Video Article

Optimization of the Wound Scratch Assay to Detect Changes in Murine Mesenchymal Stromal Cell Migration After Damage by Soluble Cigarette Smoke Extract

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Abstract

Cell migration is vital to many physiological and pathological processes including tissue development, repair, and regeneration, cancer metastasis, and inflammatory responses. Given the current interest in the role of mesenchymal stromal cells in mediating tissue repair, we are interested in quantifying the migratory capacity of these cells, and understanding how migratory capacity may be altered after damage. Optimization of a rigorously quantitative migration assay that is both easy to customize and cost-effective to perform is key to answering questions concerning alterations in cell migration in response to various stimuli. Current methods for quantifying cell migration, including scratch assays, trans-well migration assays (Boyden chambers), micropillar arrays, and cell exclusion zone assays, possess a range of limitations in reproducibility, customizability, quantification, and cost-effectiveness. Despite its prominent use, the scratch assay is confounded by issues with reproducibility related to damage of the cell microenvironment, impediments to cell migration, influence of neighboring senescent cells, and cell proliferation, as well as lack of rigorous quantification. The optimized scratch assay described here demonstrates robust outcomes, quantifiable and image-based analysis capabilities, cost-effectiveness, and adaptability to other applications.

Video Link

The video component of this article can be found at http://www.jove.com/video/53414/

Introduction

Cell migration is highly coordinated and vital to many physiological processes such as tissue development, repair, and regeneration, as well as pathological processes such as cancer metastasis and arteriosclerosis [1]. An understanding of cell migration is particularly relevant to emerging therapies used to repair damaged tissues and treat pathological conditions, including cell transplantation technologies and artificial tissue grafting [2]. Given the current interest in the role of mesenchymal stromal cells (MSCs) in mediating tissue repair [3], quantifying the migratory capacity of these cells using an assay that is rigorously quantifiable, adaptable, and cost-effective is of interest. Importantly, such an assay must be sufficiently sensitive to detect relatively subtle changes in cellular migratory capacity after damage. Current methods for quantifying cell migration, including scratch assays, trans-well migration assays (Boyden chambers), micropillar arrays, and cell exclusion zone assays possess a range of limitations in reproducibility, customizability, quantification, and cost-effectiveness [1,4,5]. The optimized scratch assay described here demonstrates robust outcomes, quantifiable and image-based analysis capabilities, cost-effectiveness, and adaptability to other applications.

Scratch assays have been used in multiple capacities to assess cell migration and proliferation under different experimental conditions [5]. The assay entails seeding the designated cells, growing them to complete or near confluence, and scratching the resultant monolayer with a sterile needle or pipet tip [6]. Analysis is most commonly performed by comparing the width of the scratch over multiple time points at randomly selected locations [7-9]. Despite its prominent use, the scratch assay is confounded by issues with reproducibility and quantification. Variability in generation of the scratch not only alters the microenvironment of the cells, but can also impede cell migration by damaging the plate surface and underlying extracellular-matrix [5]. Assays are frequently conducted over 7 to 12 hr, however for cell lines displaying slower migration and longer assay times, proliferation becomes a confounding variable [7,10]. Lastly, senescent cells generated by the scratching process can release factors that interfere with the extracellular signaling required to close the gap in the monolayer [1]. Optimizing the scratch assay requires creation of a consistent gap that does not interfere with surface properties, minimizing assay time length, and preventing unwanted cell death during the manipulation. The stopper based assay is an optimization of a cell exclusion zone assay. This assay utilizes a stopper placed in the middle of the well that excludes cell growth, but allows cells to be plated around the central exclusion zone. To assess migration, the stopper is removed, and the resultant exclusion zone provides a surface for migration to occur. However, this assay is difficult to customize or adapt [10] and for some applications, this technique can also be cost prohibitive.

In contrast to scratch assays and their derivatives, trans-well migration assays (or Boyden chamber assays) assess migration by quantifying the number of cells that move from one chamber, through a microporous filter membrane, into a chamber containing chemotactic agents

[8,11,12]. This technique has limited utility for adherent cells like MSCs because following migration through the porous membrane, cells adhere to the membrane side exposed to the chemotactic agents, and can be hard to accurately quantify. While the assay is able to examine some three-dimensional migration patterns, the restricted cell types for which it is able to accurately quantify cell migration limit its utility [10]. Another alternative to scratch assays uses a micro-pillar array, which measures cellular motility through a three-dimensional space using the ability of cells to deform and migrate into the array as a surrogate. Polydimethlysiloxane (PDMS) elastomers cured over a precise mold and treated with ozone and fibronectin produces a homogenous and non-degradable microenvironment. Micro-pillar spacing can also be varied as needed to gauge the ability of cells to enter the array [4]. The mold is created through deep reactive ion etching of silicon wafers to create a negative version of the high aspect-ratio array [13]. While the assay is strengthened by its customizability, ability to model three-dimensional migration, and analysis through direct visualization of migrating cells, difficulty in creating micro-pillar arrays economically impedes its widespread use.

