Video Article

3-D Cell Culture System for Studying Invasion and Evaluating Therapeutics in Bladder Cancer

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Abstract

Bladder cancer is a significant health problem. It is estimated that more than 16,000 people will die this year in the United States from bladder cancer. While 75% of bladder cancers are non-invasive and unlikely to metastasize, about 25% progress to an invasive growth pattern. Up to half of the patients with invasive cancers will develop lethal metastatic relapse. Thus, understanding the mechanism of invasive progression in bladder cancer is crucial to predict patient outcomes and prevent lethal metastases. In this article, we present a three-dimensional cancer invasion model which allows incorporation of tumor cells and stromal components to mimic *in vivo* conditions occurring in the bladder tumor microenvironment. This model provides the opportunity to observe the invasive process in real time using time-lapse imaging, interrogate the molecular pathways involved using confocal immunofluorescent imaging and screen compounds with the potential to block invasion. While this protocol focuses on bladder cancer, it is likely that similar methods could be used to examine invasion and motility in other tumor types as well.

Video Link

The video component of this article can be found at https://www.jove.com/video/58345/

Introduction

Invasion is a critical step in cancer progression, which is required for metastasis, and is associated with lower survival and poor prognosis in patients. In human bladder cancer, the most common malignancy of the urinary tract which causes about 165,000 deaths per year worldwide, cancer stage, treatment and prognosis are directly related to the presence or absence of invasion[1]. Around 75% of the cases of bladder cancer are non-muscle invasive and are managed with local resection. In contrast, muscle-invasive bladder cancers (about 25% of all cases) are aggressive tumors with high metastatic rates and are treated with aggressive multimodality therapy[2,3]. Therefore, understanding the molecular pathways that trigger invasion is essential to better characterize the risk of invasive progression and to develop therapeutic interventions which can prevent invasive progression.

Tumor invasive progression occurs in a complex three-dimensional (3-D) environment and involves tumor cell interaction with other tumor cells, stroma, basement membrane, and other types of cells including immune cells, fibroblasts, muscle cells and vascular endothelial cells. Permeable support (*e.g.*, Transwell) assay systems are commonly employed to quantitate cancer cell invasion[4], but these systems are limited because they do not allow microscopic monitoring of the invasion process in real-time and the retrieval of samples for further staining and molecular analysis is challenging. Development of a 3-D bladder tumor spheroid system to study invasion is desirable because it allows the incorporation of defined microenvironmental components with the convenience of *in vitro* systems.

In this protocol, we describe a system to interrogate the invasive processes of human bladder cancer cells using a 3-D spheroid invasion assay incorporating collagen-based gel matrices and confocal microscopy to allow investigators to monitor cell motility and invasion in real-time (**Figure 1A**). This system is versatile and can be modified to interrogate various stromal/tumor settings. It can incorporate most bladder cancer cell lines or primary bladder tumors and additional stromal cells such as cancer associated fibroblasts and immune cells[5,6,7]. This protocol describes a matrix composed of type-1 collagen, but can be modified to incorporate other molecules such as fibronectin, laminin, or other collagen proteins. Invasive processes can be followed for 72 h or longer depending on the capability of the microscope and system used. Fixation and immunofluorescence staining of the tumor embedded in the 3-D matrix before, during, and after invasion allows the interrogation of proteins upregulated in invasive cells, thus providing crucial information that usually absent or difficult to gather using other 3-D culture models. This system can also be utilized to screen compounds which block invasion, and to delineate signaling pathways affected by such compounds.

Protocol

1. Growing Cancer Spheroids

1. **Growing from cell lines**
   1. Culture human bladder cancer cells under conventional adherent cell culture conditions and maintain in a 37 °C incubator supplied with 5% CO_2. Maintain cells at <90% confluency.
      NOTE: Culture media used is Dulbecco's modified minimum essential media (DMEM) containing 4.5 g/L D-glucose, L-Glutamine, 110 mg/L sodium pyruvate, and supplied with 10% fetal bovine serum (FBS) throughout this protocol.
   2. One day prior to the start of the experiment, trypsinize (by using 0.25% Trypsin-EDTA) cells, quantify cell concentration and distribute 1 x 10^6 cells in 3 mL culture media in each well of a 6-well ultra-low attachment plate.
   3. Incubate cells in low attachment conditions at 37 °C for ≥16 h. This should allow adequate time for cells to aggregate into spheroids for most bladder cancer cell lines.
      NOTE: The formation of spheroids can be observed by inverted bright field microscope. The optimal size and number of spheroids may depend on individual experiments, but we find that spheroids with diameters from 50 µm to 150 µm are generally suitable for time-lapse imaging using a 20X objective lens.
   4. To incorporate additional cell types, such as fibroblasts or immune cells into the spheroids, mix the desired cells with cancer cells at desired ratio (1:1, 1:10, *etc*.) in an ultra-low attachment plate and incubate at 37 °C for ≥16 h to allow formation of mixed cell type spheroids.

