Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay

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Collective cell migration is a hallmark of wound repair, cancer invasion and metastasis, immune responses, angiogenesis, and embryonic morphogenesis. Wound healing is a complex cellular and biochemical process necessary to restore structurally damaged tissue. It involves dynamic interactions and crosstalk between various cell types, interaction with extracellular matrix molecules, and regulated production of soluble mediators and cytokines. In cutaneous wound healing, skin cells migrate from the wound edges into the wound to restore skin integrity. Analysis of cell migration in vitro is a useful assay to quantify alterations in cell migratory capacity in response to experimental manipulations. Although several methods exist to study cell migration (such as Boyden chamber assay, barrier assays, and microfluidics-based assays), in this short report we will explain the wound healing assay, also known as the "in vitro scratch assay" as a simple, versatile, and cost-effective method to study collective cell migration and wound healing.

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Description: This article, designed for dermatologists, residents, fellows, and related healthcare providers, seeks to reduce the growing divide between dermatology clinical practice and the basic science/current research methodologies on which many diagnostic and therapeutic advances are built.

Objectives: At the conclusion of this activity, learners should be better able to:

- Recognize the newest techniques in biomedical research.
- Describe how these techniques can be utilized and their limitations.
- Describe the potential impact of these techniques.

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Abbreviation: RWD, relative wound density

COLLECTIVE CELL MIGRATION

Cell migration is defined as the actual movement of individual cells, cell sheets, and clusters from one location to another. The term "cell motility" is often used interchangeably, but may technically imply a less coordinated and purposeful movement of cells. Two principal types of cell migration have been identified: single cell migration and collective cell migration. Depending on the cell type, cytoskeletal structure, and the context in which it is migrating, the cell can migrate in different morphological variants such as mesenchymal, amoeboid motility modes (Friedl and Wolf, 2003). Collective migration is

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SUMMARY

Advantages:

- Relatively inexpensive and easy to perform.
- Allows observation of cell movement and morphology throughout the experiment.
- Testing conditions can be easily adjusted for different purposes.
- Creates a strong directional migratory response.
- Ability to coat assay surface with an appropriate extracellular matrix.
- Amenable to high throughput screening platforms.

Limitations:

- May not be suitable for studying specialized primary cells because a relatively large number of cells are required for the assay.
- Not suitable for chemotaxis studies or for nonadherent cells.
- Lack of standardization in its application makes it difficult to reproduce experiments.
- Scratching introduces mechanical injury to the cells, leading to release of cellular contents into the surroundings and potentially influencing the migration process.
- Cell proliferation may interfere with the measurement of cell migration. Therefore, suppression of proliferation is a recommended intervention.

the coordinated movement of a group of cells that maintain their intercellular connections and collective polarity. Depending on the anatomical and physiological context, collective migration can manifest as (i) two-dimensional locomotion across a tissue surface (also known as sheet migration) where cells migrate as flat monolayer sheets, such as epidermal keratinocytes during wound healing, or (ii) three-dimensional locomotion across a tissue scaffold where cells are organized as a network of multicellular strands (Friedl and Gilmour, 2009).

WOUND HEALING

There are four main phases in wound healing: coagulation, inflammation, migration-proliferation (including matrix deposition), and remodeling (Falanga, 2005). These phases do not represent distinct events, but rather overlap and are continuous. After tissue injury and under the influence of various growth factors and cytokines, keratinocytes at the rear of the wound margins may display a high proliferative activity. These cells then migrate forward onto the wound bed and help restore the epidermal barrier structure and function. This overall process involves cell migration, proliferation, and differentiation. In smaller wounds, the critical event is keratinocyte migration rather than proliferation (Falanga, 2005). Cell migration begins several hours after injury. Epidermal cells adjacent to the wound margin become polarized (driven by the actin cytoskeleton) and develop pseudopodium-like projections preferentially oriented outward, into the free space, and within 24 hours, the cells detach from the basal lamina and are ready for migration. Lamellipodial crawling refers to the pattern of motion that epidermal cells exhibit during migration (Ridley et al., 2003). Although we have predominantly referred to keratinocytes, one must recognize that studies of cell migration in skin processes and disease also involve other resident skin cells, including fibroblasts, microvascular endothelial cells, and melanocytes, among others.

