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A Cost-Effective and Adaptable Scratch Migration Assay

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Corresponding Author:	Stephanie Burr University of Mississippi Oxford, MS UNITED STATES
Corresponding Author's Institution:	University of Mississippi
Corresponding Author E-Mail:	sburr@go.olemiss.edu
Order of Authors:	Stephanie Burr
	James A Stewart, Jr.
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TITLE:
A Cost Effective and Adaptable Scratch Migration Assay

AUTHORS:
Stephanie D. Burr*, James A. Stewart, Jr.

Department of BioMolecular Sciences, University of Mississippi, University, MS, USA

Email addresses of co-authors:
Stephanie D. Burr (sburr@go.olemiss.edu)
James A. Stewart, Jr. (jastewa7@olemiss.edu)

*Corresponding author:
Stephanie D. Burr (sburr@go.olemiss.edu)

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SUMMARY:
We present a cost-effective method to the scratch migration assay that provides a new approach for determining cell migration without the use of equipment-intensive methods. While fibroblasts were used in this protocol, it can be adapted and utilized to study additional cell types and influences on cell migration.

ABSTRACT:
Cell migration is a key component in both physiological and pathological events. Normal cell migration is required for essential functions such as development and mounting an immune response. When a defect or alteration occurs with the cell migration process, it can have detrimental outcomes (i.e., cancer metastasis, wound healing, and scar formation). Due to the importance of cell migration, it is necessary to have access to a cell migration assay that is affordable, adaptable, and repeatable. Utilizing the common scratch migration assay, we have developed a new approach to analyzing cell migration that uses general laboratory equipment. The method described uses visual markers that allow for recapturing specific areas of interest without the use of time-lapse microscopy. In addition, it provides flexibility in the experimental design, ranging from altering the migration matrix substrate to the addition of pharmacological modifiers. Furthermore, this protocol outlines a way to account for the area of cell migration, which is not considered by several methods when examining cell migration. This new approach offers a scratch migration assay to a larger audience and will provide greater opportunity for researchers to examine the physiological and pathophysiological impact of cell migration.

INTRODUCTION
Cell migration is crucial for many physiological as well as pathological events. It is required during development, for mounting an immune response, and for proper wound healing[1–3]. Many of

these cell migration events can be triggered by physical or chemical signals. For example, during an immune response, leukocytes will migrate towards a site of injury in response to a chemoattractant[2]. Additionally, leukocytes will also release cytokines to induce migration of additional immune cells, as well as other cell types, such as fibroblasts, which are involved in the wound healing process, and thus, initiating a multicellular response[4]. The ability of cells to migrate is essential for proper physiological function; however, when cell migration goes unchecked, it can have an adverse response and contribute to pathological events, such as chronic inflammation, vascular disease, cancer metastasis, and impaired wound healing[2,5–7]. Impaired wound healing is a common affliction of diabetics due to defects in cell migration, and if these defects are not addressed, it could lead to further complications (e.g., amputation[8,9]). This study, as well as others, have indicated the need to further understand the process by which cell migration occurs, either under normal physiological or pathological conditions, and it is vital for furthering this field of research. In order to accomplish this, there needs to be migration assays available that are both accessible and affordable to those researchers, who may not possess the equipment needed to conduct these assays.

Currently, there are a variety of migration assays available to examine a wide range of topics regarding cell migration. Both 2D and 3D migration models have been developed, each targeting specific areas impacting cell migration. 3D migration models are typically associated with cell invasion studies and assess the impact of extracellular matrix on cell migration[10–12], whereas 2D migration assays have a greater range of application and are primarily used to study chemotactic migration, wound healing, and functional changes during cell migration[13–16]. Several of these assays require additional equipment, such as Boyden chambers or exclusion rings, which can reduce the availability of these assays to certain researchers. One of the more cost-efficient assays is the scratch assay, which is typically used to assess wound healing and general changes in cell migration[14,17]. While most laboratories are equipped to conduct a scratch assay, the equipment used to track cell migration tend to either be unavailable or too expensive to purchase. This includes time-lapse microscopy, which requires an inverted microscope and a live imaging system. These expensive pieces of equipment are not commonly accessible to every laboratory. Therefore, this observation highlights the need for a new protocol that allows assessment of cell migration with more readily available equipment.

The protocol presented here provides a new and affordable way to assess cell migration. This method follows the same procedure associated with scratch assays but differs in the analysis of examining cell migration by utilizing equipment more commonly available in a basic sciences laboratory setting. This protocol using common equipment allows for a more accurate determination of cell migration without the use of time-lapse microscopy. In addition to determining migration, this method also accounts for variable factors in the scratch area that has been noted to greatly impact cell migration. Overall, this new protocol for cell migration analysis provides an opportunity for more laboratories to explore and contribute to the field of cell migration.

PROTOCOL

1. General cell culture

1.1. Culture cardiac fibroblasts in Dulbecco's Modified Eagles Medium (DMEM) containing 1 g/L glucose, sodium pyruvate, L-glutamine, and supplemented with 14.2 mM $NaHCO_3$, 14.9 mM HEPES, 15% heat-inactivated fetal bovine serum (FBS), 2% L-glutamine, and 0.02% antimicrobial reagent (see **Table of Materials**) and maintained in CO_2 incubator at 37 °C.

1.2. Culture cardiac fibroblasts until 90-95% confluency is reached at passage 0 (P0). At this point the fibroblasts are ready to be split into a 48-well plate, which is used for the migration assay.

2. Preparation of the migration plate

2.1. Prepare a 48-well cell culture plate by drawing a line, using a yellow or light-colored permanent marker, down the center of the well. Then, draw three hash marks dividing the well into three separate areas of interest. These sections will be used for imaging.

NOTE: Using a light color permanent marker will allow for easy imaging of migrating cells. Dark markers such as black or blue will prevent visualization of migrating cells.

2.2. If assessing migration on a substrate (e.g., collagen), coat the well at this time following instructions and concentrations used for the specific substrate.

2.3. Plate ~15,000-20,000 cardiac fibroblasts, P1, into each well and culture cells, under normal culturing conditions (conditions listed in previous section), until they reach 90-95% confluency. Confluency should be reached between 24-48 hours.

NOTE: When setting up the migration plate make sure to include a positive control (a known migratory cell, such as 3T3 cells[18]), a negative control (unscratched cells) and a blank well.

3. Scratch migration assay & fixation

3.1. Once cells reach 90-95% confluency, remove the media and scratch along the drawn line using a sterile P200 pipette tip.

NOTE: A P200 pipette tip is the common pipette tip sized used with the scratch assay[10,14,19]. Also, only make one pass with the P200 tip as more than one attempt may result in multiple scratch lines.

