

Accurate region-of-interest recovery improves the measurement of the cell migration rate in the in-vitro wound healing assay.

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KEYWORDS

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ABSTRACT: The wound healing assay is widely used for the quantitative analysis of highly-regulated cellular events. In this essay, a wound is voluntarily produced on a confluent cell monolayer and then, the rate of wound reduction (WR) is characterized by processing images of the same regions-of-interest (ROIs) recorded at different time intervals. In this method, sharp image ROI recovery is indispensable to compensate for displacements of the cell cultures due either to the exploration of multiple sites of the same culture or to transfers from the microscope stage to a cell incubator. ROI recovery is usually done manually and, despite a low magnification microscope objective is generally used (10x), repositioning imperfections constitute a major source of errors detrimental to the WR measurement accuracy. We address this ROI recovering issue by using pseudo-periodic-patterns fixed onto the cell culture dishes, allowing the easy localization of ROIs and the accurate quantification of positioning errors. The method is applied to a tumor-derived cell line and the wound reduction rates are measured by means of two different image processing software. Sharp ROI recovery based on the proposed method is found to improve significantly the accuracy of the WR measurement and the positioning under the microscope.

Introduction

Migration and proliferation are highly-regulated cellular events that play essential roles in many normal physiological processes such as angiogenesis, immune response, embryogenesis, repairing of intestinal mucosal damage, and others^{1,2}. Also, those events are present during pathological processes such as cancer

proliferation². Understanding the molecular basis of these cellular events is of great significance³⁻⁵.

In-vitro methods for studying cellular migration and proliferation include: the wound healing assay, methods based on chemotaxis such as the use of carriers with chemo-attractants, videomicroscopy, and more complex systems

such as transwell cell migration and invasion assays, among others³. The main drawbacks of these methods is the lack of standardization, and, in the case of wound healing assay, the lack of a tool that provides accurate position data suitable for precise image analysis and registration⁶.

For its simplicity and low cost, the wound healing assay is the most widely used method. This assay is simple and generally involves the growth of a confluent cell monolayer where a small area of the culture is interrupted by a mechanical injury. As a result, a group of cells is destroyed or displaced. The free-cell area or "wound" is inspected under an optical microscope. Images of the same region of interest (ROI) of the cell culture are acquired at different time intervals until the cells cover completely the wound area. This healing may take several hours or even days, depending on the extent of the "wound", cell type, and conditions of the cell culture^{4, 2, 7}. The images acquired at the same ROI are processed in order

to calculate the percentage of wound reduction or area (%WR or %WA) and evaluate the migration rate of the cells⁷. The wound healing assay requires tools for measuring the %WR in an objective way. This implies: i) recovering in a precise way the same ROI across all observations under the microscope for further precise image registration^{8, 9}; ii) suitable software routines to compare images and quantify changes in the size of cell-covered areas.

For ROI recovering, different methodologies and tools have been developed over the years to contribute to enhance this assay. Some of those methods are: i) alpha-numeric grids that are adapted to divide the Pétéri dish surface into labeled small-sized regions and, ii) chambers with treated plastic inserts in order to generate small observation areas¹⁰. Another method, which is widely used, is the one described by Chun-Chi Liang, *et al.*². In this method, marks on the cell culture dishes are created in a manual way in order to recover the same ROI at each visualization. All the mentioned methods

for ROI recovering only provide a coarse positioning and are user-dependent in some aspects like experience and skills. Therefore, those methods do not allow high-accurate image registration, as well as cell migration and proliferation evaluation (which is measured by the %WR).

This paper addresses the problem of accurate ROI recovering for accurate and objective cell migration/proliferation evaluation, by means of an accurate ROI registration technique called Position-Referenced Microscopy (PRM). The technique was developed for high-magnification events but in this work it has been adapted for low magnification events such as the wound healing assay. As presented in section “*PRM for ROI recovering in wound healing assay*”, PRM is based on position reference patterns (PRP) that were integrated into a 12 well cell-culture plate. They allow the accurate retrieval of several ROI of a wound healing assay. The method is compared with the manual technique proposed by Chun-Chi Liang, *et al.*².

A recall of this technique is presented in section “*Manual method for ROI recovering in wound healing assay*”. The images acquired by both methodologies of ROI recovering were segmented twice: once with a commonly-used commercial software and once with the segmentation method developed by Cardona *et al.*¹¹ (Bio-EdIP). An explanation of the way as the software evaluate the performance of the wound healing is presented in section “*%WR measurement in wound healing assay*”. The comparison of the obtained results demonstrate significant improvements in both ROI recovering and %WR when using the proposed ROI recovering method. Those results are presented in section “*Results*”.

Materials and methods

Cell culture and wound healing

For the experiments presented in this article, a human hepatoma cell line SkHep1 was used for the in vitro wound healing assay. SkHep1 cells were grown in DMEM/F12 (1:1) medium

with 10% heat-inactivated fetal bovine serum and 1x penicillin-streptomycin solution, at 37 °C in a humidified atmosphere with 5% CO₂.

Before acquisitions, cells were harvested and quantified in a Neubauer chamber. Then, cells were seeded into 12-well plates at a final density of 7×10^4 viable cells/well. After 24 h, cell monolayers were scraped, with a 100µl or 1000µl pipette tip, and washed twice to remove cellular debris^{2, 9}.

