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Title: A Cost Effective and Adaptable Scratch Migration Assay

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

Videographer: All video files have been uploaded, do not film screen captures.

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **45**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **James Stewart Jr.**: This protocol provides a new approach for an old method that will increase its availability to researchers **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **James Stewart Jr.**: The main advantages of this technique are its cost-effective nature and adaptability, which allow for a wider use by researchers **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Introduction of Demonstrator on Camera

1.3. **James Stewart Jr.**: Demonstrating the procedure will be Stephanie Burr, a PhD graduate student from my laboratory **[1][2]**.

1.3.1. INTERVIEW: Author saying the above

1.3.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera

Protocol

2. Migration Plate Preparation

2.1. To prepare a migration plate, use a light-colored permanent marker to draw a line down the center of the back of each well of a 48-well plate **[1]** and to draw three hash marks to divide each well into three separate areas of interest **[2]**.

2.1.1. WIDE: Talent drawing line in well

2.1.2. Well being divided

2.2. Then plate 1.5-2 x 10^4 single-passage cardiac fibroblasts into each well **[1-TXT]** and culture the cells under normal culture conditions for 24-48 hours until they reach 90-95% confluency **[2-TXT]**.

2.2.1. Talent adding cells to well(s), with medium container visible in frame **TEXT: Include positive control (known migratory cell), negative control (unscratched cells), and blank wells**

2.2.2. Talent placing plate into incubator **TEXT: See text for all medium and solution preparation details**

3. Scratch Migration Assay

3.1. When the cells are ready, remove the supernatant from each well **[1]** and use a sterile P200 pipette tip to make a one-pass scratch along the drawn lines **[2]**.

3.1.1. Supernatant being removed

3.1.2. Cells being scratched along line

3.2. Rinse the wells with low serum medium to remove any unattached cardiac fibroblasts **[1]** and add 500 microliters of low serum medium to each well **[2]**.

3.2.1. Well being rinsed, with medium container visible in frame

3.2.2. Talent adding medium to well(s), with medium container visible in frame

3.3. If pharmacological agents are being used, add the agents to the appropriate wells **[1]**.

3.3.1. Talent adding agent, with agent container visible in frame

3.4. Next, use an inverted microscope with a 20x objective to capture two, 0-hour images per well **[1]**, using the markings to position each well to allow imaging of the top half of the scratched line **[2-TXT]**.

3.4.1. Talent placing plate onto microscope stage *Videographer: Important step*
3.4.2. SCREEN: screenshot_7: 00:10-00:18 **TEXT: Avoid imaging middle dash to ensure same area not imaged twice**

3.5. Then move the plate to position the bottom half of the scratched line into view to collect the second image **[1]** and place the plate in the cell culture incubator for 24 hours **[2]**.

3.5.1. SCREEN: screenshot_7: 00:30-00:38
3.5.2. Talent placing plate into incubator

4. Fixation

4.1. At the end of the incubation, wash each well with non-sterile PBS **[1]** and add 500 microliters of 4% paraformaldehyde to each well in a fume hood **[2]**.

4.1.1. WIDE: Talent washing well(s), with PBS container visible in frame
4.1.2. Talent adding PFA to well, with PFA container visible in frame

4.2. After 10 minutes at room temperature, wash each well three times in fresh PBS for 5 minutes per wash **[1-TXT]**.

4.2.1. Talent washing well(s), with PBS container visible in frame **TEXT: Optional: Store plate at 4 °C for 1-2 wks before imaging**

4.3. After the last wash, permeabilize the cells with 300 microliters of permeabilizing solution per well and gentle rocking for 30 minutes at room temperature **[1]**.

4.3.1. Talent adding solution to well, with solution container visible in frame

4.4. At the end of the incubation, replace the permeabilizing solution with 1% Coomassie Brilliant Blue stain for a 10-minute incubation with gentle rocking at room temperature **[1]** followed by three, 5-minute washes in PBS with rocking **[2]**.

