Chapter 12

A 3D Culture Model to Study How Fluid Pressure and Flow Affect the Behavior of Aggregates of Epithelial Cells

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Abstract

Cells are surrounded by mechanical stimuli in their microenvironment. It is important to determine how cells respond to the mechanical information that surrounds them in order to understand both development and disease progression, as well as to be able to predict cell behavior in response to physical stimuli. Here we describe a protocol to determine the effects of interstitial fluid flow on the migratory behavior of an aggregate of epithelial cells in a three-dimensional (3D) culture model. This protocol includes detailed methods for the fabrication of a 3D cell culture chamber with hydrostatic pressure control, the culture of epithelial cells as an aggregate in a collagen gel, and the analysis of collective cell behavior in response to pressure-driven flow.

Key words Mechanical stress, Fluid flow, Micropatterning, 3D culture, Fluid pressure

1 Introduction

1.1 Studying the Physical Microenvironment

In addition to the biochemical signals in the microenvironment, many physical aspects of the microenvironment can affect cell behavior [1, 2]. Physical factors including stiffness, pressure, flow, shear stress, and stretch can cause changes in cell behavior that help maintain tissue homeostasis during development, and alterations in these physical factors are frequently associated with initiation and progression of disease [3–5]. For example, increased matrix stiffness is a telltale sign of cancer, and can promote tumorigenesis [6]. A stiffer microenvironment can alter epithelial plasticity by promoting epithelial-to-mesenchymal transition (EMT) in mammary epithelial cells [7], a process linked to cancer invasion. Furthermore, most malignant solid tumors have elevated interstitial fluid pressure (IFP) compared to normal tissue [8]. High IFP has been associated with poor prognosis and metastasis to lymph nodes [9, 10].

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Because of the connection between physical factors and disease, it is necessary to develop experimental models that recapitulate the various physical properties of the microenvironment [11]. Cells exist primarily in 3D within living tissues (though epithelial and endothelial cells can exist as quasi-2D monolayers). Interactions between cells, their neighbors, and the surrounding extracellular matrix (ECM) are crucial for maintaining normal tissue function and homeostasis [12]. Therefore, 3D culture models are often used to mimic the structure and function of the tissue microenvironment [13, 14].

1.2 Interstitial Fluid Pressure (IFP)

Solid tumors have a high IFP that results from abnormal, leaky blood vessels and impaired lymphatic drainage [15, 16]. This feature has poor implications in cancer, as elevated IFP can lead to therapeutic resistance by hindering the delivery of drugs into solid tumors [10, 17–19]. IFP has also been shown to influence the migratory and invasive behaviors of single-cell suspensions of MDA-MB-231 breast cancer cells in collagen gels. In one study, a hydrostatic pressure differential of culture medium was established across the suspensions. Single-cell tracking showed that IFP increased the percentage of migratory cells and the speed at which they moved, and cells were observed to migrate primarily in the direction of flow via autologous chemotaxis, a phenomenon that has been previously reported by the same group using modified 3D Boyden chambers [20, 21]. In similar studies, IFP was also found to affect the migratory behavior of MDA-MB-231 cells, except that cells migrated against the flow direction, particularly when seeded densely [22, 23]. The response of cancer cells to physical cues such as elevated IFP seems to depend on the context in which the signals are presented, and neither of the previous models captures the behavior of an intact aggregate of tumor cells, suggesting the need for a new model. Here we describe a microfluidic approach to model the effects of IFP on an aggregate of tumor cells in 3D.

We have developed a culture model in which a defined hydrostatic pressure differential of culture medium may be applied across an aggregate of epithelial cells surrounded by a gel of type I collagen [24]. Our technique allows for the generation and control of the fluid pressure profile experienced by the aggregate of epithelial cells, and enables us to examine the effects of IFP on collective migration of those cells. An ideal model system is as close to in vivo conditions as possible; here, the 3D model mimics the physiological conditions of a dense tumor tissue. This approach allows one to visualize directly cell migration and tumor invasion in 3D from a preexisting aggregate, as well as to analyze changes in gene expression using in situ assays including immunostaining, and bulk analyses including immunoblotting and real-time RT-PCR. The model can also be used as a platform to screen for therapeutics that inhibit cancer cell invasion under different pressure conditions. Although

we use this model in the context of cancer, it could also be used to study the effects of pressure and/or fluid flow in other physiological or pathological contexts.