The optimized scratch assay described in this protocol provides an efficient, cost-effective method for producing consistent scratches that can be analyzed using freely available software. Instead of simple width measurements made across the scratch before and after cell migration, the software enables the user to determine total scratch areas before and after migration. This advancement limits the issue of trying to determine where the scratch the width measurements should be taken, and whether the width of the scratch is uniform along it's length. In addition, careful optimization of cell numbers, cell confluence and the type and degree of damage inflicted on the cells is discussed in order to further optimize the assay.

Protocol

Note: For this study, lung mesenchymal stromal cells (LR-MSCs) from distal lung tissue were isolated either based on their expression of cell surface markers ($CD45^{neg}$, $CD31^{neg}$, $Sca\text{-}1^{high}$, $Epcam^{neg}$) [14,15], using explant out-growth [16], or using enzymatic digestion [17]. Adherent LR-MSCs were cultured in DMEM with high glucose and no glutamine, 15% FBS, 1x antibiotic/antimycotic, and 2 mM glutamine (henceforth complete media) and incubated at 37 °C, 5%CO_2. LR-MSCs were passaged 3-4 times to ensure a population of cells with relatively homogenous growth characteristics for the migration assay.

1. Preparing a Confluent Monolayer

1. To detach MSCs from standard 100 mm culture dishes, wash cells with 10 ml of 37 °C pre-warmed PBS, aspirate the PBS and add 4 ml of trypsin (0.25%). Incubate 3-5 min in a 37 °C incubator. Aspirate the cells into a conical tube, add equal volume of complete media and centrifuge at 300 g for 5 min to pellet down the cells.
   Note: Longer incubation in trypsin can alter the cell surface receptors and potentially affect migration.
2. Aspirate media and resuspend cells in fresh complete media. Count cells, excluding dead cells using trypan blue exclusion and plate 500,000 mesenchymal stromal cells in 4 ml of complete media on a 60 mm cell culture dish.
   Note: Depending on the source of the cells, tests may be necessary to determine the optimal number of cells needed.
3. Incubate cells until plates are 90% confluent for optimal cell attachment and proliferation, usually 48-72 hr for MSCs.
   Note: 100% confluence is not recommended, since many anchorage-dependent cells experience contact inhibition, which may alter their migratory capacity. Plates with less than 80% confluence will result in unusable photos because the image analysis software will interpret gaps between cells as part of the perimeter of the scratch (see **Figure 1D**).
4. After the cells have reached 90% confluence, damage the cells as appropriate for the application. To assess cell damage after exposure to soluble cigarette smoke extract (CSE), generate 100% CSE by drawing the smoke from 1 University of Kentucky research cigarette (3R4F) through 25 ml of complete media in a Büchner flask over 2 min [18].
5. Incubate the cells for 24 hr in 4 ml 1-4% CSE diluted in complete media. After incubation, wash the cells twice with 4 ml of warm sterile PBS, before replacing with 4 ml fresh complete media.
   Note: Damaging cells with high concentrations of cigarette smoke extract (>5%) may substantially reduce confluence.

2. Creating the Scratch

1. In a sterile environment, remove the lid from the 60 mm plate of damaged MSCs, and lay a straight edge (for example a sterilized plastic ruler) across the top rim of the plate. Without removing the media, use a sterile 200 µl pipet tip guided by the sterile straight-edge to make one to four parallel scratches across the plate approximately 5 mm apart and 50 mm long.
   Note: A straight edge minimizes the amount of rotational adjustments needed to the plate when it is on the microscope stage. It is imperative that the scratches appear completely vertical (or horizontal) on the screen. Generating multiple scratches on a 60 mm plate does not alter the microenvironment in a way that affects cell migration (data not shown), but may be a consideration for other cell types. The use of smaller wells is not recommended since the variability of cells growing close to the edge of the well becomes more pronounced and may affect uniform generation of scratches.
2. Aspirate the media and gently wash the plates with 2 ml of pre-warmed complete media to prevent cells from adhering to the center of the scratch, and to remove cells that have been loosened by the scratching. Replace with 4 ml of fresh complete media.
3. Using a sterile permanent marker, label each scratch from 1-4 on the underside of the plate at location that will not interfere with imaging the scratches.
   Note: It is important that images from the same scratch are compared before and after migration, since there can be variation in scratch width.