Manual method for ROI recovering in wound healing assay

ROI selection

In order to get reference results from state of the art techniques, we implemented a manual method as proposed by Chun-Chi Liang, *et al.*². In this method, once the confluent cell-culture monolayer is obtained and scrapped, the ROI of the cell culture is selected under the microscope (see Figure 1). Then, a first image of the wound area is acquired and a mark is created

near the ROI. This mark is used as reference point for further ROI retrieval. The reference points are made by etching the culture dish with a razor blade on the outer bottom of the dish or with an ultrafine tip marker.

Once the ROIs are selected, the cell culture is returned back to the incubator until the next ROI follow-up under the microscope. For this article, 6 ROIs were acquired at each cultivated plate by using the above-mentioned manual method.

ROI recovering

Images of each cell culture ROI were acquired every 6 hours until the wounded area closed completely. This was done by using a microscope objective with 10X magnification and 0.25 numerical aperture. For ROI retrieval, the microscope diaphragm is closed in order to create a beam of light focused on the cell culture dish. Then, the dish is shifted until the user observes that the marked reference point is illuminated by the focused beam, which

means that a ROI has been recovered. At this time, the microscope diaphragm is opened and an image of the ROI cell culture is acquired.

In the experiments presented later, plates instrumented with PRP were used and images of those patterns were always acquired as necessitated for comparison with results obtained with the proposed PRM method (cf. section “ROI selection”).

PRM for ROI recovering in wound healing assay

PRM is a technique aimed to allow the easy and accurate recovery of ROI on specimens observed under a microscope¹². For that purpose, a PRP is put on the cell container and used as a position reference. Then the procedure requires the recording of two images: one of the cell culture and one of the PRP which is localized at a different depth. The position derived from the PRP image is also relevant for the position of the observed ROI since the shift in the

focus depth (z) does not change lateral (x, y) coordinates.

In practice, the PRPs are made of glass coverslips on which a thin layer of aluminum was micro-structured with pseudo-periodic patterns by photolithography. The pattern is made of dots of $2\mu m \times 2\mu m$ of size, with a period of $4\mu m$ and covers an area of $1cm \times 1cm$. Those PRP are attached to the bottom surface of the culture plates for use in the wound healing assay on hepatic cancer cell lines.

ROI selection

The procedure is performed at the same time than the manual method and is as follows: **as presented in the previous section**, the SKHep1 cell line is grown until the formation of a confluent cell monolayer. Then the monolayer is scraped and a ROI is selected **and an image is acquired** (corresponding to the same ROI chosen during the previously mentioned manual method) (**Figure 2(a)**). Then, as presented in **Figure 2(b)**, the microscope objective is focused on the PRP and an image of this pattern

is acquired and processed. Those PRP images are used as reference positions when returning back the culture dish to the microscope for a new observation. **As in the manual method, a total of 6 ROIs were registered at each cultivated plate.**

ROI recovering

As in the manual method, images of each cell culture ROI were acquired every 6 hours until the wounded area was completely closed.

For these visualizations, the microscope objective is focused on the PRP and an image of this pattern is acquired. The obtained image is processed and compared to the one obtained during the ROI selection step. This processing puts both images within a common coordinate reference system allowing their superimposition (**Figure 2(c)**). The position mismatch is then available and indicates the shift necessary to recover the expected ROI under the microscope. Then, the culture dish is shifted (manually or with a motorized stage) until reaching the exact ROI absolute position (**Figure 2(d)**). Once the ROI has been recovered, the focus of

the microscope is shifted to the cell culture and an image of the "wound" is acquired, **which can be then superimposed with the one obtained during the ROI selection step (Figure 2(e)) and which common area (yellow color) is the one that is used for the wound rate analysis.**

This procedure is repeated for all observation sessions. The images of the PRP were recorded with a magnification of $40\times$ while the cell culture ROI images were acquired with a $10\times$ objective. This was done because the spatial resolution of the $10\times$ objective lens does not allow obtaining sharp images of $2\mu\text{m}$ -sized features as on the PRP.

Processing of PRP images

General concept

The processing of PRP images, for absolute high accurate position estimation, involves two **phases:**

i). **Evaluation of the distance from the area of interest: in this first phase the pseudo-periodic**

pattern images are used to evaluate the distance from the area of interest. Results indicate the displacement necessary to retrieve the desired zone. The required displacement can be realized manually or with a motorized stage. In both cases, unavoidable imperfections could produce residual errors for accurate positioning at the ROI (in the case of a motorized stage, these residual errors could be caused by mechanical backlash). This first phase can be repeated if the residual positioning error remains significant but some error remains necessarily.

ii). Image registration for compensation of residual errors: the aim of the second phase is to deal with these residual errors by means of a software procedure of image registration. The residual distance between the positions of, respectively, the previous and the current images of the sample is evaluated from phase computations applied to the images of the pseudo-periodic pattern associated to the previous and current cell-culture positions. In this way, the

unavoidable residual positioning errors are estimated and compensated for by a software procedure that provides the final image registration. This software adjustment does not require any displacement of cell-culture since it is merely numerical. Demonstrated resolutions are lower than 10 nm^2 in lateral positioning and this level of performance makes residual errors negligible regarding the definition of recorded images (about $3.4 \mu\text{m}$ per pixel).

