Video Article

Concentric Gel System to Study the Biophysical Role of Matrix Microenvironment on 3D Cell Migration

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Abstract

The ability of cells to migrate is crucial in a wide variety of cell functions throughout life from embryonic development and wound healing to tumor and cancer metastasis. Despite intense research efforts, the basic biochemical and biophysical principles of cell migration are still not fully understood, especially in the physiologically relevant three-dimensional (3D) microenvironments. Here, we describe an *in vitro* assay designed to allow quantitative examination of 3D cell migration behaviors. The method exploits the cell's mechanosensing ability and propensity to migrate into previously unoccupied extracellular matrix (ECM). We use the invasion of highly invasive breast cancer cells, MDA-MB-231, in collagen gels as a model system. The spread of cell population and the migration dynamics of individual cells over weeks of culture can be monitored using live-cell imaging and analyzed to extract spatiotemporally-resolved data. Furthermore, the method is easily adaptable for diverse extracellular matrices, thus offering a simple yet powerful way to investigate the role of biophysical factors in the microenvironment on cell migration.

Video Link

The video component of this article can be found at https://www.jove.com/video/52735/

Introduction

Migration of cells plays a key role in various physiological responses such as embryonic development, haemostasis, and immune response as well as in pathological processes such as vascular diseases, inflammation, and cancer[1]. Dissecting the biochemical and biophysical factors underlying cell migration is therefore fundamentally important not only to understand the basic principles of cellular functions, but also to advance various biomedical applications, such as in tissue engineering, anti-metastasis and anti-inflammatory drug development. Since *in vivo* observation is technically challenging, a lot of efforts has been focused on *in vitro* recapitulation of cell migration.

In vitro methods to study cell migration have largely been designed for assays on two dimensional (2D) surfaces, most notably the scratch or wound healing assay[2]. Such assays offer simple experimental setup, easy live-cell imaging, and provide useful insights into various biochemical mechanisms underlying cell migration. However, these assays do not account for extracellular matrix (ECM) architecture and remodeling, which are critical aspects in understanding *in vivo* migration. Recently, it has been increasingly appreciated that a 3D culture model, often in collagen-based matrices[3], provides a platform that better resembles the *in vivo* situation. Indeed, cells exhibit migrational dynamics that are distinct from those on 2D surfaces, especially due to the different dimensionality of the environment[4]. Moreover, the biophysical and mechanical properties of the matrix sensitively affect cell migration[5], including in the context of tumor cell invasion[6].

Here, we present a method to study 3D cell migration behavior in ECM with biophysical properties that can be easily varied with preparation conditions. The cells are seeded in an "inner gel" and are allowed to escape into and invade the initially acellular "outer gel". The method relies on the cell's ability to recognize the presence of, and propensity to migrate into, cell-free regions in the outer gel, which is closely linked to cell mechanosensing[7]. In this study, we employ collagen networks as the ECMs invaded by highly invasive breast cancer cells, MD-MBA-231. The mechanical properties and microstructure of both the inner and outer gels can be tuned[8] and characterized[9] to achieve physiologically relevant conditions. Reconstruction and analysis of the cell tracks allow detailed quantitative examination of the spatiotemporal migration behavior at both population level and individual cell level. Importantly, the setup of the concentric gel system mimics the *in vivo* tissue topology faced by migrating cells, especially invading cancer cells, thus offering important insights into the physical mechanisms of cell migration and metastasis.

Protocol

1. Cell Harvesting

1. Obtain MD-MBA-231 cells from the 37 °C, 5% CO_2 incubator. Detach cells from tissue culture plate using 0.5% Trypsin-EDTA solution. Use 1 ml of Trypsin-EDTA solution for cell cultured in a T25 flask.
2. Pellet cells in a 15 ml conical tube by centrifugation at 200 × g for 4 min, aspirate the supernatant, and resuspend cells in 5 ml of culture media.
3. Count cell density, ρ, using a hemocytometer.
   Note: To prepare the cell-seeded inner gel, the cell suspension will later on be diluted 10× to reach the final cell seeding density. Therefore, 10× concentrated cell suspension is required.
4. Calculate the volume of medium required to achieve a 10× cell concentration:
   $$V_{\text{medium}}(\text{in ml}) = \frac{\rho \times 5\ \text{ml}}{10 \times c_{\text{final}}}$$
   Note: A final cell seeding density, c_{final}, of around 2 × 10^6 cells/ml is recommended for MD-MBA-231 cells and is used in this protocol. Other seeding densities can also be explored for other cell types.
5. Pellet cells one more time in a 15 ml conical tube by centrifugation at 200 × g for 4 min, and aspirate the supernatant.
6. Resuspend the cells in the required amount (V_{medium} calculated in step 1.3) of serum-free cell culture medium thoroughly to minimize cell clumping.
   Note: Phenol red is auto-fluorescent, and can interfere with fluorescence/reflectance imaging. Use of phenol red-free medium may be considered to achieve best image quality.