2. **Growing from primary tumors**
   NOTE: Tumor spheroids can also be derived from primary bladder tumor sources such as tumors developed from carcinogen-induced bladder tumor models[8]. For example, bladder tumors generated from mice fed with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), can be harvested and minced into small pieces (about 0.5 mm^3) using sterile surgical instruments. Digested primary samples of human bladder cancers can also be studied in this system.
   1. Collect and wash 0.5 mm^3 tumor pieces in 5 mL ice-cold phosphate-buffered saline (PBS). Centrifugation at 200 x *g* for 5 min at 4 °C. Repeat this washing step once.
   2. Collect tumor pieces by centrifugation at 200 x *g* for 10 min at 4 °C. Remove the supernatant and add 5 mL of DMEM containing 10% FBS. Transfer tumor spheroid/media mixture to a 6-well ultra-low attachment plate. Incubate at 37 °C for ≥16 h before embedding into collagen matrix (see step 2.3).

2. Preparing the 3-D culture chamber

1. **Dilute type-1 collagen (derived from rat tail) in DMEM containing 10% FBS to make a 2 mg/mL mixture according to manufacturer's instructions.**
   1. In short, mix collagen with appropriate amount of 1 N NaOH solution and DMEM by gentle pipetting based upon the manufacturer's instructions to achieve physiologic pH.
   2. After mixing collagen and DMEM, quickly coat the wells of the chamber slide (for most applications, chamber slides with a number 1.5 cover-glass is optimal) with the collagen-DMEM mixture prior to solidification (200 µL/well).Allow the coated chamber slide to be stationary at room temperature for 15 min.
      NOTE: The composition of collagen-based matrix can be modified by adding other extracellular matrix ingredients, such as fibronectin or laminin, according to the purposes of experiment.

2. Gently pipet 500 µL of media containing spheroids (or tumor pieces, if primary tumor sample is used) from the 6-well ultra-low attachment plate into an empty 1.5 mL microcentrifuge tube. Wait for 2 min to let the spheroids settle to the bottom of the tube.
3. Carefully remove the supernatant from the tube. Prepare another tube of type-1 collagen (2 mg/mL) mixed with DMEM containing 10% FBS and quickly add 500 µL of this mixture to the spheroids. Gently mix the spheroids and collagen by slow pipetting.
4. Add 250 µL of spheroids/collagen mixture to a well of collagen-pre-coated chamber slide. Allow the collagen matrix containing spheroids to solidify completely (about 30 min at 37 °C).
5. After the collagen matrix is solidified, add 1 mL of DMEM containing 10% FBS to each well (**Figure 1B**). Incubate the chamber slide in a 37 °C incubator supplied with 5% CO_2 until ready for imaging.

3. Live Cell Time-lapse Imaging

1. Turn on the confocal microscope following the manufacturer's instructions and ensure that the climate chamber reaches 37 °C and is supplied with 5% CO_2.
   NOTE: Our microscope and chamber usually require 1 h to reach system equilibrium.
2. Carefully transfer the chamber slide to the slide adaptor attached to the microscope.
3. Locate the spheroids of interest using a low power objective (*e.g.*, 5X) and start imaging with the higher power objective (in current protocol, 20X objective is used for most of the live cell imaging for its better image quality).
   NOTE: For primary bladder tumor samples, 5X and 10X objectives may be needed in order to cover larger imaging area. For our time-lapse imaging, the imaging interval is set as 30 min and a Z stack is used to image the whole spheroid. Typically the distance between Z-slices is set to 4 µm for the 20X objective, and the total distance of Z axis is around 200 µm. Images can be obtained using DIC and with fluorescence if cells/tissues harbor fluorescent protein markers.
4. Perform time-lapse imaging for 24–72 h.

