of each timeline are saved in the output folder, with the calculated initial scratch area appropriately highlighted.

An overview of the different parameters utilized by the application are listed in table 1. Furthermore, a save and load option of the settings is implemented, which allows the user an easier use of the program for different datasets. For demonstration purposes, it is also possible to generate a gif animation, which visualizes the workflow and the calculated wound healing progress.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Top-hat-kernel</td>
<td>Sets the kernel size for the top-hat transformation used in the image preprocessing module to correct uneven illumination.</td>
</tr>
<tr>
<td>2 Dilation-Kernel</td>
<td>Sets the kernel size for morphological operation dilation in the scratch detection module. The lower the cell density, or for Hoechst33342-stained cells, a larger kernel is necessary.</td>
</tr>
<tr>
<td>3 Blurring-Kernel</td>
<td>Sets the kernel size for smoothing after the dilation operation. The higher the value the more homogeneous the image content becomes, which leads to improved thresholding but can also cause unwanted smoothing of the wound edges.</td>
</tr>
<tr>
<td>4 Threshold-Value</td>
<td>Sets the threshold for the detection of the initial wounded area.</td>
</tr>
<tr>
<td>5 Linear-scaling</td>
<td>Sets the factor with which the detected wound is scaled to counteract any shrinking of the region induced by the previous analysis steps.</td>
</tr>
<tr>
<td>6 Threshold final</td>
<td>Sets the threshold for the detection of migrated cells in the wound area.</td>
</tr>
</tbody>
</table>

Table 1 : Overview of the parameters used in the python application.

**Cell counting**

We utilized the CellProfiler image analysis platform to count single cells in the acquired images to estimate the contribution of possible cell proliferation to the observed wound closure. The pipeline consisted of the IdentifyPrimaryObjects module with diameter restrictions of 8 pixels to 40 pixels and default thresholding parameters. Identified objects outside the diameter range or those touching image borders were excluded. Next, object outlines were drawn on the original image and saved for manual inspection, if needed. Object counts were exported to a spreadsheet.

**Statistics**

Statistical analysis was done with R (version 4.2.2). If not stated otherwise, data are presented as
means ± 95%-confidence intervals (CIs). Two-way analysis of variance (ANOVA) with post hoc-Dunnett-corrected multiple comparisons (“emmeans” package) with vehicle (veh) group as reference was chosen to analyze the generated datasets containing more than two groups. Shapiro-Wilk test was consulted to verify normal distribution and Levene test was applied to evaluate homoscedasticity (both “rstatix” package). P -values below 0.05 were considered statistically significant and are plotted above respective comparisons. All experiments were performed within \( n = 3 \) biological replicates with three to four technical replicates each.

Half maximal effective concentrations (EC\(_{50}\)) of nintedanib were calculated based on a 4-parameter logistic model fitted to respective datasets using the “drm” function (“drc” package).

Results

Generation of a comprehensive image dataset

To evaluate wound closure detection accuracy of the different tools, we generated a dataset containing 216 sequences (18 conditions, \( n = 3 \)) of 13 images each in this project. We introduced known pro- and anti-migratory factors and conditions to HUVEC cultures to assess whether resulting effects are positively recapitulated in the assay and can be correctly identified by our analyses. Besides VEGF, a frequently used factor to trigger angiogenic activity of endothelial cells in various assays (Alexander et al., 2012; Boos et al., 2023), we selected IL-1\(_\beta\) which has been previously described to have pro-angiogenic effects (Garlanda et al., 2013; Afonina et al., 2015; Chen et al., 2022). To investigate whether potential migration regulating effects of IL-1\(_\beta\) were mediated via triggered pro-inflammatory pathways, we also included polyI:C, a viral double-stranded RNA analog that instigates toll-like receptor 3 (TLR3) activation. Of great interest, interference with TGF-\(\beta\) signaling has been shown to modulate migratory activity of several cell types (Zhang et al., 2019a; Puthdee et al., 2022). To block migration, we kept HUVECs in starvation medium devoid of the usual supplements and containing only 0.1% FBS. We then again introduced previously described cytokines to screen for potential rescue effects. Of the resulting 216 image sequences, three had to be excluded due to experimental errors prior to analysis. In summary, we could test the different analysis tools on a set of 213 image sequences encompassing low and high migration conditions.