2 Materials

Prepare collagen mixture on ice. Keep reagents at 4 °C.

2.1 Preparation of PDMS Chamber and Cavity Surrounded by Collagen

1. Polydimethylsiloxane (PDMS).
2. PDMS curing agent.
3. Lithographically patterned silicon master.
4. ¼″ hole punch.
5. 150-mm petri dishes.
6. 100-mm petri dishes.
7. 100-mm tissue-grade polystyrene culture dishes.
8. 24 mm × 50 mm #1½ glass coverslips.
9. 18 mm × 18 mm #2 glass coverslips.
10. 70 % (v:v) ethanol.
11. 0.12 mm × 30 mm acupuncture needles.
12. 1.5 ml microcentrifuge tubes.
13. 10× Hank's balanced salt solution (HBSS).
14. 0.1 N NaOH.
15. Bovine dermal type I collagen (non-pepsinized).
16. Cell culture medium. For example, 1:1 Dulbecco's Modified Eagle's Medium : Ham's F12 Nutrient Mixture (DMEM/F12 (1:1)) supplemented with: 10 % fetal bovine serum (FBS), and 50 μg/ml gentamicin.
17. Sterile phosphate-buffered saline (PBS).
18. 1 % (w:v) bovine serum albumin (BSA) in PBS. Store at 4 °C.
19. Handheld drill.

2.2 Formation of 3D Epithelial Cell Aggregates

1. Culture medium (*see* Subheading 2.1, **item 16**).
2. 0.05 % 1× trypsin–EDTA.
3. Collagen gels with channels, assembled between PDMS chambers and glass coverslips.
4. PDMS.
5. PDMS curing agent.
6. ¼″ hole punch.

2.3 Immunofluorescence Imaging

1. 16 % paraformaldehyde.
2. PBS.

3. 1.5 ml microcentrifuge tubes.
4. PBS with 0.3% (v:v) Triton X-100 (0.3% PBST).
5. Normal goat serum.
6. Rabbit anti-E-cadherin antibody.
7. Alexa 488 goat anti-rabbit antibody.
8. Nuclear counterstain, such as Hoechst 33342.
9. Aluminum foil.
10. Inverted microscope with phase-contrast and fluorescence capabilities and a 10×/0.30 NA objective.

2.4 Image Analysis: Measuring the Extent of Collective Migration

1. ImageJ (National Institutes of Health, Bethesda, MD).

3 Methods

Here we describe an engineered 3D culture model that can be used to study the effects of pressure gradients and fluid flow on the migratory/invasive behavior and gene expression profile of an aggregate of epithelial cells.

3.1 Preparation of PDMS Chamber and Cavity Surrounded by Collagen

1. Mix the PDMS prepolymer and curing agent at a 10:1 (w:w) ratio. Aim for a total weight of approximately 50 g. Remove the entrapped air bubbles by degassing in a vacuum chamber (~15 min). Pour the bubble-free mixture onto a lithographically patterned silicon master in a 150-mm petri dish. The silicon master should have features that produce channels that are approximately 20 mm long, 1 mm wide, and 1 mm tall spaced approximately 1.5 cm apart. Cure the PDMS in an oven at 60 °C for at least 2 h.
2. Once the PDMS is cured, carefully peel the PDMS from the silicon wafer, removing any PDMS from the bottom of the master. Using a clean razor blade, cut off the excess PDMS from around the molded features.
3. Using a clean razor blade, cut the polymerized PDMS into chambers containing individual channels ~1.25 cm wide and ~2.5 cm long (Fig. 1a). Use a ¼″ hole punch to bore holes on either side of each channel well. Bore one of the holes (hole B in Fig. 1b) in the middle of the channel such that the distance between the holes is 8–10 mm. Sterilize the chambers carefully in a biosafety cabinet (cell culture hood) by sonicating the chambers in 70% ethanol, washing briefly with 100% ethanol, and aspirating the excess liquid (*see* **Note 1**).
4. Drill a rectangular hole (~15 mm × ~40 mm) in the middle of a 100-mm tissue culture dish.