NOTE: The duration is determined by the cell type and the needs of the experiment.

4. Preparation of Sample Block Containing Cancer Spheroids for Frozen Tissue Sectioning

1. Carefully lift the block of collagen gel and spheroids from the chamber slides by using small forceps. Place the collagen gel block in a plastic histology mold.
2. Rinse the collagen gel with 1x PBS briefly, and then fix it with 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature.
   CAUTION: PFA is potential carcinogen and should be handled with care.
3. Wash the collagen gel with 1.5 mL of 1x PBS on a shaker for 15 min. Replace the PBS and repeat the previous washing step 3x.
4. Apply a thin layer (about 3 mm) of optimal cutting temperature (OCT) compound to cover the bottom of a new plastic histology mold. Place the fixed and washed collagen gel on top of the OCT compound, then carefully embed the whole gel by filling the mold with OCT compound while avoiding the formation of any air bubbles in the OCT.
5. Leave the mold at 4 °C for 1 h.
6. Place the mold containing the sample and OCT compound on a 100 mm Petri dish floating on liquid nitrogen. Allow the sample to flash-freeze completely.
7. Store the frozen sample block at -80 °C for future use.

5. Immunofluorescence Imaging for Frozen Sectioned Cancer Spheroids

1. Transfer the frozen sample block from the -80 °C freezer to the -20 °C chamber of a cryostat.
2. **Perform conventional frozen sectioning by setting the section interval to 7 µm. Let sectioned samples attach to the glass slide without wrinkles or trapped air.**
   1. Store the slides at -80 °C before performing further staining.
      NOTE: Different intervals may be used to fit experimental needs.
3. Air dry the slides for 1 h at room temperature.
4. Permeabilize the samples with PBS containing 0.5% Triton X-100 for 15 min.
5. Wash samples 3x with PBS for 10 min per wash.
6. Encircle the samples on the slide with hydrophobic barriers by using a hydrophobic barrier pen.
7. Treat the samples with blocking solution (1x PBS containing 5% bovine serum albumin (BSA)), for 1 h at room temperature.
8. Apply 40 µL of primary antibody-containing solution (1x PBS containing 5% BSA with 1:100 dilution of primary antibody) to each sample on the slide.
9. Incubate the slides in primary antibody at 37 °C for 1 h or at room temperature overnight (the incubation time and conditions vary depending on primary antibody).
10. Wash samples 3x with PBS for 15 min (per wash).
11. Apply 40 µL of secondary antibody-containing solution (1x PBS containing 5% of BSA with 1:300 secondary antibody dilution) to each sample on the slide.
12. Wash samples 3x with PBS for 15 min (per wash).
13. Stain the samples with Hoechst 33342 solution (1 µg/mL, diluted in PBS) at room temperature for 10 min. Then wash samples with PBS for 5 min.
14. Mount the samples with mounting medium and cover them with appropriately sized cover slips. Leave the slides in dark at room temperature for 24 h and then perform confocal microscopy.

Representative Results

Successful creation of invasive bladder cancer tumor spheroid requires the formation of appropriately sized tumor spheroids from cell lines or primary tumors. **Figure 2A** shows appropriately sized spheroids developed from four human bladder cancer cell lines (UM-UC9, UM-UC13, UM-UC14, 253J, and UM-UC18). **Figure 2B** shows a tumor spheroid from a BBN-generated mouse bladder tumor embedded in collagen matrix. These spheroids were embedded as described above and representative images were captured using a microscope equipped for live cell imaging. The 20X objective lens was used for imaging UM-UC9, UM-UC13, UM-UC14, and UM-UC18 spheroids, whereas mouse bladder tumor spheroids were examined using 5X and 10X objective lenses.

Representative images of human bladder cell line spheroids after 24–72 h (**Figure 2A**) and mouse BBN primary bladder tumor spheroids (**Figure 2B**) demonstrate bladder cancer migration into the collagen matrix. 253J, a non-invasive cell line, remain largely intact with little or no migration (**Figure 2A**). **Videos 1**, **2**, and **3** demonstrate the time-lapse invasion process for UM-UC9, UM-UC13, and UM-UC14 spheroids, respectively. **Video 4** shows the invasion properties of a UM-UC18 spheroid. **Video 5** shows the two areas of mouse bladder tumor from 66 h to 87 h post-collagen embedding.