3.2. Rinse the well with low serum media (1.5% FBS) to remove any unattached cardiac fibroblasts.

3.3. Add 500 µL of low serum media to each well. If using any pharmacological agents, add them at this time.

NOTE: Low serum media is used because it allows/promotes cell migration to occur and prevents fibroblasts from proliferating which could skew the results of the migration assay[20]. For this protocol, 1.5% FBS was used.

3.4. Capture 0 h images before incubating the fibroblasts in a 5% CO_2 incubator at 37 °C for 24 h.

3.4.1. Capture 0 h images using an inverted microscope with a 20x objective. Take two 0 h images per well. This will allow for a more complete coverage of the line of migration.

3.4.2. Using the markings (line and dashes), position the well to capture the top half of the line of scratch. Avoid imaging the middle dash to ensure that the same area of migration is not imaged twice, which could skew results.

3.4.3. Use imaging software (**Table of Materials**) to capture 0 h image #1. Then move the plate to position the bottom half of the well/line of scratch in view of the camera. Again, avoid capturing the middle dash. Once in place, capture 0 h image #2 (**Figure 1**).

NOTE: Avoiding the middle dash in both 0 h images will prevent capturing areas of migration twice which if done could produce repeating results and skew the data.

3.5. After incubating for 24 h, remove the media from the well, and wash the well with 1x nonsterile PBS (henceforth all 1x PBS used is nonsterile).

3.6. In the fume hood, add 500 µL of 4% paraformaldehyde to the well and incubate for 10 minutes at room temperature (RT).

3.7. Remove paraformaldehyde and wash 3x with 1x PBS for 5 min each at RT.

3.8. Once cells are washed, proceed to capturing 24 h images or add 1x PBS to each well and place at 4 °C until a later time. Cells can stay at 4 °C for 1-2 weeks until ready to image.

3.8.1. Permeabilize the cells by adding 300 µL of permeabilizing solution (1x PBS and 0.01% triton X-100) to each well. Incubate cells/plate with gentle, continual rocking for 30 min at RT.

3.8.2. Remove blocking solution and add 1% Coomassie Brilliant Blue stain (3% Coomassie Brilliant Blue, 10% acetic acid, 45% methanol, and 45% dH_2O) for 10 min with gentle, continual rocking at RT.

NOTE: If plates are coated with an extracellular matrix substrate make sure to test a coated plate with Coomassie staining before conducting migration assay. **Figure 2** demonstrates that a matrix substrate can be used with this assay and not impact visualization of cells.

3.8.3. Wash wells 3x with 1x PBS at RT with continual rocking for 5 min each.

3.8.4. After washes, added 300 µL of 1x PBS and capture 24 h images.

3.8.5. Capture 24 h images by aligning the well into the same position that was used to capture the 0 h images. In order to accomplish this, open the previously captured 0 h images and using the marking made with the permanent marker, align the well into the same position. The line down the middle and additional dash marks should allow close alignment between the 24 h image and the 0 h image (**Figure 1**).

4. Preparation of 0 h and 24 h images

4.1. Open 0 h and 24 h images taken of the same well and position in imaging software (**Table of Materials**).

4.2. Create a new layer on the 0 h image. Then, click the new layer and change the name of the layer to “line layer” by double clicking on the text.

NOTE: This layer will be referred to as line layer from this point forth.

4.3. Click the **Brush** Tool (brush icon) and set the size at 10 px and the color to red.

4.4. With the line layer selected, draw two, separate lines that outline the area of the scratch. The lines should not touch any cells due to these lines marking the area of migration.

4.5. Click the **Move** Tool (arrow icon) and then press down **Ctrl** button and click on both the line and background layers.

4.6. Click in the center of the 0 h image and drag both these layers to the middle of the 24 h image.

4.7. Now using the 24 h image, click on the 0 h background layer and change the opacity to 50%.

4.8. Holding the **Ctrl** button, click on both the 0 h background and line layer and then free transform the layers (**Edit>Free Transform**). Using free transformation, align the 0 h background and line image to the 24 h background image. This step results in the overlaying of the 0 h and 24 h image, which is necessary to position the lines that mark the area of migration in the correct position on the 24 h image.

4.9. After successful overlaying of the 0 h background and line layers onto the 24 h background image, delete the 0 h background layer. Delete by clicking the 0 h background layer, then right click, and click delete layer.

4.10. Deleting the 0 h background image will leave just the line and the 24 h background layer (now referred to as migration image). The line layer will indicate the area of migration/scratch on the 24 h image and can be used for determining the number of migrating cells.

4.11. Save as the new migration image as both a photoshop file and a TIFF/JPEG. NOTE: a representative figure that depicts this process is presented in **Figure 3**.

5. Counting the number of migrating cells

5.1. Open the migration image. This can be done in a program that accepts TIFF/JPEG files (**Table of Materials**).

5.2. Count the number of cells that are located in between as well as touching the two red lines.

5.3. Record the number of migrating cells per image. There will be two migrating images per well, and these values should not be combined until after the values have been corrected for area of migration (detailed in next section).

6. Determine area of migration

6.1. Open the 24 h image that contains the lines of migration in the imaging software program in section 5 (**Table of Materials**).

6.2. Save As this image as “Migration Area Image”.

6.3. After saving, click the background layer, right click, and click delete layer. This should leave only the scratch lines on the image (**Figure 3**).

6.4. Using the Brush Tool (brush icon) fill in the area between the lines of scratch. Match the color of the brush with the color used to draw the lines for migration.

6.5. Change the image to a grayscale to generate a black and white image (**Image>Mode>Grayscale**) and then save the image (**Figure 4**).

6.6. Start the analysis software (**Table of Materials**) and then open the “Area of Migration” file within analysis software.

6.7. To determine the area of the line of migration, click **Image>Adjust>Threshold**. The black migration area will turn red and the percent area will be indicated in the Threshold box. The area will be reported as the percent of area the line of migration covers compared to the entire area captured in the image.

6.8. Record the percent area for the line of migration and make sure that this value is paired with the number of migrating cells for the same image.

7. Data analysis

7.1. Normalize the number of migrating cells to the percent area of the scratch. Divide the number of migrating cells by the percent area of the line of migration (# migrated cells ÷ % Area of migration). Do this per image and not an average based on migration per well.

7.2. After normalizing values per image, add the values of the two images, which represents an individual well. This value will be used for graphical and statistical analysis. If the experimental design contained multiple replicates. Average replicate values before statistical analysis.