2. Preparation of Collagen Solutions

1. Obtain the stock collagen solution, 10× PBS buffer, Milli-Q H_2O, 0.1 M NaOH, and several microcentrifuge tubes. Keep all on ice to prevent premature collagen polymerization, and maintain sterile condition.
2. Equilibrate a sterile glass-bottom dish by pre-warming it in the 37 °C incubator.
   Note: All volumes in this protocol have been optimized for a glass-bottom dish with 12 mm well. If other dish type is used, adjust the volumes correspondingly.
3. Calculate the required volume needed to prepare 50 µl of 2.4 mg/ml collagen solution for the inner gel (solution I) based on the collagen stock concentration.
   Note: Other collagen concentrations for the inner gel can also be used.
4. In a sterile environment (typically a biosafety hood), slowly add 5 µl of 10× PBS buffer to the required amount of collagen stock solution (calculated in step 2.3) with gentle swirling. Take care to avoid air bubble formation.
5. Adjust the pH of the mixture to 7.4 using 0.1 M NaOH using calibrated pH-meter. As a rough guide, use about 5 µl to bring the pH close to 7.4 (the amount varies depending on the stock concentration and pH).
   Note: Note that the volumes involved in this step is too small for the use of standard pH meter. Use one of the following tricks:
   1. Prepare collagen solutions for multiple samples. Adjust the pH in bulk using standard pH meter and distribute the collagen solutions across the samples.
   2. Alternatively, adjust the pH of a collagen solution in larger volume (*i.e.*, volume that allow the use of standard pH meter). Note the amount of NaOH needed to bring the pH to the final pH. Scale down the volumes and use the appropriate amount of NaOH for the experiment. Confirm the pH value using Litmus paper.
   3. Otherwise, use a micro pH electrode to more accurately adjust the pH of small quantities.
6. Bring the solution to a volume of 45 µl using H_2O. Perform all steps on ice to prevent premature collagen polymerization.

3. Formation of Concentric Gel Culture

1. Take the pre-warmed glass-bottom dish (see step 2.2) from the incubator.
2. Add 5 µl of the 10× concentrated cell suspension (prepared in step 1.5) to solution I. Resuspend thoroughly. Take care to avoid air bubble formation. The mixture now has a volume of 50 µl and contains the final collagen concentration (2.4 mg/ml) and cell density (c_{final} = 2 × 10^6 cells/ml).
3. Add 20 µl of the cell-containing solution I slowly to the center of the well, so that it forms a dome-shaped droplet (**Figure 1A**). Take care to avoid bubble formation at this step. If a bubble forms, carefully but quickly try to either rupture it or suck it out using a pipette tip. Gently place the dish back in the incubator to let the inner gel polymerize for 45 min.
4. Prepare solution O (for the outer, acellular collagen gel) about 15 min before the end of this incubation step.
   Note: The outer gel condition can be varied in terms of collagen concentration and polymerization pH to obtain networks with different microstructures[10]. In this protocol, focus on a 1.5 – 4.0 mg/ml collagen gel polymerized at a pH of 7.4.
   1. Based on the collagen stock concentration, calculate the required volume needed to prepare 200 µl of collagen solution O at the final concentration.
5. Add 20 µl of 10× PBS buffer slowly to the required amount of collagen stock solution (calculated in step 3.4) with gentle swirling. Adjust the pH of the mixture to the final pH using 0.1 M NaOH with the use of calibrated pH-meter. See note to step 2.5 regarding pH adjustment.
6. Bring the solution to a final volume of 200 µl using H_2O. Perform all steps on ice to prevent premature collagen polymerization.

