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The wound healing assay revisited: A transport phenomena approach

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ABSTRACT

The Wound Healing (WH) assay is one of the most popular methods for the analysis of cell migration *in vitro*, widely used to investigate physiological and pathological processes. Several experimental factors of difficult control, such as variation of cell density, hinder a reliable and reproducible application of this assay. We investigate the effect of cell density (from very low values to complete surface occupation) on WH assays on human fibrosarcoma cells, by using *in vitro* time-lapse microscopy. We found that wound closure velocity is linear with cell density, and explained this dependence by analyzing wound closure as a diffusion-reaction process, according to available models. This finding leads to a simple scaling of the experimental data to account for cell density differences, obtaining a significant improvement in the quantitative assessment of results. We also suggest a simple way to evaluate whether cell motility or proliferation drive the process, based on a non-dimensional parameter.

1. Introduction

The dynamic behavior of the cells, driven by proliferation and migration mechanisms (Lauffenburger, 1989; Lauffenburger and Horwitz, 1996), is essential for a wide spectrum of physiological and pathological processes, including morphogenesis, angiogenesis, immune response, tissue repair, tumor growth and invasion (Horwitz and Webb, 2003; Ziebert and Aranson, 2013). In order to achieve a better comprehension of these complex processes (Lauffenburger, 1989; Veltman, 2014) a rigorous approach, based on the quantitative measurement of well-defined cell movement and proliferation indices, is required. For this reason, the development of quantitative analyses is nowadays within the core business of Chemical Engineering (Ottino, 2011), which can contribute to the building of mathematical models, based on a transport phenomena approach, useful to describe and predict the mechanisms driving cell dynamics (Love, 2010).

The wound healing (WH) assay is widely used to quantitatively investigate the dynamical aspects of cell behavior in vitro (Liang et al., 2007; Kramer et al., 2013). In the classical WH assay, also referred as scratch test, the cells are grown on a two-dimensional surface; in principle cell growth can reach a condition defined as confluency, where the available space is completely covered by cells (Sheardown and Cheng, 1996). An artificial scratch is then created on the confluent cell layer by mechanically scraping off an area of cells with a sharp-tipped instrument (Rodriguez et al., 2005). Then the cells are washed with medium to remove cell debris and floating cells. Several alternative wounding techniques, including laser ablation (Kiehart et al., 2000; Zordan et al., 2011), electric field application (Keese et al., 2004) and microfluidic approaches (Murrell et al., 2011; Nie et al., 2007), have been developed to implement the WH assay in a more controllable way (Riahi et al., 2012).

In response to the stimulus arising from the availability of free space (Block et al., 2004), the cells at the wound edges, which are no longer contact-inhibited, proliferate and move toward the center of the denuded region to cover the wound area (Tremel et al., 2009; Poujade et al., 2007). Depending on the type of cells involved, two main mechanisms of wound healing have been identified (Kramer et al., 2013; Liang et al., 2007; Rodriguez et al., 2005; Rørth, 2009). In fact, epithelial-like cells repopulate the wound area in a collective mode, ensured by strong cell-cell interactions, moving as two coherent sheets (Nikolic et al., 2006; Poujade et al., 2007; Vitorino and Meyer, 2008). Fibroblast-like cells cover the wound region moving isotropically as dispersed individual units (Liang et al., 2007), due to the lack of strong cell-cell contacts.

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The wound healing process can be mathematically described using the Fisher-Kolmorgoroff equation (Arnold and Adam, 1999; Cai et al., 2007; Johnston et al., 2014; Sherratt and Murray, 1990), which includes terms for modeling cell motility and proliferation. Both these mechanisms are involved in the spatial spreading of the cells invading the wound area. The Fisher-Kolmorgoroff equation (Eq. (1)) describes the evolution in space and time of the cell density u(x,t) as a reaction-diffusion system, function of two contributions. In Eq. (1) u is cell density at time t at a given distance x, measured from the wound edge, D is the constant diffusivity (also referred as random motility coefficient), k is the proliferation rate and û is cell density at confluency (Maini et al., 2004b):

$$\frac{\partial u}{\partial t}=D\frac{\partial^2 u}{\partial x^2}+ku\left(1-\frac{u}{\hat{u}}\right) \quad (1)$$

The diffusion term of the equation models cell motility as a Fickian diffusion with a cell flux proportional to the concentration gradients, in which the driving force that determines the spreading of the two cell sheets in the denuded area is the difference between cell density on the wound edge and in the wound region. The reaction term of the equation, that is a parabolic function of cell density, mathematically describes cell proliferation as a logistic growth, and includes crowding effects by reducing the growth rate as the cell density approaches confluency (û). Overall, the Fisher-Kolmorgoroff equation predicts that after a short transient, the movement of the invading cell front can be observed in terms of a traveling wave, that propagates with constant speed $s=\sqrt{4\hat{u}kD}$ in the direction perpendicular to the wound (Maini et al., 2004a; Tremel et al., 2009).