This phase of image registration provides the high accuracy to the PRM method and justifies that: i) the eventual accuracy in image registration does not depend on the manual positioning of the cell culture and ii) the PRM method can be used as a gold standard reference to estimate errors due to manual positioning, which are presented in further sections of the article.

Phase 1: evaluation of the distance from the area of interest

This phase is composed of two steps. The first one is based on Fourier transform and allows high-accurate relative position retrieval in both x and y directions as well as the in-plane orientation (β). The second step is based on the binary decoding of PRP images and provides the coarse but absolute (x, y) position. The results of those two steps are combined to obtain the final (x, y, β) absolute-high-accurate position of the area under observation on the microscope.

The processing of PRP images by Fourier transform gives as a result two phase planes corresponding to the vertical and horizontal directions. Those planes can be fitted by first degree equations due to the linear distribution of the phase with respect to the image pixel frame. A least square fitting for the two directions φ_V and φ_H that corresponds to the phase in the vertical and horizontal directions is then obtained as shown by equations Eq. (1) and Eq. (2) below ¹³:

$$\varphi_V(u, v) = A_V \cdot u + B_V \cdot v + C_V \quad (1)$$

$$\varphi_H(u, v) = A_H \cdot u + B_H \cdot v + C_H \quad (2)$$

With

$$A_V = \frac{2\pi}{pM} \cos(\beta) \quad A_H = \frac{2\pi}{pM} \sin(\beta)$$

$$B_V = \frac{2\pi}{pM} \sin(\beta) \quad B_H = \frac{2\pi}{pM} \cos(\beta)$$

$$C_V = 2k\pi + \Delta\varphi_V \quad C_H = 2k\pi + \Delta\varphi_H$$

where, p is the physical period of the PRP, M is the magnification, $\Delta\varphi_V$ and $\Delta\varphi_H$ are phase shifts in the vertical and horizontal directions respectively. C_H and C_V parameters are obtained with an ambiguity of a multiple of 2π because of the phase unwrapping procedure involved in the Fourier processing ¹⁴⁻¹⁶. (u, v) are image coordinates in pixels, and β is the angle of the pattern with respect to the horizontal axis of the pixel frame.

Once that the phase equations have been calculated, the values of the constants A_H , B_H , and

A_V, B_V that correspond to equations Eq. (1) and Eq. (2) were multiplied by the outcome of the relationship between $10 \times / 40 \times$ to compensate for differences in magnification between the PRP and cell-culture images as follows:

$$A_V = \frac{10}{40} \frac{2\pi}{p} \cos(\beta) \quad A_H = \frac{10}{40} \frac{2\pi}{p} \sin(\beta)$$

$$B_V = \frac{10}{40} \frac{2\pi}{p} \sin(\beta) \quad B_H = \frac{10}{40} \frac{2\pi}{p} \cos(\beta)$$

In the second step, we obtain the binary code encrypted in the missing points of the PRP by means of a local contrast evaluation where absent dots are distinguished from the present ones¹³. The binary values compensate the ambiguity of the constants C_H and C_V from equations Eq. (1) and Eq. (2). Then, in conjunction with the high-resolution position obtained from the Fourier processing, the high-accurate absolute coordinates are obtained. **These coordinates indicate the displacement necessary to retrieve the desired zone (in which residual errors**

could remain). They are also necessary for cell culture image registration.

Phase 2: Image registration for compensation of residual errors

Image registration is an important procedure in cell migration analysis since it allows placing all the acquired data in a common imaging frame (independent of the time of acquisition). In this way, time-lapsed images can be analyzed in an objective way. This is a factor not only useful for the wound healing assay but also for other microscopy procedures such histological analysis^{17,18}.

In this phase 2, we take advantage of the information given by the phase map equations (obtained during the phase 1), for superimposing two different images. From the phase equations (Eq. (1) and Eq. (2)), we use the compensated phase constants, which give the absolute phase values with respect to the total encoded area in both vertical and horizontal directions. These phase constants are transformed into

pixels by means of the slope of the corresponding phase equations. The obtained pixel values give the shift necessary to superimpose images within a same frame and thus to obtain the common area between them (as represented in **Figure 2(c)**). Image superimposition is obtained numerically and does not depend on residual errors tied to the actual positioning of the cultureplate on the microscope.

It is important to note that the procedure of image superimposition is performed once the implied images have been numerically rotated by the value obtained during the Fourier analysis(β).

More details on PRM processing algorithms and procedures can be found elsewhere ^{12,13} for the case where a single magnification is used.

%WR measurement in wound healing assay

The acquired images of the cellular events were segmented with WimScratch (a web-based software from Wimasis) ¹⁹ and Bio-EdIP

(a software developed by Cardona et al ¹¹). This is done in order to compare the effect of ROI recovering methods together with segmentation techniques on %WR calculations.

The WimScratch program is a specialized image processing tool used to quantify cell migration features. The recognition of a gap in an area is based on edge detection techniques ¹⁹. In the case of the program developed by Cardona et al ¹¹ (Bio-EdIP), the program is based on the intensity level of pixels in the image under evaluation in order to distinguish areas covered by cells from uncovered areas. Initially, the images are converted to grayscale and segmented using a region growing algorithm.