7. Take the dish from the incubator after 45 min polymerization of the inner gel (see step 3.3). Gently add 180 µl of solution O on top of the inner gel, so that the solution completely covers the inner gel and fills up the well (**Figure 1B**).
    1. Perform this step carefully without stirring the solution, which can lead to non-uniform fiber orientations in the outer gel. Take care to avoid touching the inner gel with a pipette tip, and to avoid the formation of bubbles or air pockets. If a bubble forms, carefully but quickly try to either rupture it or suck it out using a pipette tip. Gently place the dish back in the incubator to let the outer gel polymerize.

8. Take the dish from the incubator after 45 min of polymerization. The gel should already be fairly solidified at this point, although it can still detach from the bottom surface if handled roughly.
    1. Gently pour 2 ml of warmed cell culture medium to the dish (**Figure 1C**). Make sure that the gel is completely submerged in the medium. Refresh the medium every 2 – 3 days throughout the duration of culture.

4. Live-cell Imaging

1. Perform imaging using an inverted confocal microscope equipped with long-term live-cell imaging capability. Include a built-in incubation chamber with a temperature (37 °C) and CO_2 (5%) control. Switch on the microscope and warm up the stage at least 1 hr before starting the experiment.
   Note: Use objective lens with long working distance to optimize the observation and localization of cells in the 3D gels.
2. Incubate the gel in medium containing 5 µl of fluorescent cell tracker dye for 30 min to allow accurate localization of the cells in the 3D system. Subsequently, remove unbound dye by washing three times with 1× PBS. Afterwards, add cell culture medium to the dish.
3. Take the dish from the incubator and place it on the microscope stage (**Figure 1D**).
   Note: Live cell imaging can start in principle right after the polymerization of the outer gel. However at this point, the cells in the inner gel have not spread yet. To examine the migration of the first cells that have invaded the outer gel, live-cell imaging can start 24 hr (depending on the cell type) after the initiation of culture. As a rough guide, around 12 - 14 days of culture are needed for most of the cells in the inner gel to enter the outer gel.
4. Select Volumes of View (VoV's) in regions in the outer gel surrounding the inner gel. After 24 hr of incubation, the cell population will have spread, crossed the interface between the inner and outer gels, and started to invade the outer gel.
    1. For the VoV's, include the gel regions immediately next to the gel interface, intermediate regions, and regions close to the outskirt of the outer gel[7]. Exclude regions closer than 50 µm from the bottom and side surfaces, as well as from the top of the gel, to avoid possible edge effects. Each VoV typically measures 647 × 647 × 100 μm^3 (in *x*, *y*, and *z* directions, respectively), with 5 µm interval in the *z*-stack.

5. Ensure imaging modes, channels/filters, exposure times, and image resolutions are correctly selected. For label-free imaging of the collagen network, use confocal reflectance microscopy simultaneously during the time-lapse live-cell imaging.
6. Take sample images, plot the intensity histograms, and adjust the gains and offsets to observe sufficient signal and avoid saturation by ensuring that the histogram lies between zero and the maximum intensity. Do not change these settings any more throughout the duration of the experiment.
7. Take time-lapse images of the selected VoV's, with a time interval, Δ*t*, of 10 min for 8 hrs (or longer if necessary).

5. Cell Tracking and Data Analysis

1. Carry out quantitative image post-processing from the *z*-stack images using appropriate image processing software.
    1. Segment the time-lapse images to automatically select the 3D cell positions in (*x*,*y*,*z*,*t*).
    2. For every frame, manually check the localization accuracy and remove false positives due to cell debris and cellular protrusions that may have been mistaken for cells. Remove proliferative cells from the analysis and split overlapping or connected cells into distinct objects.

2. Generate 3D time-lapse cell tracks from the cell coordinates (*x*,*y*,*z*,*t*) obtained in the previous step by linking the location of each cell in the time sequence.
3. Eliminate random and system noise by removing tracks shorter than a threshold track length (typically 20 min).
4. Correct for sample drift by subtracting the overall net displacements from the tracks if necessary.
5. Calculate cell displacement, $\Delta d = \left|\overrightarrow{d_n} - \overrightarrow{d_0}\right|$, and cell migration distance, $d = \sum_{i=0}^{n-1}\left|\overrightarrow{d_{i+1}} - \overrightarrow{d_i}\right|$ from the observed cell trajectories, where is a vector representing the 3D location of a cell at time and is the total number of time points.
    1. Calculate cell speed as $S = d/(n \cdot \Delta t)$, where Δ*t* is the time interval between frames. Calculate cell migration directionality (or persistence) using $P = \Delta d/d$. This simple measure of persistence implies that for $P = 0$ the net displacement is zero and for $P = 1$ the trajectory is a straight directional line.

