Review

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In vitro wound healing assays – state of the art

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Abstract: Wound healing is essential for the restoration of the barrier function of the skin. During this process, cells at the wound edges proliferate and migrate, leading to re-epithelialization of the wound surface. Wound healing assays are used to study the molecular mechanisms of wound repair, as well as in the investigation of potential therapeutics and treatments for improved healing. Numerous models of wound healing have been developed in recent years. In this review, we focus on in vitro assays, as they allow a fast, cost-efficient and ethical alternative to animal models. This paper gives a general overview of 2-dimensional (2D) cell monolayer assays by providing a description of injury methods, as well as an evaluation of each assay's strengths and limitations. We include a section reviewing assays performed in 3-dimensional (3D) culture, which employ bioengineered skin models to capture complex wound healing mechanics like cell-matrix interactions and the interplay of different cell types in the healing process. Finally, we discuss in detail available software tools and algorithms for data analysis.

Keywords: 3D assay; image processing tools; in vitro; scratch assay; skin model.

Introduction

The skin constitutes the largest organ of the human body and maintenance of its barrier function is indispensable for any functional organism. The dynamic process of wound healing can be divided into five stages, namely hemostasis, inflammation, proliferation, migration and maturation/remodeling [1, 2]. During this process, complications can occur, such as infections, chronic wound formation and excessive wound healing, leading to hypertrophic scars and keloids [3]. Research on wound healing elucidates the basic underlying mechanisms of this wound repair process, but also paves the way for the improvement of already existing clinical treatments.

During healing, cells proliferate and migrate into the wounded area. The migration phase is the limiting event in wound healing. Therefore, assays studying wound healing are based on migration assays. In wound healing assays, a confluent cell layer is wounded under defined conditions and the migration of cells into the formerly cell-populated area is documented and analyzed [4]. The cell culture surface is frequently coated with extracellular matrix components to simulate cell-matrix interactions. By studying the wound healing process, possible medical treatments can be screened in vitro before starting in vivo experiments. Therefore, in vitro assays are an excellent tool to study the impact of specific compounds, as well as the influence of genetic alternations on the healing process.

Wound healing assays are usually performed in the conventional two-dimensional (2D) cell monolayer format. This format is the most widely used, since 2D wound healing assays can be conducted under standard cultivation conditions using equipment available in most cell culture laboratories. It has become increasingly recognized, however, that the complexity of the wound healing process is beyond what 2D cell culture is able to depict. Hence, the development of novel three-dimensional (3D) in vitro models to study healing processes marks the next step in wound healing research. 3D cell culture employs scaffolds seeded with cells to capture complex wound healing mechanics like cell-matrix and cell-cell interactions in the healing process.

This review paper covers the latest state of the art in the field of wound healing assays with a special focus on 2D and 3D in vitro models. Commonly used assays and

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modes of injury are examined in detail, and strengths and weakness of each assay procedure are presented. We clearly distinguish wound assays from migration assays, in that we define as requirement the wounding of a cell layer to be a prerequisite for this type of studies. Furthermore, an additional focus of the review is set on the importance of data analysis.

2D wound healing assays

The basic principle of each 2D wound healing assay is a deliberate destruction of a confluent cell monolayer, thus creating a cell-free region, which is then available for cells to bridge and repair. Therefore, most 2D wound healing assays are composed of three basic steps: cell injury (wounding), monitoring of the healing process and data acquisition (e.g. using a microscope or impedance measurement) and finally, data evaluation. Data acquisition with time-lapse microscopy is an especially attractive option for documenting a wound assay, since micrographs are obtained under defined conditions in the stable environment of an incubator. Microscopic procedures are standard laboratory protocols and will not be discussed further in this paper. For additional information on time-lapse microscopy imaging for wound healing assays, we refer to the technical review by Jonkman et al. [5]. Aspects involving algorithms and data analysis are covered in a later part of this review. This chapter describes different cell-wounding methods in more detail. Table 1 summarizes all cell-injury methods described, lists references and also briefly discusses the advantages and disadvantages of each given method. The most common 2D wound healing assay is the "scratch assay" which introduces a wound mechanically to a confluent cell layer by scratching. Destroying cells mechanically is also possible by "stamping" methods. Additional methods for cell injury include thermal, electrical and optical wounding (see Figure 1).