For a particular image, those programs provide the value of both the cell-covered area and scratched area or wound reduction (WR). The results are presented in terms of percentages of the total area, as presented in equation Eq. (3) for the case of Bio-Edip^{9,11}.

$$WR[\%] = \frac{WS}{FIS} 100 \quad (3)$$

where WS and FIS are the Wound region Size and Full Image Size respectively, both in pixels.

Manual ROI recovery performances

The parameters recall, specificity, and accuracy were calculated in order to evaluate the position recovery performance of the manual method. Those parameters were calculated by comparing the cell culture images acquired by the manual method with the ones obtained by the PRM (the method proposed in this work). Assuming the latter images as the gold standard, the changes in the parameter recall, specificity, and accuracy are related with the regions of the images that are different due to their position recovery. A definition of the previously mentioned parameters is presented as follow:

- Recall: measures the proportion of positives that are correctly identified as such. This parameter represents the segmented areas that are coincident in both images (manual and PRM). This parameter is

measured from the image background (areas without cells that are coincident).

- Specificity: measures the proportion of negatives that are correctly identified as such. This parameter measures the areas with cells that are coincident between both types of images.
- Accuracy: is defined as percent of correctly tagged pixels (true positive and true negative pixels) out of the total number of pixels.

Results

As presents in the section “Materials and methods”, Human liver tumor-derived cell line SKHep1 was used to acquire images of wound healing assay in order to compare the performance of both ROI recovery methods. 2 wells from the 12-well plate were cultivated with this kind of cells. Each well corresponds to a cell culture, which were scraped with 100 μ l and 1000 μ l tips respectively. Then, a total of 6 ROIs were selected at each SKHep1 cell cultures.

For this wound healing assay, the manual and the PRM methods for ROI recovering were compared by means of its positioning recovery performance and %WR. The aim of these experiments is to quantify the improvement allowed by the RPM method in comparison with the usual manual ROI recovering method. Results are presented in the following sections.

Positioning error in manual and PRM ROI recovering methods

The ROI recovering capacities of the manual method were analyzed through the results given by the processing of the PRP images obtained during the manual ROI recovery. Since the PRP processing provides both the x and y coordinate values of the observed region under the microscope, the values obtained for each recovered region were compared with the corresponding reference values (obtained at the ROI selections). The PRM technique can be used here as the reference tool to evaluate the error of the manual ROI recovering since its

performances were demonstrated to be sufficient elsewhere^{12,13}.

Figure 3(a) shows the error in both the x and y coordinates for a set of 24 manual ROIs recovering (corresponding to the data presented in tables 1 to 2). It is possible to observe in the figure that the ROI recovery capacity of the manual method shows random errors throughout the duration of the assay. This is due to the dependence of the method on the observer's experience. According to the figure **Figure 3(a)**, the average error in manual ROI recovering is of $28.7\mu\text{m}$ and $24.7\mu\text{m}$ for both the x and y axes, with a standard deviation of $200.0\mu\text{m}$, and $113.4\mu\text{m}$ respectively. The minimum and maximum values are $1.6\mu\text{m} - 884.7\mu\text{m}$ and $0.4\mu\text{m} - 426.7\mu\text{m}$ for the x and y axes respectively.

On the contrary, the use of the RPM method should result in perfect ROI retrieval. However, since manual (without the use of a motorized stage) position adjustments were performed,

the positioning cannot be exactly as those suggested by the digital processing of PRP images and therefore some errors remain. **Figure 3(b)** presents the errors observed for a set of 24 ROIs recovering (corresponding to the data presented in tables 1 to 2). The results present an average of $0.9 \mu m$ and $1.2 \mu m$, and a standard deviation of $1.5 \mu m$ and $1.6 \mu m$ for the x and y coordinates respectively. The minimum and maximum values are $0.01 \mu m - 7.7 \mu m$ and $0.13 \mu m - 5.9 \mu m$ for the x and y axes respectively. (Those residual errors are then compensated numerically and do not impact final results).

Compared to the manual method for ROI recovering, PRM method is more accurate and therefore it is more appropriate for ROI position recovering during wound healing assays.

Statistical analysis in positioning error

In order to support differences between manual and semi-automatic (PRM) ROI recovery methods, a statistical analysis of $x - y$ errors in ROI recovering by both methodologies was

conducted in R 3.2.5 software (<https://www.R-project.org/>). Position errors were analyzed from normal distribution using the Shapiro-Wilk test and quantile-quantile plots. Then, differences between error means were evaluated using a paired two-sided Mann-Whitney U-test. A $p - value < 0.01$ was considered statistically significant. With this tool, some comparisons were carried out:

- Error in x PRM vs. Error in x manual: $p - value = 7.629e - 06$. Since this value is less than 0.01 , it means that the means of those data present statistical significant difference.
- Error in y PRM vs. Error in y manual: $p - value = 1.526e - 05$. There is a statistical significant difference between both ROI recovery methods.
- Error in x, y PRM vs. Error in x, y manual: $p - value = 5.821e - 11$. There is a statistical significant difference between both ROI recovery methods.

Also, the methods were analyzed within themselves. Such analysis allowed us to determine if the particular method has or not the same performance in both axes, i.e. x and y . The results of those analyses are:

- Error in x PRM vs. Error in y PRM: p – value = 0.2837. This data does not represent a statistical significance difference. This means that the semi-automatic method has the same performance in both axes.
- Error in x manual vs. Error in y manual: p – value = 0.3927. This data does not represent a statistical significance difference. This means that the manual method has the same performance in both axes.