Representative Results

The concentric gel assay presented here was performed using highly invasive breast cancer cells, MDA-MB-231, with 2.4 mg/ml inner collagen gel and a cell seeding density of = 2 × 10^6 cells/ml, as an example. As shown in **Figure 2**, typically after a few days of culture, the cells breached the inner–outer gel interface and started to invade the outer gel. The cell population spread predominantly radially outwards.

The polymerization conditions of the outer gel can be modified to study the role of the density and mechanical properties of the matrix on cell migration characteristics. **Figure 3** shows the movements of individual cells in outer gels of 1.5 mg/ml, 2.4 mg/ml, and 4.0 mg/ml collagen, polymerized at pH 7.4. Confocal time-lapse images were acquired at the boundary between the inner and outer gel for 8 hr, and the

displacement of the cells in the three different collagen concentrations is indicated in white arrows. Depending on the cell seeding density and proliferation rate, ~200 individual cell trajectories can typically be extracted and analyzed in each sample.

We quantitatively analyzed the cell trajectories in the 3 different collagen concentrations in terms of mean net displacement, distance travelled, directional persistence, and mean speed over 8 h of imaging (**Figure 4**). It was observed that the mean displacement and distance were highest for 4.0 mg/ml concentration at 58 µm and 141.5 µm, respectively, although there was no significant difference in the displacement and distance between cells in 2.4 mg/ml and 4.0 mg/ml gels. In 1.5 mg/ml gel, the mean displacement and distance were smallest. This observation may appear to contradict recent reports that show that invasion efficiency decreases in gels with increasing collagen concentration[11,12]. Note, however, that the displacements and distances in our assay are measured from all individual cell tracks within predominantly radially outward migrating population. These parameters are therefore much less susceptible to the fastest-moving cell subpopulation and are not completely dominated by stochastic movements.

In all cases, the total distance covered (**Figure 4B**) was greater than the net displacement (**Figure 4A**). Defining persistence simply as the ratio between displacement to distance, we obtained persistence of ~0.4 across all gel conditions (**Figure 4C**). This relatively low persistence reflects the intrinsically weak directional migration of the cells in our assay. We anticipate that the presence of directional chemotactic stimuli or biochemical gradients will increase the persistence, potentially in collagen concentration-dependent manner. Furthermore, we also observed that the mean speed of cell migration did not significantly change with collagen concentration (**Figure 4D**), likely because of the interplay between matrix stiffness (which increases with collagen concentration) and pore size (which decreases with collagen concentration)[10], as well as the spatiotemporal variation of migration characteristics within the cell population[7]. The mean speed varied between 31 µm/hr and 37.5 µm/hr, with a minimum individual cell speed of 7 µm/hr and a maximum individual cell speed of 114 µm/hr.

