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Image acquisition and planimetry systems to develop wounding techniques in 3D wound model

Abstract: Wound healing represents a complex biological repair process. Established 2D monolayers and wounding techniques investigate cell migration, but do not represent coordinated multi-cellular systems. We aim to use wound surface area measurements obtained from image acquisition and planimetry systems to establish our wounding technique and in vitro organotypic tissue. These systems will be used in our future wound healing treatment studies to assess the rate of wound closure in response to wound healing treatment with light therapy (photobiomodulation). The image acquisition and planimetry systems were developed, calibrated, and verified to measure wound surface area in vitro. The system consists of a recording system (Sony DSC HX60, 20.4 M Pixel, 1/2.3'' CMOS sensor) and calibrated with 1mm scale paper. Macro photography with an optical zoom magnification of 2:1 achieves sufficient resolution to evaluate the 3mm wound size and healing growth. The camera system was leveled with an aluminum construction to ensure constant distance and orientation of the images. The JPG-format images were processed with a planimetry system in MATLAB. Edge detection enables definition of the wounded area. Wound area can be calculated with surface integrals. To separate the wounded area from the background, the image was filtered in several steps. Agar models, injured through several test persons with different levels of experience, were used as pilot data to test the planimetry software. These image acquisition and planimetry systems support the development of our wound healing research. The reproducibility of our wounding technique can be assessed by the variability in initial wound surface area. Also, wound healing treatment effects can be assessed by the change in rate of wound closure. These techniques represent the foundations of our wound model, wounding technique, and analysis systems in our ongoing studies in wound healing and therapy.

1 Introduction

Conventional wound healing assays are performed in 2D cell monolayers. Several techniques for wounding 2D models are sufficiently established, for example the in vitro scratch assay [1]. However, the important three-dimensional cell and matrix structures are missing in these models. The development of 3D skin models is therefore important progress for wound healing studies [2]. The mechanical wounding of novel 3D models is still an insufficiently outlined topic.

Reproducible techniques like laser-wounding [3] or the usage of automated drilling systems [4] are already established for 3D models. However, all these methods need expensive and bulky constructions and result in low applicability for daily laboratory work. The advantage of these methods is the inclusion of important interactions between the cells in the model.

In this article, we summarize the results of a reproducibility pilot study to compare the variance of wound size of two wounding techniques analyzed by a developed image acquisition and planimetry system. We further highlight the outcome of different pilot data from agar models to establish the wounding technique in a 3D in vitro cell model.

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2 Methods and materials

An image acquisition system and planimetry system was developed to capture images, calculate wound surface area, and analyze data from three pilot studies.

2.1 Image acquisition system

The image acquisition system consists of a camera system and fixation frame, which is used to make reproducible images of the objects of interest. The digital camera Sony DSC-HX60 with 20.4 M pixel and an EXMOR R® CMOS sensor type 1/2,3″ with a focal length between 4.3 mm and 129 mm was used. The camera was fixed parallel and level to the tissue within an aluminum profile (1.5 mm).

Table 1: Camera properties and settings

Settings and Properties	Value
Self-Timer	10s
ISO	200
Exposure Time	1/3 s
Ratio	4:3
Bezel	f/8.0
Resolution	20.4 MP (5184 x 3888)
Focal length	4.3 mm

To enable a high depth of focus, a nearly closed bezel was used. Therefore, an external light source was necessary. To focus on an object of 100 mm, the distance between the lens and model need to be calculated. Using eq. 1 with an object size o [mm], focal length f [mm] and image size b [mm], the x1 = 73.99 mm was calculated. Thus, the camera system captures JPG images of wounded models for wound surface area calculation using a planimetry system.

$$x = f * \left(\frac{o}{b} + 1\right) \tag{1}$$

2.2 Planimetry system

Wound surface area of the captured images is calculated in MATLAB (R2016a). First, the wound edges are reliably detected with a tolerance method. Afterwards, the detected area is calculated in pixels.

The thresholding method detects an object in an image and separates it from the background (segmentation). However, thresholding performance decreases for images with low contrast [5]. Therefore, we implemented a tolerance method because we receive low-contrast images. A tolerance method would solve this problem of low performance in low-contrast images due to the fact, that intensity values included in the mask can be set individually from image to image. The darkest pixel of the grey image is set as reference. Afterwards, the editor specifies a tolerance value and the mean filter size. For our application, grey pixel tolerance with mean filtering was found to be a reliable method compared to thresholding, while superimposed images show a better fit of the original image and the calculated surface area for the tolerance method.