3. Taking Initial Images of the Scratch

Note: If the scratch assay is performed on multiple plates, it is recommended that initial images of all the scratches on a single plate are taken before scratches are made on the next plate.

1. Use an inverted phase contrast microscope with a camera to generate the images needed for analysis.
2. Position the culture plate on the microscope, and use the low power objective (10X) to locate scratch #1. If condensation on the lid of the culture plate is impeding a clear image of the cells, use a heated stage set to 37 °C.
3. Keeping the scratch completely horizontal and in the center of the field of view, obtain as many images as necessary to encompass 90% of the scratch's length. Obtain images of distinct portions of the scratch with no overlap between images.
   Note: The refraction of light at the edges of the culture dish overexposes the images, making them impossible to analyze. Therefore, do not take images from the first and last 5% of the scratch's length.
4. Save images from each scratch in a separate folder.
5. Repeat steps 3.2-3.4 for scratches 2, 3, and 4.
6. Return plates to the incubator immediately after initial imaging. Let cells migrate for 4-7 hr.
   Note: 7 hr is optimal when assessing MSCs damaged with CSE. Incubating cells for greater than 7 hr is not recommended because cell proliferation can act as a confounding variable for cell migration (see Optional Note 8.2 below).

4. Analyzing Initial Scratch Images

1. Download ImageJ and install the Wound Healing Tool plugin (refer to the Table of **Materials/Equipment**).
2. Open the image file in ImageJ, then click "Process" and "Find Edges" to highlight the cells surrounding the scratch for the Wound Healing Tool to calculate the scratch area (**Figure 2B**). Next, click "Image", "Adjust", "Color Threshold" and in the dropdown menu labeled "Threshold Color" change the value to "B&W."
3. Within the "Color Threshold" menu, adjust both brightness threshold sliders until optimal contrast between the scratch and cell front is achieved. To allow for the greatest range when trying to create contrast in the image, move the bottom slider completely to the right (completely black image), and then adjust the top slider to generate maximum contrast.
4. Slowly adjust the top brightness slider from left to right until the image cleanly displays the contour of the scratch (**Figure 2C**). Once the edges of the scratch have been identified, click "More Tools" in the ImageJ toolbar, select "MRI_Wound_Healing_Tool" from the dropdown menu, and press the "m" button (that will appear in the ImageJ toolbar) to highlight the scratch area and output the computed area in a results pop-out window (**Figure 2D**).
5. Copy and paste all numbers from the results window (**Figure 2E**) into an excel spreadsheet. Make sure to separate data from individual scratches.

5. Taking Final Images of the Scratch

1. After the cells have migrated (4-7 hr), repeat steps 3.2-3.4. Image the plates in the same order they were scratched so that cells on each plate have had equal time to migrate.
   Note: The cells can be imaged at multiple time points, depending on their sensitivity to both temperature and pH changes. Alternatively, live cell imaging can used to follow the cells in real time as they migrate.

6. Analyzing Final Scratch Images

1. Repeat steps 4.2-4.5 for final scratch images.

7. Calculating the Scratch Area to Quantify Migration

1. Compute the mean pre-migration area by averaging the initial area values of the respective scratches.
2. Compute the total amount of migration by subtracting the final migration area for each image, calculated in step 6, from the average pre-migration area (step 7.1). Generate a list of values for each scratch representing the change in migration.
3. Pool the data from multiple scratches within the same test group (*i.e.* all plates and all scratches on cells exposed to 0% CSE). Use an Analysis of Variance test to analyze the differences between experimental groups.
4. If the cells are slow to migrate and require a longer incubation time after scratching (greater than 10-12 hr), perform a BrDU or EdU incorporation assay [19] to determine whether cells are undergoing any proliferation during the period in which the migration assay is occurring. Choose a migration assay time to avoid significant cell proliferation.

8. Optional

1. To assess whether significant cell senescence is occurring as a result of the scratching procedure, perform a senescence-associated Beta-Galactosidase assay on a test plate at predetermined times after scratching [19].