Mechanical wounding

One of the main reasons for the prevalence of the scratch assay is the fairly simple protocol. In this assay, a scratch is introduced into the confluent cell monolayer by using a sharp object such as a pipette tip [4, 21]. Other scratching devices like special cell scrapers [6, 7], metallic microindenters [9] and toothpicks [8] are also used. After this wounding step, the migration behavior of cells into the wounded area is recorded in micrograph data, which are analyzed afterwards. As the scratch assay is the most established wounding healing assay, new methods are often compared to it.

Many scratch methods described in the current literature are modifications of existing scratch assays, but employ automation for high throughput and reproducibility [11, 10, 12].

Major disadvantages of the scratch assay include irregular scratches when performing manual wounding and the possible destruction of the extracellular matrix coatings on the cell culture dish when introducing scratches [22]. Moreover, removed cells can accumulate on the edge of the artificially introduced gap, which might impede data analysis, as well as affect consequent proliferation and migration necessary for wound closure [23].

Another possible mechanical cell destruction method is stamping. By placing a stamp mold on a confluent cell monolayer and applying a force, either manually or in an automated process, cells covered by the mold are destroyed. The remaining cell debris is either removed, if the mold is shifted laterally during force application, or the debris remains in the wounded area. For most stamping assays, a mold made of rubber [14] or polymers like Poly(dimethyl)siloxane (PDMS) [15] is used. The mold can also be engraved with regular patterns such as squares [15], concentric circles [14] or parallel lines [14]. If cell debris is left behind on the stamp site, the influence of debris on cell migration can be studied [15]. A disadvantage of this assay, similar to other mechanical methods, is the irregularity of the stamped area, especially if the pressure on the mold is applied manually. However, in contrast to the scratch assay, an advantage of stamping is that the cell culture matrix coating is not affected by the wounding process.

Since thermo-mechanical damage is one of the most common injuries of the skin e.g. electrocautery or unprotected contact with hot objects, an in vitro model to study the effects of this type of damage on cells is very useful. For example, by using temperature-controlled stamps, the effects of thermo-mechanical and mechanical damage can be differentiated from one another [16]. Drawbacks of this method include possible heat transfer beyond the actual wounding area and therefore low reproducibility in defined wounding.

Electrical wound ing

Electric cell-substrate impedance sensing (ECIS™) is a method utilizing the association of impedance

Table 1: Common wound healing assays in 2D.