To calculate the surface area, the implemented user-interface in MATLAB was used. In a first step, the wanted grey value tolerance need to be set as well as the values for mean filtering. After the edge definition, the MATLAB 'bwarea' function was used to estimate the white pixels of a binary picture 0.

2.3 Pilot studies

The pilot studies consist of three parts. The *Method Comparison Test* detects possible errors due to the operator who is analysing the surface area. In the *Variance Study*, agar models were wounded with a sharp spoon and pipette-tip, aiming to detect variances in wound size. In a third part, a *Training Effect* due to wounding repetitions is considered.

2.3.1 Method comparison test

With the help of this study, significant bias through editors can be detected. For a *Method Comparison Test*, scaled millimeter paper was used as reference. A working distance of 40 mm between lens and paper was defined. For the test, 15 pictures were taken under normal conditions; in each image, a marked reference square with 1 mm^2 was analyzed.

Two different operators were requested to calculate the surface area of the reference objects using the planimetry system. For analyzing the normal distributed data, MATLAB R2016a was used. A two-sample t-test for independent random samples ($p = 0.05$) was carried out to compare mean and variance values.

2.3.2 Variance study

The aim of the *Variance Study* was to evaluate the reproducibility of the wounding techniques using a Volkmann 3 mm sharp spoon and a single use pipette tip ($\emptyset$2 mm) for 3D cell models.

Single-use petri dishes (84 mm x 16 mm, Polystyrene) and disposable syringes (50 ml) were used. After labeling the petri dishes, the agar models were produced. For the agar models, 500 ml water and 15 g agar were mixed and boiled for two minutes. With the help of the syringe, each petri dish was filled with 15 ml agar. To ensure an even surface level, the dishes were placed on a planar ground for the hardening process.

After curing, ten test subjects were requested to wound the agar model ten times with a sharp spoon (3 mm) and ten times with the pipette tip ($\varnothing$2 mm). For analyzing the normal distributed wound site areas, MATLAB 2016a was used. The mean values of all pipette wounds and all spoon wounds were compared using ANOVA. To exclude pure random chance, pipette wounds of three persons were repeated two times and mean and variance were compared to the first results.

2.3.3 Training effect

Measuring the impact of repetitions to the wound size was determined as *Training Effect*. To figure out if a *Training Effect* will take place, two of the test subjects were requested to repeat ten spoon wounds daily for eight days. The normal distributed data was analyzed by using the ANOVA to compare mean values.

3 Results

3.1.1 Method comparison test

We tested the differences in the calculated surface area of a 1 mm^2 size square with 15 different pictures and two editors (see **Figure 1**). By using the two-sample t-test (used significance level: p = 0.05), no significant difference between the mean values of the two test persons can be observed.

3.1.2 Variance study

As described in 2, the study was performed on ten test subjects. In **Figure 3A**, the blue bars represent the pipette wounds (median: 4532 pixels) whereas the yellow ones represent the spoon wounds (median: 12893 pixels). The size of the spoon wounds is with an average of 14378 pixels more than three times bigger than the pipettes with averaged 4532

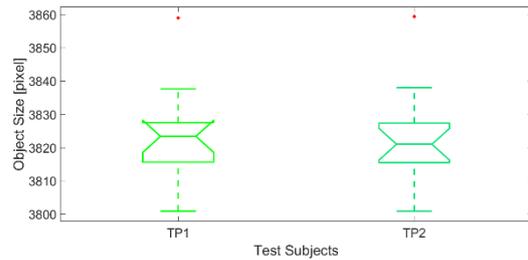pixels (highly significant differences between the two methods).

Figure 1: Boxplots of surface area of 1 mm^2 reference objects, analyzed by both test subjects, do not show significant differences in mean and variance using the two-sample t-test (p = 0.94).

The averaged variance of the pipette wounds of all individual test persons is 131230 pixels, for the spoon wounds 6928629 pixels (>50%). While considering the overall variance of all pipette wounds, the difference is insignificant, whereas the overall variance of the spoon wounds is with $5.5*10^{12}$ significantly bigger than the average of the several spoon wounds.