Representative Results

The scratch assays presented here were performed using murine lung resident mesenchymal stromal cells (LR-MSCs) and isolated as referenced in the protocol notes. The LR-MSCs were seeded at a density of 500,000 cells in a 60 mm tissue culture dish, and grown to 90% confluence in 48 hr. To generate damage, 4% CSE (described above) was incubated with the cells for 24 hr following the initial seeding period but before the scratch assay. For each assay, the scratch was generated using a 200 µl pipet tip. Initial images were taken after the scratch had been generated, and any loose cells and debris had been washed away with complete media. As shown in **Figure 1A** and **B**, appropriate confluence is important to ensure that the imaging software can correctly identify the boundaries of the scratch. As shown in **Figure 1C**, cells are not sufficiently confluent (either because of inadequate seeding densities, inadequate incubation times, or excessive damage), which can result in scratches with heterogeneous boundaries that are not easily distinguished by the imaging software (**Figure 1D**). **Figure 2** demonstrates

image acquisition and analysis. **Figure 2A** depicts the original image before analysis. **Figure 2B** shows the result of the "Find Edges" tool under the "Process" menu (Step 4.2) and **Figure 2C** shows the image following adjustment of the "Color Threshold" slidebar (Step 4.3). **Figure 2D** depicts the final analyzed image after running the MRI_Wound_Healing_Tool plugin (Step 4.4). **Figure 2E** depicts the results pop-out window, highlighting (red box) the numbers that are generated. Representative pre- and post-migration images are shown in **Figure 3**, with corresponding scratch area boundaries as defined by the imaging software. **Figure 4** graphically illustrates the altered migratory capacity seen in LR-MSCs after damage with 4% CSE. In **Figure 4A** and **B**, the average calculated scratch areas (in pixels) for each of 4 scratches made across a plate of LR-MSCs at 95% confluence either pre- or post-migration, with exposure to either 0% CSE or 4% CSE are compared. **Figure 4C** compares the calculated average change in scratch area (post-migration area subtracted from pre-migration area) in LR-MSCs exposed to either 0% or 4% CSE, demonstrating less migration (lower average change in scratch area) after exposure to 4% CSE.