IN VITRO WOUND HEALING ASSAY

Studying the collective migration of cells in a two-dimensional confluent monolayer in highly controlled in vitro conditions allows investigators to simulate and explore critical mechanisms of action involved in the process. A variation of this method that tracks the migration of individual cells has been described in the literature (Rodriguez et al., 2005). There is argument about whether the assay can be equated to an actual wound, which is obviously more complex, but the assay does allow modeling and testing of cell movement under well-defined conditions. This assay is suitable for cell types such as keratinocytes and skin fibroblasts that exhibit collective migration, also known as "sheet migration" (Bindschadler and McGrath, 2007). The technique involves making a linear thin scratch "wound" (creating a gap) in a confluent cell monolayer (Figure 1) and subsequently capturing at regular time intervals images of the cells filling the gap (Cory, 2011). One can then analyze the images to quantify migration. Live cell imaging using time-lapse microscopy allows recording of spatial and temporal information and allows for investigation of dynamic processes in living cells (Supplementary Movie S1 online). The measurements are generally taken for 24 hours in an attempt to limit the study to migration and minimize the contribution of cell proliferation to gap filling. However, the time frame should be adjusted according to the particular cell type to be studied. To further reduce the risk of cell proliferation confounding the study of migration, a low dose of the proliferation inhibitor mitomycin C can be used. Mitomycin C is an antitumor antibiotic that inhibits DNA synthesis. The dose needs to be carefully optimized to avoid toxic effects that may affect cell migration. Using low serum concentrations in cell medium (serum starvation) is the most common method to suppress cell proliferation in wound healing assays. However, the duration of serum starvation and the required serum concentrations need to be rigorously determined for each studied cell line. Serum starvation can elicit complex, unpredictable time-dependent, and cell-type-dependent effects (Pirkmajer and Chibalin, 2011).

APPLICATIONS OF THE WOUND HEALING ASSAY

- Quantitative and qualitative analysis of collective cell migration under different experimental conditions.
- Studying the effects of cell-matrix and cell-cell interactions on cell migration.
- High-throughput screening for genes involved in cancer cell migration (Simpson et al., 2008), small molecule screening (Yarrow et al., 2005), and drug discovery (Hulkower and Herber, 2011).

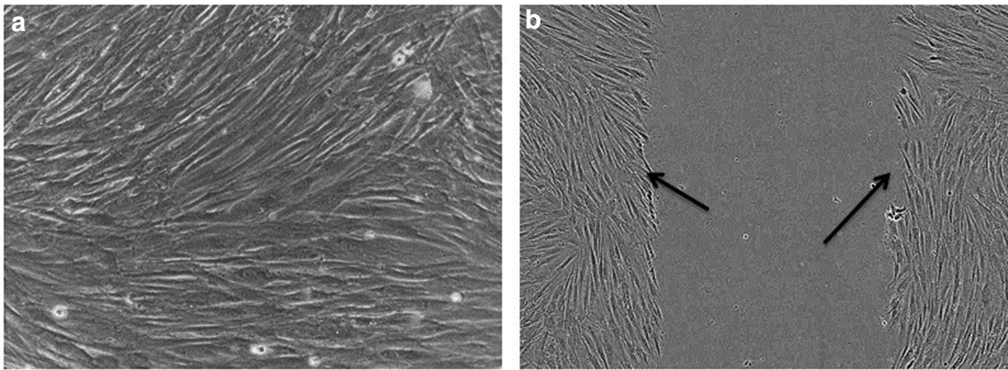

Figure 1. The in vitro wound healing assay. (**a**) Human skin fibroblasts forming a confluent monolayer. (**b**) In vitro "wound" was created by a straight line scratch across the fibroblast monolayer. Black arrows are pointed toward wound edges.

