Cell Image Velocimetry (CIV): boosting the automated quantification of cell migration in wound healing assays

Florian Milde, Davide Franco, Aldo Ferrari, Vartan Kurtcuoglu, Dimos Poulikakos and Petros Koumoutsakos

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Cell migration is commonly quantified by tracking the speed of the cell layer interface in wound healing assays. This quantification is often hampered by low signal to noise ratio, in particular when complex substrates are employed to emulate in vivo cell migration in geometrically complex environments. Moreover, information about the cell motion, readily available inside the migrating cell layers, is not usually harvested. We introduce Cell Image Velocimetry (CIV), a combination of cell layer segmentation and image velocimetry algorithms, to drastically enhance the quantification of cell migration by wound healing assays. The resulting software analyses the speed of the interface as well as the detailed velocity field inside the cell layers in an automated fashion. CIV is shown to be highly robust for images with low signal to noise ratio, low contrast and frame shifting and it is portable across various experimental settings. The modular design and parametrization of CIV is not restricted to wound healing assays and allows for the exploration and quantification of flow phenomena in any optical microscopy dataset. Here, we demonstrate the capabilities of CIV in wound healing assays over topographically engineered surfaces and quantify the relative merits of differently aligned gratings on cell migration.

Introduction

Cell migration is an essential component of biological processes ranging from embryonic development to wound healing and cancer. The quantification of the migratory potential of cells under varying conditions is usually performed by wound healing assays, a rather simple albeit ubiquitous tool. In wound healing assays, epithelial or endothelial cells in culture are grown to create a confluent monolayer and a wound is inflicted by scraping the monolayer with a pipette tip. Following the wounding, migrating cells invade the open area. Cell migration is quantified as the rate of change of the open area, an approximation to the wound front migration speed. In addition to examining the front propagation, recent work has examined the dynamics at the interior of the migrating cell monolayer. These dynamics are important in determining cell–cell interactions (signaling, mechanics, adhesion) and they ultimately provide multidimensional information regarding cell motility.

In recent years, a number of computational tools have been developed to automate the quantification of cell migration in wound healing assays. We distinguish methods for wound interface tracking, cell segmentation and tracking and flow detection inside the cell layer and inside the deforming substrates (traction force microscopy). These computational tools aim to overcome hurdles associated with the image

Insight, innovation, integration

We introduce Cell Image Velocimetry (CIV), a method to extract and analyze detailed spatiotemporal information for cell migration, as studied by wound healing assays. CIV combines a cell layer segmentation and image velocimetry algorithms and it is shown to be highly robust for images with low signal to noise ratios. The associated software enables the quantification of the advancing front as well as the velocity field inside the migrating layer in an automated fashion. The modular design of CIV further enables flow analysis on in vitro and in vivo time-lapse datasets beyond wound healing. We demonstrate the capabilities of the presented software by applying it to a set of wound healing assays over topographically modified surfaces.
acquisition process and imply a problem specific design for the underlying algorithms and software. We note that no method to date quantifies the wound front and the interior of the cell layer for low contrast and low detail images. Furthermore the proposed methods for wound healing quantification are not integrated into a software toolbox and therefore they are not readily available to experimentalists. We note also that enhanced spatiotemporal information for cell migration can contribute significantly to the development of corresponding computational models. The methods and software presented herein aim to resolve this situation.

Wound interface tracking methods quantitatively quantify the collective migration in the direction perpendicular to the wound. These methods are quite robust and readily applicable to variable conditions. On the other hand, cell segmentation and tracking methods resolve the migration path of each cell inside the monolayer, providing additional information for cell migration in wound healing assays. However these methods impose stringent conditions on the image acquisition process. The effective cell tracking in phase contrast images relies on minimal background noise and a constant contrast throughout all time frames, requirements that are not always met in wound healing assays. The robustness of automated cell tracking can be increased by introducing fluorescent nuclear markers at the expense of possibly altering cellular activities.

Optical methods for flow detection, originally developed for experimental fluid mechanics, have been recently adopted to quantify the velocity field inside the cell monolayer. These methods have been used to quantify cell migration in phase contrast, time-lapse images of confluent monolayers and wound healing assays as well as to capture substrate displacement in confluent cell layers and during wound healing, a method referred to as traction force microscopy.

In order to capture accurately the velocity field inside migrating cell layers, the phase contrast images should feature high contrast and a high signal to noise ratio. Such conditions are possible in the case of flat glass coverslips but more difficult to attain in assays that employ more complex substrates. Wound healing assays evolving on topographically modified substrates are becoming increasingly common, as they emulate cell migration in the presence of the Extracellular Matrix or artificially embedded guiding structures. Wound healing on topographically modified substrates made of biocompatible polymers, as considered in this work, drastically reduce the quality of phase contrast images, due to scattering and reduced transparency. Fluorescent cell markers have been introduced in order to enhance the analysis of cell migration under such conditions. However, for long term recordings in high throughput setups, this method requires low excitation and magnification in order to minimize photobleaching and photo-damaging effects on the cell layer. In turn, this leads to time-lapses with low contrast and very low signal-to-noise ratio.