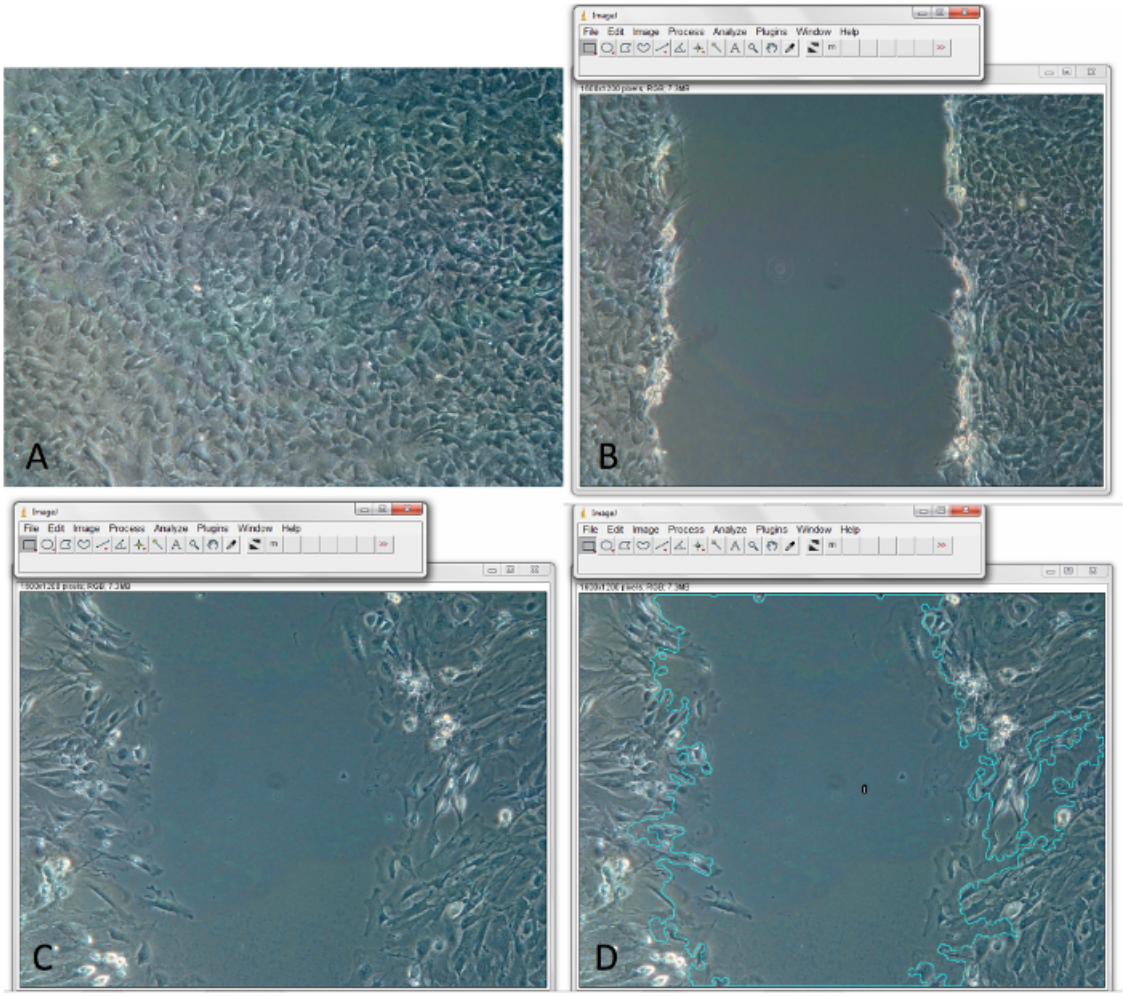

Figure 1: Correct confluence is critical for the generation of the robust reproducible scratch assay. (**A**) Murine LR-MSCs at 95% confluence, appropriate for scratching. (**B**) A successful scratch at 95% confluence. (**C**) LR-MSCs at 70% confluence, too low for a successful scratch. (**D**) Image acquisition at 70% confluence can produce false boundaries. Please click here to view a larger version of this figure.