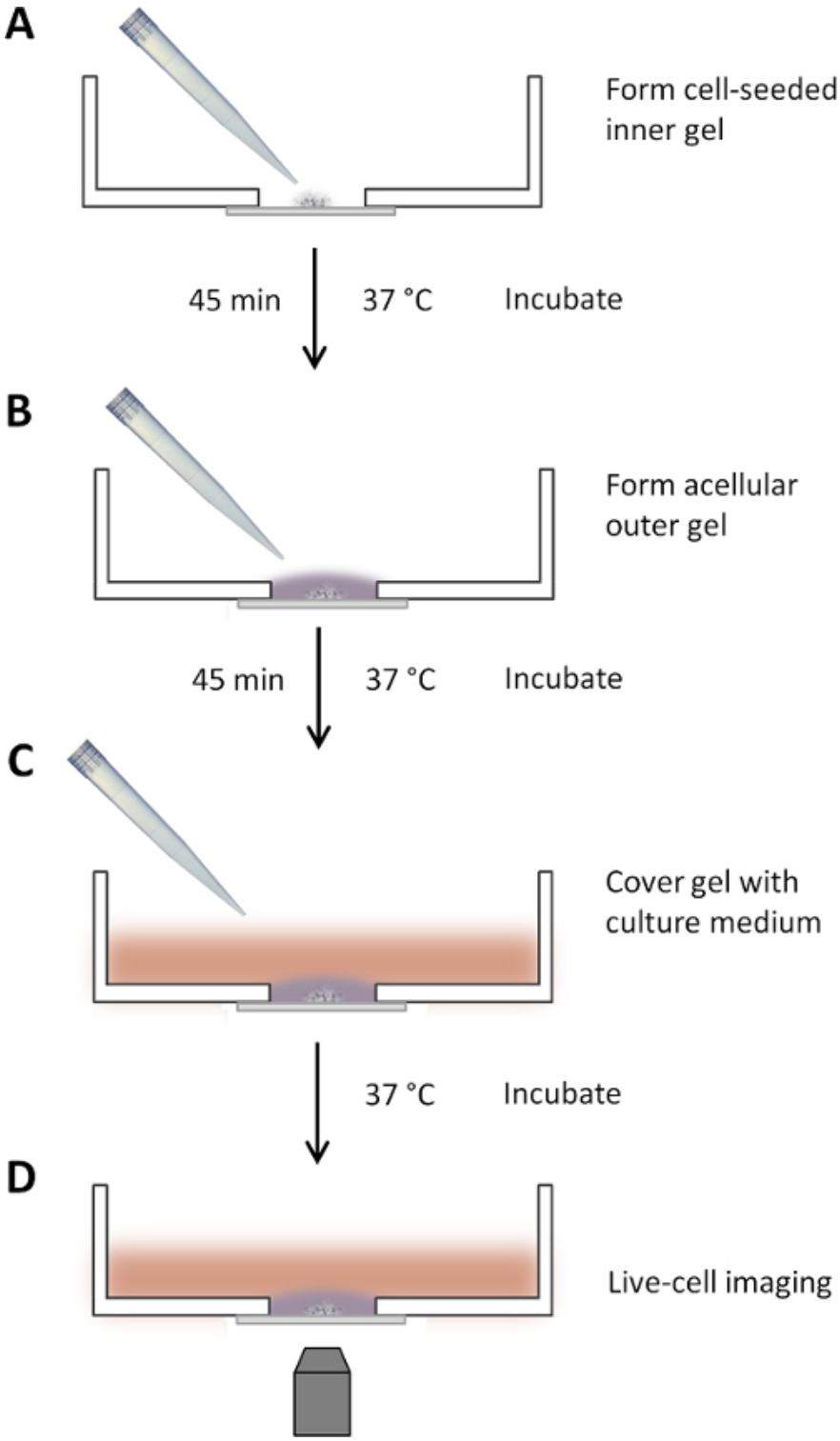

Figure 1. Schematic of the steps involved in setting up the concentric gel 3D migration assay. (A) Formation of the inner collagen gel at the center of the well in the glass-bottom dish, containing the cells. **(B)** Formation of the (acellular) outer collagen gel encapsulating the inner gel. **(C)** Immersion of the gels in cell culture medium. The cells are then allowed to equilibrate and attach to the surrounding fibrous matrix. This step can take a few hours up to 1 day after the initiation of gel polymerization. **(D)** Live-cell imaging, monitoring the spreading of cell population into the outer gel.

