Chapter 14

Determination of Breast Cancer Cell Migratory Ability

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Abstract

Cell migration is defined as the movement of individual cells, sheets of cells, or clusters of cells from one location to another (Friedl et al., Int J Dev Biol 48:441–449, 2004). This ability of cells to migrate is critical to a wide variety of normal and pathological processes, including embryonic development, wound healing, immune responses, and cancer (Leber et al., Int J Oncol 34:881–895, 2009). Migration of tumor cells is widely thought to be an essential component of the metastatic spread of tumor cells to new sites, and inhibiting metastasis is an important therapeutic goal in cancer treatments (Horwitz and Webb, Curr Biol 13:R756–759, 2003). Therefore, the ability to observe and quantify migration in cancer cells is critical not only for basic cancer biology but especially for drug development (Friedl and Gilmour, Nat Rev Mol Cell Biol 10: 445–457, 2009). Researchers continue to develop new techniques for measuring cell migration in vitro. This chapter will discuss two techniques commonly used to study cell migration: wound healing and Boyden chamber migration assays.

Key words Migration, Wound healing, Time-lapse microscopy

1 Introduction

1.1 Wound-Healing Assay Using Time-Lapse Microscopy

Tissue wounds initiate a complex series of cellular events that promote the proliferation and migration of cells in order to close the wound [5]. Since these changes may be observed on a microscopic scale, researchers have taken advantage of these phenomena to study the migration of cells. Such studies are especially pertinent to cancer cells, since migratory ability is a major factor in determining cancer aggressiveness [6]. Researchers believe determining migratory ability in vitro may translate to the metastatic potential the breast cancer cells possess in humans [7]. Therefore, wound-healing assays, or "scratch" assays, have been performed for many years as a way to study the migratory ability of breast cancer cells (as well as other cell types) and whether experimental treatments may be able to inhibit this ability [8].

Wound-healing assays typically involve growing a confluent monolayer of breast cancer cells. A scratch is then made in the monolayer, which disrupts cell–cell interactions and initiates a complex

and ordered series of events to repair the tissue. As the cells migrate in an effort to close the "wound" that was made in the cell monolayer, the scratch is visualized over time with the use of a microscope. Complete "healing" of the wound usually takes anywhere from several hours to over a day, depending on the cell type and treatments being studied, as well as the width of the initial scratch.

Although scratch assays may be imaged and analyzed manually, a number of steps can be automated, including focus, acquisition, and analysis. Automated microscopes equipped with a motorized stage can be used to collect time-lapse data of the wounds at multiple locations within the culture dish over the course of an experiment. The location of each image field is saved into a file, allowing the stage to return to that precise point for each acquisition. This has the advantage that the exact same field is imaged at each time point, and the collected images can be made into a time-lapse video. This also greatly reduces the time spent by the researcher in data collection.

Analysis of scratch assays can be performed manually, as described below, or by using an automated algorithm. Such algorithms are often included in image analysis software packages and operate by first segmenting the image into cell and non-cell areas and then measuring the changes in non-cell area between subsequent images. Although this removes some of the subjectivity in measuring scratch width, care must be taken to ensure that the segmentation of the image corresponds to the actual presence or absence of cells. Additionally, this technique may be less useful for cells that do not maintain contact with their neighbors, or migrate as a sheet. Many plate-based imaging systems, such as the Celigo (Nexelcom, Lawrence, MA), include software modules for performing this task.

The major benefit of the wound-healing assay is the relative ease with which it can be performed and quantified. This assay is simple, inexpensive, and uses common laboratory supplies. It has an additional advantage in that it preserves the context of cell–cell interactions, unlike the Boyden chamber assay discussed below, which requires the disruption of cell–cell junctions before cells can be added to the chamber.