Migration analysis Fiji Wound Healing Size Tool

First, we analyzed obtained images with the previously described Wound Healing Size Tool (Suarez-Arnedo et al., 2020). We observed an accurate detection in the earlier stages of wound healing when the left and right layers in wells were fully separated by the cell-free area. However, with partial wound closure the algorithm was unable to detect the wound in its entirety but rather chose the biggest cohesive cell-free area as the remaining wound (Figure 4A ). This issue was also represented in the high 95%-CIs shown in the RAC graphs between 0 h and 12 h in growth medium conditions and from 12 h onwards in starvation medium conditions (Figure 4B , C ). Expectedly, this issue became negligible as RAC values approached 1.0. Upon growth medium conditions, the analysis suggested IL-1\(_\beta\) as a potential migration promoting factor as cells exposed to either IL-1\(_\beta\) in combination with TGF-\(\beta\) or VEGF showed faster wound closure (Figure 4B ,C ). HUVECs exposed to IL-1\(_\beta\) alone or a mixture of all assessed growth factors (GFs) also showed a tendency towards higher migratory capacity but did not achieve statistical significance (\( p = 0.0910 \) and \( p = 0.0954 \), respectively). However, the missing statistical confirmation of pro-migratory action of IL-1\(_\beta\) alone suggested by the analysis might ensue from the high deviation within the control and IL-1\(_\beta\) datasets caused by the incomplete wound detection as mentioned before. Expectedly, HUVEC migration was significantly reduced under starvation conditions (two-way ANOVA (medium) < 0.0001). IL-1\(_\beta\) reestablished HUVEC migration partly when applied either alone or in combination with other GFs (Figure 4B ,C ). Interestingly, none of the other applied putatively pro-migratory substances seemed to accelerate would closure.

We additionally investigated the influence of cell proliferation on the observed wound closure in our experimental setup. We utilized a simple CellProfiler pipeline to count Hoechst33342-labeled nuclei in the images and counts were normalized to the respective first image of the time sequence (Supplemental Figure S1 ). We observed a slow decline in cell counts over time in growth medium conditions and a slightly steeper decrease under starvation conditions. This cell count reduction can be in part explained by dying cells, the
rate of which would be expectedly higher under nutrient deprivation. Another contributing factor could be bleaching or further dilution of Hoechst33342 over time. Therefore, we concluded that the contribution of cellular proliferation to wound closure could be neglected in our assay.

Migration analysis FijiMigration Assay Analysis Macro

We analyzed the image dataset with our Migration Assay Analysis Macro to see whether the results including a putative pro-migratory effect of IL-1β from before could be confirmed with this method. As described in detail in the methods section, we created a wound detection algorithm utilizing assessment of pixel-lane intensity plots instead of cohesive particle identification in a binary mask. This design should be able to circumvent the aforementioned issue of incomplete wound area detection. As depicted in Figure 5A, even partially closed wounds were successfully identified throughout the full image span. Another difference we identified when comparing the analyzed images of both tools was particle exclusion within the wound area. While the Wound Healing Size Tool includes a hole-filling step deleting any singularized particles of a certain size from the image, our tool evaluates the intensity and width of such objects and draws the wound border accordingly. The advantage here is that single cells sitting in or migrating into the wound are also accounted for (arrows) while a disadvantage is a higher risk for false positive identification (filled arrowheads). However, due to the relatively minimal error such false identification causes, we decided to accept this risk for a greater benefit.

As a result of this, RAC curves had much smoother courses and 95%-CIs were smaller (Figure 5B, C). Therefore, conditions that have been identified previously upon analysis with the Wound Healing Size Tool to have pro-migratory influence could not only be confirmed with this analysis method with a higher confidence (growth versus starvation medium, two-way ANOVA \( p_{\text{medium}} < 0.0001 \)), but also IL-1β and the mixture of all tested GFs now showed statistically significant influences on HUVEC migration.

Migration analysis Python Scratch-Assay-Tool

The Python-based application for automated cell migration analysis was integrated into a graphical user interface (GUI) using the tkinter library (Figure 6A-C). Assuming consistent image quality across the time series, users can easily adjust relevant parameters for optimal scratch detection. To support the high throughput approach, it is sufficient to set the parameters for the first timeline, the following image series will use the same settings. The effects of parameter changes can be tracked in real time by the preview window on the right side of the application tremendously simplifying the selection of values for the different parameters.