To demonstrate the feasibility of using immunofluorescence staining of protein markers in our invasive tumor spheroids, they were fixed and stained at 24 or 72 h. **Figure 3** shows representative samples stained for Ataxia-Telangiectasia Group D-Associated Protein (ATDC, also known as TRIM29), a protein which plays a significant role in the tumorigenesis and cancer progression of human bladder and pancreatic cancers[9,10], tubulins (α and β), which form microtubules and play a key role in shaping the cell and cellular movement[11,12], keratin 14 (KRT14), a basal epithelial marker involved in invasion[13], and vimentin (VIM), a mesenchymal marker[14,15,16]. These images demonstrate that visualization of cellular and subcellular structures can be performed using this methodology. The higher magnification view of UM-UC14 shows filamentous staining for ATDC (**Figure 3A**). The highly invasive cells disseminated from UM-UC18 spheroids after 24 h of invasion express high level of VIM, a marker of epithelial-to-mesenchymal transition (EMT, **Figure 3B**).

Incorporation of different cell types (cancer-associated fibroblasts) into the tumor spheroids is feasible and provides a way to examine heterologous cell-cell interactions (**Figure 4** and **Video 6**). In this example we used red fluorescence protein (RFP)-labeled human fibroblasts and the 253J bladder cancer cell line transduced with a vector for expression of green fluorescent protein (GFP). These cells were mixed to form tumor spheroids and embedded in collagen. The invasion process monitored by confocal microscopy. **Figure 4** demonstrates that interaction with fibroblasts can modulate 253J's invasive behavior.

Figure 5 demonstrates the utility of the assay system to test inhibitors of invasion. Cytochalasin D is a known inhibitor of cell migration and invasion which acts by blocking actin polymerization[17,18] and was used as an example. Cytochalasin D treatment inhibited the invasion of UM-UC9 spheroids (**Figure 5A**). The same concentration of cytochalasin D (0.2 µM) is able to partially inhibit invasion of UM-UC18 spheroids (**Figure 5B**). Since the system can be used to image multiple wells and tumor organoids in parallel, it is amenable to screening a limited panel of pharmacologic agents for effect on invasion.

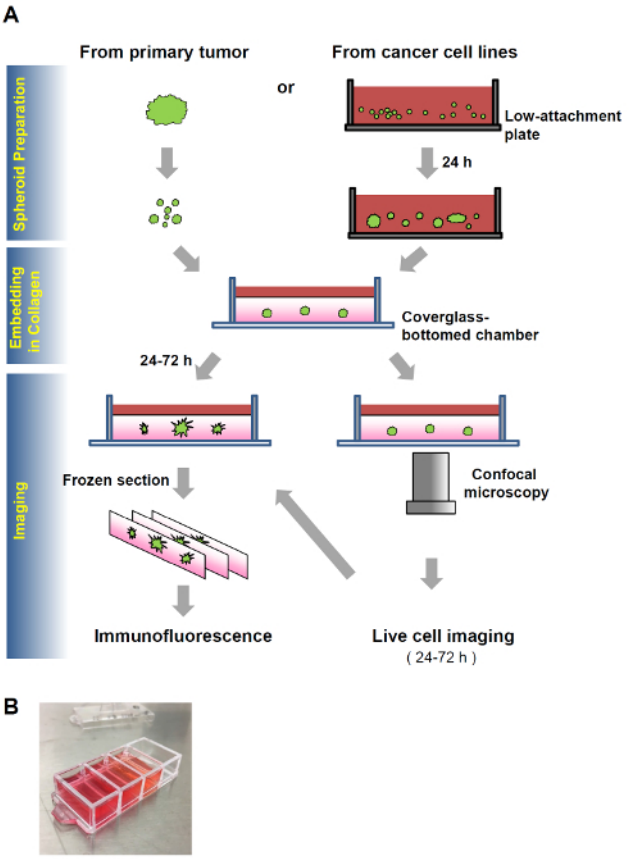


Figure 1: Setup for 3-D invasion assay with live cell imaging. (**A**) Schematic overview for 3-D live cell imaging and immunofluorescence. (**B**) Coverslip chamber with collagen and media used in this experiment. Please click here to view a larger version of this figure.