REPRESENTATIVE RESULTS

This procedure documents a new approach to studying cell migration that is both cost-effective and easily adaptable for most labs. Many studies have used time-lapse microscopy to assess cell migration, but the equipment required for this method is not readily available to many laboratories. Whereas utilizing lines and dashes for demarcation allows for the ability to recapture specific areas of interest at different time points without the use of expensive equipment (**Figure 1 & Figure 3**). While the use of demarcations is essential for this new approach, there are many areas of this method that can be adapted to suit individual researcher needs. The protocol indicated a 24 h endpoint; however, that endpoint can be extended based upon an individual lab's needs. Adjusting the endpoint of the protocol can allow for continued culturing of cells for further use. In addition, this protocol permits the flexibility to test the impact of pharmacological modifiers as well as extracellular matrix substrates on migration. Lastly, the costliest component of this method is the use of imaging software, which may be licensed software, but the use of licensed software is not the only option. Other imaging software that allow the generation of lines and overlaying images can be utilized with this method. In addition, to the cost effective and adaptable nature of this method, it presents a new approach to examining cell migration by factoring the area of the scratch.

This new approach was recently used in Burr et al. 2020 to assess differences in cardiac fibroblast migration between cells isolated from non-diabetic and diabetic hearts[20]. **Figure 3** presents representative images used to assess fibroblast migration. From these images, it was determined that 46 fibroblasts from non-diabetic hearts and 129 fibroblasts from diabetic hearts had migrated during the 24 h time period of the experiment. Upon group comparisons, the number of cells from the diabetic hearts had migrated 2.8x greater than cells from non-diabetic hearts (**Table 1**). While these results indicated cells from diabetic hearts had migrated more, the numbers were misleading, because the area of the scratch was different for each of the two groups. The non-diabetic scratch area was 24.78% of the total area measured, whereas the diabetic migration scratch was 16.77% of the total area measured. When the area of the scratch was considered, it provided a ratio (cell number/% scratch area) indicating that fibroblasts from diabetic hearts actually migrated 4.13x greater than cells from non-diabetic hearts. These results highlighted the importance for considering area of migration when conducting migration assays.

Normalizing to the area of migration provides a better and more rigorous assessment of cell migration and negates potential human error. While the described method uses a P200 pipette tip, which should provide a uniform and consistent scratch, uneven scratches can occur due to human inconsistencies. **Figure 5** highlights the importance of factoring differences in scratch area. If one was to compare only the number of migrated fibroblasts, it would show that **Figure 5A** has twice the number of migrated cells compared to **Figure 5B**. Whereas when the area of the scratch is used to normalize the data, it indicates that the ratio of fibroblasts to migration area is similar in both **Figure 5A** and **Figure 5B**. For this example, we used untreated non-diabetic cardiac fibroblasts from different fibroblast isolations; therefore, a similar migration ratio should be the expected outcome due to the nature of the cells used in **Figure 5**. If one were presenting only the number of migrated cells, these finding could be misrepresentative if the scratched area is not uniform and consistent across all samples. Therefore, it is important to account for the scratched area with this method as well as other scratch migration assays. These represented results presented in **Figure 5** demonstrated how normalizing the number of migrated cells to the scratched area can present accurate, repeatable, and reliable migration data.

Figure 1: Diagram of the experimental design for the fibroblast scratch migration assay. Setup: Before plating cells into well, draw a line and 3 dash marks on the bottom on the plate. 0 h: Culture cells until 95% confluency and administer a scratch paralleling the depicted line. Capture 0 h images by selecting a section above and below the middle dash. 24 h: Allow cells to migrate for 24 h before fixing and staining. For 24 h images, align well into the same position as 0 h images to capture migrated cells. Analysis: Outline the area of migration on 0 h image and then overlay the 0 h image onto the 24 h image to generate migration and area images.

Figure 2: Representative images that show Coomassie staining does not interfere with visualization of cells. Cardiac fibroblasts were plated on either no collagen (plastic dish), collagen isolated from non-diabetic mice tails, or collagen isolated from diabetic mice tails. The cells were used in the scratch migration assay following the methods described here. Cells were stained with 1% Coomassie Brilliant Blue stain and then images of migrating cells were captured. The scale bar depicted on the image is 100 µm. Details on collagen isolation and/or cell migration on collagen are presented in Burr et al.[20].

Figure 3: Representative data demonstrating the difference in cardiac fibroblast migration between non-diabetic and diabetic cells. 0 h and 24 h images used to calculate the number of migrated cardiac fibroblasts isolated from non-diabetic and diabetic mice (red line depicts the area of migration and images taken at 20x with scale bar = 100 µm). Diabetic cardiac cells had 129 cells migrate in an area of 16.77%, which produced a migration ratio of 7.69. Non-diabetic fibroblasts had 46 cells migrate with an area of 24.78% which led to a ratio of 1.86. The images presented in this figure were used in the results presented in Burr et al.[20] but the images depicted here were not shown in Burr et al.[20].

Figure 4: Diagram that depicts the generation of area of migration image. Step #1: Open migration image that contains lines of migration. Step #2: The 24 h image is removed, leaving on

the lines of migration in the image field. Step #3: The brush tool was used to fill in the area of migration. Step #4: The image is converted to grayscale and then saved as a new image. Scale bar represents 100 µm.

Figure 5: An example of the impact of different migration areas on fibroblast migration. Non-diabetic cardiac fibroblasts were plated on plastic culture dishes and used in the scratch migration assay, as described above (scale bar = 100 µm). (**A**) 92 migrated fibroblasts with a percent area of 35.4% which resulted in a migration ratio of 2.60. (**B**) 45 fibroblasts migrated with an area of 17.57% that calculates a ratio of 2.56.

Table 1: Migration data from migration scratch assay using non-diabetic and diabetic cardiac fibroblasts. The number of non-diabetic and diabetic fibroblasts that migrated were determined using **Figure 4**. The percent area for each image was calculated using described methods and ImageJ. The ratio of migration was calculated by dividing the number of migrated fibroblasts by the percent migration area.

DISCUSSION

This new approach to the scratch migration assay provides a more accessible method for researchers to examine changes in cell migration. While this assay follows the same procedure for administering a scratch similar to other scratch assays, it does provide a new method for imaging and accurate analysis of cell migration[10]. Instead of using equipment-intensive methods of time-lapse microscopy and live cell imaging chambers, this method details the use of commonly available lab equipment. Utilizing a general inverted microscope and camera, one can capture migration images at the same time while maintaining consistent culture conditions. In addition, this method provides a precise imaging of the same region of interest without the use of advanced equipment. Capturing the same area of migration will reduce the inconsistencies in determining cell migration and provide a more rigorous and accurate measurement of cell migration. Lastly, this method takes into consideration the area of the scratch. While caution is taken to minimize human variations in scratches, inconsistencies still can occur, demonstrating the importance of using area as a normalizing factor into the analysis of cell migration. Overall, the protocol detailed above provides a new approach to a powerful tool commonly used to assess cell migration.