Method	Injury description	Reference	Pros	Cons
Scratch assay	Scratch confluent monolayer with pipette tip or similar object	– General protocol [4] – Usage of cell scraper [6] – Usage of Nunc™ cell scraper [Thermo Fisher Scientific, USA] [7] – Usage of toothpick [8] – Usage of micro-indenter [9] – Automated 96-well WoundMaker™ [Essen BioScience, USA] [10] – Biomek® FXP Laboratory Automated Workstation [Beckman Coulter, USA] [11] – Self designed 96-well wounding device [12] – Pipette tip on vacuum pump and further automation [13]	Easy to use; widely available culture conditions and equipment	Irregular scratches; Underlying matrix substrate coating might be destroyed; accumulation of removed cells on the edges of the scratch
Stamping	Apply pressure on cells in a defined area; Combination with thermal wounding possible	– Flat circular Neoprene spring rubber disks with ridges and grooves and pneumatic actuator [14] – PDMS mold [15]	Any shape possible; influence of cell debris on migration can be monitored; cell culture matrix coating remains intact	Irregularities in manual pressure applied to stamp
Thermal wounding	Apply excessive heat to a restricted area of a confluent cell layer	– Studies thermo-mechanical vs. mechanical damage [16]	Possibility to study thermo-mechanical damage	Heat might not be restricted to a certain area; heat transfers to cells nearby (no defined wounding area)
Electrical wounding [Electric cell-substrate Impedance sensing (ECIS™)]	Apply pulses of high voltage to a restricted area	– First use of impedance to determine attachment and spreading of cells [17] – Usage of ECIS™ models 100 or 1600R [Applied BioPhysics, USA] for wound healing assay [18] – Protocol paper with video [19]	Measurement and control of cell destruction and regrowth by impedance; real-time measurement; automatic wounding eliminating human errors; quantitative and highly reproducible	Detachment/destruction of confluent cell layer of keratinocytes and fibroblasts difficult; changes in adhesion and cell density alter impedance; low throughput; specialized equipment; heat development can affect cell viability
Optical wounding	Creates a wounded area with a laser	– High-throughput method using the LEAP™ system [Cytellect®, USA] [20]	High reproducibility; High throughput; sterile environment	Acquisition of LEAP™ instrument necessary

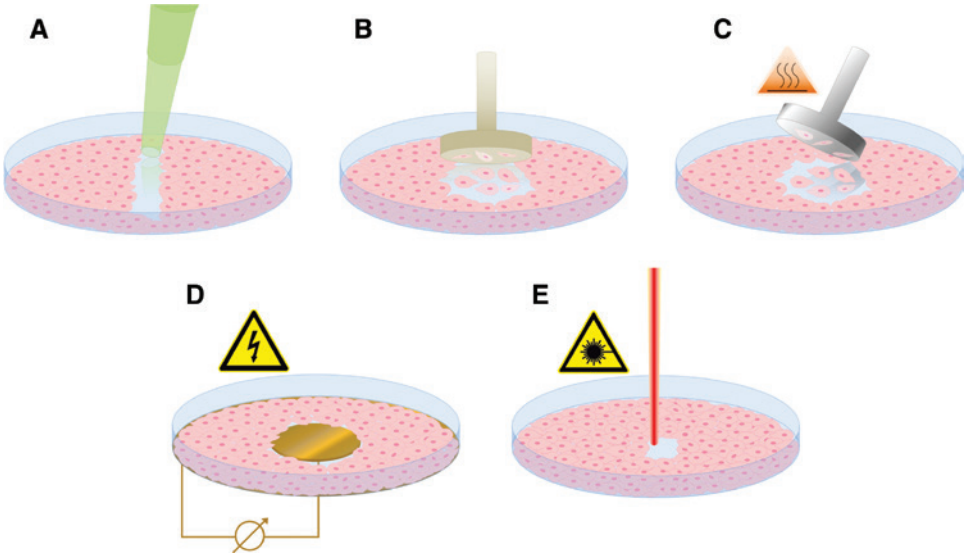

Figure 1: Cell injury methods used in wound healing assays.
(A) scratch assay, (B) stamp assay, (C) thermal wounding, (D) electrical wounding, (E) optical wounding using laser. In contrast to migration assays, where a barrier method will suffice, a form of cell injury is required for a true wound healing assay.

spectroscopy data and biological cell parameters, such as attachment and spreading [17]. To perform this assay, cells grown in a confluent cell monolayer cover an electrode at the bottom of a multi-well array. Wounding of the cells in the area of the electrode is achieved by applying an elevated current pulse which leads to electroporation and cell death [18, 19]. A constant alternating current is applied on the electrode, which allows the monitoring of initial cell growth, as well as the wounding and regrowth of the confluent cell layer. This measurement is possible due to the insulating properties of the cell membrane leading to an increase in impedance. Advantages of this method include real-time measurement options, as well as the elimination of human errors due to its automation. High comparability of results between different experiments is provided because destruction and regrowth are measured by using the physical parameter of impedance instead of optical results. Therefore, this method provides quantitative and highly reproducible results. Drawbacks include difficult detachment of confluent cell layers of strongly attached cells such as keratinocytes and fibroblasts. Additionally, changes in adhesion (cell-cell adhesion and cell-substrate adhesion) and density of the cell layer might alter the impedance measurement. Since a high current is applied during wounding, heat develops locally, which can affect the viability of cells attached to the electrode and nearby areas. In comparison to the traditional scratch assay, specialized equipment is required for performing experiments using ECIS™.