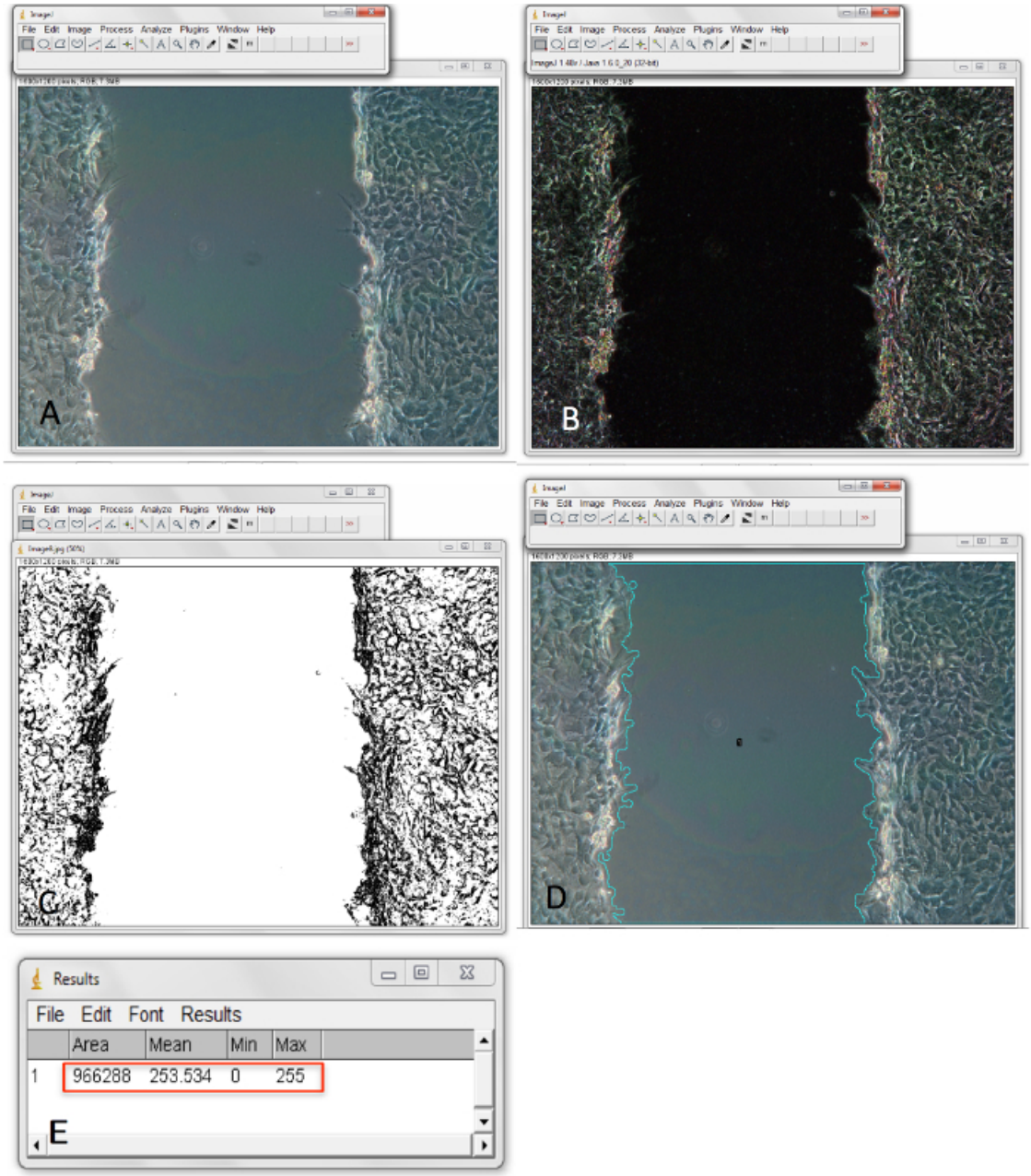

Figure 2: Representative images demonstrating image acquisition and analysis. (**A**) Original image. (**B**) Image following application of the "Find Edges" tool under the "Process" menu (Step 4.2). (**C**) Image following adjustment of the "Color Threshold" slidebar (Step 4.3). (**D**) Image after running the MRI_Wound_Healing_Tool plugin (Step 4.4). (**E**) Results pop-up window highlighting (red box) the numbers that are generated for each area. Please click here to view a larger version of this figure.

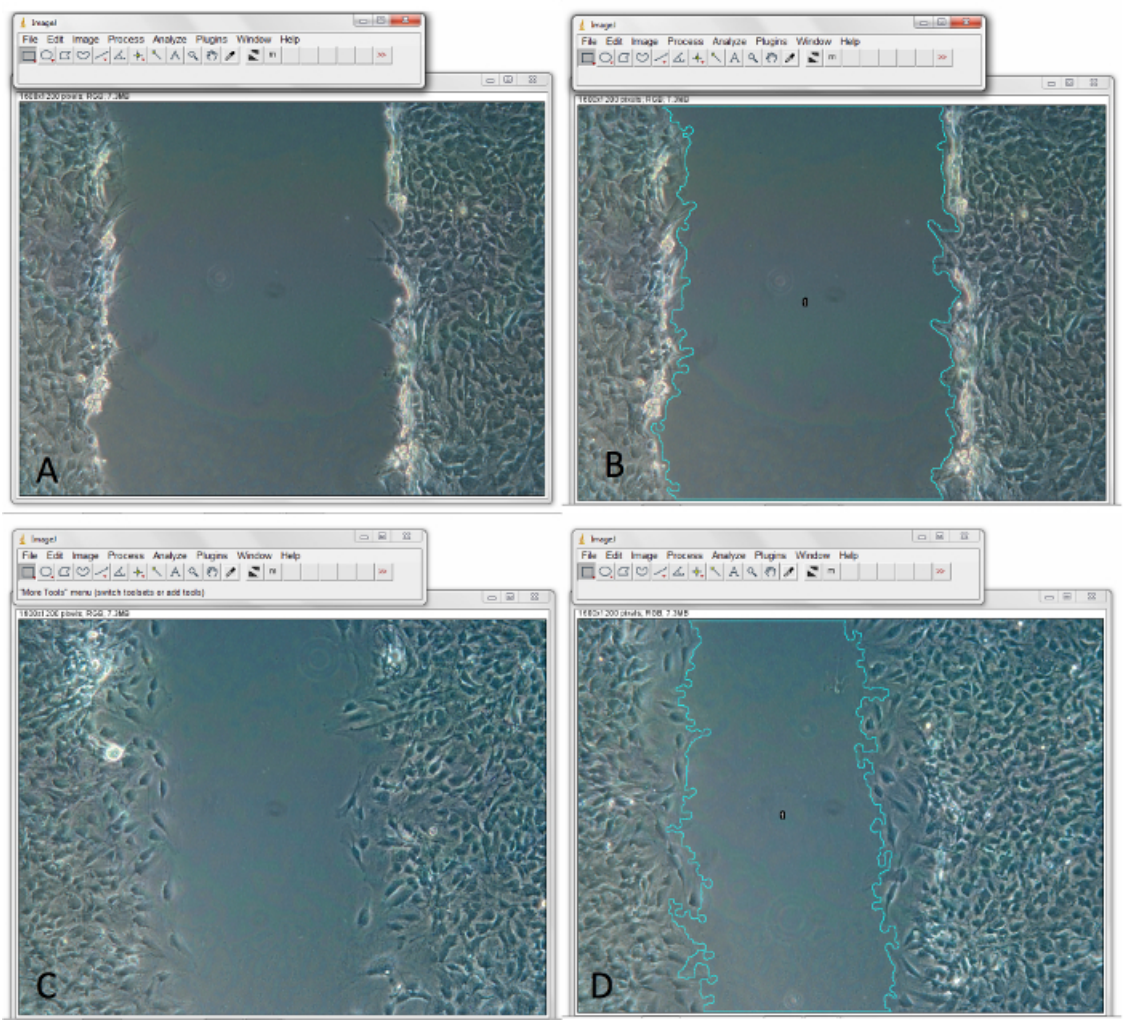

Figure 3: Representative images from scratch assay comparing scratched LR-MSCs before and after migration. (**A**) Representative original image pre-migration. (**B**) Outline of calculated scratch area pre-migration. (**C**) Representative original image post-migration. (**D**) Outline of calculated scratch area post-migration. Please click here to view a larger version of this figure.