Developing an adaptable migration assay provides new avenues for research. The migration assay presented here has the ability to be modified in order to examine specific research questions. Modifications can be made regarding activating or inhibiting specific proteins of interest. Reagents such as pharmacological modifiers (agonists or antagonists) and RNA interference can be applied to migration assay before, during, or even after migration to address questions about migration and specific proteins. The small volume of the 48 well dish also allows for lower amounts of modifiers to be added, which is another cost-effective method. In addition, this assay can be modified to study the impact of extracellular matrix components (ECM) on cell migration. Recently we applied this method, where cell culture plates were coated with collagen isolated from diabetic and non-diabetic mice to assess the impact of diabetic extracellular matrix has on cardiac fibroblast migration[20]. While this study utilized isolated collagen, this method can

be adapted to other extracellular components that may be of interest. The ability to assess the impact of ECM migration is very useful due to multiple studies indicating the importance of ECM on migration[20–22]. A potential complication that may occur with the use of ECM proteins and coating the wells with highly concentrated ECM solution is an impact on visualization of cells. It is recommended if using ECM, like collagen, to coat a well and stain with Coomassie blue to see if the ECM could visually impair the imaging of cells. If ECM impairs visualizing cells, diluting ECM solution will improve this issue and allow for visualizing cells on ECM.

This new approach on an old technique does present some limitations. This method has been adapted to a small scale (48-well cell culture plate) and may not transition to larger plates easily. Due to the size of the well and the area captured in the images this protocol can document a large portion of the area of migration. However, expanding this method to larger well dimensions may result in capturing a lower portion of migration area. This can be potentially resolved by increasing the number of captured images, but additional methods may need to be applied to ensure the area of migration imaged can be reidentified for the 24 h images. In addition, this method is limited to cells that can be utilized in a scratch migration assay. Cells that do not respond in a traditional scratch migration assay may not be ideal for the approach presented within this manuscript. While there are some limitations with this approach, modifying the methods detailed in this manuscript could alleviate some of the limitations.

The scratch migration assay follows a simple approach but there are some critical steps that need to be followed to produce a successful assay. A crucial step is to draw the indicating marks on the bottom of the well. If the indicators are not drawn on the wells it will be very difficult/impossible to differentiate the area that needs to be imaged for the 24 h image as well as preventing recapturing the same area of migration. Also, it is important to recapture the same area of migration in the 24 h image that was captured in the 0 h image. If the same area is not captured at 24 h then overlaying the images for migration will not be feasible. Without overlaid images it will not be possible to determine which cells have migrated. The overlaid images are critical for this approach, because they provide an accurate determination for cell migration. Since the scratch method does not always provide straight scratch lines, it is critical that the correct areas be imaged to generate the overlaid images. The overlaid images provide the foundation for the accuracy of this migration assay presented within this manuscript.

A new adaptation of the scratch migration assay provides a more accessible and flexible approach to examining cell migration. Previous cell migration studies have used equipment-intensive methods that are not commonly available to every laboratory. Indicating that the development of a migration assay that has a wider range of accessibility is essential. This manuscript described a new approach to an old technique that will increase the accessibility to researchers interested in cell migration. In addition, this method provides the ability to alter the cell culture environment, whether via extracellular matrix components or the use of pharmacological modifiers, to determine the impact that has on cell migration.

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DISCLOSURES

The authors have nothing to disclose.

REFERENCES

1. Gilbert, S.F. *Developmental biology*. Sinauer Associates, Incorporated (1997).
2. Luster, A.D., Alon, R., Von Andrian, U.H. Immune cell migration in inflammation: present and future therapeutic targets. *Nature Immunology*. **6**, (2005).
3. Yahata, Y. et al. A Novel Function of Angiotensin II in Skin Wound Healing Induction of Fibroblast and Keratinocyte Migration by Angiotensin II via Heparin-Binding Epidermal Growth Factor (EGF)-like Growth Factor-Mediated EGF Receptor Transactivation. *The Journal of Biological Chemistry*. **281** (19), 13209–13216 (2006).
4. Trepat, X., Chen, Z., Jacobson, K. Cell Migration Single-Cell Migration. C*omprehensive Physiology*. **2** (4) (2012).
5. Brand, S. et al. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. *American Journal of Physiology: Gastrointestinal and Liver Physiology*. **290**, 827–838 (2006).
6. Chi, Z., Melendez, A.J. Role of Cell Adhesion Molecules and Immune-Cell Migration in the Initiation, Onset and Development of Atherosclerosis. *Cell Adhesion & Migration*. **1** (4), 171–175 (2007).
7. Chen, H., Nalbantoglu, J. Ring cell migration assay identifies distinct effects of extracellular matrix proteins on cancer cell migration. *BMC Research Notes*. **7** (183), 1–9 (2014).
8. Stewart, J.A., Massey, E.P., Fix, C., Zhu, J., Goldsmith, E.C., Carver, W. Temporal alterations in cardiac fibroblast function following induction of pressure overload. *Cell and tissue research*. **340** (1), 117–126 (2010).
9. Darby, I.A., Laverdet, B., Bonté, F., Desmoulière, A. Fibroblasts and myofibroblasts in wound healing. *Clinical, Cosmetic and Investigational Dermatology*. 7–301 (2014).
10. Kramer, N. et al. In vitro cell migration and invasion assays. *Mutation Research - Reviews in Mutation Research*. **752** (1), 10–24 (2013).
11. Even-Ram, S., Yamada, K.M. Cell migration in 3D matrix. *Current Opinion in Cell Biology*. (17), 524–532 (2005).
12. Valster, A. et al. Cell migration and invasion assays. *Methods*. **37**, 208–215 (2005).
13. Pijuan, J. et al. In vitro cell migration, invasion, and adhesion assays: From cell imaging to data analysis. *Frontiers in Cell and Developmental Biology*. **7** (JUN), 1–16 (2019).
14. Liang, C.-C., Park, A.Y., Guan, J.-L. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols*. **2** (2), 329–333 (2007).
15. Hulkower, K.I., Herber, R.L. Cell migration and invasion assays as tools for drug discovery. *Pharmaceutics*. **3** (1), 107–124 (2011).
16. Walter, M.N.M., Wright, K.T., Fuller, H.R., MacNeil, S., Johnson, W.E.B. Mesenchymal stem cell-conditioned medium accelerates skin wound healing: An in vitro study of fibroblast and keratinocyte scratch assays. *Experimental Cell Research*. **316** (7), 1271–1281 (2010).
17. Chaudhary, A., Bag, S., Barui, A., Banerjee, P., Chatterjee, J. Honey dilution impact on in

vitro wound healing: Normoxic and hypoxic condition. *Wound Repair and Regeneration*. **23** (3), 412–422 (2015).
18. Lipton, A., Klinger, I., Paul, D., Holleyt, R.W. *Migration of Mouse 3T3 Fibroblasts in Response to a Serum Factor*. *Proceedings of the National Academy of Sciences of the United States of America*. **68** (11) (1971).
19. Ascione, F., Guarino, A.M., Calabrò, V., Guido, S., Caserta, S. A novel approach to quantify the wound closure dynamic. *Experimental Cell Research*. (352), 175–183 (2017).
20. Burr, S.D., Harmon, M.B., Jr, J.A.S. The Impact of Diabetic Conditions and AGE/RAGE Signaling on Cardiac Fibroblast Migration. *Frontiers in Cell and Developmental Biology*. **8** (2020).
21. Chang, S.S., Guo, W.-H., Kim, Y., Wang, Y.-L. Guidance of Cell Migration by Substrate Dimension. *Biophysical Journal*. **104**, 313–321 (2013).
22. Nguyen-Ngoc, K.-V. et al. ECM microenvironment regulates collective migration and local dissemination in normal and malignant mammary epithelium. *Proceedings of the National Academy of Sciences of the United States of America*. **109** (39), 2595–2604 (2012).