Optical wounding

Cell ablation via a laser beam can also be used to introduce a defined wound in a confluent cell monolayer. Injuries created by defined laser pulses offer high reproducibility, as well as high throughput. An instrument used by Zordan and colleagues for the purpose of wounding is the Laser Enabled Analysis and Processing instrument (LEAP™). Using laser pulses, cells were removed in a defined circular area. Data collection of bright-field microscopy images at certain time points and subsequent determination of the wound areas using customized algorithms (see also “Tools and algorithms for data analysis”) were carried out for final data analysis [20]. Optical wounding offers a high throughput process under sterile conditions leading to reproducible injuries. A downside when choosing this method is the necessary acquisition of the LEAP™ instrument.

3D wound healing assays

In recent years, there has been an increased awareness of the fact that cells cultured in 3D assume a completely different morphology, signaling, migration behavior and metabolic function compared to their 2D-cultured counterparts [24, 25]. Indeed, cells in a true in vivo situation migrate while surrounded by other cells and the ECM in all directions. This complexity is much better served by

a 3D cell culture experiment. Therefore, there have been efforts to transfer the in vitro wound healing assay to a 3D format.

The constructs used for 3D wound healing assays are typically various models of artificial skin. They are also referred to as skin substitutes, skin equivalents or bioengineered skin. Many products, developed as grafts for the healing of chronic and acute wounds (e.g. Hyalograft 3D, Apligraf and TissueTech Autograft System) or as models for dermatological testing (e.g. EpiDermFT™, Phenion® FT Model and StrataTest®), have been used for 3D wound healing assays [26]. Most constructs used in wound healing assays consist of a bilayer structure combining dermal and epidermal components. The first layer mimics the dermis and consists of a scaffold embedded with fibroblasts (here hydrogels like collagen are usually used, but decellularized dermis, synthetic polymers, glycosaminoglycans are also employed). Over the first structure, an epidermis-like layer of keratinocytes is cultivated, either from immortalized cell lines or primary cells. The epidermal keratinocytes mature on the air-liquid interface forming several layers, thus mimicking true skin structure. Such constructs only approximate true skin organization and lack immune and vascular systems, as well as sweat glands and hair follicles. Depending on the experimental hypothesis and the intended complexity, additional types of cells like melanocytes and tumor cells can be co-cultured in the 3D model.

Several groups have made attempts to standardize the 3D wound healing assay procedure [27, 28]. After the construct has matured, some form of wounding is carried out. Typical instruments for mechanical injury include scalpels, mashers and punch biopsies. Thermal wounding using electrical cauterization is also possible. The lesions can range from small-scale epidermal-only injuries to full-skin wounds. Frequent problems associated with both mechanical and thermal wounding are poor reproducibility of wounds or unintentional detachment of epidermal layers with the mechanical device. As a result, there have been attempts to perform wounding in an automated manner, using devices (e.g. a rotating drill) [28] or lasers already used in dermatology [27] which inflict damage to the layer in a reproducible manner.