We demonstrated the usability of our GUI on our image dataset, first analyzing cellular migration without background scaling (Figure 6D, E). In the resulting curves (Figure 6D), we noticed that cellular density within the scratch wound in growth medium receiving HUVECs slightly declined after reaching the maximum. This might be due to the fact that staining intensity slightly declines over time as it is also partly reflected in the decrease in cell counts (Supplemental Figure S1). Nevertheless, we could reproduce the results from the Fiji Migration Assay Analysis Macro in cells kept in growth medium as significant increase in HUVEC migration under IL-1β exposure was observed. However, while both of the other tools identified the same effects of IL-1β in the starvation medium conditions, cellular densities within the wound scratch apparently did not differ among the investigated groups.

Applying background scaling according to equation (6), corrected for the decline in cellular density described before for later time points (Figure 6F). In contrast to results reported for analyses excluding background scaling, effects of IL-1β were significant solely when applied in combination with TGF-β. Interestingly, unlike analysis of the unscaled data from starved HUVECs, scaling lead to statistical significance of impacts of IL-1β on HUVEC migration when administered in combination with other GFs but not alone (Figure 6G).

Nintedanib reduces HUVEC migration

To validate our models for drug screening purposes, we treated HUVECs with the receptor tyrosine kinase inhibitor nintedanib, which is nowadays used to combat idiopathic pulmonary fibrosis (Keating, 2015). A
direct inhibition of various GF receptors, including VEGFR, is well known for nintedanib leading to reduced endothelial cell proliferation and angiogenesis (Hilberg et al., 2008). Applying our high throughput approach, we could identify a significant reduction of HUVEC migration upon exposure to 0.6 μM nintedanib or higher (Figure 7A, B). Interestingly, simultaneous application of the previously used GF cocktail doubled the calculated EC50 (veh: 2.252 μM; GFs: 5.294 μM). Comparable results were achieved upon analysis of images with our second tool using either unscaled (Figure 7C, D) or scaled (Figure 7E, F) approaches. Scaling significantly reduced the variance of data within groups enabling a more robust detection of active concentrations in this experimental design. In general, the highest applied concentration of nintedanib (15 μM) led to excessive cell death (Supplemental Figure S2), which is also represented in the aberrant migration curves and high 95%-CIs (Figure 7).

Discussion

Influence of various GFs and nintedanib on HUVEC migration in vitro

The observed influence of IL-1β on HUVECs is comparable to previously published results in different cell types. As such, exposure to IL-1β has been shown to stimulate migratory behavior of corneal endothelial cells in a scratch wound healing assay via induction of fibroblast growth factor 2 and ensuing autocrine stimulation (Lee and Heur, 2013) as well as increased matrix metalloproteinase 9 expression (Tseng et al., 2013). Apart from the two dimensional migration measured in wound healing assays, IL-1β has been reported to also enhance trans-well migration in murine (Sullivan et al., 2014) and human (Carrero et al., 2012) mesenchymal stem cells, various human peripheral blood leukocytes (Carrero et al., 2012) and rat cortical neurons (Ma et al., 2014). Interestingly, considering a missing effect of polyI:C on HUVEC migration, it is likely that the pro-migratory effect of IL-1β is not mediated via downstream pathways shared by IL-1 receptor 1 and TLR3 such as the nuclear factor κ-light-chain-enhancer of activated B cells axis. Concordantly, He et al. (He et al., 2021) recently reported a differential effect of two inflammatory triggers, lipopolysaccharide (LPS) and polyI:C, on mouse primary microglia migration which the authors accredited to discrepancies in treatment-elicted cytokine expression. They reported an increase in IL-1β and IL-6 expression in response to LPS whereas polyI:C rather enhanced type-I interferon production. The observed stimulatory effects of LPS but not polyI:C in chemotaxis assays correspond to our findings in HUVECs treated with IL-1β and polyI:C.

Even though being widely used to successfully enhance endothelial cell migration in wound healing assays (Shizukuda et al., 1999; Cerezo et al., 2017; Nareshkumar et al., 2018), we could not see an effect of VEGF. While the lack of effect in growth medium conditions could be explained by the already present VEGF supplement in the EGM-2 pack, VEGF did also not affect HUVECs kept in starvation medium during the wound closure experiment. However, cultivation of HUVECs over longer time in our growth medium might have rendered the cells irresponsible to additional VEGF stimulation. An increase in the applied concentration might resolve this issue or alternatively, endothelial cells specifically tested for VEGF responsiveness could be used.