Given the accurate results obtained by PRM in positioning error, this method can be considered as the reference when analyzing the performance of the manual ROI recovery method.

Calculation of %WR

Tables 1 to 2 show the %WR values calculated by WinScratch and Bio-EdIP software, for the SKHep1 cell line assay scratched by a tip of a 1000 μ l and a 100 μ l pipettes. Each table presents the %WR for different ROIs recovered by both the manual (M) and the PRM method (A). Taking as a reference the results obtained with PRM, the average error in %WR obtained by the manual method in the 1000 μ l assay is of 0.4 and 0.63 for the segmentation obtained by WinScratch and Bio-Edip respectively. In the case of the 100 μ l assay, the average errors are higher: 1.5 and 0.8 in %WR obtained by the respective software.

Figure 4 (a to d). shows a comparison of the segmentation results obtained using winscratah and Bio-Edip software for images of a ROI of the cell line scratched with a tip of a 1000 μ l. Also, the values of percentage of wound area %WA and cell-covered area are presented in the same figure. The evaluated images corre-

respond to one retrieval of the ROI by the manual and the PRM method. In the segmented images, it is possible to observe a shift of the cell culture image (**Figure 4 (a-b)** and **Figure 4 (c-d)**). This is due to the method of ROI recovering, which is a fact that has implications over the evaluation of %WR. For images segmented using WinScratch, there is not a significant difference between images of the same ROI recovered by both methods (**Figure 4 (a-b)**); the average error in the calculation of %WR between the manual method and the PRM is barely 1%. On the other hand, the segmentation using Bio-EdIP shows a difference between the images that corresponds to the same ROI recovered by the two methods. In this case, the error in the calculation of %WR is approximately 9% (**Figure 4 (c-d)**). The same comparison applied to other ROIs lead to similar results so a single test is commented.

In the case of the data obtained for the cell lines scratched with the 100 μ l pipette, it is possible to observe that the difference of %WR evaluation can lead to misinterpretation when

evaluating the time necessary for a cell culture to heal. As presented in table 2 at the ROI 3, Wimscratch segmentation method evaluates that SkHep1 cells spent 24 hours to cover the wound area using the manual ROI recovering method. On the other hand, Bio-EDIP shows that the same cells spent more than 24 hours to cover the wound area, independent of the ROI recovery method. A graphical representation is presented in **Figure 4(e to h)**. Therefore, the combined use of the ROI recovery method together with a segmentation method for %WR measurement, lead to a significant reduction or increase in the evaluation of the time necessary to the cells to cover the wound area.

Performance of manual ROI registration

Table 3 presents the results of the parameters recall, specificity, and accuracy calculated for the measurement of the manual ROI registration performance.

The values from table Table 3 corresponding to the 1000 μ l pipette are higher than the ones

from the corresponding data of the $100\mu\text{l}$. This is due to the fact that a tip of a $1000\mu\text{l}$ pipette produces a bigger wound area than a tip of a $100\mu\text{l}$ one. In consequence, the process of wound healing lasts more time. In return, this implies that at the moment of first-time lapsed images segmentation (which in most correspond to wound areas), the results are unaffected by the positioning errors when retrieving the same ROI.

On the other hand, the results presented for the tip of a $100\mu\text{l}$ pipette are low, which indicates that the performance in image segmentation for identifying cell and non-cell covered areas are affected by the ROI recovering method. It is important to note that in the wound healing assay, a tip of a $100\mu\text{l}$ pipette is the most widely used tool for wound generation.

Discussion

In this paper, the authors report on an improved technique for ROI recovery for the *in-vitro* wound healing assay. The proposed

method is based on PRP and on image processing techniques involving Fourier Transform and codification of 2D in-plane positions.

Compared to the widely-used method for ROI recovery in the wound healing assay, the proposed method allows more accurate cell migration analysis, which is evaluated in terms of %WR.

By analyzing Human liver tumor-derived cell line, the results report that for ROI recovery, the proposed technique has an average positioning error of only $0.9\mu\text{m}$ and $1.2\mu\text{m}$ in x and y respectively, while the manual technique reports average errors of $28.7\mu\text{m}$ and $24.7\mu\text{m}$ in the same directions.

In terms of %WR, for an assay developed with the tip of a $100\mu\text{l}$ pipette, it is shown that a manual ROI recovery method can result in erroneous evaluations that can lead to misinterpretations when analyzing a cell migration/proliferation event. It is also presented that the combination of a ROI recovery method together with a segmentation one can produce results that show significant reduction

or increase in the obtained value of the time necessary for the cells to cover a wound area. This is a factor that can lead to an overestimation or underestimation when evaluating, for example, the effectiveness of drugs by means of the wound healing assay.

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Table 1. Calculation of the %WR for SkHep1 cells by using two segmentation methods. Cell culture scratched with a tip of a 1000µl pipette.