The wound healing process in the 3D construct is normally followed by histological analysis and the data obtained is usually qualitative. Additional data can be obtained by using immunoassays and RT-PCR for the analysis of signaling molecules and gene expression profiles accompanying the healing process [29]. Wound coverage in skin equivalents can be quantified as "percent healing" by the NIH Image software, by dividing the migration distance of cells by the wound size [30]. Some groups perform advanced time-lapse microscopic techniques for 3D cell tracking like multiphoton and confocal microscopy. However, these non-invasive imaging techniques are limited to the tracking of a small group of cells and require sophisticated equipment and complex data analysis [31]. Transparent hydrogels are of special interest here. Topman et al. performed multi-tracking of fluorescently-stained cells within a transparent hydrogel of hyaluronic acid and adipic acid dihydrazine. The group adapted an image processing algorithm used for a 2D assay to 3D conditions [32] for the calculation of in-depth migration rate. The algorithm for the 2D assay developed by this group is explained in greater detail in the data analysis section of this review paper. The development of standardized and automated analysis for the calculation of migration rates in 3D wound healing assays is a major hurdle to be overcome for the establishment of high throughput screenings.

Tools and algorithms for data analysis

The key elements of each wounding assay include the preparation of cell material, the wounding process, subsequent data acquisition, as well as the analysis of the healing process. The achievement of high throughput, reproducibility and comparability between experiments is challenging for all the above-mentioned stages of a wound assay. However, this aspect is especially crucial for data acquisition and analysis.

One of the most common methods to calculate migration rates in wounding assays is the analysis of bright field microscopic images by determining the size of the wound area or the distance between wound edges over time compared to a control. In addition to these spatial parameters, the total cell count inside the wound area can also be used to define healing.

Data acquisition and analysis in most wound healing assays is done at least to some extent manually. The manual extraction of data from micrographs is always time-consuming, error-prone and the data output is very subjective depending on the individual researcher. Thus, analyzing a large set of data still remains the bottleneck of many assays. Also, the comparison of data from different tools and/or different experimenters is not very reliable. Apart from missing standards for the interpretation of wound healing assays, the additional problem of insufficient reporting also exists in the literature: some studies

show results from cell migration and wound healing assays without really giving details on the methods with which data analysis has been performed.

Alternatives to manual data analysis are possible due to automated image analysis software such as ImageJ (http://imagej.nih.gov/ij/) and the Image Processing Toolbox by MATLAB® [The MathWorks, USA] (http://www.mathworks.com/products/image/) utilizing edge detection and segmentation to detect the leading edge in a wound assay. In addition, extensions for ImageJ (White Wave Model) and MATLAB® (Texture Segmentation algorithm and Image Processing algorithm) have been developed. The White Wave Model visualizes the migration of cells during healing, whereas the Texture Segmentation and the Image Processing algorithm are used to segment an image into cell-populated areas and non-populated areas. Furthermore, two standalone software tools using MATLAB® code are presented in more detail below. These tools use statistical learning (MultiCellSeg) and a parameter-tuned algorithm (TScratch) to detect the wounding area in a micrograph. Table 2 presents an overview of automated data analysis tools available for quantifying wound assays. Each tool is listed along with a short description, as well as a concise evaluation of its strengths and limitations.

The ImageJ macro for the White Wave Model visualizes and quantifies cell migration by identifying differences in images taken at a certain time interval. By subtracting an image from another image taken one interval later, an absolute value of difference |d| for each pixel can be determined. This value |d| is then visualized in a new image. If $|d|>0$, cells captured in those pixels moved and the pixels appear white in the resulting new image, but if cells did not move and $|d|=0$, then the respective pixel remains black in the resulting image. It could be shown that this visualization of cell migration leads to a "white wave" spreading [33]. A unique feature of this tool is the visualization of cell migration over time. However, it cannot distinguish the direction of cell migration and also cannot distinguish if a change in one pixel is due to cell displacement or due to changes in the shape and size of cells. Furthermore, imaging conditions such as view perspective and orientation need to be stable throughout the whole experiment.