METRICS TO QUANTIFY CELL MIGRATION

The rate of cell migration can be quantfied using a single metric or a combination of metrics. The following are the most commonly used metrics:

1. *Wound width* can be calculated as the average distance between the edges of the scratch. Manual quantification can be time consuming. The wound width should decrease as cell migration progresses over time. Migration rate can be quantified by dividing the change in wound width by the time spent in migration:

$$R_M = \frac{W_i - W_f}{t}$$

R_M = Rate of cell migration (nm/h)

W_i = initial wound width (nm)

W_f = final wound width (nm)

t = duration of migration (hour)

2. *Wound area* can be calculated by manually tracing the cell-free area in captured images using the ImageJ public domain software (NIH, Bethesda, MD). Under normal conditions, the wound area will decrease over time. The migration rate can be expressed as the change in the wound area over time. Rotzer et al. (2016) showed how the scratch wound assay can be used to assess the migration capacity of keratinocytes under different experimental conditions (Figure 2). Alternatively, the migration rate can be expressed as the percentage of area reduction or wound closure. The closure percentage will increase as cells migrate over time:

$$\text{Wound Closure \%} = \left[\frac{A_{t=0h} - A_{t=\Delta h}}{A_{t=0h}}\right] \times 100\%$$

$A_{t=0h}$ is the area of the wound measured immediately after scratching ($t = 0h$)

$A_{t=\Delta h}$ is the area of the wound measured h hours after the scratch is performed

3. *Relative wound density* (RWD) can also be used to measure cell migration. This metric is employed in live cell imaging platforms such as the IncuCyte system (Essen BioScience, Ann Arbor, MI), and is the percentage of spatial cell density in the wound area relative to the spatial cell density outside of the wound area at each time point (Johnston et al., 2015). Under normal conditions, RWD will increase as cells migrate over time. This metric has the advantage of allowing normalization for changes in cell density caused by proliferation and pharmacological effects.

$$\%\ \text{RWD}\ (t) = \frac{w_t - w_0}{c_t - w_0} \times 100$$

w_t = Density of the wound area at time t

c_t = Density of the cell area at time t

MATERIALS AND EQUIPMENT

- Standard cell culture protocols are available for most cell lines and can be obtained from the online literature.
- Plastic-bottomed multiwell tissue culture plates, 10 µl pipette tips, razor or extra fine permanent marker, cell culture incubator, CO_2 supply, and, in the case of testing hypoxic conditions, a nitrogen supply.
- Imaging equipment: bright-field microscope connected to a digital camera. Recently, an automated live cell content imaging platform has become available that allows time-lapse microscopy (including of green fluorescent protein [GFP+] cells) and quantitative image analysis. In this system, the entire unit is placed on a tray in the tissue culture incubator connected to an external dedicated computer.

PROCEDURE

Protocols will of course vary according to the cell type being studied. However, there are some basic fundamental steps (Figure 3) that are applicable for almost all cell types: (i) cell culture preparation, (ii) scratch-making, (iii) data acquisition, and (iv) data analysis.