4.4.1. Talent adding stain to well(s), with stain container visible in frame
4.4.2. Plate on rocker, with PBS container visible in frame

4.5. After the last wash, add 300 microliters of PBS to each well **[1]** and carefully position the plate in the same position as for the 0-hour image **[2]** before capturing two, 24-hour images per well as demonstrated **[3]**.

4.5.1. Talent adding PBS to well(s), with PBS container visible in frame
4.5.2. Talent lining up plate *Videographer: Difficult step*
4.5.3. Talent at microscope, imaging plate

5. Image Analysis

5.1. At the end of the experiment, open the images in an appropriate imaging analysis software program **[1]** and create a new layer on the 0-hour image **[2]**.

5.1.1. WIDE: Talent opening image(s), with monitor visible in frame
5.1.2. SCREEN: screenshot_1: 00:15-00:19

5.2. Double-click to change the name of the new layer "line layer" and select the **Brush** tool **[1]**.

5.2.1. SCREEN: screenshot_1: 00:19-00:30

5.3. Change the pixel size to 10 and the color to red and draw two separate lines to outline the scratch. The lines should not touch any cells **[1]**.

5.3.1. SCREEN: screenshot_1: 00:31-01:10 *Video Editor: please speed up*

5.4. Open the 24-hour image in the imaging software and select the **Move** tool **[1]**.

5.4.1. SCREEN: screenshot_2: 00:07-00:28 *Video Editor: please speed up*

5.5. Holding down the **Control** button, click both the lines and the background layers **[1]**.

5.5.1. SCREEN: screenshot_2: 00:28-00:35

5.6. Click in the center of the 0-hour image and drag both layers to the middle of the 24-hour image **[1]**.

5.6.1. SCREEN: screenshot_2: 00:35-00:42

5.7. In 24-hour image, click the 0-hour background layer and change the opacity to 50% **[1]**.

5.7.1. SCREEN: screenshot_2: 00:43-00:54

5.8. Holding the **Control** button, click both the 0-hour background and line layers and click **Edit** and **Free Transform** to free transform the layers **[1]**.

5.8.1. SCREEN: screenshot_2: 00:55-01:06

5.9. Using free transformation, align the 0-hour background and line images to the 24-hour background image so that the area migration lines are positioned correctly in the 24-hour image **[1]**.

5.9.1. SCREEN: screenshot_2: 01:07-1:20

5.10. When the line layer has been successfully overlaid, right-click to delete the 0-hour background layer. The remaining line layer can be used determine the number of cells that migrated into the scratch area over 24 hours **[1]**.

5.10.1. SCREEN: screenshot_2: 01:21-01:32

5.11. Then save the new migration image as both a photoshop and a tiff or jpeg file **[1]**.

5.11.1. SCREEN: screenshot_2: 01:33-01:57 *Video Editor: please speed up*

6. Migrating Cell Quantification and Migration Area Determination

6.1. To quantify to the number of migrating cells, open the migration image in a program that accepts tiff or jpeg files **[1]** and manually count the number of cells located in between and touching the two red lines for both images for each well **[2]**.

6.1.1. WIDE: Talent opening image(s), with monitor visible in frame
6.1.2. SCREEN: screenshot_3: 00:15-00:25

6.2. Then record the number of migrating cells per image **[1]**.

6.2.1. SCREEN: screenshot_3: 00:32-00:40

6.3. To determine the area of migration, open the 24-hour image that contains the lines of migration in the imaging software and save the image as “Migration Area Image” **[1]**.

6.3.1. SCREEN: screenshot_4: 00:05-00:28 *Video Editor: please speed up*

6.4. After saving, click the background layer and right-click to delete the layer **[1]**.

6.4.1. SCREEN: screenshot_4: 00:34-00:39

6.5. Use the **Brush** tool to fill in the area between the scratch lines with the same color that was used for the lines **[1]** and click **Image**, **Mode**, and **Greyscale** to change the image to black and white **[2]**.