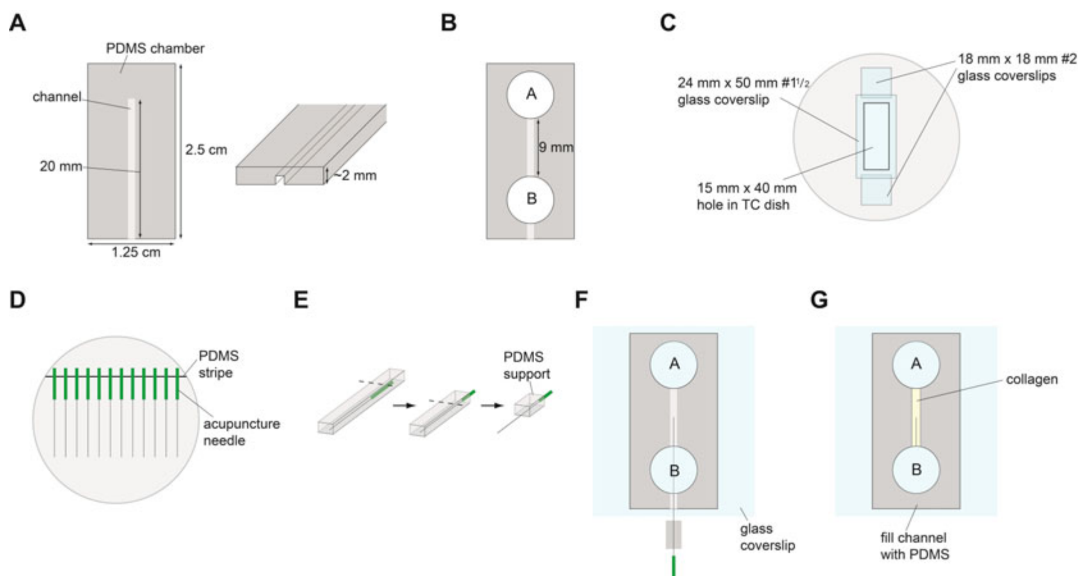

Fig. 1 Schematic diagrams detailing preparation of PDMS chamber and channel surrounded by collagen. (**a**) Top and perspective views of a PDMS chamber with a single channel, including dimensions. (**b**) Top view of a PDMS chamber with a single channel showing locations of holes A and B. (**c**) Top view of 100 mm tissue culture dish showing the placement of glass coverslips over a rectangular hole drilled in the middle of the dish. (**d**) Top view of a 100 mm petri dish containing acupuncture needles held in place by a stripe of PDMS. (**e**) Schematic detailing the preparation of an acupuncture needle with a PDMS support block. (**f**) Top view of a PDMS chamber conformally adhered to a glass surface with acupuncture needle setup prior to the addition of collagen into hole A. (**g**) Top view of a PDMS chamber containing a collagen channel with a cavity in the shape of the acupuncture needle from (**f**), with the channel adjacent to well B filled with PDMS

5. In a biosafety cabinet, wash the modified tissue culture dish containing the rectangular hole with 100% ethanol to sterilize.
6. In a biosafety cabinet, carefully sterilize one 24 mm × 50 mm #1½ glass coverslip and two 18 mm × 18 mm #2 glass coverslips by sonicating in 70% ethanol, washing briefly with 100% ethanol, and aspirating the excess liquid.
7. Lay down the 18 mm × 18 mm #2 glass coverslips parallel to the ~15 mm sides of the hole in the 100-mm tissue culture dish, leaving a few mm of space between the coverslips and the hole (Fig. 1c).
8. Mix the PDMS prepolymer and curing agent and remove air bubbles as described previously. Aim for a total weight of approximately 5 g. Using a 200 μl pipet tip, add a layer of PDMS on top of the modified 100 mm tissue culture dish around the hole and between the hole and the coverslips. Then, carefully lay the 24 mm × 50 mm #1½ coverslip on top of the two 18 mm × 18 mm #2 coverslips such that it covers the hole forming a seal with the PDMS. Cure the PDMS in an oven at 60 °C for at least 2 h.