One of the most significant drawbacks to this technique is that it is two-dimensional and thus fails to faithfully replicate the three-dimensional environment in which cells normally live. It has been established that culturing cells in a 3D environment can drastically change a number of cell characteristics, including gene expression, morphology, and drug responsiveness [9, 10]. An additional drawback to the scratch assay is that it requires the physical disruption of the monolayer, which releases the contents of the scratched cells into the media. Although the scratched monolayer is washed in an effort to remove dislodged and damaged cells, the potential still exists for damaged cells and cell contents to remain in the wells

and potentially affect the remaining cells in an uncontrolled manner. To counter this, some companies offer removable or biodegradable inserts which are placed into the culture plate before cells are added, such as the Oris and Oris Pro systems (Platypus Technologies, Madison, WI). These inserts are either manually removed or simply dissolve, and cell migration into the previously occupied area is measured, thus avoiding the presence of potentially complicating factors in the media.

It should also be noted that the wound-healing assay cannot be used to study chemotaxis due to the lack of a defined chemical gradient. Therefore, for chemotaxis studies, the Boyden chamber assay would be more appropriate. Additionally, large numbers of cells are required since the wound-healing assay is most commonly performed in cell culture plates. This usually is not a hindrance for breast cancer researchers, however, considering the relative ease and rapid in vitro growth of most breast cancer cell lines.

1.2 Introduction: Boyden Chamber Assay

The Boyden chamber assay, also commonly called the trans-well migration assay or chemotaxis assay, is a tool used to study cell migration [13]. This technique was originally developed by Stephen Boyden for analyzing the chemotaxis of leukocytes [14]. The driving force behind cell migration is chemotaxis, which is cell motility distinguished by movement toward higher concentrations of chemoattractants, such as growth factors. Overall, this assay consists of a cell culture insert that is placed inside a well of a multi-well cell culture plate. The cell culture insert described here is a PET (polyethylene terephthalate) membrane, with pores of a known, uniform size. For breast cancer cells, the appropriate pore size is 8 μm. Breast cancer cells are seeded into the insert in serum-free medium. The bottom well, which is the well of the multi-well plate, contains a chemoattractant. Due to its convenience and effectiveness, a commonly used chemoattractant is 20 % fetal bovine serum (FBS) in normal cell culture medium. After a given incubation time, breast cancer cells that have migrated to the bottom side of the membrane are fixed, stained, and quantified under a light microscope.

One notable benefit of the Boyden chamber assay is that it is a time-efficient method to analyze cell motility/migration independent of the effects of cell proliferation. This benefit is based on the fact that the time required for cells to migrate through the porous membrane is less than the time required for cells to progress through the cell cycle. For this reason, cell proliferation rates are typically not a factor during the Boyden chamber assay. Boyden chamber assays also have the advantage of being three-dimensional, in contrast to the scratch assay. One consideration that the user should be aware of is that the contributions of cell–cell interactions to cell migration are not amenable to study with the Boyden chamber assay. The reasons for this are twofold; first, the cells need to be

dissociated into a suspension in order to be counted and equally seeded into the wells, and second, if any cell–cell interactions do form after seeding, these cell–cell junctions must be released in order for the cells to efficiently migrate through the porous membrane used in this assay. If the user desires to consider the effect of cell–cell interactions on cell migration, the wound-healing assay also described in this chapter would be more appropriate. However, it is typically warranted to analyze cell migration using both assays, given that the specific treatments or gene manipulations under study may affect cell migration in different ways. A final benefit of the Boyden chamber assay is its ease of use. Users can quickly master the techniques involved and produce repeatable results.

Several Boyden chamber devices of various sizes and compositions are commercially available. Boyden chamber assays may also be supplemented by the addition of a layer of the basement membrane proteins collagen or laminin that must be degraded before cells can pass through. Such a setup is used to assay invasive potential along with migratory ability. Regardless of the specific chamber used, cell migration is usually quantified by counting the number of migrated cells under a light microscope. The user may also use image analysis software to detect the total area of each membrane that is covered by migrated cells. This method of quantification is especially useful for assays with high numbers of migrating cells, in which counting individual cells would be time-consuming and may introduce user error.