		[T(h)]	ROI 1		ROI 2		ROI 3		ROI 4		ROI 5		ROI 6	
			M	A	M	A	M	A	M	A	M	A	M	A
Method of Segmentation	WinScratch	0	71.2		81.3		100		96.4		81.8		67.2	
		6	65.8	66.1	72.2	75.3	100	100	87.1	90.6	87.5	89.1	60.1	60.6
		12	56.3	57.3	60.8	60.4	88.7	84.2	82.7	82	79.4	79.9	44.5	44.2
		24	30.3	30.2	27.7	29.1	77.2	76.8	46.7	47.5	58.7	54.5	11.5	11.6
	Bio-EdIP	0	82.4		84.9		98.2		95.9		94.5		70.2	
		6	74.1	77.6	79.5	74	100	100	87.5	91.9	90.3	89.6	62	62.1
		12	60.8	64.6	62.8	63.6	93.5	94.3	87.6	84.5	81.2	81.9	46.4	46.2
		24	31.8	31.3	33.6	34.3	79.6	80.5	47.5	45.1	55.7	56.1	13.8	14.4

T: time in hours. M: manual acquisition. A: acquisition using PRM.

Median error of 0.4 %WR and 0.63%WR for WinScratch and Bio-EdIP, respectively. STD = 1.4 and 1.6 for WinScratch and Bio-EdIP, respectively. The median error is calculated between images acquired using manual method and the PRM method.

Table 2. Calculation of the %WR for SkHep1 cells by using two segmentation methods. Cell culture scratched with a tip of a 100µl pipette.

		[T(h)]	ROI ₁		ROI ₂		ROI ₃		ROI ₄		ROI ₅		ROI ₆	
			M	A	M	A	M	A	M	A	M	A	M	A
Segmentation Method	WinScratch	0	60.4		60.9		65.3		64.7		62.3		50.2	
		24	24.4	37.7	11.3	19.3	19.2	0	22.8	24.4	44.6	37.8	8.5	12.8
	Bio-EdIP	0	81.6		77.9		81.3		84.5		87		77.6	
		24	48.9	55.2	37.4	45.8	56.7	18.7	59.8	56.8	68.9	72.8	40	48.8

T: time in hours. M: manual acquisition. A: acquisition using PRM.

Median error of 1.5 %WR and 0.8%WR for WinScratch and Bio-EdIP, respectively. STD = 10.7 and 6.3 for WinScratch and Bio-EdIP, respectively. The median error is calculated between im-ages acquired using manual method and the PRM method.

Table 3. Performance of manual ROI registration for the wounds generated with the tip of two types of pipettes

Tip	Mean	ROI₁	ROI₂	ROI₃	ROI₄	ROI₅	ROI₆
100 μ l pipette	Accuracy	0.69	0.63	0.47	0.59	0.61	0.57
	Specificity	0.72	0.74	0.44	0.49	0.36	0.67
	Recall	0.66	0.51	0.59	0.66	0.7	0.47
1000 μ l pipette	Accuracy	0.89	0.9	0.93	0.92	0.92	0.94
	Specificity	0.85	0.84	0.57	0.83	0.77	0.94
	Recall	0.88	0.92	0.96	0.93	0.94	0.87

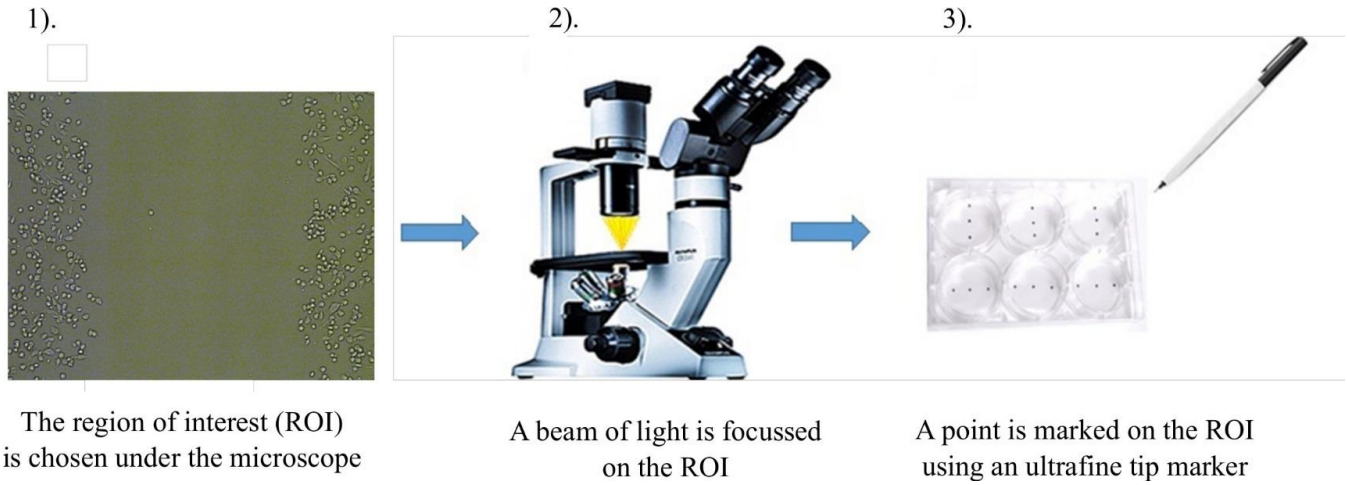
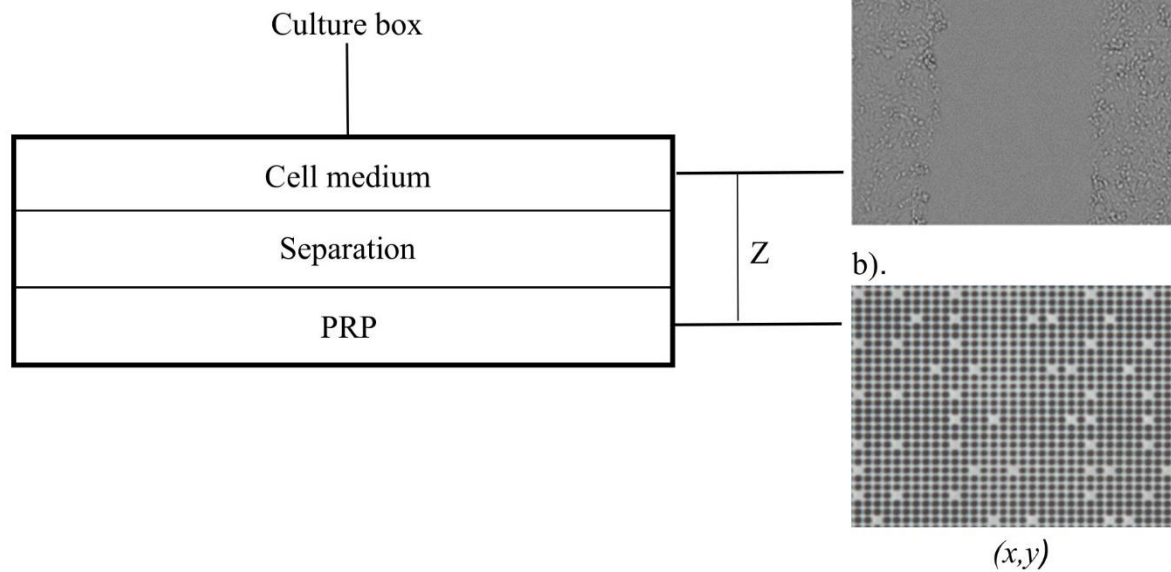


