Chapter 15

A Novel Collagen Dot Assay for Monitoring Cancer Cell Migration

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Abstract

Cell migration is a critical determinant of cancer invasion and metastasis. Drugs targeting cancer cell migration have been hindered due to the lack of effective assays for monitoring cancer cell migration. Here we describe a novel method to microscopically monitor cell migration in a quantitative fashion. This assay can be used to study genes involved in cancer cell migration, as well as screening anticancer drugs that target this cellular process.

Key words Type I collagen, Migration, Two-dimensional culture, Non-tissue culture 96-well plate, Microscope

1 Introduction

Cancer cell migration is a dynamic, multistep process, which involves the rearrangement of the cytoskeleton with adhesion protein composition to form membrane protrusions at the leading edge of the cell membrane (lamellipodia) to promote translocation through adjacent tissues and structures [1]. The migratory capacity of cancer cells is often correlated with poor prognosis in patients as this cellular process is required for metastasis, which accounts for 90 % of all human cancer-related deaths [2].

Due to the relevance of cell migration in cancer dissemination, multiple systems have been established to study this cellular process. One example is the Boyden chamber assay in which a polycarbonate membrane with a defined pore size is nested between the upper and lower quadrants of the cylindrical transwell chamber. Cells are then seeded in the top chamber in serum-free media, while a chemoattractant is placed in the bottom chamber. Cells that migrate through the pores of the membrane are then stained and quantified through microscopic approaches [3]. Although a reliable, sensitive, and useful approach, this assay has disadvantages relative to the 2-D migration assay described in this chapter such as

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prolonged drug-screening studies which are complicated by the test agent concentrations quickly equilibrating between the two transwell compartments. Another popular method adopted by many scientists is the in vitro scratch wound assay which involves generating a "scratch" in a cell monolayer and subsequently capturing images at evenly spaced time intervals. The simulated "wound healing" is then compared between the images by quantifying the migration rates of the cells [4]. Although this assay is also a useful technique, it has disadvantages relative to the 2-D migration assay described in this chapter due to the lack of a defined wound surface between cells.

Herein, we describe a new method in which cells are mixed with a type I collagen mixture and are subsequently doted onto a non-tissue culture 96-well plate. Migrating cells at the cell–collagen interface can be microscopically counted after at least an 8 h incubation period. Furthermore, by using a tooled plate to standardize the size and shape of the cell-matrix dot, this 2-D migration assay can be used in a high-throughput screening fashion. The 2-D migration assay protocol described is a simple, reproducible, and effective way to identify compounds that inhibit cancer cell migration, study pathways relevant to this cellular process, and expand our understanding of cancer dissemination.

2 Materials

Prepare all solutions for this assay in sterile conditions. All reagents used to make the dot collagen mixture, especially the type I collagen, should be kept on ice to prevent premature solidification.

2.1 Cell Culture Components

1. 0.05 % Trypsin–EDTA (1×).
2. Dulbecco's Modified Eagle Medium (DMEM): Supplemented with 10 % fetal bovine serum (FBS) and 1 % PenStrep (P/S).
3. 96-well non-tissue culture plate.
4. 37 °C CO_2 incubator.
5. Tissue culture hood.

2.2 Dot Collagen

1. 2 N NaOH: Add 8.0 g of sodium hydroxide to 100.0 mL of water.
2. Dot Collagen (3 mg/mL): Add 70 μL of sterile water, 50 μL 5× DMEM medium, and 125 μL type I collagen 6 mg/ml (acetic acid-extracted native collagen from rat tail tendon) to a 1.5 mL microcentrifuge tube and mix until contents go into solution. Adjust the pH of the dot collagen mixture using 2 N NaOH until it reaches a neutral pH of 7.4. Make sure to check pH using a pH indicator strip as a too acidic or basic solution will kill the cells (*see* **Note 1**).

2.3 Cell Fixation/ Nuclei Staining Solution

1. 16 % Paraformaldehyde (PFA) Solution: Dissolve 16 g of PFA powder in 84 mL phosphate-buffered saline (PBS) in a 250 mL beaker. Place beaker on hot plate (don't bring temp above 80 °C) and let contents stir for 30 min. Allow mixture to cool to room temperature before using.
2. 1× PBS Solution: Dissolve 8.00 g NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 mL of H_2O. Adjust the solution with HCl until a pH of 7.4 is achieved. Add water until contents reach 1 L. Sterilize the solution by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle or by filter sterilization.
3. PFA Hoechst Solution: In a 15 mL Falcon tube, add 1000 μL 16 % PFA and 998 μL 1× PBS. For every 2 mL of solution made, add 2 μL of Hoechst and/or DAPI (4′,6-diamidino-2-phenylindole) (*see* **Note 2**).