The Texture Segmentation algorithm, on the other hand, does not visualize the migration during wound healing but rather specifies the wound region by identifying variations in the vicinity of each pixel. Using a MATLAB® script, a texture filter implemented in the algorithm determines the pixel intensities variation in an 11×11 pixel window surrounding each pixel analyzed. Pixels with surrounding areas having large pixel intensity variations (areas populated with cells) will appear bright, whereas areas with low pixel intensity variation (smooth areas) will appear dark. By applying a pixel threshold, the entropy image is converted into a binary image, which is inverted and further processed by removing noisy areas and smoothing out the surface of the wound area to create a continuous wound area to be quantified [20]. As a MATLAB® script is used, this tool is fairly easy to implement. Another advantage is that there is no need for manual intervention such as parameter setting or pre-calibration in any way. Furthermore, this tool is standardized for optimal imaging conditions and results are reproducible. Nevertheless, this algorithm also comes to its limits when challenging imaging conditions occur.

Another algorithm with a similar concept to the Texture Segmentation algorithm is the Image Processing algorithm using MATLAB®. For this algorithm the standard deviation (SD) of pixel intensities is calculated for a moving window throughout the micrograph. The micrograph is converted into a SD map and a threshold is applied to this SD map to define populated and non-populated areas. This calculation using a moving window is performed twice, once using large and once using small window size. Using two windows is necessary, because a small window size would result in image noise and a big window size would result in rough boundaries of the wound area. The final wound area is determined by an intersection of both window sizes followed by a morphological operator [9]. Since the Image Processing algorithm and the Texture Segmentation algorithm share the same principle concept, advantages and drawbacks already mentioned also apply to the Image Processing Algorithm.

In contrast to the above-mentioned algorithms, MultiCellSeg and TScratch are standalone software tools. MultiCellSeg uses statistical learning of Support Vector Machines (SVMs) to classify local patches in a micrograph. The general idea of this tool is the statistical learning of the appearance of cell-populated areas vs. non-populated areas, followed by applying this trained knowledge on a large amount of data that is subsequently automatically annotated. The pre-trained SVMs classify the patches using basic image features. The training phase implements a separation of the whole image into patches, which are manually tagged either as cell area or background. Based on those tagged patches, a linear statistical model called *patch classifier* is calculated. This model can be used for analyzing data sets and gives a confidence score for each patch being cell-populated or background. After this step, an automatically-selected threshold is applied on the confidence scores to produce

Table 2: Modern data analysis tools for wound healing assays.

Tool/Software	Description	Reference	Pros	Cons
White Wave Model with ImageJ	Difference in each pixel of two consecutive micrographs is visualized in a binary picture	[33]	Shows cell migration over time in form of "White Wave"	Cannot distinguish direction of single cell migration; Cannot distinguish true cell displacement from change of shape or size of cell; Visualization of cells needs to be done very consistently (perspective and orientation)
Texture Segmentation algorithm	Algorithm uses a texture filter to determine pixel intensity variations around each pixel	[20]	Easy to implement (MATLAB® script); No manual intervention or pre-calibration necessary; Standardized and reproducible	Low robustness to challenging imaging conditions
Image Processing algorithm	Standard deviation (SD) of pixel intensities as a measure of texture; a high SD of pixel intensities in a defined window leads to assumption that this area is occupied by cells	[9]	Easy to implement (MATLAB® script); No manual intervention or pre-calibration necessary; Standardized and reproducible	Low robustness to challenging imaging conditions
MultiCellSeg	Software tool based on "training and testing" using Support Vector Machines (SVMs) to classify patches	[34]	Higher spatial resolution because of confidence score for each patch; No parameter tuning necessary	"Training and testing" is time consuming; Fitting for each new experiment necessary (e.g. different cells lines, different imaging conditions)
TScratch	Software tool with graphical and statistical output using discrete curvelet transfom	[35]	User interface; Very fast tool	Dependence on parameter settings; Low robustness to different cell types or challenging imaging conditions; Smaller wound regions difficult to detect

