Video Article

Evaluation of the Cell Invasion and Migration Process: A Comparison of the Video Microscope-based Scratch Wound Assay and the Boyden Chamber Assay

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Abstract

The invasion and migration abilities of tumor cells are main contributors to cancer progression and recurrence. Many studies have explored the migration and invasion abilities to understand how cancer cells disseminate, with the aim of developing new treatment strategies. Analysis of the cellular and molecular basis of these abilities has led to the characterization of cell mobility and the physicochemical properties of the cytoskeleton and cellular microenvironment. For many years, the Boyden chamber assay and the scratch wound assay have been the standard techniques to study cell invasion and migration. However, these two techniques have limitations. The Boyden chamber assay is difficult and time consuming, and the scratch wound assay has low reproducibility. Development of modern technologies, especially in microscopy, has increased the reproducibility of the scratch wound assay. Using powerful analysis systems, an "in-incubator" video microscope can be used to provide automatic and real-time analysis of cell migration and invasion. The aim of this paper is to report and compare the two assays used to study cell invasion and migration: the Boyden chamber assay and an optimized *in vitro* video microscope-based scratch wound assay.

Video Link

The video component of this article can be found at https://www.jove.com/video/56337/

Introduction

Cell invasion and migration are involved in the dissemination of cancer cells, which is the main cause of resistance to treatment[1] and can lead to locoregional or metastatic recurrence after cancer treatment[2]. The epithelial-mesenchymal transition (EMT) is the initial process of cell invasion-migration in which cancer cells switch from an epithelial to a mesenchymal phenotype. E-Cadherin is an extracellular marker of the epithelial phenotype[3], and increased expression of N-cadherin and vimentin is characteristic of the mesenchymal phenotype[4]. Migration also depends on the intrinsic capacity of cancer cells to invade the extracellular matrix (ECM) through the action of matrix metalloproteases[5].

This invasion–migration mechanism has been described for cancer at many locations, particularly in head and neck cancer[6]. Many researchers have focused on the migration and invasion processes to understand better how cancer cells disseminate in the hope that this knowledge will lead to new treatment strategies. It is crucial that these studies are performed using reliable and reproducible assays.

In vitro analysis of cell motility can be challenging. Developed many years ago, the Boyden chamber assay is considered to be the standard for invasion–migration analysis[7]. However, it is time consuming and is often inaccurate. A second test is the wound-healing assay[8], which involves making a scratch on a cell monolayer culture and capturing images of cell invasion and migration at fixed time intervals. This technique has been criticized widely because of the large variations between the results of two successive tests. However, the application of modern technologies, especially in microscopy, has improved the reproducibility of the scratch wound assay. Video microscopes can be easily introduced in incubators and can generate real-time images of cell migration. These devices record microscopic data and provide automatic analysis of wound cell confluence over time. The aim of this paper is to describe the Boyden chamber assay and the optimized scratch wound assay, and to discuss the advantages and weaknesses of each approach.

Protocol

NOTE: The Boyden chamber and scratch assays without inclusion of the ECM are referred to as the migration assay, and the same assays with the ECM is referred to as the invasion assay.

1. Boyden Chamber Assay

NOTE: This protocol is adapted for the SQ20B cell line, which is derived from a recurrent Head and Neck Squamous Cell Carcinoma (HNSCC) laryngeal cancer and was obtained from John Little (Boston, MA, USA).

Day 1

1. **Cell Seeding**
    1. Seed 2 10^6 SQ20B cells in 12 mL of culture medium (CM) in a 175 cm^2 flask 72 h before day one and allow the cells to grow to 80% cell confluence.
        1. To prepare CM for SQ20B cells, supplement Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS), 0.04 mg/mL hydrocortisone, 100 U/mL penicillin, and 0.1 g/L streptomycin.
    2. Under a laminar flow hood, trypsinize the cells. Remove the medium, wash the cells with sterile phosphate-buffered saline (PBS), and add 2.5 mL of trypsin ethylenediaminetetraacetic acid (EDTA) (0.5 g/L). Incubate the cells for 5 min at 37 °C and then stop the reaction by adding 12.5 mL of CM warmed to 37 °C. Count the number of cells using a cell counter.
    3. Seed the cells in a 25 cm^2 flask at a cell density of 6 10^5 cells for each condition to be evaluated. Incubate the cells for 24 h in 3 mL of CM.