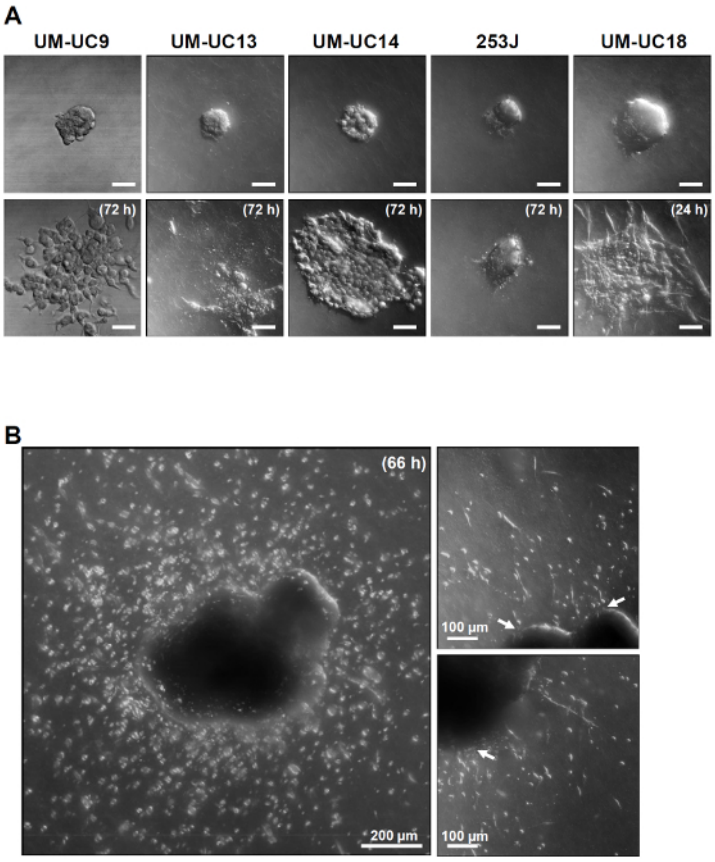


Figure 2: Bladder cancer spheroid invasion. (**A**) Examples of human bladder cancer cell line spheroids embedded in a collagen gel matrix. Spheroids are shown at the time of embedding (0 h, top row) or after 72 h (UM-UC9, UM-UC13, UM-UC14, 253J) or 24 h (UM-UC18). Scale bar = 50 µm. (**B**) Examples of BBN-induced mouse bladder tumor invasion into the collagen matrix. Arrows indicate the invasive edge of tumor spheroid. Please click here to view a larger version of this figure.

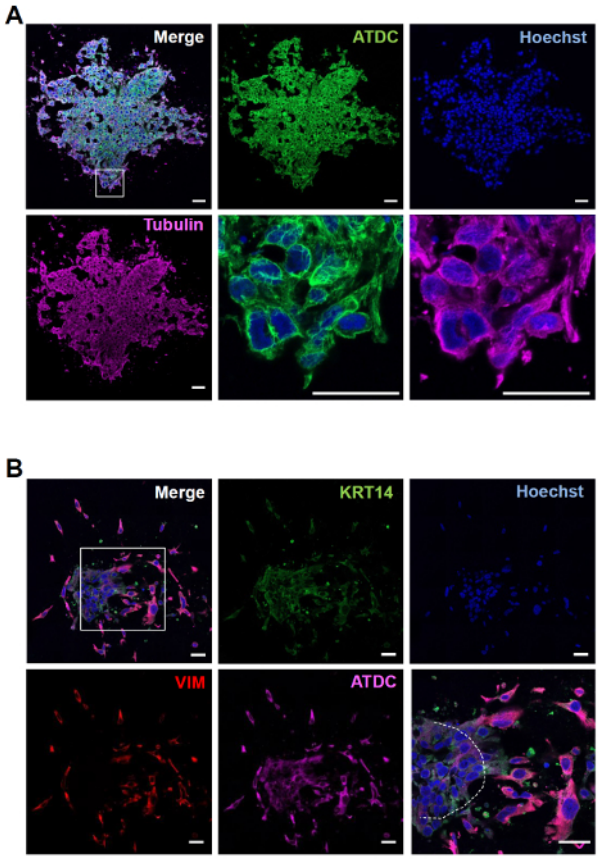


Figure 3: Immunofluorescence analysis of invasive bladder cancer spheroids. (**A**) UM-UC14 tumor spheroid after 72 h of invasion into collagen. Samples were stained by conventional immunofluorescence assay for ATDC (green), tubulin (violet), and nuclei (Hoechst stain blue). White square indicates the area with higher magnification view shown in bottom row right two panels. Scale bar = 50 µm. (**B**) UM-UC18 tumor spheroids stained for KRT14 (green), VIM (red), ATDC (violet), and nuclei (Hoechst stain blue) after 24 h of invasion into collagen. Scale bar = 50 µm. White square indicates the area with higher magnification view shown in bottom right panel. Dashed line roughly indicates the boundary of the original tumor spheroid. Please click here to view a larger version of this figure.