In **Figure 3B** the distribution of the pipette wounds surface area (range: 3674 pixels to 5587 pixels) is represented through a boxplot. With the one-way ANOVA, no significant difference (p = 0.2) of the mean values was detected. The boxplot of the spoon wounds is illustrated in **Figure 3C**. Highly significant differences in the mean values (p = $2.7*10^{-31}$) were observed.

The hypothesis of equal mean was confirmed while no significant differences were detected.

3.1.3 Training effect

To analyze a possible training effect, two test subjects repeated the spoon wound procedure (10 wounds, daily for eight days). Highly significant differences in the mean of wound size were observed during the Day 1 to Day 4, whereas lower variances and no significant changes can be detected from Day 5 to Day 8 (see **Figure 2**).
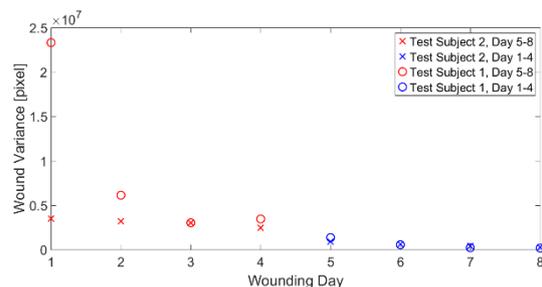

Figure 2: Falling variances of surface area (wound size) due to repetitioning spoon wounds for eight days. No significant differences after a training phase of four days (p_1 = 0.2; p_2 = 0.1).

4 Discussion

In this study, we developed an image acquisition and planimetry system to evaluate wounding techniques in a 3D wound model.

For the *Method Comparison Test* we cannot observe a significant bias due to the editor. Therefore, the editor will not influence the resulting wound surface areas.

In the *Variance Study*, wounding with the sharp spoon delivers more varied wound sizes, while wounding with the pipette results in more reliable wound surface areas even without the need for training experience.

Due to the variance in surface areas it can be mentioned, that the pipette wounds of different test subjects are comparable, whereas the spoon wounds deliver significant differences. To confirm the accuracy of the results of pipette wounds, repeated measurements ensure the reliability.

Concerning *Training Effect* in spoon wounds we can remark, that after a training phase of four days, a reproducibility without significant differences of wound size can be achieved for two test subjects.
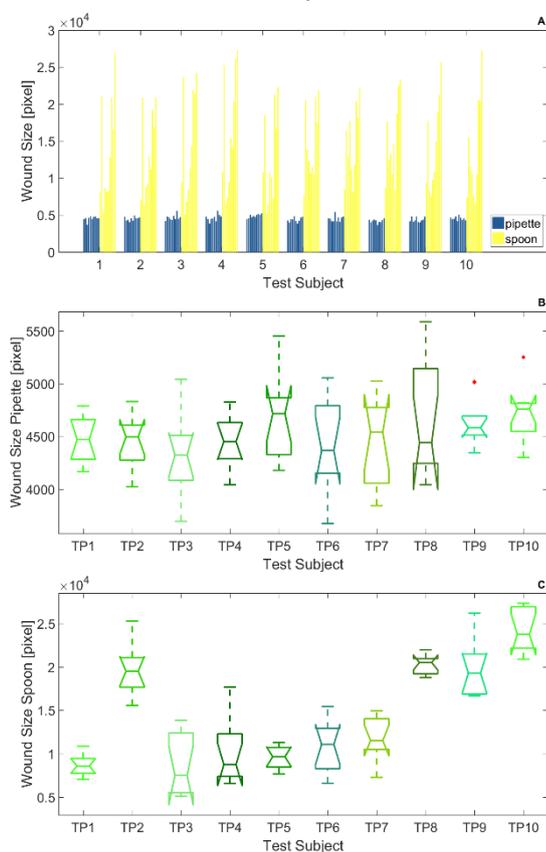

Figure 3: A: Wound surface areas (blue: pipette; yellow: spoon). **B:** Pipette wounds; no significant differences (p = 0.2). **C:** Spoon wounds; highly significant differences (p = $2.75*10^{-31}$)

5 Conclusion

In conclusion, the established system is suitable for investigations of in vitro wound healing processes. The wounds produced with the pipette tip are highly reproducible due to none significant differences in mean. Additionally, the set-up could easily be realized in every laboratory without complex constructions. We have demonstrated that different wounding techniques show different ad- and disadvantages. Although we restricted our experiments to agar models, we would assume that the results are also suitable for multilayer cell cultures.

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