Day 2

2. **Cell starvation and preparation of coated chambers**
    1. Starve the cells by replacing the medium in each flask with 3 mL of low-fetal bovine serum (FBS) medium using 0.1% bovine serum albumin (BSA) instead of FBS. Starve the cells for 24 h in the incubator.
        1. To prepare starvation CM (s-CM), supplement DMEM with 0.1% BSA, 0.04 mg/mL hydrocortisone, 100 U/mL penicillin, and 0.1 g/L streptomycin.
    2. For the migration assay, go to step 1.3.
    3. For the invasion assay, 12 h before day 3, prepare the coated Boyden chambers by adding 500 µL of s-CM to each coated chamber. Use commercially prepared coated Boyden chambers and keep them at 4 °C before use. Place each Boyden chamber in the companion plate and set it in the incubator at 37 °C overnight.

Day 3

3. **Cell seeding in the Boyden chamber**
    1. Use the companion plate to prepare the chemoattractant.Fill each well of the 24-well companion plate with 750 µL of complete medium with 10% FBS, 0.04 mg/mL hydrocortisone, 100 U/mL penicillin, and 0.1 g/L streptomycin.
        1. Adapt the chemoattractant for each cell line and each treatment condition. To prepare the chemoattractant CM (ca-CM), supplement DMEM with 10% FCS, 0.4 mg/mL hydrocortisone, 100 U/mL penicillin, and 0.1 g/L streptomycin.
    2. Transfer the upper chamber into the prefilled companion plate, taking care to avoid bubbles.
    3. For the invasion assay, carefully remove 450 µL of CM from each Boyden chamber.
    4. Under a laminar flow hood, trypsinize the cells. Remove the medium, wash the cells with sterile PBS, add 0.5 mL of trypsin EDTA (0.5 g/L), and then incubate the cells for 5 min at 37 °C. Stop the reaction by adding CM warmed to 37 °C. Count the number of cells using a cell counter.
    5. Seed 3 10^4 SQ20B cells in 500 µL of 0.1% BSA medium, giving a final dilution of 6 10^4 cells/mL.
    NOTE: The cell concentration should be adapted for each cell line.
    6. Place the plate into the incubator at 37 °C for 24 h.

Day 4

4. **Cell fixation and staining**
    1. Fix the cells before the doubling time specific to that cell line. Fix SQ20B cells before 24 h.
    2. Remove each insert from the companion plate and carefully remove the cells from the upper chamber using a cotton swab. It is particularly important to remove all cells located in the upper chamber.
    3. Fix and stain each insert individually to obtain May-Grunwald Giemsa coloration[9] using the staining kit provided. Alternatively, use 4% paraformaldehyde in another companion plate for a 30 min fixation.
    4. Keep the inserts in an empty 24-well companion plate under a laboratory hood to dry each chamber.

Day 5

5. **Microscopic analysis**
    1. Insert the companion plate with the inserts onto a 20 phase-contrast microscope and count each migrated cell on the lower part of the membrane. Optimize the right focus position by moving the objective from the bottom to the top, and stop the position on the bottom part of the membrane.
    2. Calculate the ratio between the numbers of migrated cells and seeded cells. Repeat each count for each treatment condition in triplicate.

2. Scratch Wound Assay: Cell Migration

NOTE: Instructions must be adapted for each type of cell.