Figure 1

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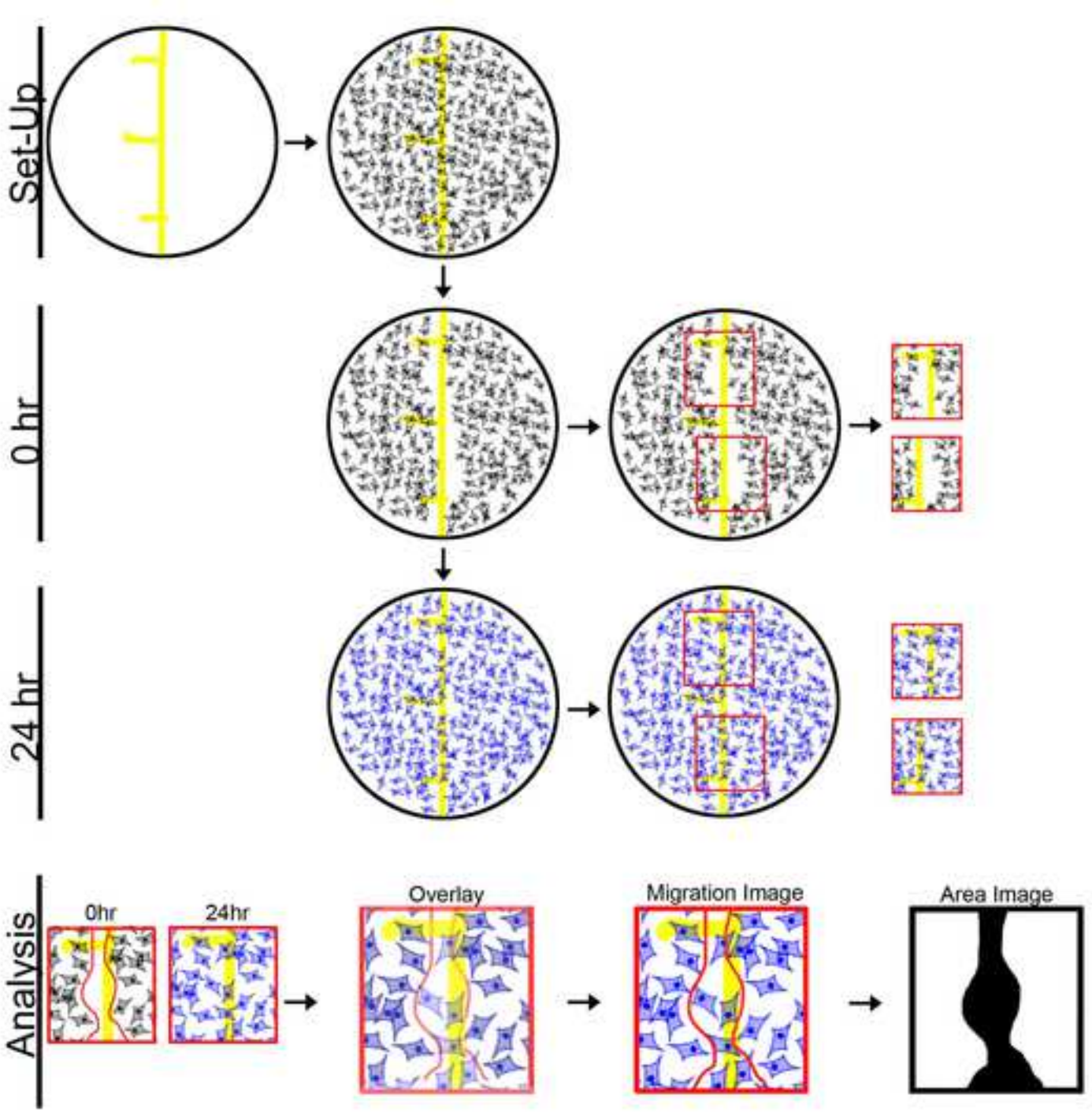