In this work, we introduce algorithms and software for the automated analysis of cell migration in wound healing assays under varying substrate conditions. The proposed Cell Image Velocimetry (CIV) combines an automated detection mechanism to trace the migrating wound front with flow detection analysis to capture transport processes inside the cell layer, drastically enhancing the extraction of information from wound healing assays. The method is highly robust against image background noise and provides quantitative analysis of cell migration in the interior of the cell layer while the associated, freely available software employs a graphical user interface to facilitate experiment analysis. The capabilities of the present methods are demonstrated by examining a set of wound healing experiments. We consider endothelial cell migration on flat and topographically modified surfaces with gratings inducing alignment of the cell shape and directional cell migration. Here we use gratings that maximize cell polarization to investigate the link between topographically driven cell polarization and directional migration in endothelial wound healing.

### Materials and methods

We use wound healing assays for the development and validation of the computational tools.

### Micro-fabrication of substrate patterns

Gratings are imprinted on 180 µm thick untreated cyclicolefin copolymer (COC) foils (Ibidi, Germany) using nanoimprint lithography (NIL). A silicon mold is fabricated with ridge and groove widths of 1 µm and ridge height of 1 µm. The height of the ridges is adjusted by tuning the etching time while the side wall steepness is kept constant. This procedure generates on the mold squared patterned areas of 1 cm side length. The COC foils are placed on top of the mold, and then the temperature is raised up to 160–180 °C to soften the COC. Afterwards, a pressure of 50 bar is applied for 10 minutes before cooling down to 40 °C. The mold is then gently detached from the COC foil using a scalpel. To finish the fabrication procedure, the samples are treated with oxygen plasma (100 W for 30 seconds) to increase the hydrophilicity of the surface and to promote cell adhesion (see Fig. 1A and B).

### Cell culture

Human umbilical vein endothelial cells (HUVEC, Invitrogen) are grown in 200PRF medium supplemented with fetal bovine serum 2% v/v, hydrocortisone 1 µg mL⁻¹, human epidermal growth factor 10 ng mL⁻¹, basic fibroblast growth factor 3 ng mL⁻¹ and heparin 10 µm L⁻¹ (all reagents from Invitrogen, USA) and are maintained at 37 °C and 5% CO₂. All reported experiments are performed using cells with less than seven passages in vitro. Up to three imprinted COC substrates (1 cm²) are individually sealed to the bottom of a well in a 12-multwell culture plate (Becton Dickinson, USA), hereafter denoted topographic chip. The topographic chips are sterilized by overnight treatment with ethanol and rinsed three times with PBS before starting the coating procedure. The topographic chips are coated with gelatin according to the following protocol: 1.5% gelatin (Merck, Darmstadt, Germany) in water is added to the samples and let to adsorb for 1 hour at room temperature (RT). Subsequently, the gelatin is cross linked by incubating with 2% glutaraldehyde (Sigma Aldrich) in water for 15 minutes at RT. After a 1 hour sterilization-step
with 70% ethanol in PBS (Sigma Aldrich), the substrates are washed 5 times with PBS and left overnight at RT in 20 mM glycine (Sigma Aldrich) in PBS to neutralize the glutaraldehyde. After washing with PBS, the topographic chip is stored at 4°C until the seeding of the cells. To generate confluent monolayers, cells are seeded on COC substrates at high density (3.5–5 × 10^4 cells per cm^2) as reported in ref. 33 and cultured for three days. Before starting the wound healing experiments, the cells are labeled using the DiD Vybrant solution (Invitrogen). The cell monolayer is incubated for 40 minutes at 37°C with 1 mL of normal growth medium supplemented with 3 μL of the labeling dye. Then the staining solution is substituted with fresh normal growth medium and the samples are kept at 37°C and 5% CO2 for 15 minutes before inflicting the wound. The wound is applied by scratching the monolayer with a pipette tip.

**Image acquisition**

Cell imaging is performed using an inverted Nikon-Ti wide-field microscope (Nikon, Japan) equipped both with an Orca R-2 CCD camera (Hamamatsu Photonics, Japan) and an incubated chamber where temperature and CO2 concentration are maintained at 37 °C and 5%, respectively (Life Imaging Services, Switzerland). Images are collected with a 20×, 0.45 NA long-distance objective (Plan Fluor, Nikon). Wound healing on topographic substrates is recorded in parallel time-lapse movies for the three different conditions (flat, orthogonal and parallel (see Fig. 1C)). Immediately after the wound infliction, the experiment is started to automatically collect images in the TRICT channel with a time resolution of 15 minutes for a total of 15 hours. Using the large image function (NIS elements, Nikon, Japan), a field of at least 3 × 3 single images is acquired for every set positions. Focal drift during the experiments is avoided using the microscopes PFS autofocus system. At the end of the experiment, the resulting time-lapses are converted into a single 16 bit movie for each field under analysis (see Fig. 1D).