9. Mix the PDMS prepolymer and curing agent as described previously. Aim for a total weight of approximately 20 g. Again, remove the entrapped air bubbles by degassing in a vacuum chamber (~15 min). Using a pipette tip, paint a stripe of PDMS in a straight line ~1.5 cm from the edge of a 100-mm petri dish. Lay down 12–14 120 μm diameter acupuncture needles with handles placed on the PDMS (Fig. 1d). Cure the PDMS in an oven at 60 °C for at least 1 h.
10. Pour the remainder of the 20 g PDMS mixture on top of the needles. Cure the PDMS in an oven at 60 °C for at least 2 h.
11. Using a clean razor blade, separate the individual needles embedded within PDMS and remove a portion (~1 cm) of the handles. Use the razor blade to cut away the PDMS surrounding the needle, leaving a small rectangular portion in the middle of the needle to be used for support (Fig. 1e). Sterilize the needles carefully in a biosafety cabinet by sonicating in 70% ethanol, washing briefly with 100% ethanol, and aspirating the excess liquid.
12. Coat the needles with 1% BSA in PBS for at least 4 h at 4 °C and then wash with PBS and ddH_2O.
13. In a biosafety cabinet, place three of the PDMS chambers in the middle of one modified glass-bottom 100-mm tissue culture dish such that the channels are parallel to one another (and perpendicular to the 50 mm sides of the 24 mm × 50 mm #1½ glass coverslip) with the open face of the channels against the glass (*see* **Note 2**). Carefully thread the cleaned needles into the side of the chamber next to hole B such that the tip of the needle is in between the two wells formed by holes A and B and in the middle of the channel (Fig. 1f). Secure the needle in place by conformally adhering the PDMS support to the tissue culture dish next to the PDMS chamber and the 24 mm × 50 mm #1½ glass coverslip. Cool the tissue culture dishes to 4 °C for at least 2 h (*see* **Note 3**).
14. In a cold (4 °C) 1.5 ml microcentrifuge tube, prepare a neutralized solution of collagen. Add 30.6 μl 10× HBSS, 18.4 μl 0.1 N NaOH, 244.8 μl collagen, and 12.2 μl cell culture medium for a final concentration of collagen of approximately 4 mg/ml (*see* **Note 4**). Mix slowly by pipetting up and down; try not to introduce bubbles. If bubbles are induced, centrifuge the mixture briefly at 15,700 rcf.
15. Add 15 μl of neutralized collagen solution to the well formed by hole A (well A) of the PDMS chamber, opposite to the side containing the needle (Fig. 1f). Tilt the culture dish on its side to allow the collagen to flow down and fill the channel, tapping the dish as necessary (*see* **Note 5**). Gently aspirate the excess collagen from well A. Incubate at 37 °C for 20 min.

16. Add 20 μl of cell culture medium to both wells at the gel surface in order to wet the gel. Gently remove the needle from the chamber: bend it 90° where it exits the PDMS support, and gently pull it straight out while holding down the PDMS support.
17. Add a small amount of PDMS (prepared as above and incubated at 60 °C for 15 min) to plug the channel next to the well formed by hole B (well B) that contained the needle (Fig. 1g). Add 20–50 μl of cell culture medium to both wells once the PDMS is cured. Incubate the channels overnight at 37 °C.

3.2 Formation of 3D Epithelial Cell Aggregates

1. Aspirate the cell culture medium from the wells in the PDMS chamber.
2. Trypsinize epithelial cells (in this case MDA-MB-231 human breast carcinoma cells) and resuspend in cell culture medium at a final concentration of approximately 10^7 cells per ml.
3. Add 50 μl of the concentrated suspension of cells to well B and allow the cells to fill the cavity by convection (Fig. 2a, b).
4. Resuspend the cells in well B after 2–5 min. Then, once the cavity is completely filled, aspirate the cell suspension from the well.
5. Wash well B twice with 50 μl of medium.
6. Add fresh medium to the rim of well A and slightly over the rim of well B. Incubate the seeded channels at 37 °C for 48 h, changing the medium every 24 h.
7. After 48 h, discard any samples in which cells have migrated outside of the original shape of the aggregate.
8. Mix the PDMS prepolymer and curing agent as described above. Aim for a total weight of approximately 50 g. Pour the bubble-free mixture onto a 150-mm petri dish. Cure the PDMS in an oven at 60 °C for at least 1 h.
9. Using a clean razor blade, cut the PDMS into blocks that are approximately 1 cm by 1 cm. Use a ¼″ hole punch to bore a hole in the center of the blocks, creating PDMS gaskets (Fig. 2c).
10. To set up a hydrostatic pressure differential across the cell aggregates in the collagen channels within the PDMS chambers, add up to four PDMS gaskets on top of one of the wells on one side of the channel. Ensure that the holes in the PDMS gaskets align with the wells in the chamber (Fig. 2d). Seal the PDMS gaskets conformally to the chamber by gently pressing down.
11. Add culture medium to the rim of both wells. Maintain the pressure differential by replenishing the medium on the higher pressure side every 12 h (*see* **Note 6**).
12. Culture the cell aggregates for up to 9 days at 37 °C.