The Boyden chamber assay will require optimization depending on the cell type, the specific type of chamber being used, and the chemoattractants. Incubation time will also need to be determined based on cell type, cell number, membrane pore size, and the composition and concentration of the chemoattractants. The method described below is specifically designed for measuring the migration of breast cancer cells using 24-well cell culture plates and 24-well Boyden chamber cell culture inserts from BD Falcon™.

2   Materials

2.1   Wound-Healing Assay

Cell lines: Human breast cancer cell lines may be purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells should be cultured in the appropriate cell culture medium, such as DMEM/F-12 supplemented with 10 % FBS. Cells should be tested to be free of mycoplasma contamination and always cultured using proper aseptic cell culture conditions.

Other reagents: Prepare all reagents using proper sterile technique under a cell culture hood. Store reagents at 4 °C. Before use, warm cell culture reagents to 37 °C in a clean water bath. Closely follow all waste disposal regulations when disposing of waste materials.

1. Cell culture media supplemented with 10 % fetal bovine serum.
2. Dulbecco's Phosphate-Buffered Saline.
3. 0.25 % trypsin–EDTA.
4. Breast cancer cell lines.
5. 200 μL pipette tips.
6. Six-well cell culture plates.
7. Actinomycin D (optional).

2.2 Boyden Chamber Assay

1. Cell culture media supplemented with 10 % fetal bovine serum.
2. Dulbecco's Phosphate-Buffered Saline.
3. 0.25 % trypsin–EDTA.
4. Breast cancer cell lines.
5. 24-well BD Falcon™ Cell Culture Inserts—8 μm pore size.
6. 24-well BD Falcon™ Cell Culture Insert Companion Plates.
7. Methanol.
8. Solution of 0.05 % crystal violet in 20 % ethanol.
9. Cotton-tipped swabs.
10. Glass beakers (250 mL or larger).

2.3 Instruments

1. 37 °C CO_2 incubator.
2. Laminar flow hood.
3. Light microscope.

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3 Methods

3.1 Wound-Healing Assay

3.1.1 Preparing the Cells

1. Seed cells in triplicate wells at approximately 80 % confluence in six-well cell culture plates in normal cell culture media. This is equal to approximately 1×10^6 cells/well; however, the exact number will vary with cell type.
2. Incubate the plates at 37 °C in a 5 % CO_2 cell culture incubator overnight.
3. The next day, observe cells under a light microscope to ensure cells have reached greater than 90 % confluence. Ideally, the cells should be 95–100 % confluent.
4. Make a "scratch" in the cell monolayer in each well of the six-well plate with a 200 μL pipette tip, making sure to hold the pipette tip fully perpendicular to the plate surface to ensure uniform scratch diameter.
5. Gently wash the cells with 1 mL of DPBS to remove the dislodged cells. If care is not used, the cell monolayer may lift or be rinsed away. The borders of the scratch are especially vulnerable to disruption.

6. Carefully suction the DPBS wash from the wells. Gently wash the cells once more with 1 mL of DPBS to remove any remaining dislodged cells and again remove the DPBS by suction.

7. Add 3 mL of fresh cell culture medium to each well. If you want to ensure that any changes in cell migration observed during this assay are due solely to differences in cell migration and not different proliferation rates, you may wish to add actinomycin D (1 μg/mL). Actinomycin D inhibits cell proliferation by inhibiting RNA synthesis.

3.1.2 Observation of Cells: Manual Method

1. On the underside of the six-well cell culture plate, use a fine point permanent marker to mark three points along the length of the scratch. These points will be the reference marks used to ensure you are imaging the same portion of the scratch at each time point.

2. Using an inverted light microscope equipped with a camera, select a 10× objective and find the first reference mark you made, focusing the image just above the reference point and making sure the reference mark is just visible in the field of view. The region of interest is the scratch in the cell monolayer, so center the image over the scratch.