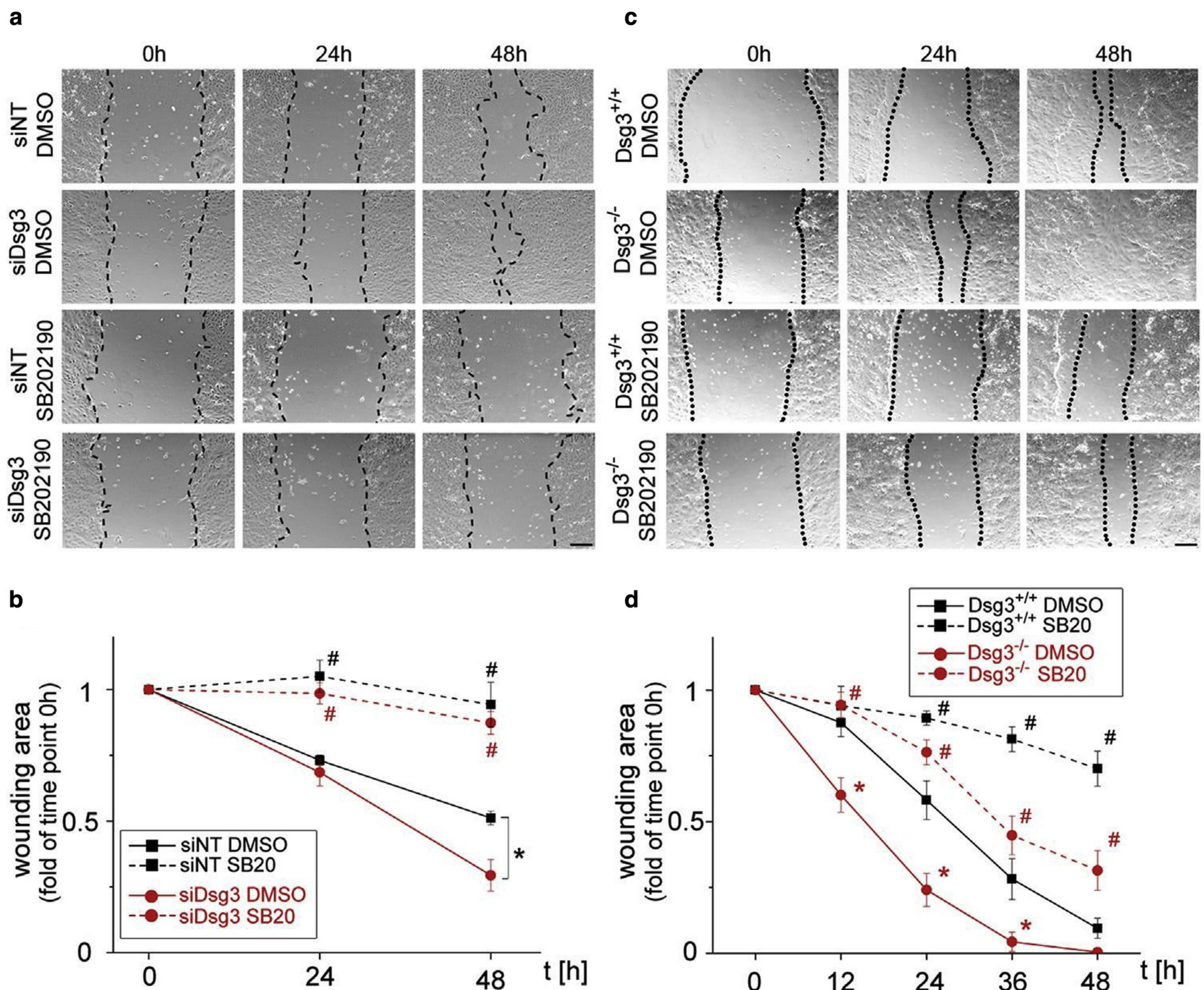

Figure 2. Migration is increased in desmoglein 3 (Dsg3)-depleted keratinocytes in a p38MAPK-dependent manner. (**a**) Representative bright-field images show that silencing of Dsg3 resulted in significantly increased migration speed compared with nontarget siRNA controls. This acceleration of gap closure was also prevented by p38MAPK inhibition using SB20. (**b**) Wound closure expressed as the remaining area uncovered by the cells. The scratch area at time point 0 hours was set to 1 (n = 4–6; $^*P < 0.05$, $^\#P < 0.05$ vs. respective DMSO condition). (**c, d**) Scratch-wound closure monitored over time in cells isolated from $Dsg3^{+/+}$ and $Dsg3^{-/-}$ mice. Bar is 150 mm (n = 10–15 from four independent isolation procedures; $^*P < 0.05$ vs. $Dsg3^{+/+}$ DMSO, $^\#P < 0.05$ vs. respective DMSO condition). The black bar at the right lower corner is 150 mm. MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA. Reprinted from Rotzer et al. (2016), with permission from Elsevier.

1. Cell culture preparation:
   a. The required number of cells to form a confluent monolayer need to be determined according to the particular cell type and the size of wells.
   b. Place the culture dishes inside the incubator until a confluent monolayer is formed (Figure 1).
   c. To inhibit cell proliferation, add an optimized (nontoxic) dose of mitomycin C for a few hours after cells reach confluence and then removing mitomycin-C by washing before making the scratch (Chen et al., 2013).
2. Scratch-making:
   a. A sterile plastic micropipette tip or razor blade can be used to simulate an in vivo wound by creating a straight-edged, cell-free zone across the cell monolayer in each well. A gap width of 0.5 mm allows observation at ×4 or ×10 magnification. It is important to angle the pipette correctly and apply consistent pressure to create a consistent gap width. The gap should have relatively smooth edges and little cellular debris. A fine permanent marker tip can be used to draw several reference points close to the scratch. Manual scratching may reduce reproducibility due to well-to-well variation in gap width. Commercially available "wound maker" allows for uniform scratch-making (Supplementary Teaching Slides online).
   b. After creating the scratch, the monolayer is washed with basal medium to remove cell debris, and complete medium is added. Most experiments are performed with cells in a tissue culture incubator set at 37 °C, 5% CO_2, and 95% air. These conditions can be altered, for example, to study