**Image processing**

An optical method for flow detection is coupled to a wound interface tracking algorithm that integrates contrast enhancing filtering techniques for microscopy images (Fig. 2B and C). Segmenting the cell layer from the background is challenging for images where the characteristic noise length scale is of the same order as the cellular structures. Neither the intensity level nor the signal frequency can clearly identify the cells from the background. We devise a difference-based thresholding method coupled to a correction step in order to accurately segment the wound interface from the image background. Furthermore, the automated multi-tile stitching routine of the microscope can introduce artificial shifts in between consecutive frames.
These shifts are automatically detected and corrected for in the reconstructed velocity field.

We observe that in a few corrupted images, single image tiles are out of focus. The software offers the capability to manually exclude such frames from the image analysis. In order to reduce the time needed for image analysis, a scaling factor is introduced to decrease the original image size. If not reported otherwise, a scaling factor of 0.3 is applied. The flow chart of the algorithm is presented in Fig. 2A.

**Image-based flow detection**

Flow detection is performed using the MATLAB toolbox MatPIV v.1.6.1.\(^3^4\) It is here integrated into a processing pipeline operating on two consecutive image frames \(F_i\) and \(F_{i+1}\) (see Fig. 2B).

**Contrast enhancement.** In time-lapse fluorescent microscopy, higher excitation of the fluorophore leads to an amplification of the signal and an increased contrast. However, high fluorescent excitation may jeopardize the integrity and homeostasis of the cell layer, ultimately leading to cell photobleaching and photodamage. Therefore, low excitation of the cell layer is desirable which in turn implies low contrast. To improve the PIV analysis on low contrast images, a local normalization filter\(^3^5\) is invoked on the target frames

\[
N(x, y) = \frac{I(x, y) - \mu^k(x, y)}{\sigma^k(x, y)},
\]

where \(I\) is the original grayscale image and \(\mu^k\) and \(\sigma^k\) are estimates for the mean and standard deviation of \(I\) inside a defined neighbourhood. Here, we calculate \(\mu^k\) and \(\sigma^k\) over the points inside a disk centered at \((x, y)\) with radius \(k\). If not stated otherwise, the filter is applied with a window radius of \(k = 35\) pixels.

In time-lapse Differential Interference Contrast (DIC) microscopy, imperfections of the substrate greatly influence the result of the image-based flow detection (Fig. S1E, ESI†). To remove such structures and enhance contrast of the cell layer, curvelet transform for image denoising\(^3^6,3^7\) can be invoked instead of local normalization filtering (Fig. S1A–D, ESI†). As the structures reside at the same scale as intracellular features picked up by DIC microscopy, cellular details are filtered along with such imperfections. The recovered
velocity profile therefore features a reduced magnitude (Fig. S1E, ESI†).

Flow analysis. Parameters of the flow analysis can be set as specified in the MatPIV v.1.6.1 user manual.34 In the presented work, we invoke the iterative multistep method implemented in MatPIV for a shrinking window size. If not stated otherwise, we employ a three step approach with \( w = [128 \times 128, 64 \times 64, 32 \times 32] \). We set a minimal window size of \( w_{\text{min}} = 32 \times 32 \) pixels (34.5 × 34.5 μm) with a window overlap of 75%. The parameters allow for velocity detection in the range of \([0.072, 2.297]\) μm min\(^{-1}\).38 In order to avoid the detection of intra-cellular mass transport, the minimal window size \( w_{\text{min}} \) should not be smaller than the estimated cell diameter. A parametric study confirms the robustness of our method with respect to perturbations around the chosen parameter values (see Fig. S2, ESI†).

Filtering. Following the flow analysis, a set of local and global filtering operations is applied.34 In order to remove outliers from the velocity field, we apply a global histogram equilibrator followed by a local filtering method and linear interpolation of non-defined velocity components in the vector field. Global filtering is performed with a threshold value of 4 standard deviations, local filtering is performed with a median filter that employs a threshold of 2.5 standard deviations and a kernel size of 3 pixels, as suggested in ref. 34. The final filtered and interpolated velocity field \( \dot{V} \) of frame \( F_i \) is denoted \( \dot{V}_i \).

Shift correction
The multi-tile image mode operated by the microscope automatically stitches individual image tiles together to produce one large image. The stitching occasionally produces a misalignment of consecutive image frames that result in a global shift in the detected velocity field. In order to correct for these artifacts, we propose an automated shift detection and correction algorithm, integrated inside the flow processing module (see Fig. 2B). Image shifts generate large mean values in the velocity field (see Fig. S3, ESI†). We use this feature and define a threshold value that indicates the level for the mean value of the velocity field (see Fig. S3, ESI†). In order to avoid the detection of intra-cellular mass transport, the minimal window size \( w_{\text{min}} \) should not be smaller than the estimated cell diameter. A parametric study confirms the robustness of our method with respect to perturbations around the chosen parameter values (see Fig. S2, ESI†).

Cell layer segmentation
We propose a semi-automated, difference-based segmentation algorithm that detects the progression of the migrating cell front in consecutive frames (see Fig. 2C). The method is designed to work for images of low contrast and high image to noise ratio as considered in this work. The automated segmentation algorithm relies on manual initiation of the mask in the first frame. CIV independently tracks up to two initially disjoint masks and detects mask reconnections. The processing pipeline is composed of three sub-steps: difference detection, mask segmentation and a correction step.