TGF-β, a hallmark factor of fibrosis, is broadly known to cause activation in proliferation and migration in fibroblasts in vivo (Frangogiannis, 2020). However, in vitro cultivation of fibroblasts can reflect pro-fibrotic gene expression changes after TGF-β treatment to some degree (Zhang et al., 2019a) but clearly lacks to display behavior of activated fibroblasts on a functional level due to their rapid activation and myofibroblast transition (Rohr, 2011). The role of TGF-β in the context of endothelial cells seems to be contradictory in literature. On the one hand, a reduction in trans-well migration of HUVECs caused by TGF-β through phosphatidylinositol 3-kinase catalytic subunit type 3 has recently been described (Zhang et al., 2019b). In line, medium transferred from TGF-β -treated dental pulp stem cells to HUVECs reduced their migratory activity compared to medium transferred from respective control cells (Zhang et al., 2021). On the other hand, Wang et al. (Wang et al., 2017) show an increase in HUVEC migration both in scratch wound as well as trans-well migration assays in response to TGF-β. The discrepancies might result from the differences in concentrations and time periods of the treatment. While in the first and second cases relatively high concentrations (5 μg/mL for 48 h and 20 ng/mL for 48 h) or long timespans (10 ng/mL for 7 days) were
chosen for incubation in TGF-β containing medium, only 5 ng/mL were used in the latter. Higher exposure to TGF-β might therefore result in an over-stimulation of the cells eliciting a negative feedback loop (Yan et al., 2018) suppressing migration and proliferation.

For demonstration of usability of the herein proposed tools for pharmacological research, we screened for effective concentrations of nintedanib on HUVEC migration. Through its triple RTK inhibitor action, nintedanib is a promising substance for interference with cellular migration, a process largely reliable on intercellular communication via cytokines and GFs. Kataria et al. could also show a significant effect of nintedanib on cellular response to IL-1β and tumor necrosis factor α stimulation as expression of various cytokines decreased compared to control cells (Kataria et al., 2022). In line, multiple studies have proven that concentrations ranging from 0.1 μM to 1 μM were able to reduce wound healing capacity of various cell types, including epithelial cells, smooth muscle cells and fibroblasts (Jamadar et al., 2021; Kataria et al., 2022; Yin et al., 2023). Comparably, we could show that HUVEC migration could be reduced upon application of similar dosages even in the presence of wound closure promoting GFs.

Comparison of methods applied for assessment of HUVEC migration in scratch wound assay

As already teased in part in the results section, we discovered various advantages and disadvantages of the herein applied tools highlighted in Table 2. The main advantage of the Wound Healing Size Tool (Suarez-Arnedo et al., 2020) for Fiji over the other two presented tools, is the possibility to analyze bright field images. However, we propose an assay protocol that includes Hoechst33342 which not only simplifies wound identification but also has been described to have anti-proliferative effects in various cell types while not interfering with viability (Fried et al., 1982) thereby removing the confounding influence of cellular proliferation on wound closure. In doubt, potentially remaining proliferative activity of investigated cells can still be estimated by simple cell counting as we demonstrated in this report utilizing the CellProfiler platform. Occasionally, DNA fragments or cellular debris remain attached to the plate surface after introducing the wound which could ensue in Hoechst33342 signals within the cell free area. These unwanted signals are removed by the hole filling step in the Wound Healing Size Tool reducing false detection of migratory activity. The major drawback of this tool however is the incapability of whole wound detection in later stages during the assay when wounds are already partially closed. This could lead to high variations among different experiments challenging reproducibility and accurate reflection of actual migratory behavior. For proper high throughput utilization of the Fiji plugin, additionally measures are necessary such as stacking the images that should be analyzed or automating single image analysis via a supplemental macro. Still, images are always analyzed individually in a timeline independent manner.