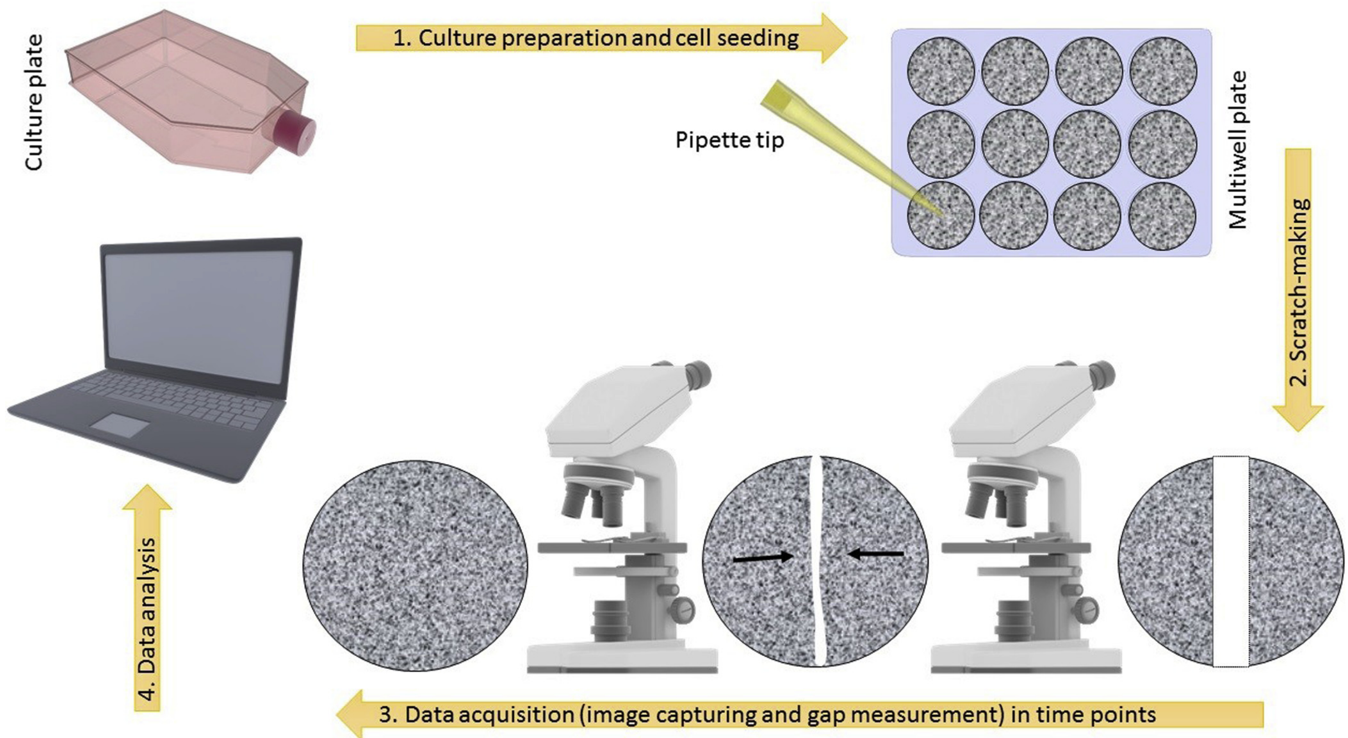

Figure 3. Graphical abstract summarizing the workflow of the in vitro wound healing assay. The technique involves basic steps applicable to almost all cell types: 1) cell seeding and preparation; 2) making a linear thin scratch "wound" (creating a gap) in a confluent cell monolayer; 3) data acquisition through microscopic image capturing and gap measurement at each time point; and 4) data analysis.

the effects of hypoxia on migration, whereby nitrogen is infused to decrease the air/oxygen concentration. The time needed for incubation should be determined empirically for the particular cell type to be investigated. Each experimental condition is typically evaluated in a triplicate.