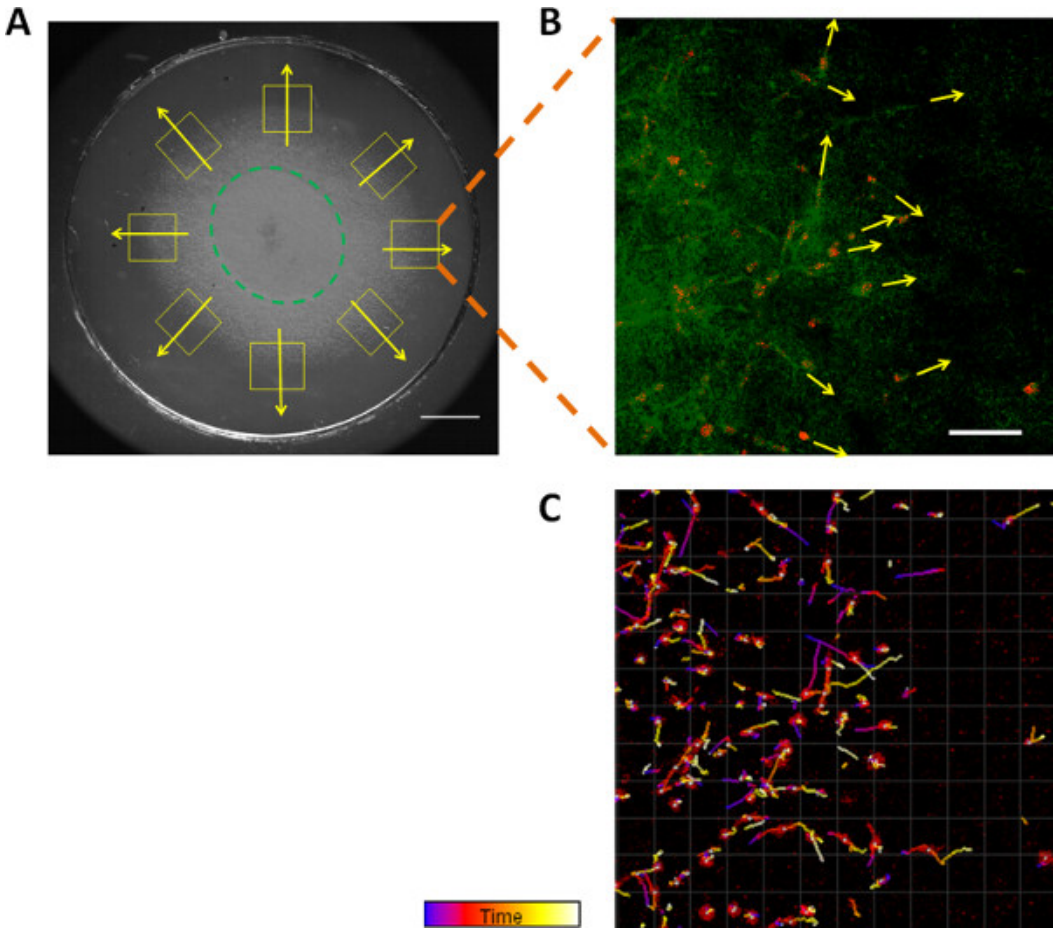

Figure 2. Spreading of cell population. (A) After 12 days of culture, MDA-MB-231 cells had breached the inner–outer gel interface (green dashed line) and invaded outwardly into the originally acellular outer gel (scale bar = 200 µm). The brighter areas are regions occupied by cells, whereas the darker areas are cell-free regions in this phase contrast image. The yellow rectangles represent the VoV's of interest, where the 3D cancer cell invasion was monitored. The cells migrated predominantly radially outward, as indicated by the arrows. **(B)** Overlay of confocal fluorescence image of the migrating MDA-MB-231 cells (red; yellow when overlaid with green pseudo-colored collagen) and reflectance image of the collagen matrix (green). The arrows point to the migration direction of individual cells within a time span of 5 h. Scale bar = 50 µm. **(C)** From the time-lapse images, tracks of the cells can be obtained. Here the cell tracks are color-coded for time, as indicated by the color bar (timescale = 8 hrs). Adapted from Sun *et al*[7,10]. Please click here to view a larger version of this figure.