Day 1

1. **Cell Seeding**
    1. Seed 2 x 10^6 SQ20B cells in 12 mL of CM in a 175 cm^2 flask 72 h before day one and allow the cells to grow to 80% cell confluence.
    2. Under a laminar flow hood, trypsinize the cells. Remove the medium, wash the cells with sterile PBS, add 2.5 mL of trypsin EDTA (0.5 g/L), and incubate the cells for 5 min at 37 °C. Stop the reaction by adding 12.5 mL of CM warmed to 37 °C. Count the number of cells using a cell counter.
    3. Generate a prediluted sample at a cell density of 4 x 10^5/mL, giving a final density of 4 10^4 cells per 100 µL in each well. This concentration must be adapted for each cell line and should be in the range of 1 x 10^4 to 6 10^4 cells.
    4. Seed cells into each well of a 96-well plate by depositing 100 µL of the solution prepared in step 2.1.2.
    5. Leave the plate at room temperature for 5 min to disperse the cells evenly on the bottom of the wells.
    6. Place the plate into the incubator and allow the cells to adhere to the plate for 12 to 16 h (maximum) at 37 °C.

Day 2

2. **Scratch wound assay**
    1. Remove the plates prepared in step 2.1 from the incubator.
    2. Under the hood, make a wound using the commercial wounding device according to the manufacturer's protocol.
    3. Immediately remove the medium from each well using a pipette with an adapted conical tip, taking care not to touch the wound.
    4. Wash the cells twice by repeating the aspiration and using 100 µL of CM warmed to 37 °C for each well.
    5. Remove the CM using a pipette with an adapted conical tip after the washing steps.
    6. Add 100 µL of medium specific for each treatment condition to each well.
    7. Try to avoid creating any bubbles in the wells. A reverse-pipetting technique may be helpful here. Alternatively, remove bubbles with a syringe needle.
    8. Place the plate into the adapted rack of the video microscope.
    9. To improve the image quality, allow the plate to warm for at least 15 min before the first scan to avoid condensation on the lower side of the plate.
    10. Program the schedule of scans, using the video microscope software at one image per well. A maximum 2-h interval is required for an invasion-migration experiment. If the objective of the experiment is to produce a video, an interval of 30 min maximum is preferred.
    11. At the end of the experiment, wash the wounding device carefully and follow each of the four washing steps indicated by the manufacturer.

Days 3, 4, and 5

3. **Analysis of migration using the video microscope**
    1. For a minimum of 24 h and up to 5 days, monitor and check the cell migration.
    2. Use an appropriate cell mask adapted for each cell type to analyze cell migration. To obtain a cell mask adapted for each cell line, generate a cell processing definition from the software using a specific cell image collection.
    3. Trace the curves and export the data to a spreadsheet, which can be used to analyze and compare the results.

3. Scratch Wound Assay: Cell Invasion

NOTE: Instructions must be adapted for each type of cell.

Day 1

1. **Cell Seeding**
    1. Use the same protocol for cell seeding as described in step 2.1.

Day 2

2. **Preparing the ECM**
    1. Defrost the matrix for at least 12 h before use at 4 °C. Make sure that no aggregates are visible. If aggregates are visible, keep the matrix at 4 °C for a longer period until the aggregates disappear. Keep the matrix on ice. Use precooled pipette tips.
    NOTE: The matrix will solidify if not kept at 4 °C.

2. Chill the microcentrifuge tubes on ice for 5 min.
3. Take CM cooled to 4 °C from the refrigerator and dilute the matrix in the precooled microcentrifuge tubes to obtain a final concentration of 300 µg/mL.
4. Return the microcentrifuge tubes with prediluted matrix to the refrigerator at 4 °C.
   NOTE: A cooling rack adapted for microcentrifuge tubes is helpful for maintaining the tubes at 4°C throughout the experiment.