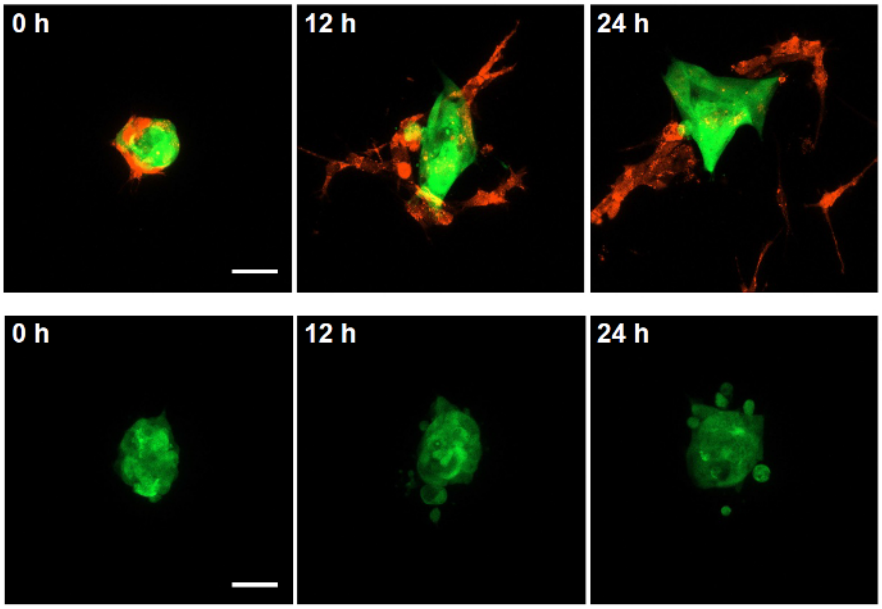


Figure 4: Fluorescently-labeled tumor spheroid incorporating fibroblasts. Invasion of GFP-labeled 253J bladder cancer cells alone (bottom panels) or co-cultured with RFP-labeled fibroblasts (top panels) monitored for 24 h. Scale bar = 50 µm. Invasion is enhanced with addition of the fibroblasts to the culture system (top panel). Please click here to view a larger version of this figure.

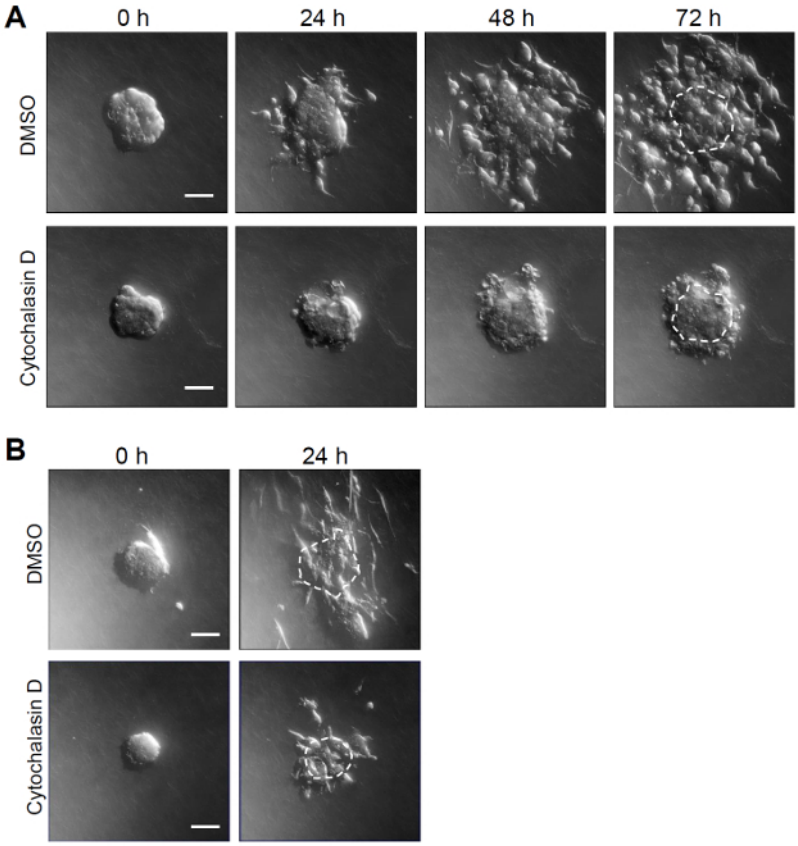
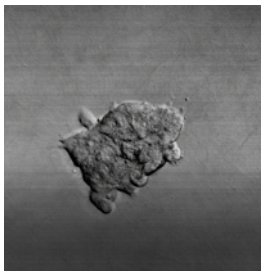