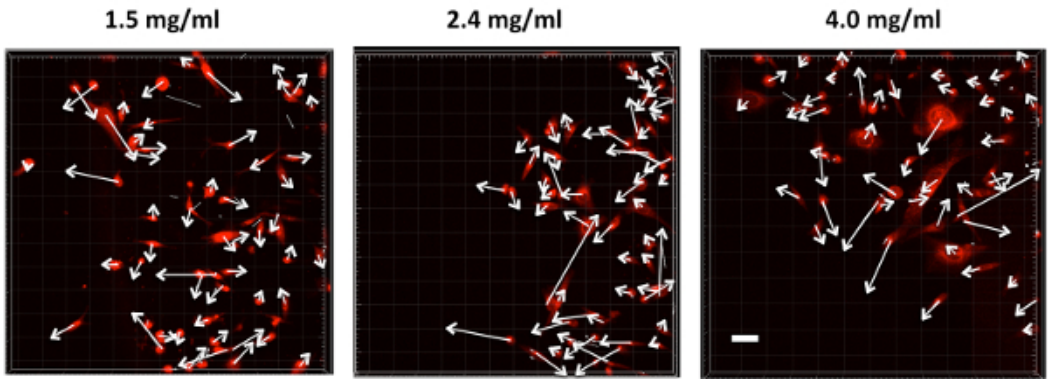

Figure 3. Cell movement in different collagen gel densities. The movement of MDA-MB-231 cells in outer gels with three different outer collagen concentrations: 1.5 mg/ml, 2.4 mg/ml, and 4.0 mg/ml. Cells were labeled with live-cell tracker CMTMR (red). The white arrows indicate the net displacement of individual cells within 8 hrs of imaging. Scale bar = 100 µm. Please click here to view a larger version of this figure.

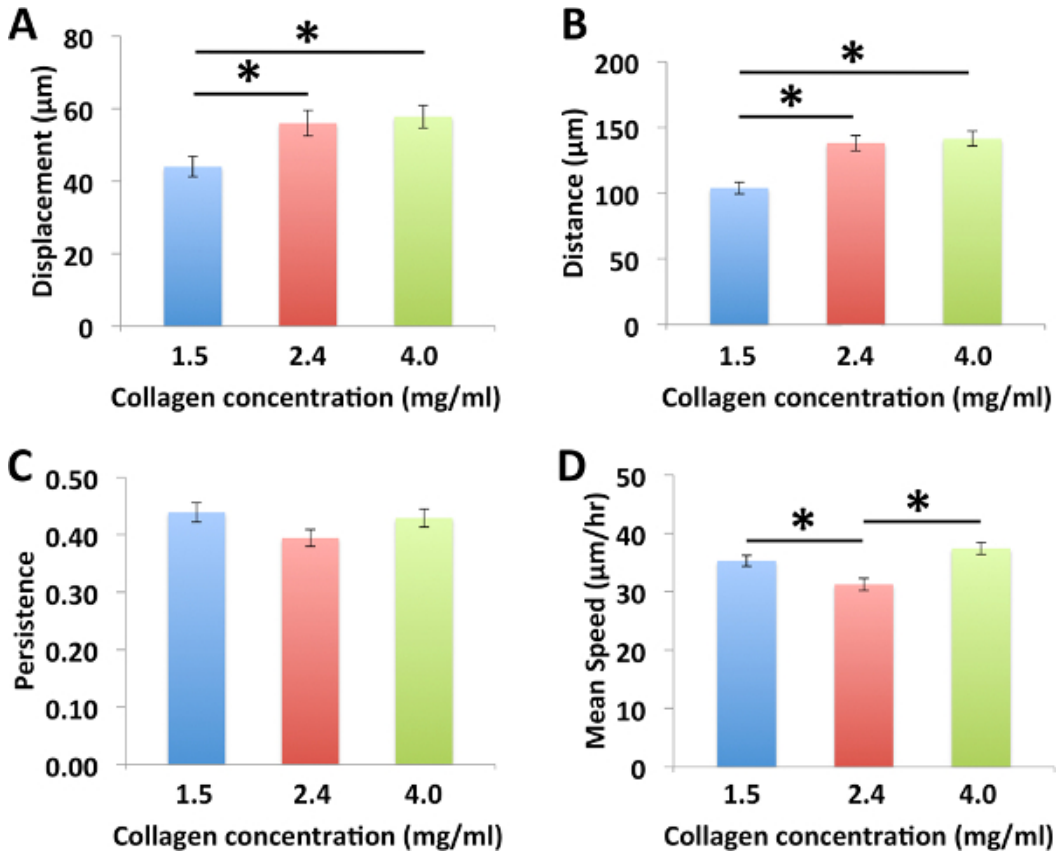

Figure 4. Analysis of cell migration trajectories. The migration trajectories of the individual MD-MBA-231 cells over 8 hrs were quantified in terms of **(A)** net displacement, **(B)** distance, **(C)** persistence, and **(D)** mean speed for different outer gel collagen concentrations: 1.5 mg/ml, 2.4 mg/ml, and 4.0 mg/ml. Time-lapse images were acquired near the interface of the inner and outer gels. Data are mean ± standard error of the mean (s.e.m.) obtained from more than 200 cell trajectories in 3 independent experiments in each condition. Asterisks (*) denote statistically significant difference (p-value < 0.01).

