Video Article

Moving Upwards: A Simple and Flexible *In Vitro* Three-dimensional Invasion Assay Protocol

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Abstract

Although 3D invasion assays have been developed, the challenge remains to study cells without affecting the integrity of their microenvironment. Traditional 3D assays such as the Boyden Chamber require that cells are displaced from the original culture location and moved to a new environment. Not only does this disrupt the cellular processes that are intrinsic to the microenvironment, but it often results in a loss of cells. These problems are especially challenging when dealing with cells that are either rare, or extremely sensitive to their microenvironment. Here, we describe the development of a 3D invasion assay that avoids both concerns. In this assay, cells are plated within a small well and an ECM matrix containing a chemoattractant is laid atop the cells. This requires no cell displacement, and allows the cells to invade upwards into the matrix. In this assay, cell invasion as well as cell morphology can be assessed within the collagen gel. Using this assay, we characterize the invasive capacity of rare and sensitive cells; the hybrid cells resulting from fusion between breast cancer cells MCF7 and mesenchymal/multipotent stem/stroma cells (MSCs).

Video Link

The video component of this article can be found at https://www.jove.com/video/56568/

Introduction

Cell motility is a normal developmental and physiological process of cells. However, changes in the pattern and the ability of cells to migrate and invade are associated with pathological conditions including inflammatory diseases and cancer metastasis[1,2,3]. Metastasis is arguably the most poorly understood aspect in cancer. To metastasize, cancer cells must degrade the extra cellular matrix (ECM), invade the local tissue and intravasate. Once in circulation, the cancer cells will disseminate to the distant site, extravasate and form secondary tumors. Therefore, the ability of cells to degrade the ECM, to migrate, and to invade is related to their metastatic potential. Different approaches have been developed to analyze and quantify the migration and invasion capabilities of cells. The most used approaches include the wound healing assay, time-lapse microscopy, and the Boyden chamber assay. The wound healing assay[4] and time lapse microscopy are simple and convenient assays that would give preliminary insight into cell migration. However, both are 2D assays that do not reflect the 3D environment in which cells exist *in vivo*. Studies have shown that cells respond differently to a 3D environment compared to a 2D one[5,6]. Moreover, wound healing assays are difficult to reproduce and to yield quantitative results[7,8,9]. The Cell Exclusion Zone assay, which is a 3D modification of the wound healing assay, is a more physiologically relevant assay but it is not suitable for cells low in number such as hybrid cells resulting from cell fusion events[10,11].

The Boyden chamber assay is a 3-dimensional assay that provides relevant microenvironments for cellular studies. However, it requires cells to be removed from their original microenvironment and to be deposited into the upper chamber of the Boyden assay system to migrate and invade downwards toward the chemoattractant containing medium. This 3D approach is not appropriate in analyzing the migration and invasion capability of cells vulnerable to their microenvironment and/or limited in number[10,11,12]. A newer approach to analyze cell migration and invasion is the microfluidic gradient chamber assay[13]. Although suitable and reliable in migration and invasion studies, this assay is more expensive and could be technically challenging for a typical laboratory. We describe here a simple inverted vertical invasion assay that is compatible with many cell types including cells sensitive to their microenvironment and/or restricted in number. This assay was adapted from the protocol proposed by Hooper *et al.*[14]. In this assay, the cells remain in their original microenvironment and their migration and invasion is monitored as they move upwards in the collagen gel containing a chemoattractant[11]. This assay is reproducible and has been optimized and validated in the 35-mm culture dish. It could be adapted to different assay conditions including different cell culture sizes, different matrices or strength of adhesion, and different chemoattractants. This assay could serve as an *in vitro* platform for initial screening in the drug discovery process.

Protocol

NOTE: This protocol is described in McArdle *et al.*[11]. A timeline is provided in **Figure 1**.

1. Preparation of the culture plate

1. Wash the 35-mm culture dish containing a 10-mm glass-bottom well with 1 mL of 1 M hydrochloric acid (HCl) for 15 min to lower the hydrophobicity of the glass-bottom.
2. Wash the culture plate twice with 1 mL of phosphate buffer saline (PBS) to get rid of all the HCl.
3. Wash the culture plate twice with 1 mL of 70% ethanol.
4. Rinse the culture plate with 1 mL of the cell specific culture medium (here, use α-MEM supplemented with 10% heat inactivated FBS and 1% non-essential amino acids).
   Note: The treated culture plate containing the culture medium can be stored in the CO_2 incubator with a humidifier at 37 °C until the next day.
5. Grow the cells (MSCs and MCF-7 co-culture) in the treated glass-bottom well of the 35-mm culture dish to 100% confluency. Co-culture 35,000 MSC cells and 75,000 of MCF7 cells for 24 h.