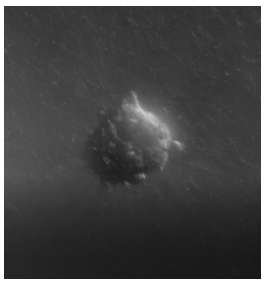
Figure 5: Effect of a small molecular drug targeting actin polymerization in invasion. (**A**) UM-UC9 tumor spheroid treated with cytochalasin D (0.2 µM) or DMSO (vehicle control) and monitored for 72 h. (B) UM-UC18 tumor spheroid treated with cytochalasin D or DMSO for 24 h. Scale bar = 50 µm. Dashed line indicates the boundary of the original spheroid. Please click here to view a larger version of this figure.

Possible problem	Recommended solution
Low number of viable spheroids	• Increase number of cells cultured in suspension • Ensure >90% cells are viable after typsinization • Check cells for contamination • Maintain cells in logarithmic growth before typsinizing • Optimize cell culture media • Modify the culture time in suspension condition • Try alternative cell lines
Spheroids are too big or too small	• Adjust the number of cells added to low attachment plate • Use gentle pipetting to break down larger cell aggregates
Hard to focus during imagining	• Adjust the thickness of collagen gel. For objectives with short working distance, try to make the collagen gel thinner. • Ensure that the chamber slide or coverslip is #1.5 • Optimize the size of spheroids (50–150 µm in diameter)
The cells migrate out of the imaging zone during invasion assay	• Reduce the size of spheroids • Re-center the imaging zone every 24 h if needed • Engaging tiling imaging technology (if applicable) to cover larger area • Consider use of alternative cell lines
The chamber slide dries out	• Ensure there is no leakage in the climate chamber setup • Ensure the moisture control mechanism is functional

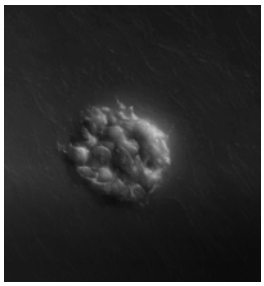
Table 1: Troubleshooting the 3-D invasion model.



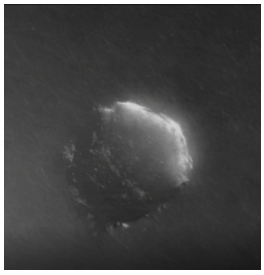
Video 1: Time-lapse video showing UM-UC9 spheroid cultured in collagen from 0 to 72 h. Please click here to view this video. (Right-click to download.)



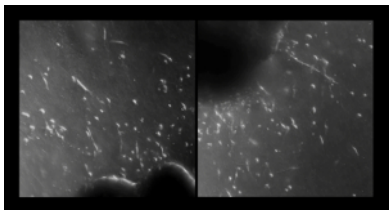
Video 2: Time-lapse video showing collective migration of UM-UC13 spheroid cultured in collagen from 0 to 72 h. Please click here to view this video. (Right-click to download.)



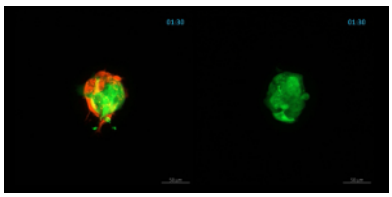
Video 3: Time-lapse video of UM-UC14 spheroid cultured in collagen from 0 to 72 h. Please click here to view this video. (Right-click to download.)



Video 4: Time-lapse video of UM-UC18 spheroid cultured in collagen from 0 to 24 h. Please click here to view this video. (Right-click to download.)