6.5.1. SCREEN: screenshot_4: 00:40-01:53 *Video Editor: please speed up*
6.5.2. SCREEN: screenshot_4: 01:59-02:08

6.6. Save the image **[1]** and open the "Migration Area Image" in an appropriate image analysis software program **[2]**.

6.6.1. Talent saving image, with monitor visible in frame
6.6.2. SCREEN: screenshot_5: 00:02-00:08

6.7. Click **Edit** and **Invert [1]**.

6.7.1. SCREEN: screenshot_5: 00:10-00:17

6.8. To determine the area of the line of migration, click **Image**, **Adjust**, and **Threshold**. The black migration area will turn red and the percent area will be indicated in the **Threshold** box **[1]**.

6.8.1. SCREEN: screenshot_5: 00:18-00:27 *Video Editor: please emphasize percent area in Threshold box when mentioned*

6.9. Then record the percent area for the line of migration and confirm that this value is paired with the number of migrating cells for the same image **[1]**.

6.9.1. SCREEN: screenshot_5: 00:30-00:38

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
3.4.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
4.5. This step may not be the most difficult, but it is the most time consuming and a very critical step. This step requires lining up the cell culture plate in the location as the cell culture plate was when the 0 hr image was captured. In order to ensure success, do not rush, take the time to make sure the plate/well is lined up in the same manner as the 0 hr image.

Results

7. Results: Representative Scratch Migration Assay Analyses

7.1. In this analysis, 46 fibroblasts from non-diabetic hearts **[1]** and 129 fibroblasts from diabetic hearts migrated during the 24-hour experimental time period **[2]**.

7.1.1. LAB MEDIA: Figure 2 and Table 1 *Video Editor: please emphasize cells between red lines in 24 hr non-diabetic Migration image and 46 data cells*
7.1.2. LAB MEDIA: Figure 2 and Table 1 *Video Editor: please emphasize cells between red lines in 24 hr diabetic Migration image*

7.2. Measurement of the migration area as demonstrated **[1]** revealed that the non-diabetic scratch area made up 24.78% of the total area measured **[2]**, while the diabetic migration scratch area made up 16.77% of the total area measured **[3]**.

7.2.1. LAB MEDIA: Figure 2 and Table 1
7.2.2. LAB MEDIA: Figure 2 and Table 1 *Video Editor: please emphasize 24.78% data cell*
7.2.3. LAB MEDIA: Figure 2 and Table 1 *Video Editor: please emphasize 16.77% data cell*

7.3. Normalizing to the area of migration provides a better and more rigorous assessment of cell migration and negates potential human error **[1]**.

7.3.1. LAB MEDIA: Figure 4

7.4. For example, if only the number of migrated fibroblasts are compared, it would appear in this analysis that the number of cells that migrated in this well **[1]** was twice that of the migrated cells in this second well **[2]**.

7.4.1. LAB MEDIA: Figure 4 *Video Editor: please emphasize Figure 4A image and 92 Fibroblasts text*
7.4.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize Figure 4B image and 45 Fibroblasts text*

7.5. When the area of the scratch was used to normalize the data **[1]**, however, the ratio of the fibroblasts to the migration area was observed to be nearly the same in both wells **[2]**.

7.5.1. LAB MEDIA: Figure 4 *Video Editor: please emphasize 35.4% and 17.57% area texts*

7.5.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize 2.60 and 2.5.6 texts*

Conclusion

8. Conclusion Interview Statements

8.1. **Stephanie Burr**: Be sure to recapture the same area of migration in the 24-hour image as was captured in the 0-hour image **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (4.5.)

8.2. **Stephanie Burr**: Following this procedure, immunohistochemistry can be conducted to examine the protein expression within the cells **[1]**.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

8.3. **James Stewart Jr.**: We believe that this new approach will allow greater access to the scratch migration assay, which could open up multiple avenues of research that might not have been previously available **[1]**.

8.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*