2. Preparation of the collagen gel

1. Store the micropipette tips used for pipetting the collagen gel in the freezer to prevent the polymerization of the collagen upon contact.
   Note: This is the most critical step.
2. Determine a volume of rat tail collagen I to be used based on the concentration of the collagen stock. Prepare 100 µL of collagen gel for this assay.
   Note: High concentration of rat tail collagen I stock works better. Determine the volume of 10x Dulbecco's Modified Eagle's Medium to be used to dilute the collagen accordingly.
3. Combine on ice rat tail collagen I (3.8 µg/mL stock), 10x Dulbecco's Modified Eagle's Medium and 1x cell culture medium supplemented with 2% fetal bovine serum and the appropriate chemoattractant to a final concentration of collagen of 2.4 mg/mL.
4. Add to the mixture 4.5% of sodium bicarbonate solution to neutralize the collagen gel.
   Note: The volume of sodium bicarbonate solution added may vary based on the exact pH of the other reagents. It is best to test the mixture with pH strips to ensure neutral solution, or use a pH indicator in the culture medium.
5. Lay 80 µL of the collagen mixture over the cells within the glass-bottom well of the culture dish.
6. Allow the collagen gel to polymerize for 30 min in a CO_2 incubator with a humidifier at 37 °C.
7. Cover the collagen gel with 2 mL of cell medium with reduced serum (2%) and incubate the cells at 37 °C in a CO_2 incubator with a humidifier for 24, 48, or 72 h.
   Caution: The plate should be handled with care to prevent the collagen gel from detaching from the glass bottom.

3. Example of preparation of collagen gel using a rat tail collagen stock of 3.8 µg /mL

1. On ice, combine 114.5 µL of rat tail collagen, 16.6 µL of 10x DMEM, and 33.1 µL of culture medium containing the chemoattractant.
2. Neutralize the collagen mixture with 14.0 µL of sodium bicarbonate.
3. Continue as in step 2.5.

4. Imaging of the cells

1. Gently remove the media from the dish.
2. Gently rinse the gel with 1 mL of PBS.
3. Fix the cells with 1 mL of 4% paraformaldehyde for 15 min.
4. Wash the cells twice with 1 mL of PBS.
5. Stain the cells with DAPI solution (100 ng/mL) for 20 min at room temperature.
6. Image the cells using a confocal microscope at different locations and taking 400 µm z-stack images of each location in 16 µm steps. The microscope used has a disk scanning unit that enables confocal imaging using a white light, arc excitation source. Use a 20X lens utilized with numerical aperture of 0.75.
7. Reconstitute images .
   1. Open images for a given gel (approximately 25 images) and create an image stack by clicking image → stacks → images to stack. The first image corresponded to the bottom of the gel (z = 0 µm), the second image corresponded to the first step in the z direction (z = 16 µm), the third image corresponded to the second step in the z direction (z = 32 µm). Assess each nucleus at each image depth and designate the depth at which the nucleus was in sharpest focus as the invasion depth for that particular cell.

Representative Results

We have used a 35-mm culture dish with a glass bottom and rat tail collagen I to develop and optimize an *in vitro* 3D invasion assay protocol. Using this assay, we showed that breast cancer cells MCF7 and MSC cells could invade into the collagen gel to an average distance of 0.04 ± 1.8 µm, and 41.3 ± 76.0 µm respectively after 48 h when IGF1 (18.6 ng/mL) was used as chemoattractant (**Figure 2**). More importantly, we showed that fusion products from MSC and MCF7 cells, which are rare cells, could invade into the collagen gel as well. Their average distance of invasion in 48 h was 10.7 ± 12.4 µm when IGF I at 18.6 ng/mL was used as chemoattractant (**Figure 2**). The difference in the invasion capability between MCF7 and MSCs was statistically significant (P <0.01)[11]. The difference between the invasion capability of the fusion products and the one of MCF7 or MCSs was not statistically significant. However, this assay consistently showed that the cancer cell fusion products had a greater migratory and invasive capability than the parental cancer cells, MCF7.

