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Quantitative image analysis of the shape and size of circular wound sites generated by vertically stamped scratches

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ABSTRACT

A protocol for quantitative image analysis of wound generation is important to better understand the integrative process of wound healing and the closure mechanism. Here, we present a method for quantitative analysis of microscopic images of circular wound sites generated by vertically stamped scratches. To demonstrate proof-of-concept validation, we used two types of mechanical stamping tools, a mechanical pencil lead (type 1; brittle) and polydimethylsiloxane (PDMS) pillars (type 2; ductile), to create circular wound sites. We also present a method for analysis of microscopic images of the generated wound sites by suggesting new parameters, such as controlled area transfer ratio, modified shape factor, and roundness index, specifically to investigate the shape and size of wounds via house-coded image processing. We believe that this approach can be potentially useful by providing a better way of studying vertical wound generation for future skin wound generation and care applications compared with its counterpart, conventional horizontal wound generation.

1. Introduction

In the field of biomedical engineering and tissue engineering, regenerative and/or reparative function at tissue and organ levels has long been a major interest among scientists due to its significance in applications ranging from organ transplant to validation of drug effectiveness (Hacking et al., 2009; Shen et al., 2002; Kladakis and Nerem, 2004; Nerem, 2006; Lewis et al., 2015). To elucidate the mechanism of full wound closure, a thorough understanding of biomaterials, tools, methods, and characteristics of the wound is needed (Lee et al., 2010; Yun et al., 2013a; Zhong and Ji, 2013; Riahi et al., 2012; napavichayanun and Aramwit, 2017). In particular, among the various features of the wound, the shape of the wound affects the recovery result (Cardinal et al., 2009; Shetty et al., 2012; Chang et al., 2011). One of the classic methods for analyzing biological response involves two-dimensional observation, in which cells are cultured *in vitro* on artificial substrates such as Petri dishes and tissue culture flasks before observation under conventional microscopes. Under such conditions, understanding the rate of cell growth as well as migration behavior becomes critical for accurate analysis and evaluation of the experimental results.

With the advent of more precise techniques that incorporate computer-assisted and automated measurement, an increasing number of observation methods for understanding cellular behavior are currently being developed (Decaestecker et al., 2007; Poujade et al., 2007; Yun et al., 2013b; Kim, 2016). Although it is hard to determine which type of observation method is more suitable for revealing the cellular response to its full extent in each situation, two-dimensional observation methods are still important as they provide insights into intracellular interactions and the effects of contact inhibition on cell movement (Walker et al., 2004; Harley et al., 2014; Kim and Lee, 2017). A wound healing cell migration assay is conveniently used to study intracellular interactions, combined with cell migration and proliferation processes (Cai et al., 2007; Nnetu et al., 2012). The experimental scheme of the wound assay involves removal of part of the monolayer cell culture and observing how cells migrate into the empty section for wound closure. Among different types of assay, the scrape wound healing assay is favored for the following reasons: (1) cost-effectiveness, (2) minimal equipment requirements, (3) ease of performance, and (4) reproducibility (Liang et al., 2007; Ascionea et al., 2016). However, cell migration is an adaptive process that is subject to cell-type and environment-specific modifications (Decaestecker et al., 2007). The scrape wound assay can only provide observations in respect to the horizontal or vertical plane. Moreover, because the scrape assay is manually performed, clear boundaries of the wound edge are not always guaranteed and then in real-life the wound shape may vary according to the generation process (Daniel et al., 1996; Qi et al., 2016; Deborah Wendland and David Taylor, 2017). Thus, it is necessary to represent the variety of wounds while observing wound generation and healing processes. There are two main methods for measuring the wound healing rate. The

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first is to subtract the size of the recovered wound from the initial size immediately after generation, to divide the value by the wound size, and then to multiply it by a factor of 100. This method is not suitable for comparing two or more samples with different sizes of wounds, because it simply compares the areas each other (Deborah Wendland and David Taylor, 2017). The other is to measure the change in the distance from the edge of the wound to the center of the wound, and to consider both the area and perimeter of the wound. The recovery rate using this method is not affected by the shape and area of the wound. It may be appropriate to compare and analyze the recovery process of the injuries with different areas and wounds. However, this method does not measure how the shape of the wound changes during wound healing (Ascionea et al., 2016; van der Meer et al., 2010; Arno et al., 2014). So we developed a method of generating and evaluating circular wounds as a model that might represent more real-life situations compared with the regular scratch wound. Here, we present a method of analyzing the shape and size of round wounds created by applying vertical pressure using a circular cross-sectional tool. Moreover, we used easily accessible tools such as mechanical pencil leads and PDMS pillars to examine which type of tool would be appropriate for this purpose.