Day 2

3. **Scratch Wound Assay and Treatment of the ECM**
   1. Remove the plate prepared in step 3.1 from the incubator.
   2. Under the hood, make the wound using the commercial wounding device according to the manufacturer's protocol.
   3. Immediately remove the medium from each well using a pipette with an adapted conical tip taking care not to touch the wound.
   4. Wash the cells twice by repeating the aspiration procedure and using 100 µL of CM warmed to 37 °C.
   5. After the second wash, place the plate on the 4 °C incubator to equilibrate its temperature for 5 min.
   6. Remove the cold medium from each well with the aspiration pipette with a conical tip, taking care to pipette carefully from the edge and to avoid touching the wound.
   7. Add 50 µL of prediluted matrix to each well using precooled conical tips at 4 °C.
   8. Place the plate in the incubator at 37 °C for 30 min.
      NOTE: A prewarmed support rack at 37 °C is helpful to accelerate the warming of the 96-well plate.
   9. Remove the plate from the incubator and add 100 µL of the adapted CM to each well as indicated for each treatment condition.
   10. Try to avoid creating any bubbles in the wells. A reverse-pipetting technique may be helpful here. Alternatively, remove bubbles with a syringe needle.
   11. Place the plate into the adapted rack in the video microscope.
   12. To improve the image quality, allow the plate to warm for at least 15 min before the first scan to avoid condensation on the lower side of the plate.
   13. Program the schedule of scans, using the video microscope software at one image per well, in the Scratch Wound scan mode. A maximum 2-h interval in required for an invasion-migration experiment. If the objective of the experiment is to produce a video, an interval of 30 min maximum is preferred.
   14. At the end of the experiment, wash the wounding device carefully and follow each of the four washing steps indicated by the manufacturer.

Days 3, 4, and 5

4. **Analysis of cell invasion using a video microscope.**
   1. Repeat step 2.3.

Representative Results

We report here two different methods to analyze cell invasion and migration. **Figure 1** shows the Boyden chamber experiment. The inserts are placed into a companion plate with chemoattractant medium, and the cells are seeded in CM. The membrane can be uncoated (migration assay) or coated (invasion assay). Cells are seeded into the upper chamber in s-CM. The lower chamber is filled with CM as a chemoattractant. The cells are fixed before the doubling time.

Figure 2 shows the Boyden membrane after cell fixation. **Figure 2A** shows a suboptimal result, with cell clusters on the upper side of the membrane. **Figure 2B** shows an optimal result with cells fixed and stained in blue on the membrane.

Figure 3 shows an optimal result of the scratch wound assay. The linear wound can be seen in **Figure 3A**. No cells are observed in the wound, and wound healing has occurred within 30 h. The time to heal the wound is dependent on the cell line and ranges from 24 to 50 h.

Figure 3B is a graphical representation of wound healing under four treatment conditions: control, ABT-199 (anti-Bcl-2), cetuximab (anti-EGFR), and ABT-199+cetuximab. The ABT-199+cetuximab combination significantly decreased cell migration. The curves are obtained using the video microscope software, which provides robust data analysis of cell migration with time. Error bars represent the standard deviation (SD) for each well.

Ninety percent confluence is the optimal cell density for the wound-healing assay. The optimal cell seeding depends on the cell line and ranges from 3×10^4 to 6×10^4 cells. **Figure 4A** shows the optimal cell density, and **Figure 4C** shows low cell density. Wells should be washed twice to avoid cell clusters, as shown in **Figure 4B**.

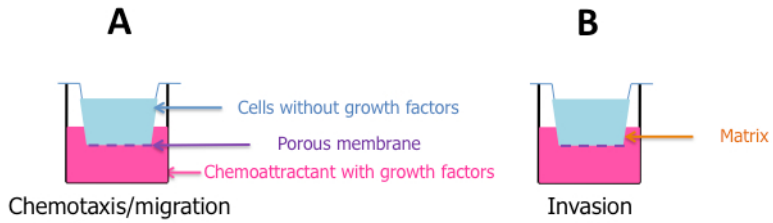

Figure 1: Schematic representation of the Boyden chamber experiment. Inserts are placed into a companion plate with chemoattractant medium, and the cells are seeded in CM. The membrane can be uncoated (**A**) or coated (**B**).