Cells remained healthy throughout the experiments as shown by their morphology and quantity 48 h post gel polymerization (**Figure 3a**). With the inverted vertical invasion assay design, the kinetics of cell migration and invasion can be analyzed (**Figure 3b**, **4**) and cell morphology can be assessed (**Figure 4**).

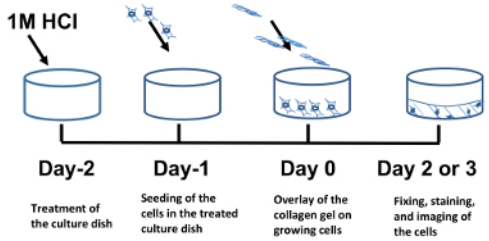

Figure 1: Timeline of the inverted vertical invasion assay design Please click here to view a larger version of this figure.

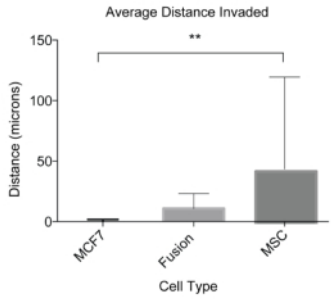

Figure 2: Invasion capability of cancer cell fusion products. The invasion capacity of cancer cell fusion products could be determined using the inverted vertical invasion assay. The numbers represent the average invasion capacity of four independent fusion products from three separate co-cultures and their parental cell lines analyzed from eight different fields within each assay. This experiment was performed in triplicate. ANOVA with Tukey's HSD post hoc test. **P <0.01. Error bars represent standard deviation (SD). This figure has been adapted from [11]. Please click here to view a larger version of this figure.

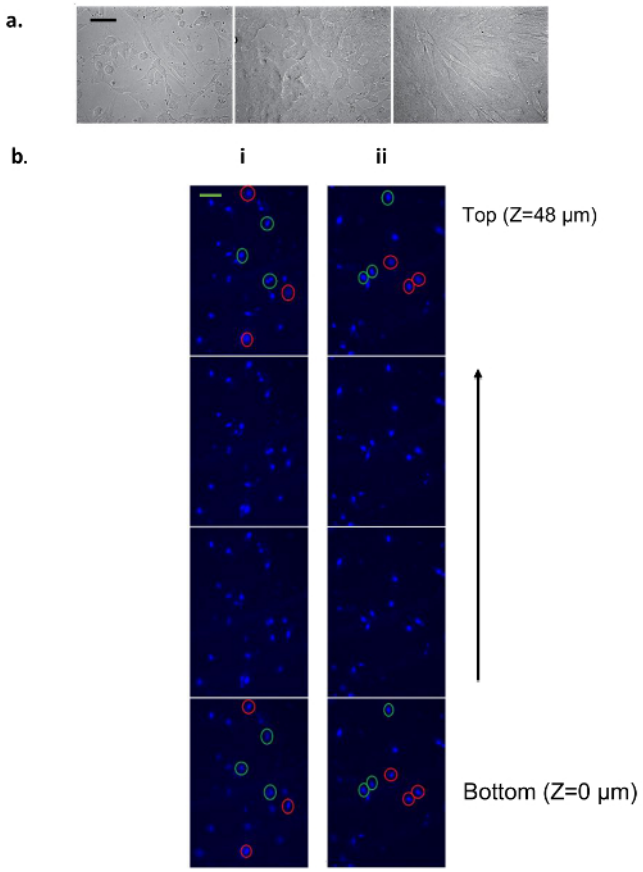

Figure 3: Cell viability and Z series of the inverted vertical invasion assay.
a) 20X bright field images showing the morphology of cells under the collagen gel after two days. From left to right MCF7 and MSC co-culture, MCF7, and MSC; b) representation of z series showing two locations (i and ii) within a dish from the bottom to the top. Successive images are 16 µm apart. 3.8% FBS was used as a chemoattractant. Green circles on both panels indicate invading cells; note, out of focus at the bottom of the gel and in focus in the top gel, invading into the gel. Red circles on both panels indicate cells remaining at the bottom layer of the z series (non-invading cells). Scale bar (a): 15 µm; Scale bar (b): 10 µm. This figure has been adapted from [11]. Please click here to view a larger version of this figure.