2. Materials and methods

2.1. Cell culture and sample preparations

Human HeLa cells (Korea Cell Line bank) were grown in culture media with the following composition (v/v): 89% Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (PS). All cells were grown in tissue culture polystyrene (TCP) flasks (25 cm^2, Falcon™) in an incubator at 37°C and 5% CO_2 before being used in the experiment. When the culture reached full confluence, the medium was removed from the flask and the cells were detached by trypsinization, mixed with 4 mL media, and centrifuged at 1300 rpm for 5 min. After centrifugation, the total number of cells was counted and cells were plated into tissue culture plates. The cell cultures were incubated at 37°C with 5% CO_2 for 17 h for 96-well plates (Falcon™) and 48 h for 24-well plates (Falcon™).

2.2. Wound generation

A schematic illustration of the overall experimental procedure is shown in Fig. 1A. Two types of easily accessible tool were used to generate wounds and the experimental results were observed for various applications of denudation methods (Fig. 1B & C). For the purpose of this experiment, the stamping surfaces of each tool were limited to a circular shape. Also, every set of experiments was conducted by vertical application of mechanical pressure to the confluent monolayer. The types of tools that were used in the experiments were (1) mechanical pencil leads and (2) polydimethylsiloxane (PDMS) pillars. For the mechanical pencil lead, we examined the effect of area and hardness of the lead on cell proliferation and movement. First, we examined the effect of area in increasing order of 0.3 mm, 0.5 mm, 0.7 mm, and 0.9 mm with a fixed hardness of HB. Second, we examined the effect of hardness of the lead with 2H, HB, and 2B for a fixed area of 0.5 mm diameter. For the PDMS pillar, we obtained a cylindrical segment from a block using a PDMS puncher and then denudated a confluent monolayer by securing the obtained segment inside the PDMS puncher. The area effect for PDMS was examined in the diameter range of 2.5 mm, 3.0 mm, and 4.0 mm, and 5.0 mm. For blood lancet devices, we denudated a confluent monolayer with 0.3 mm in diameter. Microscopic images of the wound generated using the above mentioned tools and methods are shown in Fig. 2. Experimental precautions for the procedures are described in Table S1.

2.2.1. Mechanical pencil leads

Mechanical pencil leads were sterilized with an alcohol lamp and rinsed with 70% ethanol. Fully confluent cell cultures in 96-well plates were perpendicularly compressed to create round wounds. The old medium was removed and the wells were rinsed with 100 μL PBS to remove any debris and dead cells. Initial images were collected immediately after the rinsing process, and further images were taken at appropriate intervals after the wells were filled with fresh medium.

2.2.2. PDMS pillars

PDMS pillars (2.5 mm, 3.0 mm, 4.0 mm, and 5.0 mm in diameter) were obtained using PDMS punchers of corresponding sizes. All PDMS punchers were sterilized with an alcohol lamp and rinsed with 70% ethanol before obtaining PDMS pillars. Fully confluent cell cultures in 24-well plates were perpendicularly compressed to create round wounds. Old medium was removed and the wells were rinsed with 1 mL PBS. Initial images were collected immediately after the rinsing process, and further images were taken after the wells were filled with fresh medium for appropriate periods and intervals.

2.3. LIVE/DEAD assay

The LIVE/DEAD assay was performed to examine the effects of the materials of each tool on the cell activity when forming the wounds using the mechanical pencil lead and PDMS pillars. The LIVE/DEAD viability kit is a bright fluorescence assay with high sensitivity composed of green fluorescent Calcein-AM (Component A) and red fluorescent ethidium homodimer-1 (Component B). For the component A trapped within the cellular membrane, esterases remove the acetomethoxy group from calcein causing emission of strong green fluorescence, whereas component B trapped within the cellular membrane undergoes 40-fold enhancement upon binding to nuclei. The protocols for measuring cell viability with the LIVE/DEAD viability kit were as follows: (1) 5 μL each of component A and B were separately dissolved in 10 mL of DPBS, (2) 200 μL of the mixture was distributed to each well and incubated for 30 min, and (3) fluorescent inverted microscope (IX71, Olympus, Japan) and CCD camera (AxioCam, Zeiss, Canada) were used to capture fluorescent cell images as shown in Fig. 2.

3. Numerical derivation of mathematical indice for quantitative wound evaluation

Synchronicity of the wound shape and tool surface was investigated by the ratio of the circumscribed circle and an inscribed circle within the wound area. Since the ratio alone is not sufficient to determine whether the wound approach to a circle, we calculated a roundness index and modified shape factor. The roundness index and modified shape factor are the indices that could be applied to validate how close the shape of the wound is to the ideal circle by calculating the correlation of generated wound area and perimeter. More details of the mathematical parameters were described in Fig. S2.

3.1. Modified shape factor (α_M)

Modified shape factor is an index for quantitative evaluation of the wound shape. When the wound matches the ideal round shape, the distance from the centroid of the wound area to the wound edge is equal to the radius of the circle that has the same area, which means that the standard deviation of the centroid to the wound edge is zero. To derive the standard deviation of the wound, we redefined the distance modifier for our calculation,

$$l_{M_i} = \frac{d_{M_i}}{d_{\max}} \tag{1}$$

where l_{M_i} is a dimensionless parameter for considering the effect of standard deviation, d_{M_i} is the distance from centroid of the wound to