Discussion

In this protocol we describe an *in vitro* assay to study the 3D migrational behavior of cells in matrix environments that topologically resemble ECMs encountered *in vivo*. There are three main strengths of this assay as compared to other currently available methods. First, this assay allows one to simultaneously examine the cell migration mechanisms at both population level and individual cell level. This opens up possibilities of studying collective cell migration[13], which has to date been largely limited to 2D assays[14], while at the same time observing the behavior of the individual cell within the population. For cells that do not naturally migrate in groups or clusters, the assay offers a way to analyze the migratory properties in detail from a large number of cells with diverse local microenvironment, which can provide important insights into the biophysical origins of migration efficiency and directional persistence[7,15].

Second, by modifying the gel conditions of the inner and outer gels, one can directly study the effect of matrix mechanics and microstructure on 3D cell migration behavior[10]. This is especially important, as it is known that cell migration and cancer invasion are highly influenced by matrix mechanics and porosity[16,17], and that soft tissues in our body have a very diverse topology[18]. Moreover, the method is readily adaptable for matrices other than collagen. This is advantageous, as not all cell lines can spread and migrate in collagen-based matrices. A critical step that must be noted is that, to ensure physical connectivity of the gel interface, the formation of the outer gel must be initiated *before* complete polymerization of the inner gel. Since this is strongly dependent on the specific polymerization kinetics of the gels (also for collagen prepared under different conditions, *e.g.*, concentration, polymerization pH, temperature, cross-linking[8]), an optimal polymerization duration should first be systematically identified. Imperfections in the interfacial connectivity between the inner and outer gel usually results in a gap and collection of the spreading cell population in the interface. Note, however, that perfect interfacial connectivity can introduce a practical problem: the boundary between the inner and outer gels becomes less obvious when imaging. If necessary, sparse fiducial markers, such as inert beads, can be used to distinguish the inner and outer gels.

Third, the possibility of long-term 3D culture (up to 3 weeks in our laboratory) and live-cell imaging allows a wide application of the assay. It is now possible to obtain spatially-resolved viscoelastic measurements of the local matrices surrounding the cells using microrheological approaches[19]. Furthermore, our recent study[10] reveals that the efficacy of various anti-migratory drugs (administered on the 7th day of the experiment) can be strongly affected by the architecture and stiffness of the matrix (*i.e.*, outer gel). In the future, we plan to use these experimental frameworks to further understand how such effects can arise from the basic molecular mechanisms of cell migration (*e.g.*, cytoskeletal contractility[20], extracellular matrix reorganization[21], and pericellular proteolysis[22]).

One limitation of this assay is the asymmetry of the mechanical support: the top surface of the gel is not attached to any solid surface, while the bottom surface adheres to the glass surface of the dish. While edge effects can be avoided simply by excluding cells near the edges, the lack of mechanical support at the top surface can potentially lead to in-plane preferential migration and therefore introduce artificial flattening of the 3D cell trajectories[7]. One way to solve this issue while still maintaining oxygen and nutrition supply to the cells is by topping the gel with holed glass plate[23]. Additionally, to further suppress any wall effect, a thin layer of collagen gel can be first made on the glass surfaces, before the formation of the inner and outer gels. This ensures complete geometrical encapsulation of the gels.

To obtain meaningful and accurate description of cell migration behavior, it is important to consider the time scale and the nature of migration, which can vary considerably between cell lines. Preliminary experiments must be systematically performed to understand these factors, and then the interval time between image captures and live-cell imaging duration can be adjusted. The interval time should be no larger than the characteristic time scale of cell turning. It is also important to consider that cell migration behavior may change both spatially and temporally, depending on the biophysical conditions of the local microenvironment[7]. It is always a good idea to simultaneously visualize the cells and the fibrous matrix, for example using multi-color confocal fluorescence or multimodal (*e.g.*, reflectance, second harmonic generation) imaging. After imaging, post-processing of the time-lapse 3D image, construction of the cell tracks, and analysis of the trajectories can in principle be done with any image processing software.

In conclusion, the concentric gel assay described here provides a simple and useful experimental system for quantitatively studying 3D cell migration, mechanosensing and mechanotransduction in diverse microenvironments.

Disclosures

The authors have nothing to disclose.

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