Difference detection. The cell layer mask \( M_i \) in frame \( F_i \) is detected based on the intensity difference in frame \( F_i \) and the reference frame \( R_i = F_{i-1} \). In case the frame \( F_{i-1} \) is explicitly excluded from the analysis, the next frame is considered as the reference frame: \( R_i = F_{i+1} \). The reference frame \( R_i \) is then de-shifted and equilibrated with respect to the histogram of \( F_i \) to remove variances in the illumination. We denote \( R^\text{eq}_i \) the result of this process. We rescale the difference in the equilibrated frames \( \tilde{D} = \max(F_i, R^\text{eq}_i) - R^\text{eq}_i \) according to

\[
D = \frac{\tilde{D} - \min (\tilde{D})}{\max (\tilde{D}) \tau_i - \min (\tilde{D})}
\]  

with \( \tau_i = 0.15, D \) in eqn (2) serves as the basis for the cell layer mask segmentation. Image differences can be optionally computed on filtered images rather than on the original, equilibrated ones. This will reduce the influence of noise and small structures (smaller than cell scale) such as debris carried by the media and cell residues from the scratching procedure.

Mask segmentation. CIV supports filters implemented by the MATLAB fspecial method. We experienced good performance employing a Laplacian of Gaussian (LoG) filter of size \( s = 20 \) pixels and standard deviation \( \sigma = 4 \) as implemented by MATLAB’s fspecial method:

\[
h_g(x, y) = e^{-\frac{x^2 + y^2}{2\sigma^2}},
\]  

\[
h_{\text{log}}(x, y) = \frac{(x^2 + y^2 - 2\sigma^2)h_g(x, y)}{2\pi\sigma^2\sum_x \sum_y h_g},
\]

where \( x \) and \( y \) are the distances to the origin in the horizontal and vertical axes in the range \([-\frac{s}{2}, \frac{s}{2}]\). The mask \( \tilde{M}_i \) is calculated by converting the difference image \( D \) into a binary mask based on the threshold level \( \tau_2 \) (normally set to \( \tau_2 = 0.15 \)). The threshold can be adapted for individual experiments by the user. For the dataset under investigation, threshold levels are in the interval \([0.1, 0.25]\). In Fig. S4 (ESI†), we report results of a parametric study on the recovered wound closing speed under variations of parameters \( s, \sigma \) and \( \tau_2 \). The study identifies \( \tau_2 \) as the most sensitive parameter where a variation of 30% results in an error of 5% with respect to the reference solution.

Morphological operations. Following the thresholding step, a set of morphological operations is applied to collapse the detected cell patches into a layer mask. Parameters to these operations will depend on the microscope resolution and the
fluorescent marker of the experiments. For all experiments considered in this work, these parameters remained constant. The morphological closing $M_c$ of the mask $M_i$ (a dilation followed by an erosion) is calculated with a disk of adjustable radius as the structural element. This operation will connect single nearby components to bigger structures. The default radius is set to 10 pixels. In the next step, components of an area smaller than 30 pixels are removed, defining $M_i$. Detected cell patches are connected by a second morphological closing operation with a disk of radius 40 pixels as the structural element, followed by an image dilation step with a disk of radius 3 pixels to correct for the vanishing cell boundary: $M_d$.

**Correction.** After mask segmentation, we perform a correction step to increase the robustness of the algorithm against over and under segmentation in the final cell layer mask $M_i$. Such segmentation errors result from frame shifts of small magnitudes, not captured by the shift detection algorithm, floating debris and uneven illumination across image frames, not removed by the image equilibration procedure. We define a maximal velocity $v_{\text{max}}$ that bounds the distance at which the cell layer is allowed to extend/retract with respect to previous frame $M_{i-1}$. For the results reported here, we set $v_{\text{max}} = 15$ pixels per frame ($\approx 1.08 \mu m$ min$^{-1}$). In the presence of a frame shift, a more conservative maximal extension/retraction speed is applied and $v_{\text{max}}$ is scaled by a factor of 0.5.

**Manual segmentation.** CIV offers the capability of manual mask augmentation in individual frames. The manual intervention can greatly improve the accuracy of the method in situations where patches of cells enter the image domain across previously cell-free image boundaries, and in cases where a series of consecutive frames is excluded from analysis due to poor image quality.

**Benchmarking.** A comparison of the automated cell layer segmentation to manually segmented images is shown in Fig. S5 (ESI†). The reported results show good agreement with the manual segmentation for different experimental conditions, not exceeding a maximal error of 4% for the estimated cell layer front speed.

The proposed algorithm has been further benchmarked against a selection of interface tracking software for wound healing.5,7,30 These software feature threshold based segmentation algorithms that rely on specific features of phase-contrast and DIC images, not present in the fluorescent images examined in this work.