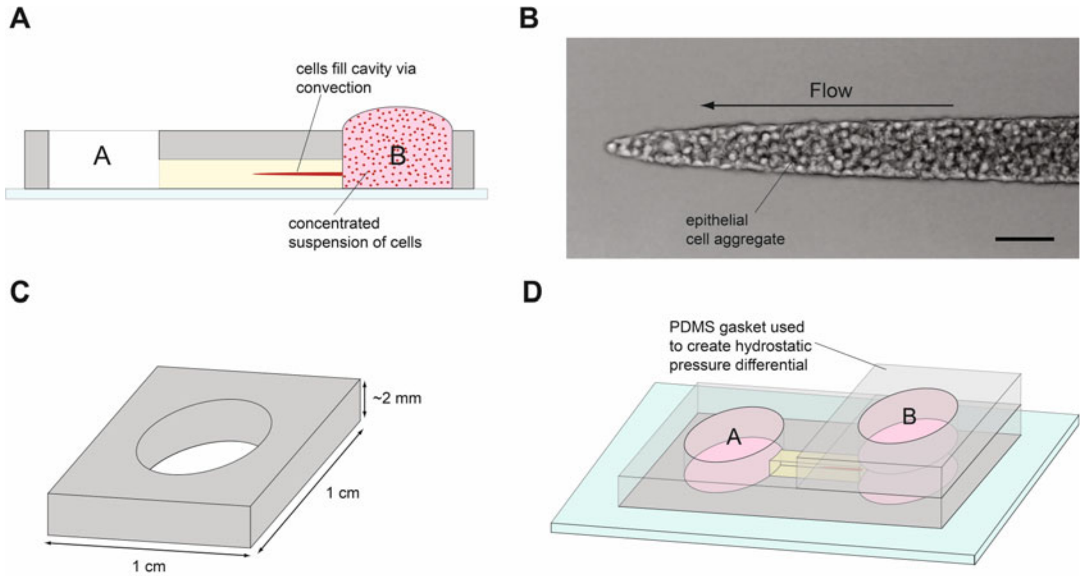

Fig. 2 Schematic diagrams detailing the formation of 3D mammary epithelial cell aggregates and the control of hydrostatic pressure profiles across the channels. (**a**) Side view of a cavity surrounded by collagen within a PDMS chamber being seeded with mammary epithelial cells via convection from a concentrated cell suspension in well B. (**b**) Phase-contrast image of a seeded cavity depicting the direction of convective flow during seeding. Scale bar 100 μm. (**c**) Perspective view of a PDMS gasket to be used to control the profile of hydrostatic pressure. (**d**) Perspective view of the culture model showing the creation of a hydrostatic pressure differential across the channel

3.3 Immunofluorescence Analysis

1. Prepare a fixative by diluting 16% paraformaldehyde 1:4 (v:v) in PBS.
2. Aspirate the medium from the PDMS chamber and add fixative to well B until the well is slightly overfilled. Incubate at room temperature for ~18 min.
3. Aspirate the fixative and fill well B to the rim with PBS. Repeat twice, each after 20 min, for three total washes.
4. *To stain for nuclei*: prepare 4 ml of a 1:1000 (v:v) solution of Hoechst 33342 in PBS. To stain for E-cadherin or another marker that is detected with antibodies, skip to **step 8**.
5. Aspirate the PBS from well B and fill it to the rim with the Hoechst solution. Incubate at room temperature for 15–20 min.
6. Aspirate the Hoechst solution and wash well B three times with PBS as in **step 3**. Stained samples can be stored in PBS at 4 °C until further use.
7. If desired, visualize the stained nuclei as described in Subheading 3.4.
8. *To stain for E-cadherin or other protein marker* (Fig. 3): aspirate PBS. Peel the PDMS chamber from the tissue culture dish.