3. Capture the image with the attached camera and repeat for the two other reference points per well. These images will be analyzed later to measure the width of each scratch at each point for each well at time point 0 h.

4. Return the six-well plate to the cell culture incubator.

5. Capture images in the same manner as described above in **steps 2** and **3** at the 6, 12, and 24 h time points. You may also wish to capture a number of images with a 40× or greater objective at the 6 and 12 h time points to examine changes in cell morphology at the leading and trailing edges of migrating cells.

3.1.3 Observation of Cells: Automated Method

1. Place the plate into an appropriate fixed stage insert, being sure to note the orientation of the plate. Using the software interface for a microscope with an automated stage, select three or more points within each well for imaging.

2. If using an incubated microscope stage, set the desired time interval and duration for image acquisition, and set the incubator to 37 °C and 5 % CO_2. It is advisable to include an autofocus step at the beginning of each acquisition if the hardware permits it or to use a system that maintains focus over the course of the acquisition. This will prevent focal drift due to thermal expansion.

3. Run the acquisition.

4. If no incubated stage setup is available, remove the plate from the microscope stage after the first set of images is acquired, and return the plate to the cell culture incubator.

5. Capture images in the same manner as the steps above at the 6, 12, and 24 h time points. If desired, capture a number of images with a 40× or greater objective at the 6 and 12 h time points to examine changes in cell morphology at the leading and trailing edges of migrating cells.

3.1.4 Quantitation and Analysis

1. Using image analysis software, either a commercial solution such as NIS-Elements (Nikon Instruments, Inc.) or free, open-source options such as CellProfiler [11] or ImageJ [12], analyze each image at each time point, measuring the width of the scratch. If the edge profile of the cells is uneven along the length of the scratch, you may wish to use a parallel line measurement tool, where the lines are set to the average edge of the cells on each side. Migration may be expressed either as absolute migration rate or percent wound closure. The absolute rate is equal to $[(D_0 - D_x)/2T]$, where D_0 is the distance between edges at time zero, D_x is the distance between edges measured at the time point of interest, and T is the elapsed time. The distance is divided by two because the change in distance between time points is the result of the migration of both sides of the scratch. The percent closure for each point in each well is equal to $[(D_0 - D_x)/D_0]$, where D_0 is the measurement taken at time zero and D_x is the measurement taken at the time point of interest. Either measurement may then be normalized to the value obtained from the control sample and reported as percent with respect to the control.

3.2 Methods

3.2.1 Boyden Chamber Assay

Preparation of the Cells

1. Remove the normal growth medium containing FBS from cell cultures. The cultures should be in logarithmic growth phase and less than 75 % confluent.

2. Gently rinse the cell cultures three times with DPBS.

3. Replace the culture medium with serum-free medium.

Seeding the Cells into the Chamber

1. Using sterile conditions under the laminar flow hood, dispense 750 μL of serum-free medium into each well of the 24-well BD Falcon™ Cell Culture Insert Companion Plate.

2. Gently place one BD Falcon™ Cell Culture Insert into each well (*see* **Note 1**).

3. Dispense 500 μL of serum-free medium into each BD Falcon™ Cell Culture Insert, and place the lid back onto the BD Falcon™ Cell Culture Companion Plate (*see* **Note 2**).

4. Place the plate containing the inserts into a 37 °C CO_2 incubator and incubate for 2 h. This allows the insert membranes to rehydrate.

5. Using vacuum suction, carefully remove the serum-free media from the lower well and the insert.

6. Trypsinize the cell cultures to obtain cell suspensions. Neutralize the trypsin with cell culture medium containing 10 % FBS.

7. Count the cells using a hemacytometer and light microscope to determine the cell concentration. Using this concentration, calculate how many total cells you will need in order to seed triplicate wells for each cell line or treatment group. Prepare enough cell suspension for extra wells to provide room for pipetting error. The number of cells seeded per well will depend on the cell line used and typically varies from 20,000–50,000 cells/well.