Video 5: Time-lapse video of BBN-induced mouse bladder tumors 66–84 h post-collagen embedding. Please click here to view this video. (Right-click to download.)



Video 6: Time-lapse video showing cancer spheroids formed from GFP-labeled 253J cells mixed with RFP-labeled human fibroblasts (left) or GFP-labeled 253J cells alone (right). Spheroids were embedded in collagen and monitored for 24 h. Please click here to view this video. (Right-click to download.)

Discussion

Here we describe a 3-D tumor spheroid model that allows real-time observation of bladder cancer invasion which is critical for cancer progression and metastasis. This system is amenable to the incorporation of various stromal and cellular components to allow investigators to better recapitulate the tissue microenvironment where bladder cancer invasion takes place. Bladder cancer spheroids can be generated from various sources such as cell lines (including genetically modified cell lines useful for the examination of signaling pathways that affect invasion processes), and mouse primary tumors (described here). It can potentially also be adapted for human primary tumor analysis. Depending on the imaging system used, fluorescence can be monitored in real-time or by fixation and immunofluorescence staining at various time-points. The system can also be used to test potential inhibitors of invasion. This makes the system a versatile and powerful tool to assess bladder cancer biology.

The confocal microscopy system with a climate chamber which provides temperature control, moisture control, and CO_2 supply is a key piece of equipment for constructing the model described in this protocol.

It has been widely reported that 3-D cultures provide specialized microenvironments which better mimic native tissues for cell biology and cancer studies[19,20]. Many studies rely on permeable membrane insert assays which do not reflect the conditions under which invasion occurs *in vivo*. Further, these conventional studies allow neither easy monitoring of the invasion process in a real-time fashion, nor detailed analysis of samples during or following invasion. Herein we described a system which is useful for both real-time and endpoint interrogation of invasive cancer biology.

Collagen is an important component of the extra cellular matrix (ECM), and exists in many forms. Type-1 collagen is the predominant form of fibrillar collagen whereas Type-4 collagen is a nonfibrillar collagen making up the basement membrane[21]. Cancer cells must penetrate the basement membrane and move through type-1 collagen during the progression from non-invasive to invasive tumors[22]. Given its ubiquitous presence in the ECM, type-1 collagen has been used as matrix for constructing 3-D cultures, which mimic the ECM better than the two-dimensional culture dish[5,6,23]. While the system outlined here is based upon a type-1 collagen matrix, it can be modified to include other stromal constituents based on experimental question and need.

The methodology we use to generate bladder cancer spheroids from cell lines involves cell culture in low-attachment conditions and cell aggregation. Not all cells can tolerate these culture conditions and some may undergo apoptosis or formation of loose aggregates that dissociate during the collagen embedding process. Modifying the culture time, cellular number or incorporating other cell types in suspension may improve the outcome. The cell lines selected for this assay depend upon the goal of the experiment. We have successfully used this technique with 7/7 unique human bladder cancer cell lines. However, it is possible that some cell lines may not be suitable for this experimental setup. Furthermore, some cell lines have a very invasive phenotype, which leads to early disassociation of spheroids, and cancer cells moving out of the observation

area during the time-lapse experiment. Pilot experiments for understanding the general behavior of cell lines are highly recommended to determine the best time frames for studies. Troubleshooting for common issues with spheroid generation and imaging is listed in **Table 1**.

This system is suitable for limited screening of pharmacologic compounds with potential to block cancer cell invasion. Herein we used Cytochalasin D, a cell-permeable inhibitor of actin polymerization, to illustrate this potential application[18]. As shown above, 0.2 µM of cytochalasin D effectively inhibits the invasion of UM-UC9 and UM-UC18 spheroids. By utilizing multi-chambers slides and automated microscopic stage control, the effect of multiple compounds on tumor spheroid invasion is feasible.

Although these assays are best suited to qualitatively describe invasive behavior, quantification of the extent of migration and invasion is also feasible. Acquisition of images of spheroids at the start of experiment and at various time points, and subsequent image analysis to measure the farthest linear distance of invasion in X, Y or Z directions can provide quantification of invasive migratory behavior. More advanced image analysis using fluorescently labelled cells could be used to define and quantitate individual cell or cell type behavior depending on the experimental need or question.

Disclosures

The authors declare no competing financial interests.

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