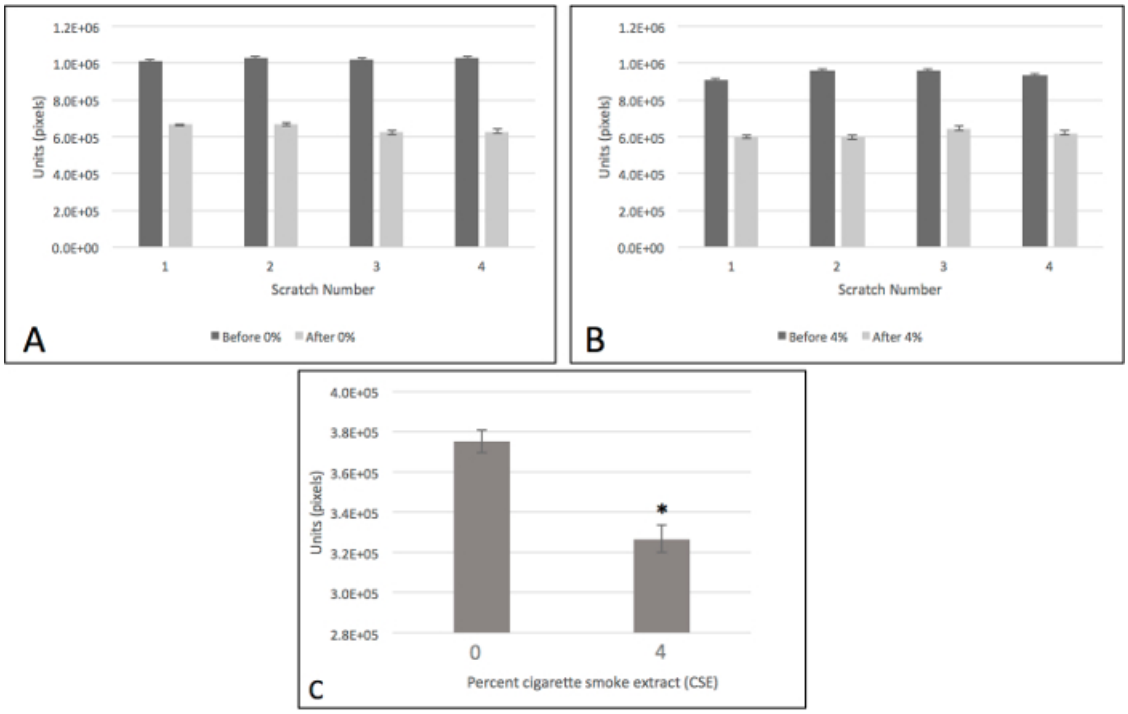

Figure 4: Representative results from scratch assay comparing quantification of scratch area before and after LR-MSC migration, and exposure to either 0% or 4% CSE. (**A**) Graphical representation of average calculated scratch area (in pixels) for each of 4 scratches made across a plate of LR-MSCs at 95% confluence either pre- or post-migration, with exposure to 0% CSE, with data presented as mean and standard deviation. (**B**) Graphical representation of average calculated scratch area (in pixels) for each of 4 scratches made across a plate of LR-MSCs at 95% confluence either pre- or post-migration, with exposure to 4% CSE, with data presented as mean and standard deviation. (**C**) Comparison of calculated average change in scratch area (post-pre migration) in LR-MSCs exposed to either 0% or 4% CSE, demonstrating less migration (lower average change in scratch area) after exposure to 4% CSE, with data presented as mean and standard deviation, *P = 2.99 x 10^{-8}. Please click here to view a larger version of this figure.

Discussion

The protocol described here provides a quantitatively robust, standardized method to perform and analyze scratch assays. Simple scratch assays are routinely used in many different areas of study to examine cellular migration. However, traditionally the scratch assay has lacked a standardized set-up and quantification protocol, which has led to issues with reproducibility [7,10]. Many of the modifications and optimizations for improving the scratch assay have reduced these issues, including individual live-cell imaging, standardized stopper-based assays, and three dimensional micro-pillar assays [4,8,10]. However, these assays can be difficult to customize or adapt [10] and for some applications, these techniques can also be cost prohibitive.