In contrast, we created a Fiji-based analysis module that offers timeline-corrected migration measurements. Here, wound identification in subsequent images is assisted by the analysis performed in previous ones reducing the risk of false wound identification drastically. Unlike in the Wound Healing Size Tool, the Migration Assay Analysis Macro algorithm is based on assessment of pixel intensities plotted across the image rather than selecting the largest cohesive cell-free area. This avoids incomplete recognition of wounds that already have been partially closed by migrating cells and increases robustness against deviations in cell layer confluence. A disadvantage of this approach is the dependency on the wound angle in the image as profiles are always plotted horizontally. Therefore, most accurate results can only be obtained in case of perfectly vertically (or horizontally, if adequate settings are applied) oriented scratch wounds. Omission of a hole-filling step on the one hand allows for taking single cell migration into account but increases the risk for false identification of allegedly migrated cells. Lastly, to maximize throughput, we included the option to deconvolve image file names for automated identification of time points, well positions, and imaging channels.

Finally, we present a Python-based application for cell migration evaluation. Maintaining the high throughput character from our Fiji tool, we propose an alternative way of wound closure quantification. Unlike previous approaches that calculate either the size of the remaining cell-free area or the distance between the borders, we estimated the cellular density within the initial wound area for each time point to reflect migratory behavior. While this model still accounts for both, single cell and collective migration, normalization to the background of the remote area removes the bias of inconsistency in cellular confluence. Even
though assured through cell counting based on Hoechst33342 staining, this background scaling would also correct for proliferation influencing wound closure rates. For this approach to work however, unambiguous delineation of coherent timelines in file names is a necessary prerequisite because images are evaluated based on the initial time point based on which the wound outline is fixed. While this approach is error prone in case of image position changes along the timeline, it greatly minimizes the risk of incomplete wound identification. Performing the analysis runs with Fiji tools, we noticed that finding optimal parameters can be time consuming. Therefore, we incorporated a preview window into the GUI for live tracking of parameter influences. This also expedites the analysis process especially for inexperienced users.

<table>
<thead>
<tr>
<th>Tool</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiji Wound Healing Size Tool (Suarez-Arnedo et al., 2020)</td>
<td>hole filling reduces noise in scratch wound no staining necessary</td>
<td>additional efforts needed to increase throughput incomplete identification of wound possible no timeline correction</td>
</tr>
<tr>
<td>Fiji Migration Assay Analysis Macro</td>
<td>high throughput timeline-based correction robust against inconsistent confluence account for single cell migration</td>
<td>false positive identification within scratch wound possible dependent on wound orientation staining required</td>
</tr>
<tr>
<td>Python Scratch-Assay-Tool</td>
<td>high throughput timeline analysis GUI with preview for easy parameter selection robust against confluence alterations via background scaling</td>
<td>clearly labeled timeline required incomplete wound identification possible susceptible to image movement over timeline</td>
</tr>
</tbody>
</table>

Table 2: Advantages and disadvantages of applied migration analysis tools.

Conclusion

In this report we created a protocol that enables high throughput assessment of cellular migration in vitro. For this, we developed two novel approaches for automated image-based wound closure calculation and validated these in scratch assays performed with HUVECs, where we discovered a pro-migratory effect of IL-1β as well as anti-migratory actions of nintedanib. While this report is confined to the endothelial context, we speculate that our pipeline is easily transferable to other cell types such as fibroblasts or epithelial cells as well and will therefore arguably simplify expeditious pre-clinical screenings not only for medications targeting the vasculature but also various other drug discovery objectives for a broad range of pathological conditions.

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Disclosures

T.T. is founder and shareholder of Cardior Pharmaceuticals GmbH (outside of the content of this manuscript).

Competing interests’ statement

None.

Author contributions

K.S. developed analysis software, performed cell culture experiments and wrote the initial draft of the manuscript as well as subsequent versions. D.L. developed analysis software and contributed to writing the initial draft. A.S. performed cell culture experiments and revised the manuscript. N.D. assisted in analysis.
software development. J.F., T.T., and M.K. supervised the project and assisted in manuscript writing and revision. All authors read and approved the manuscript.

Data availability
The source code for the Fiji Migration Assay Analysis Macro is available here: https://github.com/schnikev/Migration-Assay-Analysis-Macro.

The Python Scratch-Assay-Tool and a test dataset can be downloaded here: https://github.com/DomiLerm/CellMigrationTool.

References


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Figure 1
Figure 4
Supplemental Figure S1
Supplemental Figure S2