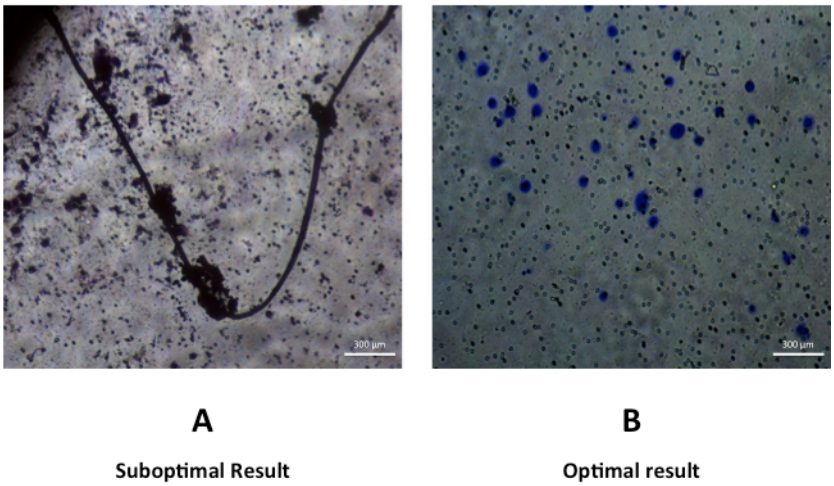

Figure 2: Suboptimal and optimal microscopic results obtained in the Boyden chamber experiment. (**A**) Suboptimal membrane with cell clusters on the upper side of the membrane and uninterpretable results. Scale bar = 300 µm. (**B**) Optimal membrane with countable cells in the lower part of the membrane stained in blue. Scale bar = 300 µm.

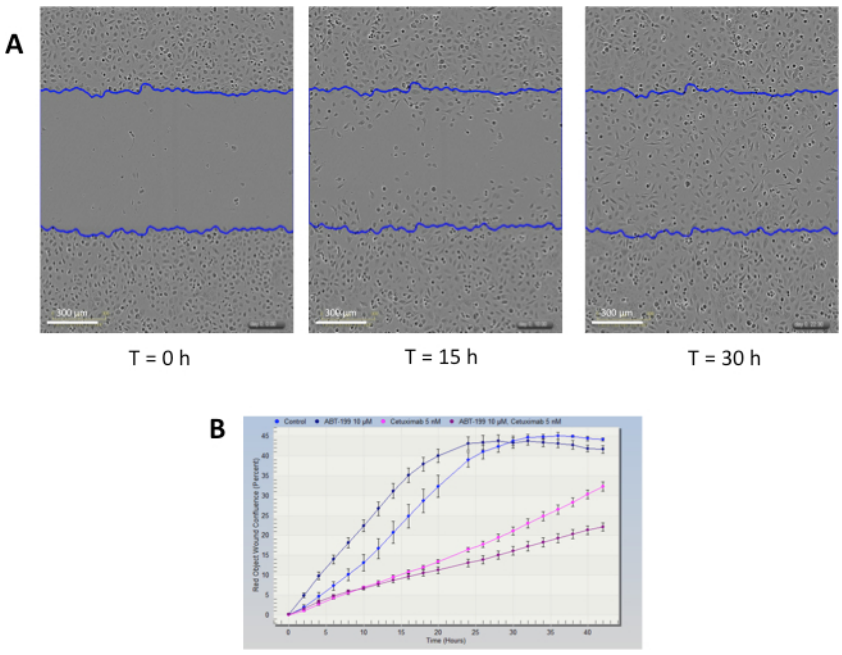

Figure 3: Optimal results obtained in the wound-healing experiment. (**A**) Representative image from the scratch wound assay shown wound healing observed at 0, 15, and 30 h. The criteria for a quality experiment are a linear wound, no cell fragments observed in the wound, and optimal cell confluence. Scale bar = 300 µm. (**B**) Graphical representation of wound healing showing quantification of parameters of wound cell confluence (percentage) according to time (h). Four treatment conditions are analyzed here, and the data are shown with the SD.