Therefore, automated threshold estimation fails in all three software packages and a manually set constant threshold was employed where possible.5,30 Furthermore, we note that these tools do not integrate information of the time-lapse image stack, e.g. using previous frames to segment subsequent ones. For these reasons, we expect the above software packages to perform worse on the tested images than the presented method. Nevertheless, these software comprise the state of the art among available software tools for wound healing quantification. The performance of the different software as compared to manually segmented images is reported in Fig. S5 (ESI†). The software toolbox MultiCellSeg was not able to provide segmentation results. The reason for this failure lies in the fully automated segmentation setup that has been trained against DIC images. Currently, this software does not feature training of the algorithm against user specific datasets.

We further tested the proposed segmentation algorithm against a set of DIC images. The recovered wound area is slightly smaller compared to manual and automated segmentation of the corresponding fluorescent images. The relative error of the estimated cell layer front speed is below 5% with respect to manual segmentation (Fig. S1F, ESI†).

**Quantification**

We quantify wound healing by a set of metrics, including the wound front speed ($\mathcal{F}$), the mean cell layer speed in orientation ($\mathcal{A}$, $\mathcal{B}$), the angular velocity distribution ($\mathcal{A}$) and a metric for coordinated cell migration, the velocity angle correlation ($\mathcal{C}$). Statistical quantities are calculated separately for both sub-masks $M^r_i$ and $M^f_i$ (disjoint components in $M_i$). Cell patches detached from the initially connected cell layers are assigned to their layer of origin until they reattach. We detect the time frame of reconnection of the two layers and only consider frames before this time point $t_{\text{rc}}$ for the statistical analysis. The initial wound orientation of the mask $M^r_i$ and $M^f_i$ is denoted $\theta^r$ and $\theta^f$. From the initial scratch wound we deduce the coordinate alignment of the wound, assign the wound length $l$ to the corresponding image side length and define the wound width as the open area divided by the scratch length, $w_i = A_i/l$ (Fig. 2D). All reported directional quantities are re-oriented to align with $\theta^r$ or $\theta^f$, respectively. We report the wound front speed ($\mathcal{F}$), which is an estimate for the wound closing speed. It is approximated by half the slope of the linear regression fit to the wound width $w_i$ for $i = 0, \ldots, t_{\text{rc}} - 1$.

In the following, a set of metrics is defined to quantify the cell velocity field $\mathbf{V}_i$ inside the detected cell layer $M_i$. We define the angular velocity distribution as

$$\mathcal{A}(z_i) = \frac{1}{T} \sum_{j=1}^{T} \frac{B^i}{N} \sum_{j=1}^{V_i} v(\theta^{j,i}, z_i) \delta^{j,i},$$

where $z_i$ denotes the angular sector $z_i = [\beta_i(i + 1)\beta_i]$ with $\beta_i = \frac{2\pi}{B}$ and $B$ is the number of angular bins and $i \in [0, B - 1]$. $T$ denotes the total number of frames, $N$ is the number of velocity vectors inside the layer mask in frame $t$ and $\theta^{j,i}$ and $r^{j,i}$ are the polar coordinates of the vector $\mathbf{v}^{j,i}$, the $j$th vector of $\mathbf{V}_i$ inside $M^r_i (M^f_i)$. The velocity contribution of vector $\mathbf{v}^{j,i}$ to bin $z_i$ is defined as

$$v(\theta^{j,i}, z_i) = \begin{cases} 1 & \theta^{j,i} \in z_i, \\ 0 & \text{else.} \end{cases}$$

Furthermore, we observe the mean cell layer speed $\mathcal{A} = \frac{1}{T} \sum_{i=1}^{T} \frac{1}{T} \sum_{j=1}^{V_i} r^{j,i}$ and the mean migration orientation $\mathcal{B} = \frac{1}{T} \sum_{i=1}^{T} \frac{1}{T} \sum_{j=1}^{V_i} \cos(\theta^{j,i})$. We quantify coordinated cell migration by measuring the velocity angle correlation as the mean inverted difference in vector orientation with respect to their spatial distance $d^{20}$

$$\mathcal{C}(d,t) = 1/|\langle \theta^{j,i} - \theta^{\text{prev}}(j) \rangle|_t,$$
for $||r' - r'|| = d$. All average values are reported for a defined subregion of the mask, extending a distance of $r$ into the cell layer (see Fig. 3) and a number of maximal time steps $\min(T, \tau_c)$. For spatiotemporal analysis, averages are calculated for overlapping sub-regions of the mask and overlapping sub-intervals in time.

**Statistical analysis**

Reported mean values and standard errors are calculated over the mean values for individual experiments. Where standard error of mean is reported, the data have been tested for normality using the Lillifors test with a 5% significance level. Where standard error of mean is reported for spatially and temporally binned data sets, the normal distribution assumption holds true for the large majority of the data bins. Where normality of the data distribution is confirmed in every bin of all conditions, statistical significance is tested via Welsh’s $t$ test, otherwise the Wilcoxon rank-sum test is employed. Only datasets that provide cell layer velocity information for locations up to $300 \mu$m into the cell layer are considered for the spatiotemporal analysis (Fig. 5, Fig. S9, ESI).

**Results and discussion**

We report CIV analysis results on a set of wound healing experiments over different substrate topographies (Fig. 1). The results highlight the capability of CIV analysis to capture and quantify flow patterns in migrating cell layers.