3 Methods

Before conducting this assay, it is necessary to ensure there are enough cells to make the desired number of cell–collagen dots for each condition. On average, a concentration of 5×10^4 cells/μL is optimal when performing this assay. It is important to note that although this is the optimal cell density, certain cell lines may be smaller than others so more cells might be necessary to perform this assay properly. The following steps are performed in a standard tissue culture hood.

3.1 Cell Preparation

1. Once cells reach log growth rate, carefully aspirate the medium from the cell culture plate or flask they reside in (*see* **Note 3**).
2. After removing media, wash the cells with 1× phosphate-buffered saline (PBS) to remove any remaining FBS-containing medium.
3. After the wash step, aspirate PBS from the dish and incubate the cells at 37 °C for 4 min in 0.05 % Trypsin–EDTA (1×) in order to cleave adherent proteins on the cell membrane, releasing them from the bottom of the plate or flask (*see* **Note 4**).
4. Remove the cells from the incubator and mix with equal volumes of 10 % FBS-containing medium to neutralize the Trypsin–EDTA solution.
5. Mechanically pipette the cells to further separate and transfer into a new 15 mL tube.
6. Determine the cell concentration with a hemocytometer or an automated cell counter.
7. Use the counted cell density to calculate how much of the cell solution is needed to obtain 1×10^6 cells. This will give a final cell–collagen dot density of 5×10^4 cells/μL in 20 μL (*see* **Note 5**).

8. Pipette the volume of cells required into a new 1.5 mL microcentrifuge tube and spin down (800×*g* for 5 min at room temperature) to remove the media.
9. Resuspend the pellet of cells in fresh complete medium to a volume of 10 μL (*see* **Note 6**).
10. Add 10 μL of dot collagen to the suspended cells.
11. Vigorously mix the cells by pipetting to ensure a homogenous mixture of cells: dot collagen solution (*see* **Note 7**).

3.2 Drop Cell–Collagen Dot into a 96-Well Plate

1. Pipette 1.0–1.5 μL of the cell–collagen dot mixture onto a non-tissue culture 96-well plate at the center of each well (*see* **Note 8**).
2. Incubate the 96-well plate at 37 °C for 5–10 min or until collagen has solidified (*see* **Note 9**).
3. Check cell–collagen dots under a compound light microscope. They should appear to be full of cells at both the center and the peripheral edges (*see* **Note 10**).
4. Once cell–collagen dots appear to be solidified, pipette 100 μL of complete medium onto the side of each well gently to prevent washing away the cell–collagen dot.
5. Incubate the 96-well plate at 37 °C for 8–16 h depending on the cell line used (*see* **Note 11**).
6. After incubation, add 8 % PFA/Hoechst solution to each well at room temperature to fix the cells and visualize them through nuclei staining. Wrap the 96-well plate in aluminum foil to avoid exposure to light and let it stand for at least 30 min before imaging (*see* **Note 12**).
7. Use microscope visualization software to center the cell–collagen dot and record a DAPI (Fig. 1a) and bright-field (Fig. 1b) image using both a 4× magnification lens to visualize the entire dot and a 10× lens to image the four sides of the cell–collagen dot. Count the migrating cells at the cell–collagen interface and compare between the different conditions (*see* **Note 13**).

4 Notes

1. It is generally acceptable to leave the dot collagen mixture on ice in the refrigerator for up to 2 h prior to use.
2. Hoechst and DAPI are fluorescent stains that bind and label nucleic acids (double-strand DNA) for visualization of nuclei when performing microscopy.
3. Verify the morphology and health of the cells before performing the experiment as abnormalities can lead to unexplained results or poor migratory capacity.