a discrete binary image. Furthermore, another pretrained *region classifier* is applied to identify patches which were falsely categorized as background. The *region classifier* groups patches and then analyzes the individual patch within its region. The algorithm then compares if this region has much more image-textural information than the individual patch. If this is the case, the patch also gets classified as cell area. A graph-cut based segmentation algorithm is finally applied, which results in the final division into cell regions and cell-free zones. MultiCellSeg is available through http://www.cs.tau.ac.il/~assafzar/ [34]. Since a training set is used to analyze micrographs with this tool, no parameter settings are necessary. The application of a confidence score on each patch also leads to a high spatial resolution. A major drawback of this tool is the mandatory training phase, which needs to be repeated each time cell lines and/or imaging conditions change.

The standalone software TScratch uses a curvelet-based image analysis algorithm for the analysis of wound healing assays. A fast discrete *curvelet transform* is utilized to distinguish between areas occupied by cells and those not occupied by cells. Therefore, the *curvelet transform* encodes a *curvelet coefficient* which contains all information about the original image. The resulting *curvelet coefficient* is processed further and the magnitudes of the different scales of the *curvelet coefficient* are added together. This generates a *curvelet magnitude image*, which gives information about the amount of detail in the original image and therefore about areas with and without cells. Finally, a threshold is applied to distinguish between cell-populated and non-populated areas. Thresholds can be adjusted manually to reduce sensitivity to local variations (e.g. small non-populated areas) and define contiguous areas further. TScratch can be downloaded on the website of the Swiss Federal Institute of Technology under http://cse-lab.ethz.ch/software/ [35]. Advantages of this tool include the speed of data analysis, as well as easy application for first-time users. Nevertheless, data output is dependent on parameter settings and results differ widely depending on cell types and image conditions. Furthermore, small wound regions are difficult to detect.

Conclusion and perspectives

The choice of a suitable wound healing assay and an appropriate data analysis method can be truly challenging when dipping your toe into the sea of wound healing assays for the first time. A good starting point when making a choice on the right assay method can be to clarify few questions beforehand. What experience on wound healing assays is available in your lab? How many experiments need to be conducted, hence what throughput is needed? Which laboratory equipment is available? Does it currently make sense to invest resources in new equipment to achieve a higher throughput and reproducibility? Apart from these general questions, it is also advisable to consider the actual healing process to be studied. For example, when investigating the healing of burn wounds, it is surely better to use an assay involving thermal destruction of cells rather than an ordinary scratch assay. A micrograph-analyzing tool needs to be able to cope with variability, owing to slight variations in imaging conditions and the different appearance of alternative cell lines. There are a number of analysis tools available which fulfill these requirements. For beginners, a software tool having a user interface like TScratch might be most suitable. On the other hand, if experiments with large datasets containing micrographs captured under reproducible conditions are conducted, MultiCellSeg might be more appropriate. Once the training phase for this tool is implemented, the whole dataset can be analyzed based on the statistical model without further parameter tuning or other manual interventions. If MATLAB® is available in your laboratory, codes for the Texture Segmentation and Image Processing Algorithm can be easily implemented.

Once some promising results are obtained with a conventional 2D wound healing assay, it might be worth considering further testing in a more complex 3D model. There is to date no universally-agreed upon, standardized model of 3D wound healing, but it is useful to establish a defined construct in your lab. High throughput data acquisition and analysis of artificial skin constructs remains a huge challenge, but reproducibility can be improved by automating some stage of the assay procedure, like wounding. Wound assays in 3D represent a cost-effective and ethical alternative to animal models of wound healing. Human cells in the skin equivalent are frequently better suited to represent human metabolism and skin anatomical structure than small animal models. Therefore, it is to be expected that 3D in vitro wound healing assays will considerably deepen the understanding of the wound healing process and will be an invaluable aid in the screening of bioactive substances which promote healing.

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