**Topography retains migrative cell potential**

We analyze the wound healing dynamics over different topographical substrates as defined in Fig. 1 (e.g. flat surface,
gratings parallel to the wound and gratings perpendicular to the wound), and consider a sub-region of the mask extending 250 μm into the cell layer and frames up to \( T = 30 (t = 7.25 \text{ h}) \); Fig. 3). A detail of the velocity field \( \mathbf{V} \) inside the cell layer at time \( t = 7.25 \text{ h} \) is shown in Fig. 3B.

Mean values over a set of at least three independent experiments for the front speed \( \langle \mathbf{v} \rangle \), layer speed \( \langle \dot{v} \rangle \) and cell layer orientation \( \langle R \rangle \) are reported in Fig. 3C. We observe that wound closure in the absence of topography is significantly faster compared to wound healing in the presence of gratings. Furthermore, wound closure is significantly faster on gratings orthogonal to the wound as compared to gratings aligned with the wound. We observe a mean front speed of 0.34 μm min\(^{-1}\) on flat surfaces, which agrees well with values reported in the literature. The mean speed inside the cell layer follows the same pattern observed for the front speed. Complementing the wound healing experiments, we measure the mean speed inside a set of confluent cell layers on flat and grated substrates. We observe no significant differences in the layer migration speed for confluent cell layers on topography and on a flat surface. Furthermore, we observe no significant change in the cell layer speed for the flat and orthogonal wound conditions as compared to the confluent conditions.

The observation suggests that the presence of open space stimulates cell migration directionality rather than cell migration speed. Only for wound healing in the presence of parallel gratings we identify significant decrease in the mean migration speed as compared to the confluent condition.

We observe that the mean orientation \( \langle R \rangle \) inside the cell layers for the flat condition as compared to the orthogonal condition does not differ significantly. Both flat and orthogonal conditions show significantly stronger orientation as compared to the parallel case. Measuring \( \langle R \rangle \) inside confluent cell layers for both flat and grated substrates indicates that orientation inside the cell layer, as captured by the metric \( \langle R \rangle \), is solely observable in the presence of a wound. We note that in the metric of orientation, migration aligned with the gratings back into the cell layer will cancel out with velocity contributions aligned with the grating into the open area. Consequently, the difference in cell orientation inside confluent cell layers on grated and flat surfaces is not reflected in this metric.

In order to account for these effects of reverse migration, we report the mean angular velocity distribution \( \mathbf{A} \) in Fig. 3D. The angular velocity distribution in wound healing experiments is often deflected to one side of the initial wound orientation \( \theta \). We account for this by mirroring the distribution along the \( x \)-axis such that all deflections are pointing towards the top of the distribution plot, when aggregating the mean \( \mathbf{A} \) (see Fig. S6, ESI†). For confluent cell layers in the absence of topography, we observe no preferential migration direction in the \( \mathbf{A} \) distribution. Considering confluent cell layers on topography, the \( \mathbf{A} \) metric reflects strong alignment of cell migration with the orientation of the gratings. In the presence of a wound, we capture global migration into the wound for all experimental conditions. In the presence of parallel gratings, however, we observe a strong contribution of migration in the direction parallel to the wound. Comparing the flat with the orthogonal condition, we observe that cell migration on orthogonal gratings is more aligned with the grating structures. Decoupling the axial components of the \( \mathbf{A} \) plot, we observe that total migration (both perpendicular and into the open area) on the flat substrate is larger than on orthogonal gratings (Fig. S7, ESI†). Furthermore, we see that the total backward migration contribution into the cell layer for the orthogonal case is significantly greater than for the flat condition.

These data indicate that wound closure on flat surface is most effective due to migration activity spanning the angular range of \( \theta \pm \frac{\pi}{2} \). Gratings hamper wound healing, however, with differences depending on the substrate geometry. In the presence of orthogonal gratings, cell migration is channeled. This leads to the loss of migrative contributions into the wound that are not strictly aligned with \( \theta \). On orthogonal gratings, migration into the open wound area is observed. However, the migrating cells are deflected by the grating structure, leading to incomplete wound closure.

**Topographical guidance confines directional migration stimulation of the wound**

We performed a temporal refinement of the statistical analysis, calculating temporal averages over time bins of six consecutive

![Fig. 4](image-url) Temporal quantification of wound healing over topography. Mean values are taken over areas extending 250 μm into the cell layer and 6 consecutive frames (1.5 h). Temporal bins overlap by 3 frames (0.75 h). Error bars show standard error of mean (for \( n \geq 4 \)). Temporal evolution of mean cell layer speed (A) and mean cell layer orientation (B). Colors denote experimental conditions: turquoise: flat substrate (\( n = 18 \)), purple: parallel topography (\( n = 8 \)), pink: orthogonal topography (\( n = 18 \)). Solid lines represent wound healing experiments, dashed lines depict observations on confluent cell layers (\( n = 2 \)). (C–E) Temporal evolution of angular velocity distribution in the absence of gratings (A), on parallel (B) and on orthogonal topography (C). Color indicates temporal order.
frames (1.5 h) overlapping by three frames (0.75 h). Reported metrics include the mean cell layer speed ($\bar{v}$), mean orientation ($\phi$) and the average velocity distribution ($\phi$) inside a sub-region of the cell-layer mask extending 250 µm into the cell layer. We present statistics for frames up to $T = 30$ ($t = 7.25$ h; see Fig. 4).