As the measurement of cell density is a challenging task from an experimental point of view, the wound closure process is often quantified in terms of change in the wound size over time (Zahm et al., 1997). In order to gain this quantitative information over long periods of time, two methodological approaches are typically used. A popular approach is based on the manual acquisition of microscope images within the sample, in almost random positions that can vary along the wound from time to time, at the beginning and at fixed time intervals (for example every 6 h) until the gap is closed (Kanazawa et al., 2010). This approach is approximate, since it doesn't allow investigating the dynamic of cell spreading in the wound area. An alternative, and more reliable, approach is based on direct visualization by time-lapse microscopy (TLM), which enables to dynamically monitor specific regions along the wound, rather than compare randomly acquired images along the wound, iteratively acquiring sample images with a defined time frequency (Terryn et al., 2009), while controlling the environmental parameters to ensure cell viability (Silano et al., 2012; Wong et al., 2013). Both methodological approaches allow to determine the number of cells in the wound region (Andújar et al., 2013; Tsai et al., 2014) or the percentage of wound closure (Lucena et al., 2011; Sumagin et al., 2013; Yue et al., 2010). However, TLM should be used to obtain accurate quantitative measurements, such as wound closure velocity, calculated by measuring the reduction of wound area over time, or the cell front propagation speed, by quantifying the position of the wound front over time (Bindschadler and McGrath, 2007; Poujade et al., 2007). WH assays are typically performed in multi-well culture plates in order to carry out several tests in parallel. This allows to investigate simultaneously the effect of various biological treatments on cell dynamics. However, as several common biological factors, including contaminations of the samples and environmental variations, can skew the experimental results, a control cell sample where no treatment has been done is necessary to provide a baseline for comparison.

Although the WH assay provides a valuable experimental approach for studying cell dynamics in vitro, the outcomes of the WH assay are somewhat influenced by several factors, that may represent a limit in accomplishing reproducible and reliable quantitative results. For example, the wound width can vary along its length and among different experiments (Grasso et al., 2007) (Riahi et al., 2012). Moreover, the scratching process may involve mechanical injures to the cells at the wound edges (Zhang et al., 2013). Some damaged cells and cell debris can also keep attached to the wound margins, perturbing the motility of other cells (Ascione et al., 2016; Cochet-Escartin et al., 2014). Additionally, the migrating surface can be damaged in the scraping process, leading to preferential paths in cell movements (Chou et al., 1995). Furthermore, the relative cell confluence ($\frac{u}{\hat{u}}$) is challenging to control and reproduce even within the same culture plate and among different cell samples (Doran et al., 2009; Jin et al., 2016). The difficulty to obtain the same cell density in the samples can arise from inaccuracy in cell counting, as well as from anisotropies in the spatial spreading of the cells, mainly due to uneven cell adhesion in the culture plate. Several biological treatments, including gene silencing, transfection, treatment with drugs at high concentration, require aggressive techniques, which may result in undesired detachment of cells from the plate. The final result is a not negligible variability in cell densities among the samples. Overall, these sources of variability might influence the outcome of the WH assay making it difficult to compare independent experiments. Furthermore, the sensing of chemotactic cues released by cells lying on the other wound edge may also play a key role in the healing process, the related signaling being also dependent on the number of cells in the sample.

In this work, we revisited the methodological approach typically used to quantify the wound closure dynamic in a WH assay, by using a novel analysis approach based on transport phenomena concepts. In particular, we explored the influence of cell density on the wound closure dynamics, and show that it strongly affects the reproducibility of the WH assay. We performed in vitro WH assays on HT1080 human fibrosarcoma cells, which exhibit fibroblast-like dynamic behavior, by using TLM image acquisition. Overall, our work is addressed to overcome, at least in part, the limitations related to the conventional quantitative analysis of the WH assay. In particular, we propose a phenomenological scaling of the experimental data, based on a transport phenomena approach, in order to account for the effect of cell density on wound closure velocity. The approach we propose suggests an easy way to describe complex phenomena, such as WH, where several biological (Matsubayashi et al., 2004) as well as physical mechanisms (Cochet-Escartin et al., 2014; Ladoux, 2009; Salm and Pismen, 2012; Tambe et al., 2011) are involved.

2. Materials and methods

2.1. Cell cultures

HT1080 human fibrosarcoma cells were maintained at 37 °C in Dulbecco's Modified Eagle Medium (Lonza, Switzerland) supplemented with 10% fetal bovine serum (Lonza, Switzerland) and antibiotics (50 units/mL penicillin and 50 μg/mL streptomycin) (Lonza, Switzerland) in a humidified atmosphere containing 5% CO_2 in air. Cultures between passages 18 and 22 were used for WH experiments.

2.2. Time-lapse microscopy

Time-lapse Microscopy (TLM) acquisitions were carried out using an inverted microscope (Zeiss Axiovert 200; Carl Zeiss, Jena, Germany) and a 10× objective (CP Achromat Ph1) in phase contrast (Caserta et al., 2013; Vasaturo et al., 2012). The microscope was enclosed in a homemade incubator keeping the sample at 37 °C and under 5% CO_2, humidified atmosphere. Live-cell imaging was performed by a high-sensitivity CCD camera (Orca AG; Hamamatsu, Japan) driven by homemade control software in Labview. The images were iteratively acquired at several locations (fields of view) within the samples using a motorized x–y stage and focus control. For each cell concentration investigated, at least three independent fields of view were selected in