Figure 1. Representation of the manual method for ROI selection.

ROI selection in PRM



ROI retrieval in PRM

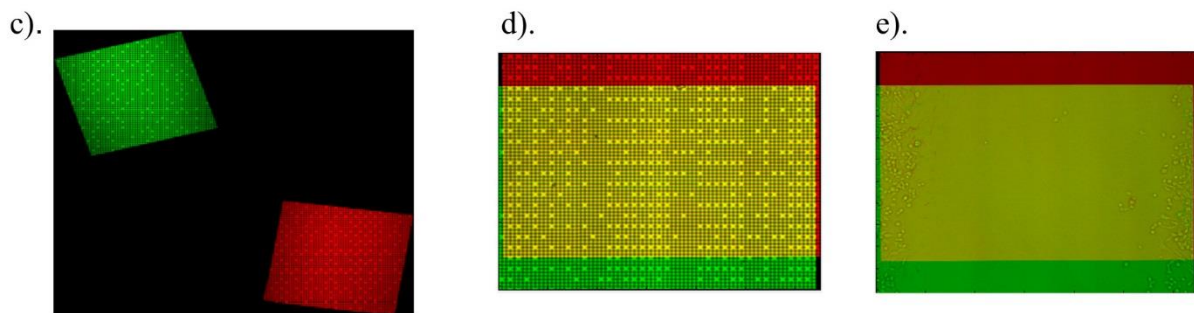


Figure 2. Principle of ROI selection in PRM: images of both cell-culture (a) and PRP are recorded with a monochromatic camera at different depth of focus of the microscope (b). Principle of ROI recovery in PRM: after a transfer from the incubator to the microscope, an image of the PRP is recorded and compared with the referenced one (image (a)) by placing them in a common coordinate system (image (c): red color represents the PRP image obtained during the ROI selection step, while the green image corresponds to the current PRP one). This comparison allows evaluating the necessary shift that should be performed in the cell-culture plate in order to achieve the desired ROI. Once this ROI has been achieved, both the PRP and the cell-culture images are superimposed in a common coordinate system (image (d) and (e) respectively). Once again, the red color represents the images obtained during the ROI selection step while the green color represents the current ones. The yellow color results from balanced mix of green and red and indicate the area common to both images.