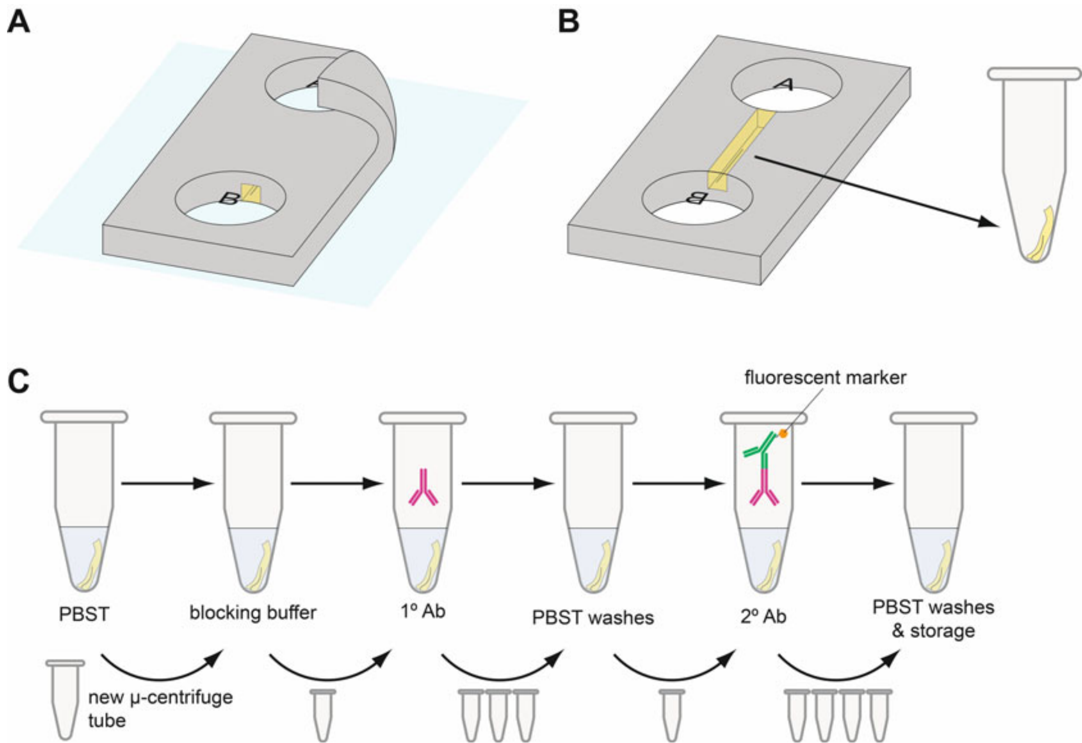

Fig. 3 Schematic of antibody staining procedure. (**a**) The PDMS chamber is peeled off of the tissue culture dish. (**b**) The collagen channel with embedded cell aggregate is carefully removed from the underside of the chamber and placed in a 1.5 ml microcentrifuge tube. (**c**) Washes and staining all take place inside new microcentrifuge tubes

Carefully remove the collagen channel and embedded cell aggregate from the underside of the PDMS chamber and place in a 1.5 ml microcentrifuge tube (*see* **Note 7**).

9. Add 300 μl of PBST to the tube and incubate the sample at room temperature for 15 min.
10. Prepare blocking buffer by diluting goat serum 1:10 (v:v) in PBST.
11. Remove the sample and place in a new 1.5 ml microcentrifuge tube. Add 300 μl of blocking buffer and incubate on a shaker at room temperature at least 4 hours.
12. Prepare a 1:200 (v:v) solution of primary rabbit anti-mouse E-cadherin antibody in blocking buffer (*see* **Note 8**).
13. Remove the sample and place in a new 1.5 ml microcentrifuge tube. Add 300 μl of the primary antibody solution and incubate on a shaker overnight at 4 °C.
14. Remove the sample and place in a new 1.5 ml microcentrifuge tube. Add 300 μl of PBST and incubate on a shaker at room temperature for 30 min. Repeat with a fresh microcentrifuge tube and PBST aliquot every 30 min for 3–4 h.