Figure 2

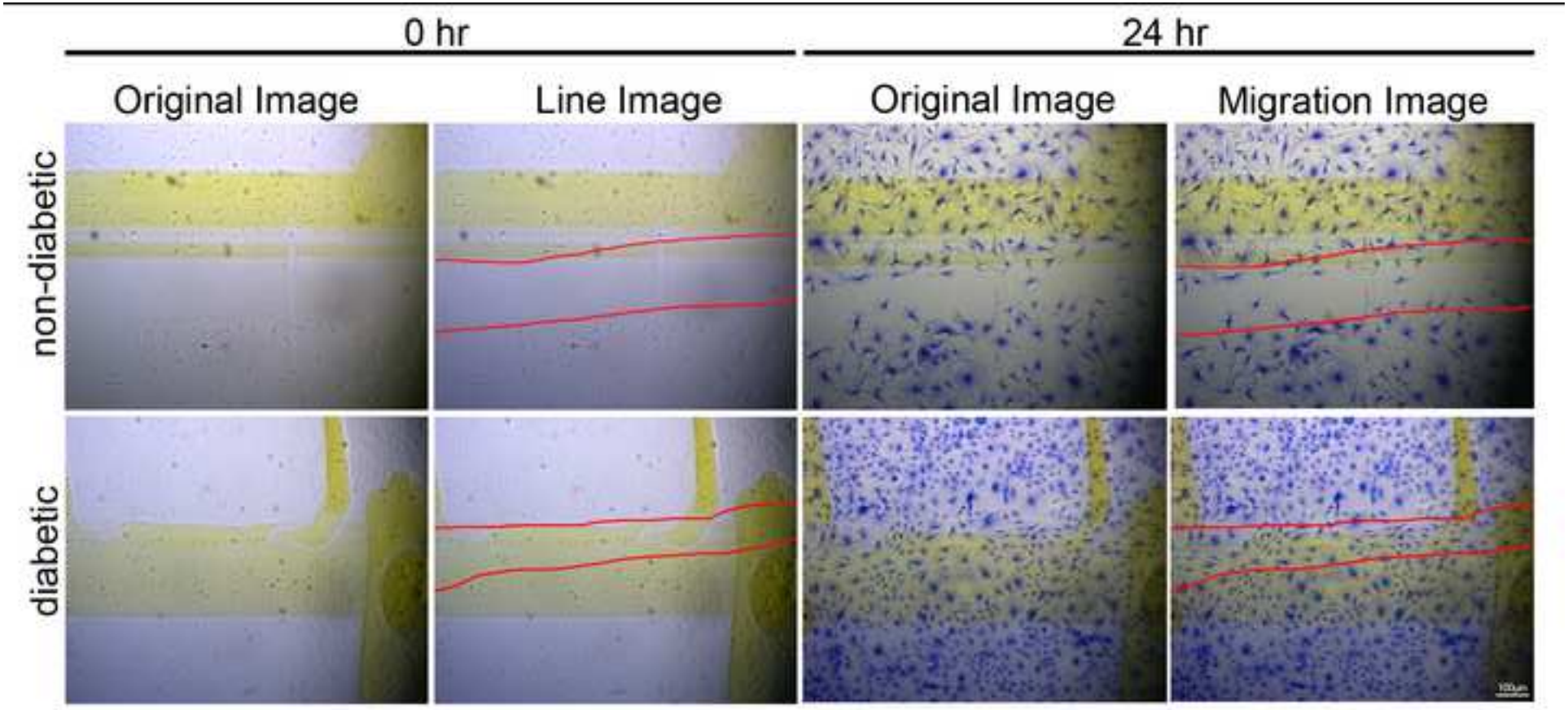

Figure 3

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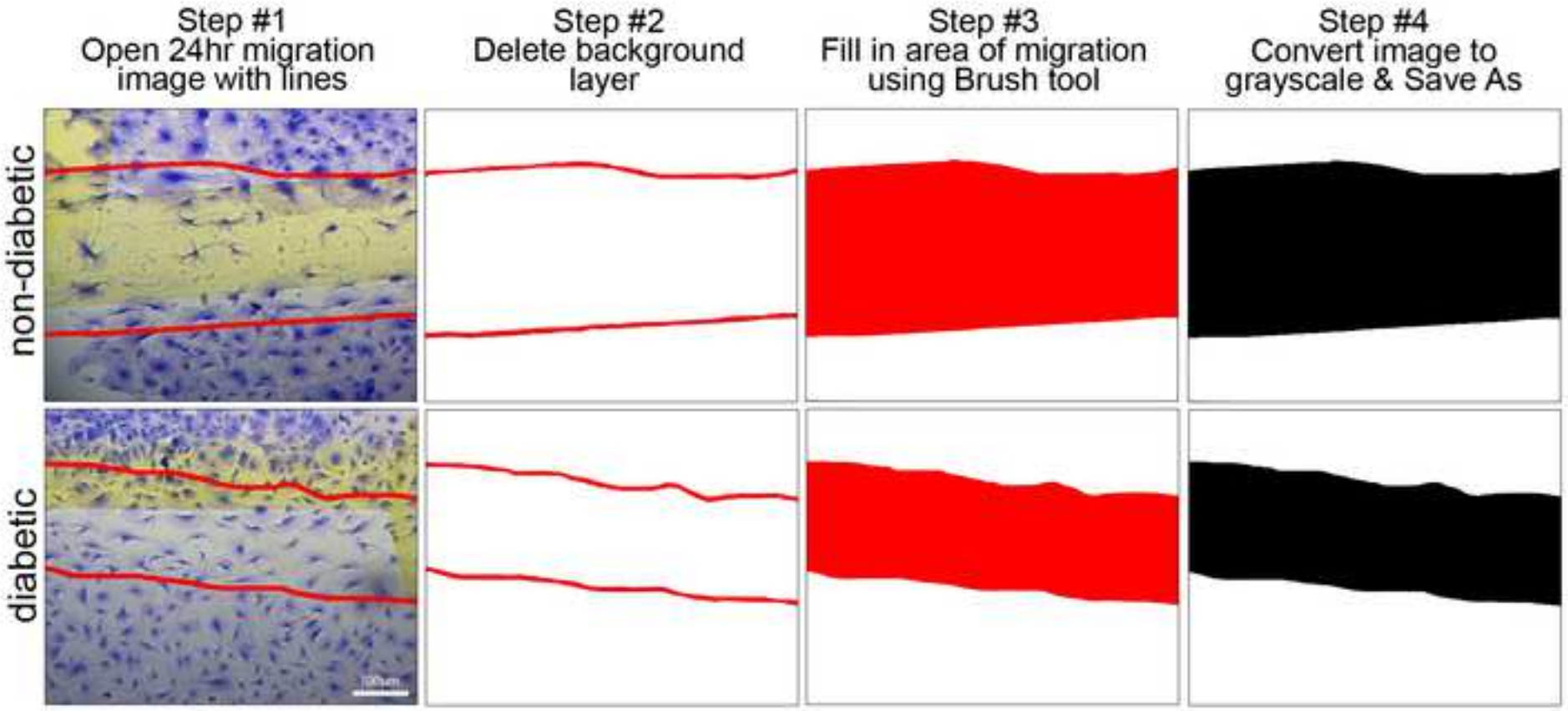