The cell layer speed in the flat condition increases linearly throughout the time course of the experiment. In the presence of orthogonal gratings, the cell layer speed shows a slight increase in time in the beginning of the experiment, saturating at a level of 0.36 mm min$^{-1}$, after approximately 4.5 hours, 0.07 mm min$^{-1}$ (16%) slower than the final speed observed for the flat condition. For wound healing on parallel gratings, we observe an initial drop in the cell layer velocity, followed by a steadily increasing speed after 2.9 hours (Fig. 4A). The profile for the cell layer orientation in the flat condition increases initially, saturating at a value around 0.5 after 2.9 hours. For the orthogonal condition we observe an initial increase in orientation, followed by a steady decrease to the level observed at the beginning of the experiment. In the presence of parallel gratings, we observe an overall decrease in the cell layer orientation (Fig. 4B).

These results show that there exist temporal regimes at which the cells behave differently depending on the substrate topography. We attribute the increase in speed and orientation in the flat and orthogonal condition at the beginning of the experiment ($0 \leq t \leq 2.1$ h) to the stimulating effect of the open space. However, whereas this effect seems to prevail throughout the course of the experiment on flat substrates, this effect disappears over time in the presence of orthogonal gratings ($t > 2.1$ h). The $\phi$ for the flat case shows a steady increase in speed in all forward oriented directional bins together with a dilation of the ellipse widths and a decrease in the backward oriented angular components. Considering the axial velocity components (Fig. S8, ESI†) underpins these observations. For the parallel condition, reorientation in the elongation of the $\phi$ ellipses along the grating structures indicates an increase in influence of the grating structures over time. In the presence of orthogonal gratings, we observe a steady regression in the $\phi$ profile. In contrast to the flat condition,

![Fig. 5](https://example.com/fig5.png)

**Fig. 5** Spatiotemporal quantification of wound healing over topography. (A) Conceptual sketch of angular correlation estimation ($\phi$). Angular correlation at $x$ is calculated inside a band of width $w_{ac} = 0.323$ µm located at a distance $d_{ac} = 150$ µm. The band is truncated to a sector of width $\alpha_{ac} = \pi$ extending into the cell layer. The metric guarantees equal treatment of regions close to the wound and regions further inside the cell layer. (B) Conceptual sketch of spatial binning. Bins are defined as bands of width $w_{sb} = 40$ µm. Neighbouring bins overlap by a region of $\alpha_{sb} = 20$ µm. Temporal binning is performed in bins of six time steps (1.5 h) and overlap by three time steps (0.75 h). (C–F) Sample sizes vary for bins: flat substrate ($10 \leq n \leq 12$), parallel topography ($n = 4$) and orthogonal topography ($8 \leq n \leq 12$). (C) Spatiotemporal evolution of $\phi$. Color represents the distance to the wound. Black lines represent observations on confluent cell layers ($n = 2$). Dotted black lines represent the base-line for uncorrelated migration. Error bars show standard error of mean. (D) $p$-Value for the Wilcoxon rank-sum test comparing angular correlation distributions inside the wound layer with the angular correlation distribution at the wound front. (E) Collapsed view of spatiotemporal evolution depicted in (C). (F) Spatiotemporal evolution of the mean contribution of the cell layer velocity. (E, F) Colored dots depict mean values, surface represents a B-spline fit to the data points.
the backward directed angular components do not regress but rather show a slight tendency to increase (Fig. S8, ES1) while no dilation of the ellipses in the $\mathcal{A}$ profile is observed.

The reorientation of the $\mathcal{A}$ ellipses agrees well with the temporal evolution of the observed layer speed and orientation (Fig. 4A and B). Together, these data demonstrate that the presence of open space stimulates migration into the open area in all considered conditions. However, topographical features restrict (parallel gratings) or channel (orthogonal gratings) this effect over time leading to a slower observed wound closing speed as compared to wound healing on flat substrates.

Migration patterns in cell monolayers during wound healing

We conduct a spatially refined statistical analysis and consider spatial bins of width $w_{sb} = 40 \, \mu m$ with respect to the wound front, overlapping by a distance of $o_{sb} = 20 \, \mu m$ (Fig. 5B). Directed migration is quantified by the angular correlation ($\mathcal{C}$) at a distance $d_{sc} = 150 \, \mu m$ (Fig. 5A). In Fig. 5C we report the spatiotemporal evolution of the angular correlation for the three wound healing conditions, along with the base-line observation on confluent cell layers. In Fig. 5D, the $p$-value of the Wilcoxon rank-sum test for the angular correlation of spatial bins towards the back of the cell layer and the first spatial bin is reported. A surface plot of the data reported in Fig. 5B is depicted in Fig. 5E. A surface plot of the orthogonal contribution of the cell layer velocity is shown in Fig. 5F.