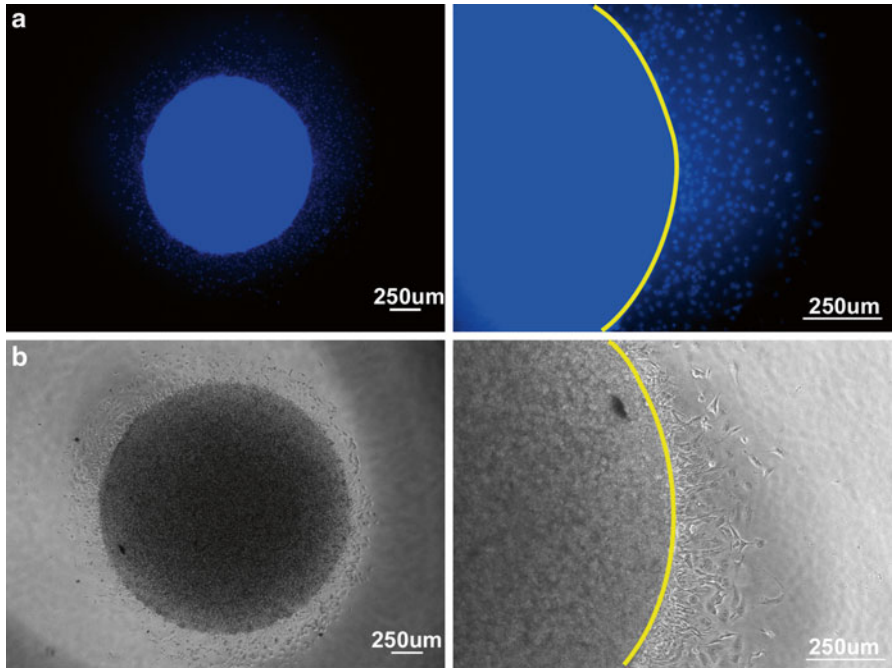

Fig. 1 2-D dot collagen migration assay using HeLA (cervical cancer) cells. (**a**) DAPI images of entire (*left* panel) and right quadrant (*right* panel) of dot collagen under 4× and 10x lens magnification respectively. (**b**) Bright-field images of entire (*left* panel) and right quadrant (*right* panel) of dot collagen under 4× and 10x lens magnification respectively.

4. The concentration and duration of treatment with Trypsin–EDTA (1×) may vary depending on the cell type.
5. It is common for small cell types to require a final cell–collagen dot density of 7×10^4 to 10×10^4 cells/μL to ensure full dots.
6. It is important to be very careful when aspirating excess media/trypsin to not disturb the pellet. It might be necessary to pipette excess liquid off mechanically to achieve a 10 μL volume when resuspending the cell pellet in fresh complete media. After resuspending the pellet, it is worth measuring the cell mixture volume by pipetting before adding the dot collagen as excessive liquid may lead to longer drying periods. Remember to account for the volume of the cells in the 10 μL resuspension mixture.
7. It is important to pipette the cell–collagen dot mixture precisely to ensure dots have a relatively even number of cells between each repeat. It is also important to not use small pipette tips (0.1–10 μL) when mixing the cell–collagen dot mixture as this may lead to mechanical disruption of the cell membrane and/or accidental cell lysis.

8. In order to optimize image quality, it is important to place dots as centered as possible. It is also important to stop pipetting the dot when the first stop is reached to avoid bubbles, as this will affect dry times and image quality. If the cell–collagen dots seem inconsistent in cell number between repeats, it may be necessary to pipette the cell–collagen dot mixture vigorously to resuspend the cells again as they may have settled. It may also help to use pipette tips that have already been pre-chilled in the refrigerator as this makes working with collagen much easier. Lastly, ensure the plate is a non-tissue culture 96-well plate as the hydrophobic surface helps maintain the collagen dot shape.
9. The drying period is highly dependent on how quickly dots are placed and will vary between experiments. The cell–collagen dots usually turn a pale opaque color when properly dried and should not be allowed to turn white in color as this means the collagen has completely dried and cells will no longer be capable of migrating.
10. Some cell lines such as HT1080 might require the addition of 2.5 % dialdehyde dextran to the final cell–collagen dot mixture (10 μL cells suspended in complete media, 8 μL dot collage mixture, 2 μL 2.5 % dextran for a 20 μL total volume) to prevent the cell–collagen mixture from contracting while drying [5–7]. This is noted as a clear empty space around the dried cell–collagen dot under microscopic visualization.
11. Note that incubation periods between cell types vary greatly, but in general shorter incubation times are best for more aggressive cell types while those less aggressive will tend to have a longer incubation period.
12. Generally, it is ok to remove the lid of the 96-well plate and invert over a designated biohazard trash can while gently tapping the side to remove old media from the wells. Instead of 8 % PFA/Hoechst solution, a 4 % PFA/Hoechst solution can be used to help remove excess background when imaging.
13. After fixation, cells should be stored in a dark place at room temperature, such as a desk drawer, and be imaged within 24–36 h.

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