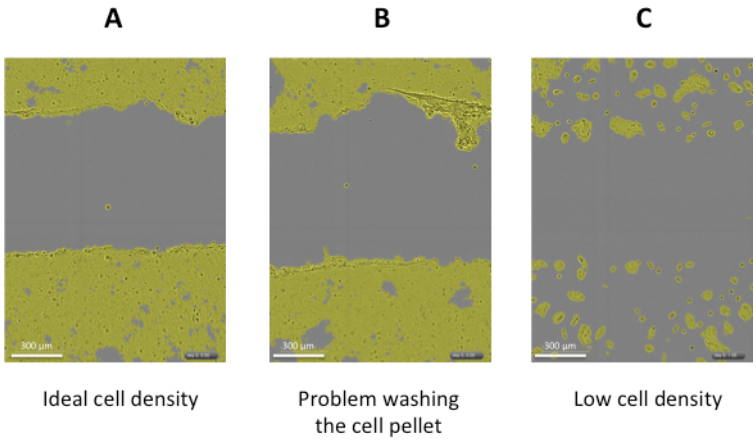

Figure 4: Suboptimal results obtained in the wound-healing experiment. (**A**) Ideal cell density. (**B**) Problems washing the cell pellet. (**C**) Low cell density. Scale bars = 300 µm

	Advantages	**Disadvantages**
Boyden Chamber Experiment	- 3D-Cell Chemotaxis	- Time-Consuming
	- Both invasion and migration with coated and non coated layer matrix	- Low reproducibility
		- Expensive inserts
		- No Time-Lapse exploration
		- Need of adherent cells
Wound Healing Assay	- Automatic Highly reproducible wound	- 2D-Cell motility
	- Both invasion and migration with coated and non coated layer matrix	- Need of adherent cells
	- Cell proliferation included in the analysis	
	- Time-Lapse microscopic visual analysis	
	- Data exportation of wound healing metrics with precise analysis	
	- Post-treatment robust software for cell migration and invasion curves	

Table 1: Advantages and disadvantages of the Boyden chamber experiment and video microscope wound-healing assay. The advantages and disadvantages of the Boyden chamber experiment and scratch wound assay.

Discussion

We report here two different modalities to study the cell invasion and migration process. The analysis of this process is important to understanding the factors involved in metastatic recurrence, which might be explained by increased motility of a subpopulation of cancer cells called cancer stem cells[10,11].

The Boyden chamber experiment is one the most frequently used techniques to explore cell invasion and migration. One advantage of this approach is its reproducibility, although this technique is highly dependent on the experimenter's expertise. Cell counting is manual and can vary between experimenters. Many critical steps must be standardized and followed with care, such as ensuring the use of the appropriate chemoattractant and starvation medium specific to each cell line. Removing cells from the upper membrane with a cotton swab is also critical to ensuring optimal results and avoiding the suboptimal results shown in **Figure 2A**. If this assay is used to evaluate cell motility, invasion, and migration capacities through a three-dimensional (3D) porous membrane, it is not possible to track cells through a time-lapse experiment. Moreover, commercial inserts with a porous membrane are often expensive.

The video microscope-based scratch wound assay presented here is a robust technique to evaluate cell migration and invasion. The assay is performed with a mechanical wound maker and provides a reproducible comparison between treatment conditions. The video microscopic analysis can be used to generate precise quantitative data for wound healing and cell confluence through a time-lapse experiment. Time-to-time analysis of different treatment conditions can be corroborated by microscopic observations. This analysis integrates cell proliferation during the assay and takes into account the cell-doubling time. Video or raw data content can be exported to obtain a highly visual representation of the results, as shown in the cell migration curves in **Figure 3B**.

Many critical steps are required to obtain interpretable results. The optimal conditions for cell seeding are dependent on the cell line and must be tested with different dilutions to obtain 90% cell confluence in the wells. To obtain a linear wound, cell plating must not exceed 16 h. The washing step must be done twice and rapidly to avoid introducing cell debris into the wound that could alter the results. For the invasion scratch wound,

proper care of the ECM is also a critical step. The cooling rates must be constant, and the ECM should be manipulated with chilled pipette tips and maintained at 4 °C.

These techniques have many advantages, which are summarized in Table 1. Adherent cells are needed for this technique. Scratching causes mechanical injury to the cells, which causes release of cellular contents into the surrounding medium and may influence the migration process. The scratch wound assay provides an estimate of 2D cell motility but not of cell chemotaxis, which cannot be studied using this technique. We have recently developed a video microscopic chemotaxis assay using a commercial plating system. This assay needs cells with a high migration ability, such as cancer stem cells, as described previously[12]. This new technique may become the gold standard for invasion-migration assays in the future.

Disclosures

The authors have nothing to disclose.

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