On a flat surface, an increase in coordinated migration from the cells in the back of the layer towards the wound front can be identified. Over the first 2.1 hours we observe an increase in coordinated cell migration for all spatial bins. After this initial phase, collective migration in the front of the cell layer reduces again and stabilizes around a value of 0.96. In the back layers, collective migration continues to increase, saturating at a level of 0.8. Throughout the experiment an increase in coordinated migration towards the wound front is maintained.

In the presence of orthogonal gratings, over the first 0.8 hours, we observe an increase in coordinated cell migration for all spatial bins comparable to the observations made for the flat condition. After the initial phase, collective coordination can be observed to reduce in the front layers while continuing to increase in the back until the angular correlation at all distances collapses to a value of roughly 0.8. For the parallel condition, we do not observe a significant difference in angular correlation at different locations inside the cell layer. We observe a mean correlation value around 0.7 across the entire cell layer.

Next, we consider the $p$-value of the Wilcoxon rank-sum test for the null hypothesis that the angular correlation in regions towards the back is drawn from the same distribution as the angular correlation in the first layer. Additionally, we report the difference in the angular correlation of the back layers as compared to the first layer (see Fig. S9, ES1). We observe a significant difference in the coordination at regions towards the back of the cell layer as compared to the front layer for both the flat and the orthogonal conditions. However, in the orthogonal condition, this difference is lost after $\sim 3$ hours, whereas in the flat condition, a significant difference in the distribution is maintained at a distance of roughly 200 $\mu m$.

In Fig. 5F, we report the spatiotemporal evolution of the mean orthogonal velocity component (with respect to the wound front orientation) inside the migrating cell layer. For the flat condition, the velocity profile increases steadily from regions in the back of the cell layer towards the wound. In the presence of orthogonal gratings, the profile indicates a traveling wave moving into the cell layer.

The reported findings suggest that for all conditions, the mechanical wounding triggers a directed migration response of the cell layer into the open space. In the presence of grating structures parallel to the wound, cell migration is counteracted and demoted by the topography. Eventually, migration is redirected to align with the gratings and wound closure is slowed down considerably. In the presence of topography directed orthogonal to the wound, we observe an initial response comparable to the flat condition. Cells at the wound front align with the gratings and migrate into the void area. In the absence of gratings, in continuation of the initial wound stimulus, we observe an increase in forward oriented migration speed towards the cells located at the front of the layer. As a consequence, cell contact will be lost, leading to a sparser cell population at the front of the wound, leaving space for the cells to migrate more freely. We can observe this effect clearly in the widening of the ellipses in $\mathcal{A}$ distribution over time (Fig. 4C). In contrast to the observation made for the flat condition, in the presence of orthogonal gratings, the initial stimuli of the wounding process are hampered. The oscillation in the forward velocity profile together with the decrease in directed migration indicates that after the initial phase, migrating cells at the front are slowed down while cells located further inside the cell layer gain in speed. Furthermore, cell migration is observed to stay aligned with the topographic structure (Fig. 6).

Conclusions

We introduce Cell Image Velocimetry (CIV) to drastically enhance the extraction and automated analysis of information for cell migration available from wound healing assays.
CIV provides a methodology and software for cell layer segmentation and flow detection inside migrating cell layers for in vitro monolayer wound healing assays. Explicit features of the CIV methods and software are:

- robustness against low signal to noise ratio
- robustness against variation in image contrast
- robustness against floating debris and residues form the scratching procedure
- autocorrection for image frame shifts
- active front tracking
- flow pattern detection inside the cell layer
- spatiotemporal statistical analysis of flow patterns inside cell layers
- a graphical user interface (GUI) that allows for manual intervention

The presented algorithms have been evaluated against manually segmented images and showed to produce results in good agreement (max. error: 4%), outperforming currently available segmentation tools on the considered image dataset.\(^5,7,39\) Next to the standardized front speed observed in traditional wound healing experiments,\(^5\) the CIV toolbox offers drastically enhanced capabilities of spatiotemporal quantification of cell migration inside connected cell layers. Unlike cell tracking methods, that either demand nuclear fluorescent markers\(^1,20\) or very standardized phase contrast imaging modalities not applicable on grated surfaces,\(^9,10,13\) the presented method is robust in images with low signal to noise ratio and can handle very low levels of fluorescence. Compared to existing flow based analysis methods for cell migration,\(^12,14\) this work combines the flow detection analysis with an algorithm for active front tracking. The toolbox allowed for a refined study of collective cell migration over different topographic conditions and led to the identification of distinct patterns inside the migrating cell layers which are not detectable with the standard cell layer segmentation methods.

The enhanced spatiotemporal information made available by CIV is expected to contribute significantly to the development of spatiotemporal models of cell migration,\(^40\) providing the necessary data for their validation and uncertainty quantification. We motivate that the modular design of the CIV software toolbox enables flow analysis on time-lapse image dataset beyond wound healing. Both the segmentation and flow analysis modules are fully parameterized to allow for adjustment to any in vivo and in vitro microscopy datasets that reveal flow patterns.

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References