Figure 4

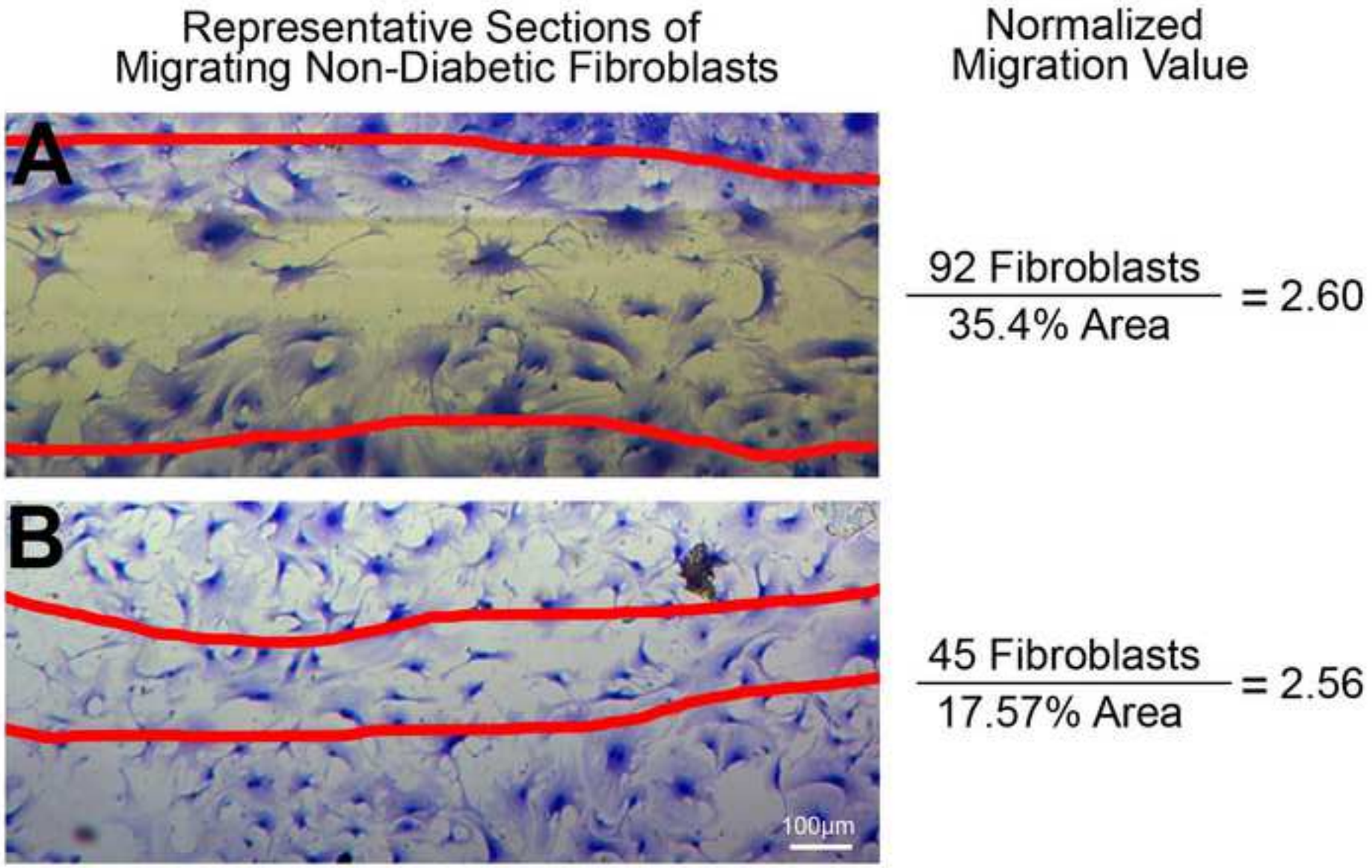

Figure 5

Click here to access/download;Figure;Figure 5.psd

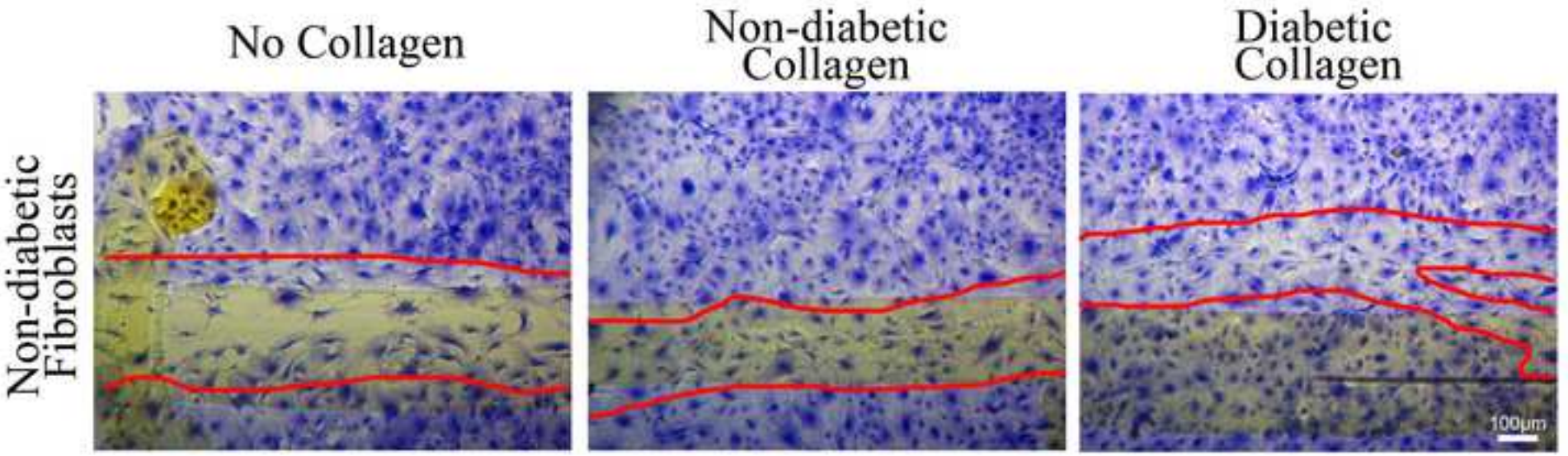

Table 1. Example Calculation of Cell Migration to Scratched Area Ratio

Fibroblast Type	Average Number of Migrated Cells	Average Area of the Scratch
Fibroblasts from Non-Diabetic Hearts	46	24.78%
Fibroblasts from Diabetic Hearts	129	16.77%

Cell Number to Scratched Area Ratio	
1.86	
7.69	

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Adobe All Apps	Adobe		This includes Adobe photoshop which is the imaging software used with this protocol
Avant Pipette Tips 200ul Binding non-sterile	MIDSCI	AVR1	Tips are autoclaved to sterilize
AxioCam Erc 5s Camera	Zeiss	426540-9901-000	
Coomassie Brilliant Blue R-250	Fisher Scientific	BP101-25	
Costar Flat Bottom Cell Culture Plates 48 Wells	Fisher Scientific	07-200-86	
DMEM with L-Glutamine, 1g/L glucose and sodium pyruvate	Fisher Scientific	MT10014CM	
Image J	NIH		This is a free software offered by the US government and is the analysis software used with this protocol
Paraformaldehyde	Fisher Scientific	AC416785000	
Premium US origin fetal bovine serum	Innovative Research	IFBS-HU	
Primocin	InvivoGEN	ant-pm-2	This is the anitmicrobial used for with this procotol to culture cardiac fibroblasts
Zeiss Primovert Microscope	Zeiss	491206-0002-000	
Zen Blue Edition 2.3 software	Zeiss		software comes with camera purchase

The authors would like to thank the editors and reviewers for their time and expertise in reviewing this manuscript. The authors' responses are included below.

Editorial Comments:

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.*

   The authors have reviewed the manuscript and have corrected for spelling and grammatical errors. Please note, should additional errors be found, we will make further corrections.

2. ***Protocol Detail:*** *Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video.* ***Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.*** *There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.*

   We have made changes to the manuscript to provide clearer method details. We believe that there is sufficient information for those to replicate this protocol.

3. ***Protocol Highlight:*** *Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.*
   1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
   2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
   3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
   4) Notes cannot be filmed and should be excluded from highlighting.

   Portions of the manuscript that we would liked to be included in the video have been highlighted.