15. Repeat **steps 12–14** with the Alexa 488 goat anti-rabbit antibody. Wrap the microcentrifuge tubes with aluminum foil to prevent photobleaching of the secondary antibody. After the final wash, stained samples can be stored in PBS at 4 °C until further use (*see* **Note 9**).
16. Visualize samples as described in Subheading 3.4.

3.4 Imaging Techniques

1. To image samples, use a 10×/0.30 NA objective focused on the midplane of the tip of the epithelial cell aggregate (*see* **Note 10**).
2. To monitor cell migration over time in the culture model under various flow conditions, capture phase-contrast images of the epithelial cell aggregates (Figs. 4a and 5a) on each day for up to 9 days using an inverted phase-contrast microscope.
3. To image stained samples, transfer the samples onto a glass slide, and place a drop of PBS on top of each sample to keep the sample hydrated.

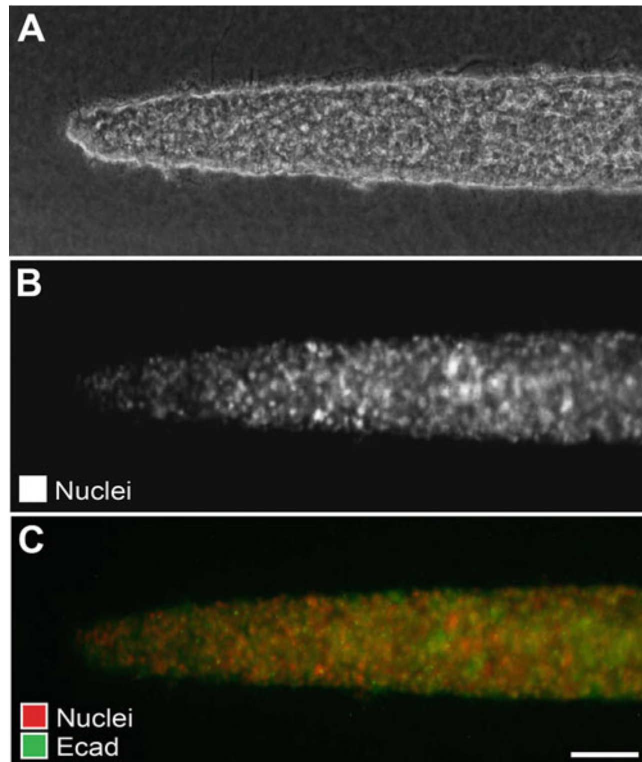

Fig. 4 Visualization of cell aggregates using phase-contrast, small molecule dyes, and fluorescent antibodies. (**a**) An epithelial cell aggregate under a control pressure profile (no hydrostatic pressure differential across the channel) on Day 6 visualized using phase-contrast imaging. (**b**) Image of the sample from (**a**) stained using the nuclear marker Hoechst 33342. Nuclei are shown in *white*. (**c**) Sample from (**a**) stained for E-cadherin (shown in *green*). Nuclei are shown in *red*. Scale bar 100 μm

4. To visualize cell nuclei, image fixed Hoechst 33342-stained samples under UV illumination (Figs. 4b and 5b).
5. To visualize samples stained for E-cadherin, image using an inverted fluorescence microscope (Fig. 4c).

3.5 Image Analysis: Measuring the Extent of Cell Invasion from the Initial Aggregate

1. Open the phase-contrast image files in ImageJ.
2. Set the scale of the image according to microscope calibrations by selecting "Set Scale…" under the "Analyze" menu.
3. Using the line tool on the main menu, draw a line along the length of a collectively migrating cohort protruding from the aggregate (Fig. 5c) (*see* **Note 11**).
4. Measure the length of the line by clicking "Measure" under the "Analyze" menu. This will output the length of the line in the units specified.