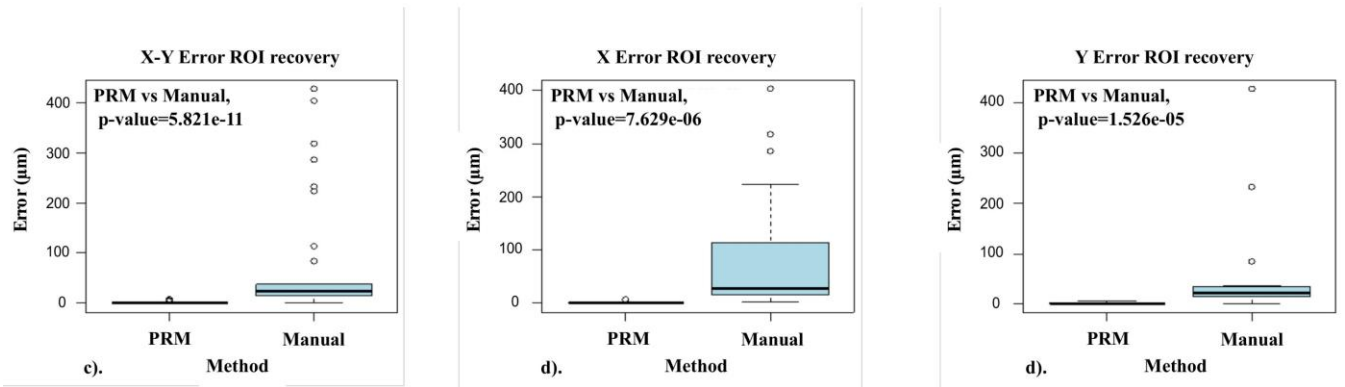
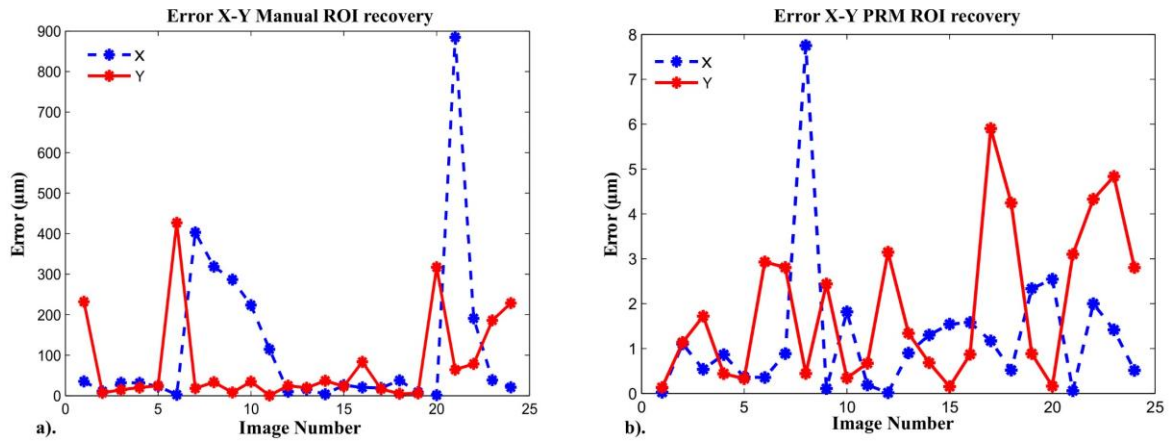


Figure 3. a). X-Y errors in ROI recovering by the manual method. b). Error in X-Y axis ROI recovering by the PRM method. Boxplots showing comparisons between error distributions in X-Y (c), X (d) and Y (e), resulting from using manual and PRM methods. Statistical results (p-values) are also shown. $p < 0.01$ was considered as statistically significant.

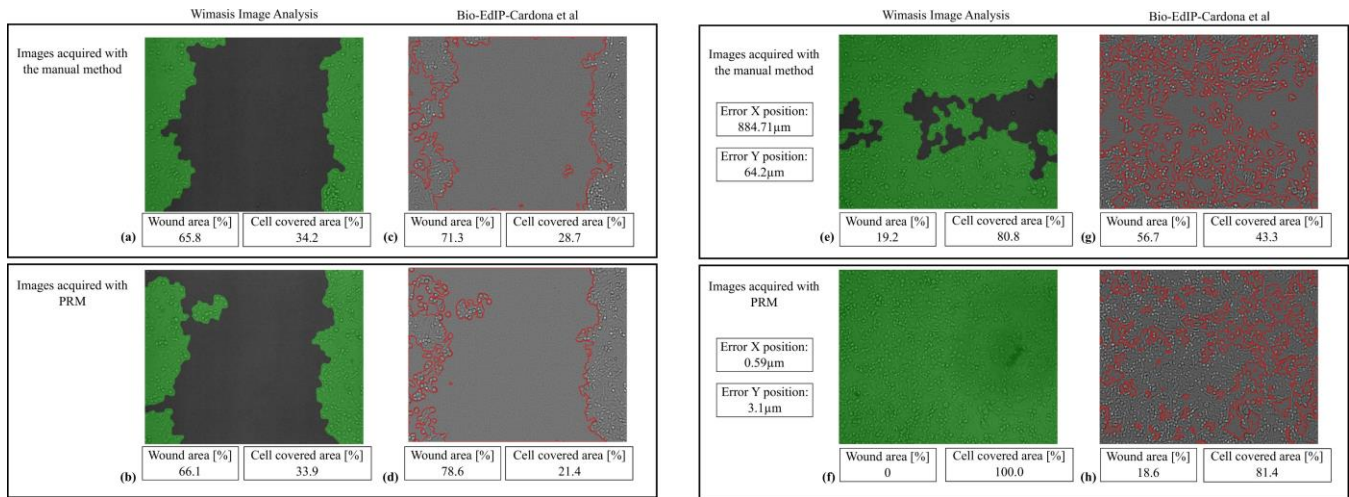


Figure 4. Calculation of %WA of a same ROI, by using Bio-EdIP and WinScratch. (a,e) ROI acquired with the manual method and segmented with WinScratch. (b,f) ROI acquired with the PRM method and segmented with WinScratch. (c,g) ROI acquired with the manual method and segmented with Bio-EdIP. (d,h) ROI acquired with the PRP method and segmented with Bio-EdIP.