8. Centrifuge the cells at $200 \times g$ for 4 min to form a cell pellet.

9. Remove and discard the medium (supernatant).

10. Carefully rinse the cell pellet with DPBS.

11. Centrifuge the cells + DPBS at $200 \times g$ for 3 min.

12. Remove and discard the DPBS (supernatant).

13. Gently resuspend the cells in serum-free medium to provide for 500 µL of serum-free medium + cells per well.

14. Pipette 750 µL of cell culture medium containing 20 % FBS into each well of the BD Falcon™ Cell Culture Companion Plate. Ensure there are no air bubbles trapped between the insert membrane and the medium in the lower well.

15. Pipette 500 µL of serum-free medium + cells into each insert.

16. Incubate the plate in a 37 °C CO_2 incubator for 6–24 h. Incubation time will be dependent on the cell type under study.

3.2.2 Fixation and Staining of Migrated Cells

1. Prepare two separate 24-well plates. In one plate, transfer 750 µL of 100 % methanol to each well. In the other plate, transfer 750 µL of 0.05 % crystal violet solution to each well.

2. Gently remove the inserts containing cells, one at a time, and remove the serum-free media out of the insert.

3. Using a cotton-tipped swab, gently, but thoroughly, scrub the top surface of the membrane to remove any nonmigrating cells from the chamber.

4. To fix the cells that have migrated to the bottom side of the membrane, place the inserts in the wells of the plate containing 100 % methanol. Incubate at room temperature for 20 min.

5. Remove the inserts from the methanol and air-dry them for 5 min.

6. To stain the cells, place the inserts into the wells of the plate containing the 0.05 % crystal violet solution. Allow the inserts to stain at room temperature for 20 min.

7. Destain the inserts by gently dipping them into consecutive beakers of distilled H_2O until no more stain comes off into the water. Allow the inserts to thoroughly air-dry (at least 1 h).

8. The inserts may be simply placed into a clean 24-well plate and imaged, or the membranes may be carefully cut from the inserts using a razor blade and mounted onto a slide for imaging.

3.2.3 Quantification of Cells

1. Survey each membrane under a light microscope at 10× magnification.

2. Capture images using a digital camera connected to the microscope using a 10× objective. For each membrane, capture three (or more) images at random positions (*see* **Note 3**).

3. To analyze the images, count the number of migrated cells manually, or using image analysis software. This may be done using an interactive software counter, where each mouse click registers as a counted cell, or by an automated object counting algorithm. If using an automated counting function, first validate the function by comparing it to a manual count performed on the same image before processing all images.

4. Calculate the mean number of migrated cells from nine images (three images per membrane × three membranes per cell line or treatment group = nine images), and calculate standard deviation.

5. Alternatively, if counting individual cells is problematic, the area occupied by stained cells may be measured instead. This is done with image analysis software by setting a threshold that will detect the presence of the stain on a per-pixel basis, thus defining a binary that represents the area occupied by migrated cells. Then calculate the mean area occupied for each treatment from nine images (three images per membrane × three membranes per cell line or treatment group = nine images) ± standard deviation.

6. Measured cell numbers or cell area may be reported directly or normalized to the control treatment and reported as percent relative to control.

4 Notes

1. When placing the cell culture inserts into the companion plate, be careful not to trap air bubbles between the insert and the media in the bottom well. To accomplish this, slowly and gently place the insert into the well at a slight angle.

2. In preparing the cells for seeding, plan for at least three replicates within each experimental group. Additionally, prepare extra volume of cell suspension in serum-free medium to allow

for slight variations in pipetting. In this protocol, since you need enough cell suspension for three replicates, prepare enough for four replicates.

3. Do not directly count the cells under a microscope. To reduce error and retain proper records, use a camera attached to the microscope to save an image file for each field of view. Cells can be counted in the desired fields from the saved images with more accuracy and objectivity.

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