To produce a standardized scratch assay protocol, there are several critical steps in the protocol presented here that require specific information about the cells involved, and optimization of the assay based on the relevant cellular characteristics. In particular, it is important to optimize the number of cells needed and the time in culture necessary in order to achieve a uniform monolayer at 90-100% confluence for the assay (Step 1.3 and Step 1.4). With too few cells (less than 90% confluent), the cells will not form a sufficient monolayer. Not only will this affect the cellular microenvironment, but it will also affect the ability of the imaging software to correctly identify the edges of the scratch. In contrast, too many cells in the monolayer (100% confluent, or stacked cells) may well result in contact inhibition, and therefore change the migratory characteristics of the cells. Some variations of the scratch assay protocol recommend cell growth to 100% confluence and do not consider this potential limitation for certain contact-sensitive cells such as MSCs [8]. The same concerns should be applied to the degree of damage that is inflicted on the cells (Step 1.5). The protocol described here is designed to assess changes in cell migration after damage by exposure to soluble cigarette smoke on the migratory capacity MSCs, but this assay could be equally useful in assessing the effect of many different types of damage on cell migration. It is important that the afflicted damage is sufficient to provide a statistical distinction between the experimental and control groups. However, too much damage may also result in reduced cell confluence, which again may affect the ability of the imaging software to correctly identify the edges of the scratch.

Both cellular senescence and cellular proliferation can be confounding factors that can falsely alter the results of the scratch assay. In particular, the generation of the scratch should be carefully considered to avoid excessive damage to surrounding cells that might result in senescence. Scratching with a plastic pipette tip rather than a hypodermic needle or scalpel avoids excess damage while also preserving the integrity of the culture plate surface in the migration zone and the extracellular-matrix (Step 2.1). In addition, the length of time over which cell migration is allowed to occur must be carefully considered to avoid significant cell proliferation as a confounding variable (Step 3.7). For analysis of LR-MSC migration, 7 hr is optimal when assessing cells damaged with CSE. Incubating cells for greater than 12 hr is generally not recommended, however this may not apply to all cell types, especially those with very low doubling rates. To assess both cellular senescence and cell proliferation, a combination of senescence-associated beta-galactosidase and BrDU or EdU incorporation can be performed on the cells at the end of the assay [19]. Importantly, the protocol described here is accessible to labs with limited technical capacity, requiring only a good quality

inverted phase contrast microscope and camera. The reagents are inexpensive, and the imaging software is open source. However, in order to ensure optimal results from this assay, including good reproducibility and the ability to perform rigorous statistical analysis, careful optimization should be considered for each new cell type or experimental damage scenario that is attempted.

Optimizations presented in this protocol address some of the limitations present in previous work done to standardize scratch assays. Whereas scratch assay protocols traditionally take scratch width measurements from a snapshot of the scratch [8,9], the protocol presented here uses area measurements from images taken along the entire length of the scratch to increase statistical rigor and to take into consideration inconsistencies in the width of the initial scratch. Based on repeated testing (data not shown), scratch width cannot be assumed to be perfectly consistent throughout its length. After migration, the edges of the scratch become more heterogeneous, making it increasingly difficult to accurately calculate a scratch width, and further necessitate area calculations. This protocol uses MRI_Wound_Healing_Tool, a specialized computer program, in order to decrease subjectivity and increase consistency when analyzing scratch area. While it is unknown whether simple width calculations could yield results specific enough to distinguish between subtle differences in migration due to prior CSE or other damaging, this protocol demonstrates this sensitivity. Optimizations presented in this protocol revamp the common scratch assay to address these limitations in an economically feasible manner, to offer options for customization, and to provide additional techniques to demonstrate sensitivity and validate results.

The optimized quantitative scratch assay described in this protocol is still limited by considering a cell population, rather than individual cells. This technique assumes that the cell population is relatively homogenous, and that this will result in relatively homogenous migration characteristics. Depending on the application, it may be more relevant to consider the migration of individual cells instead [8]. Moreover, the scratch assay protocol described here is dependent on a population of cells that will not proliferate rapidly enough to confound analysis of migration over a 4-7 hr period. Cell lines that may proliferate extensively during this short time period would not be suitable for analyzing using this technique. Despite these limitations, this quantitative scratch assay protocol has the potential for use in many different applications. For example, a cell damage-based assay might be optimized and then used to assess the ability of particular treatments to limit cell damage. Alternatively, this assay might be used with cancer cell lines to assess potential treatments to limit migratory capacity, and therefore metastatic potential, of these cells. This optimized scratch assay is also still limited by possible disturbances to the cell microenvironment that are unavoidable in generating the scratch. However, by carefully selecting the cell confluence level, increased apoptosis and the generation of sheets of cells that have partially separated on the edge of the scratch can be avoided.

Disclosures

The authors declare that they have no competing financial interests.

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