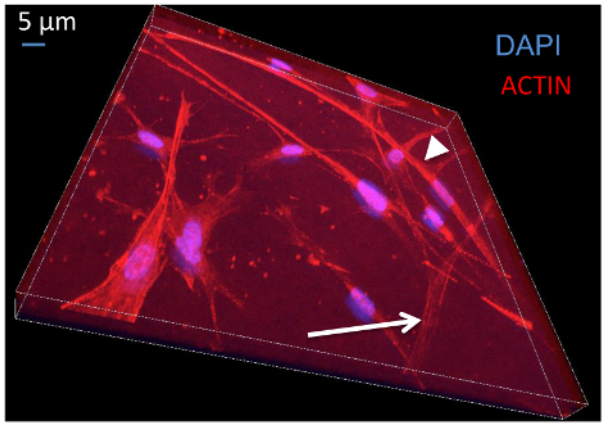

Figure 4: Cell morphology in the inverted vertical invasion assay. Morphology of MSCs in the collagen gel at one location from the bottom (Z = 0 µm) to the Z = 20 µm. 3.8% FBS was used as a chemoattractant. The long arrow indicates the appendage of an MSC invading into the gel. The arrowhead indicates an MSC, which is aligned along the bottom of the gel, and not invading. The blue shows the nuclear DAPI stain while the red shows the phalloidin stained actin filaments.This figure has been adapted from [11]. Please click here to view a larger version of this figure.

Discussion

Here, we describe a simple inverted vertical invasion assay which is convenient for a variety of cell types including cells that are rare and/or dependent on their microenvironment. In this 3D invasion assay, cells remain in their original microenvironment and are covered by the collagen gel containing a chemoattractant. The cells then migrate and invade in the collagen. In this assay, the cells can be stained and easily monitored within the gel. The simplicity and reproducibility of this assay makes it a convenient tool for studying cancer cell migration and invasion in a 3D system. However, strong chemoattractants seem to be required to break the cell affinity to the bottom of the culture dish.

We optimized the inverted vertical invasion assay in the 35-mm culture dish. This culture dish has a 10-mm glass-bottom well which is well fitted for imaging. One limitation of this culture dish is the considerable amount of collagen gel required to perform an assay. Finding smaller culture dishes with a design compatible with imaging would be more economical. The gel layer appropriate for the 35-mm dish design is very delicate due to its strength and can easily detach from the bottom of the plate if not handled carefully. A different cell culture design might improve the stability of the collagen gel layer but the compatibility between the collagen gel strength and cell viability should be optimized.

Despite these limitations, the inverted vertical invasion assay presents many advantages compared to other 3D invasion assay formats. The main benefit is that cells remain in their original culture environment, which is not the case in the Boyden chamber assay. This protocol preserves the cell microenvironment that is advantageous for cells stringently controlled by their microenvironment such as stem cells[15]. This assay also prevents cell damage or cell loss especially for cells that are delicate or rare[10,11]. In addition, with this assay, the cell morphology and associated kinetics could be assessed within the gel, which is not possible with the Boyden chamber assay. Moreover, the non-physiologic polycarbonate or polypropylene filter of the Boyden chamber assay system is not used in the inverted vertical invasion assay. The inverted vertical invasion assay is a static design, it does not mimic the *in vivo* dynamic environment that the microfluidic invasion assay design offers but it provides a simpler, less challenging and cost-effective 3D environment to quantify migration and invasion of a variety of cell types in a preserved and controlled microenvironment.

The inverted vertical invasion assay has many potential applications in pathophysiological studies. This approach could serve as an *in vitro* platform to study cell motility during embryonic development, wound healing, and/or inflammatory responses[3]. Cell interaction with the ECM and other cell types during migration and invasion could also be investigated using this protocol. This assay could also represent a useful tool for initial drug screening of anti-metastatic drugs. A model system of organ-specific cancer metastasis could be developed using the inverted vertical invasion assay as well.

The inverted vertical invasion assay was developed to circumvent the challenge that the displacement of some cells from their original microenvironment presents in the Boyden chamber assay system. This assay is easy, reliable, and reproducible; it is flexible and can be used to quantify the invasive potential of a wide variety of cell types including sensitive and rare cell types. This assay can be applied to detect the migratory activity associated with matrix degradation and can also be adapted to study the selective degrading activity on different matrix substrates.

Disclosures

The authors have declared that no competing interest exists.

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