3. Data acquisition:
   a. *Snapshot method:* Migration progress can be documented by taking sequential digital photographs of the gap using bright-field microscopy. A reasonable approach is to captures three images per well per time point. Wound area can be calculated using the ImageJ public domain software.
   b. *Live cell imaging:* Automated live-cell imaging platforms are well suited for long-term monitoring of cell behavior because the microscope can be placed inside the incubator itself. Cells are therefore imaged under optimal physiological conditions for the duration of the experiment. The system allows the end-point readout of cellular events and exploration of kinetic, functional, and quantitative measurement of living cells' behavior.
4. Data analysis:
   Summary statistics can be calculated, and a line chart can be used to plot the mean migration rate versus time. To test a hypothesis or compare migration rates of different cell populations, Student's *t*-test is used to compare two samples and analysis of variance with multiple testing corrections should be performed for comparing three or more groups of data. A *P*-value < 0.05 is used to define statistical significance. If the data satisfy tests for normality and equal variance, then a *t*-test should be performed to compare the mean of two groups; a two-tailed unpaired *t*-test (if the samples are independent), or a two-tailed paired *t*-test (if the samples are related) can be performed. If the data fail the normality test, then nonparametric tests such as the Wilcoxon-Mann-Whitney test may be used or Kruskal-Wallis nonparametric when comparing more than two groups. One drawback of nonparametric tests is that they have less power than conventional tests such as Student's *t*-test and analysis of variance. To mitigate this issue, a smaller *P*-value can be used to define significance ($P < 0.01$). To help users perform statistical analysis, we provided instructions and R code in the Supplementary Materials (online). Recent advances in network science, machine learning, and computational modeling can be utilized to model and simulate collective cell migration and "learn" patterns of complex cellular behavior on a large scale, and perhaps predict responses to perturbations. Such in silico models can complement traditional experimental research and help in narrowing research questions (Masuzzo et al., 2016).

CONCLUSIONS

The in vitro wound healing assay is a convenient and economical method to assess and quantify collective cell migration under different experimental conditions. Collective cell migration is a hallmark of many physiological and pathological processes pertaining to skin such as wound repair and cancer metastasis. One can enhance accuracy and reproducibility of the assay by creating cell monolayers with the same degree of confluence and making uniform in vitro "wounds" in terms of size and geometry.

MULTIPLE CHOICE QUESTIONS

1. Which of the following treatments can be used to suppress cell proliferation so that it does not interfere with in vitro measurement of cell migration?
   A. Mitomycin C
   B. Paclitaxel
   C. Serum starvation
   D. Vinblastine
   E. A and C

2. The wound healing assay is performed in the following sequence:
   A. Cell culture, image collection, scratch-making, sequencing
   B. Cell culture, scratch-making, data acquisition, data analysis
   C. Scratch-making, cell culture, freezing, image collection
   D. Cell coating, DNA sequencing, alignment to a reference genome
   E. Data acquisition, culture preparation, scratch-making, data analysis

3. Advantages of the wound healing assay include all of the following except:
   A. Affordable and easy to set up
   B. High reproducibility
   C. It does not require the use of specific chemoattractants or gradient chambers
   D. Suitable for chemotaxis studies
   E. B and D

4. Applications of the wound healing assay may include:
   A. Helping to identify therapies to promote cell migration in wound healing
   B. Evaluation of the effects of inhibitors/ enhancers on the migratory capacity of a particular cell population
   C. Investigating the mechanisms regulating cancer cell migration and evaluating the efficacy of potential therapeutic drugs
   D. Studying regulation of actin cytoskeletal structures and cell polarity
   E. All of the above

5. Which of the following measures can enhance reproducibility of results when performing the in vitro wound healing assay?
   A. Always seed cells at the same density and start the assay at the same degree of confluence.
   B. If a manual scratch must be made, use consistent pressure and pipette tip angle to create uniform scratch sizes and shapes.
   C. Incubate cells for no more than 24 hours.
   D. Increase the sample number.
   E. A and B

CONFLICT OF INTEREST
The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to this paper. Teaching slides and R Code files are available as supplementary material.

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