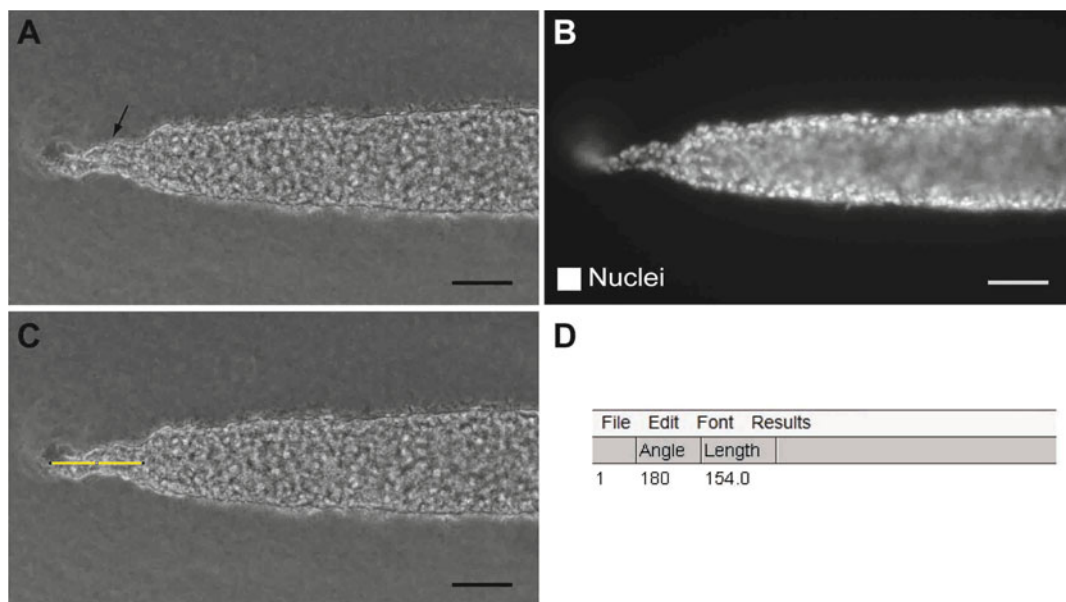

Fig. 5 Measurement of the invasion of cell aggregates. (**a**) Phase-contrast image of an epithelial cell aggregate on Day 6 under a pressure profile, obtained by holding well A at a higher hydrostatic pressure than well B. Invasion from the tip of the cell aggregate is shown with an *arrow*. (**b**) Image of the sample from (**a**) stained using Hoechst 33342. Nuclei are shown in *white*. (**c**) Example of using the line tool in ImageJ to measure the length of the invasive protrusion from the epithelial cell aggregate tip in the sample from (**a**). (**d**) Measurement output from ImageJ. Scale bars 100 μm

4 Notes

1. Cleaning should eliminate all leftover debris and dust particles.
2. Press down on the PDMS chambers to ensure that they are conformally adherent to the tissue culture dish.
3. The PDMS chambers must be chilled prior to the addition of collagen to prevent premature gelation.
4. When using a new bottle of collagen, check the pH of the final mixture. We used a pH of 8.5–9. To alter the pH, adjust the volume of NaOH accordingly.
5. To help the collagen mixture flow into the channels, turn the tissue culture dish on its side (with the channels perpendicular to the work surface) while tapping.
6. The hydrostatic pressure differential across the aggregate can range from 0.4 to 1.6 cm H_2O depending on how many PDMS blocks are used. Interstitial flow velocities are on the order of 1 μm/s (average flow rates of 20–100 μl/day).
7. Remove the collagen channel containing the epithelial cell aggregate from the PDMS chamber with forceps. Be sure to grip the channel from the end adjacent to well A.
8. Here, we stained for E-cadherin, but the same protocol may be used for other primary antibodies.
9. In addition to staining, one can also use real time RT-PCR analysis to quantify changes in gene expression. For this analysis, it is necessary to combine at least six samples per condition to obtain an adequate amount of RNA. Samples must also be incubated with collagenase (we recommend a 2 mg/ml solution of collagenase from *Clostridium histolyticum* (Sigma) in culture medium) prior to RNA extraction using an RNeasy Mini Kit (Qiagen).
10. To visualize 3D features of the mammary epithelial cell aggregates (live or fixed), capture confocal stacks of the samples using an inverted spinning disk confocal microscope (200 images, 1 μm apart).
11. We defined collective migration as protrusions from the primary aggregate, still attached to the latter, containing multiple nuclei.

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