4. ***Discussion:*** *JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.*

   Substantial edits have been made to the discussion to focus on the protocol presented within the manuscript and covers the topics listed above.

5. ***Figures:***
   a. *Add scale bars to all micrographs.*
   b. *Please list figures in the order of reference. Currently Fig 4 is called out before 2, 3.*

   Scale bars have been added to the appropriate figures, and the order of the figures presented within the manuscript have been changed.

6. ***References:*** *Please spell out journal names.*

   The authors have changed the citation format to the format required by JoVE, which includes spelling out names of journals cited.

7. ***Commercial Language:*** *JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Primocin™, Zeiss Zen Blue Software,*
   1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
   2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

   The authors have removed all commercial sounding language and TM/R from the manuscript. All commercial products necessary for the protocol are listed in the table of materials and are referred to as "see table of materials" within the manuscript.

8. ***Table of Materials:*** *Please sort alphabetically.*

   All materials required for the protocol have been listed alphabetically in the table of materials.

   • *If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."*

   All figures that presented within this manuscript are original and have not been presented or published elsewhere.

<u>Comments from Peer-Reviewers:</u>

Reviewer #1:
Manuscript Summary:
Overall good

Major Concerns:

1. *Include scale bars in the given images as well as in figure legends.*

   The authors have added scale bars to all appropriate images, and the scale bars have been presented in the figure legends.

2. *The title A COST EFFECTIVE AND ADAPTABLE FIBROBLAST SCRATCH MIGRATION ASSAY is not reflective, Author should clarify whether the present work only for Fibroblast or any in vitro adherent cell type, If this protocol is for only fibroblasts, why fibroblasts excluded in abstract.*

   The reviewer makes a good point. This method is not exclusive to fibroblasts but can be used with any cell type that will respond using a scratch migration assay. Due to this poin,t we have removed the fibroblast word from the title to better reflect the methods described within the protocol.

3. *What is the author's take home message from this article?*

   The main message of this manuscript is to provide a scratch migration assay that is both cost effect and adaptable. We have better highlighted this main idea within the introduction and the discussion.

4. *How author insure that only Pipette Tips 200ul is appropriate for making scratches. Justify.*

   A P200 pipette is commonly used with the scratch migration assay as documented by cited references due to the surface area of the tip. While other tips could be utilized with this protocol we decided not to use a different sized tip. Instead we used a P200 which has been documented within the literature to be appropriate for the scratch migration assay. We have included additional references within the manuscript to provide further support for the use of a P200 pipette tip.

5. *Author should justify the overlaying of images in more detail.*

   The authors have provided more justification for the importance of overlaying images within the discussion.

6. *Author should include wound healing/Scratch migration rate quantification/formula.*

   The authors have included the migration rate quantification formula as well as added more details concerning the quantification method used in the manuscript.

7. *Kindly refer articles like: Walter et al (2010). Mesenchymal … scratch assays. Experimental cell research, 316(7), 1271-1281; Chaudhary et al. (2015). Honey dilution impact… condition. Wound Repair and Regeneration, 23(3), 412-422 etc.*

   These additional articles have been referenced within the manuscript.

8. *Author should mention positive, negative controls and blank.*

   The authors have included the use of positive and negative controls as well as a blank well to serve as a more stringent control.

9. *Critical steps not highlighted.*

   The authors have highlighted and noted the critical steps within this assay.

Minor Concerns:

1. *Errors in formatting of references. Current references should be included.*

The authors have reformatted the references according to JoVE requirements.

Reviewer #2:
Manuscript Summary:
This manuscript describes a new approach of an old technique increasing its accessibility and precision in the evaluation of cell migration, but although he states that the method can also be used when the cell culture plates were coated with collagen, the efficacy of the protocol is not demonstrated.

Major Concerns:

1. *The concentration of the Coomassie Brilliant Blue dye used (1%) is very high compared to the methods described in the literature (see Yohichi Mochizuki and Kazunori Furukawa, Application of Coomassie Brilliant Blue Staining to Cultured Hepatocytes, Cell Biology International Reports, Vol. 11, No. 5, May 1987). In addition, the composition of the dye solution is not given and at point 4.2.2. the name of the dye is reversed. The reason for using this concentration should be explained in relation with the literature data. Also, it must be demonstrated that the 1% Coomassie Brilliant Blue dye does not stain the collagen used in the coating of the cell culture plates and does not interfere with the cells visualization method. Taking into account that the staining method is used in collagen zymography approach (Seniz Inanc, Didem Keles, and Gulgun Oktay, An improved collagen zymography approach for evaluating the collagenases MMP-1, MMP-8, and MMP-13, BioTechniques 2017 63:4, 174-180), images of collagen-coated plates stained according to the protocol in the presence and in the absence of cultured cells should be provided.*

   The reviewer makes a very good point. Highly concentrated matrix components stained with 1% Coomassie Brilliant Blue stain would impact the visualization of cells hindering interpretation of migration assay results. The authors have included additional information in both the protocol and discussion sections that highlight the need to test coated plates with 1% Coomassie Brilliant Blue stain prior to conducting migration assay. In the manuscript we recommend highly concentrated matrix components be diluted to a concentration whereby interpretation of migration results will not be impacted. The authors have also included an additional figure illustrating plates coated with matrix components, in this case collagen, and stained with 1% Coomassie Brilliant Blue stain. As demonstrated staining of the collaged does not impair visualization of cells. Also, the authors have included details regarding the materials and protocol to replicate Coomassie Brilliant Blue staining within the protocol section.

Minor Concerns:

2. *The figure 4 is mentioned before figures 2 and 3.*

   The order of the figures has been corrected.

3. *In the case of figures 2, 3 and 4 the magnitude and bar scale are omitted, inducing the impression that the compared images had different magnitudes and bar scales.*

   Scale bars have been added to all the appropriate images/figures.

Reviewer #3:
Manuscript Summary:
This paper handles interesting topic "a cost effective and adaptable fibroblast scratch migration assay". I think that using marking to improve analysis accuracy is a good idea. Although live cell imaging technique is widely used in many laboratories, it requires a little bit expensive equipment.

It will be a good paper if some issues are resolved.

Major Concerns:

1. *The described method has already been introduced in author's other paper. Are there any duplication issue?*

   There are not no issues with duplication between this manuscript and the previous published manuscript. The published manuscript provides a general outline of the methods used. This manuscript provides a detailed methodology. All figures are original and not duplicated from previously published work.

Minor Concerns:

2. *How do they differentiate between proliferated cells and migrated cells?*

   The review makes a valid point concerning differentiating between proliferating versus migrating cells. This concern has been addressed within the published manuscript. The use of a low fetal bovine serum in the media will reduce the amount of cell proliferation occurring to levels that would not impact the values of cell migration. The authors have included the reference for this published manuscript within the protocol to highlight this point as well as provided justification for the use of low serum within the assay.